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Minimal Residual Disease and Circulating Tumor Cells in Breast Cancer

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Minimal Residual Disease and Circulating Tumor Cells in Breast Cancer

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Preface

While many notable advances have occurred on the detection and characterization of minimal residual disease and circulating tumor cells in breast cancer, no book has ever summarized all the progress made until know. Here, we have invited leading investigators in the field to address relevant questions including:

- How can the study of minimal residual disease and CTCs help us to better understand breast cancer metastasis?
- What technologies are available for the detection and characterization of CTCs and DTCs, and what are their relative merits?
- What is the role of other blood-based biomarkers such as circulating endothelial cells and circulating nucleic acids?
- How are DTCs and CTCs relevant to clinical research and practice?
- What are the challenges in drug and biomarker co-development and the use of CTCs for companion diagnostic development?

We hope this book will be a reference for researchers and clinicians that are interested in minimal residual disease and circulating tumor cells in breast cancer and that it will further stimulate research in the field.

Michail Ignatiadis
Christos Sotiriou
Klaus Pantel

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Contents

Part I
Introduction

Minimal Residual Disease and Circulating Tumor Cells in Breast Cancer: Open Questions for Research

Michail Ignatiadis, Christos Sotiriou and Klaus Pantel

Contents

Most deaths from carcinomas are caused by the hematogenous dissemination of cancer cells to distant organs and eventually the development of metastasis. Occult cancer cells when found in the bone marrow or peripheral blood of carcinoma patients are defined as disseminated tumor cells (DTCs) or circulating tumor cells

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(CTCs) [1, 2]. Minimal residual disease (MRD) is defined by the presence of malignant cells in distant organs that are undetectable by conventional imaging and laboratory tests used for tumor staging after curative surgery of the primary tumor. CTCs and DTCs are considered surrogates of MRD and potentially metastasis-initiating cells [1]. In this book, we have invited leading investigators in the field to address the following questions:

1 How can the Study of MRD and CTCs Help Us to Better Understand Breast Cancer Metastasis?

The new "self seeding" theory of breast cancer progression challenges the dogma of unidirectional metastatic progression by providing evidence that circulating cancer cells can seed not only to regional and distant sites in the body but can also return to their original source, the primary tumor site [3, 4]. Beyond the study of MRD, the role of distant microenvironments (e.g., bone marrow) is very important for the fate of these cells. Currently, the mechanisms regulating the switch between dormancy and expansion of DTCs remain largely unknown, although experimental evidence supports different potential scenarios contributing to dormancy [5, 6]. DTC dormancy is ultimately thought to be a survival strategy that when targeted will eradicate dormant DTCs preventing metastasis [5, 7, 8].

2 Is There any Preferred Technology for CTC Detection and Characterization?

There are many different technologies for CTC detection and characterization [9–25]. These technologies use either physical separation or affinity-based methods for CTC enrichment [26]. As a result, the different technologies do not always detect the same subpopulations of CTCs. CellSearch®, a technology based on EpCAM-positive enrichment, is the only one that has received US Food and Drug Administration (FDA) approval for CTC detection as an aid in monitoring patients with metastatic breast, colorectal and prostate cancer [27–29]. It is anticipated that this and other technologies will be further validated in different clinically relevant scenarios in the near future.

3 What is the Role of Other Blood-Based Biomarkers like Circulating Enothelial Cells and Circulating Nucleic Acids?

Preliminary preclinical and clinical evidence suggest that the detection of circulating endothelial cells (CECs) and circulating endothelial progenitors (CEPs) may be useful in monitoring patients receiving anti-angiogenic treatments [46, 47]. Recent studies of mutations, genomic rearrangements or epigenetic alterations in

circulating DNA [48, 49] and studies of serum plasma microRNAs [50, 51] hold great promise for non-invasive monitoring of MRD in breast cancer. Although the source of circulating nucleic acids (CNAs) is still under debate, there is preliminary evidence that changes in CNAs levels correlate with tumor burden, disease progression and resistance to therapy [52]. These technologies might be used complementary to the current CTC/DTC assays [52].

4 Should DTC/CTC Detection and Characterization be Used in Current Clinical Practice?

There is solid evidence from two pooled meta-analyses on the adverse prognostic value of bone marrow DTCs detected at the time of surgery or during follow-up in early breast cancer [30–32]. Moreover, several studies have provided solid evidence about the adverse prognostic value of CTC detection by CellSearch® in metastatic breast cancer [27, 33, 34]. A single center has reported on the prognostic value of CTC detection in primary breast cancer using a reverse transcriptase polymerase chain reaction for Cytokeratin-19 [35, 36]. The SUCCESS group has conducted the largest study that has demonstrated the prognostic value of CTCs in primary breast cancer using the CellSearch technology [37, 38]. Finally, the characterization of CTC/DTC HER2 status as compared to HER2 status of the primary tumor is an example of how the characterization of these cells can be used as an additional tool for real-time monitoring of tumor genotype [39–41]. However, for adoption of CTC/DTC detection and characterization in clinical practice further prospective evidence is needed so that they can improve treatment decision and patient management in a cost-effective way.

5 What are the Challenges in Drug and CTC Co-Development?

All biomarker assays that are ultimately cleared by regulators for use in the care of patients must meet certain criteria of analytic validity, clinical validity and clinical utility [42–44]. There is an urgent need for biomarkers predicting benefit of new targeted agents. A simple example of how CTCs can accelerate drug development is clinical trials in which investigators study CTCs response as a surrogate for survival for regulatory purposes. Such an effort is ongoing in a phase 3 registration trial of abiraterone acetate in metastatic prostate cancer [45].

6 Future Perspectives

Overall the evidence presented in this series of articles suggests that DTC/CTC detection and characterization hold the promise to lead to a better understanding of breast cancer metastatic process and toward personalized treatment of breast

cancer patients. Standardization of the assays is always the first step. Most importantly, the clinical utility of CTCs/DTCs, CECs and CNAs need to be tested in large-scale trials with defined therapies and endpoints. Introduction into clinical practice will largely depend on the critical question of how MRD monitoring will influence treatment decisions in cancer patients.

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Part II
Minimal Residual Disease and Breast Cancer Metastasis

Self-Seeding in Cancer

Elizabeth Comen and Larry Norton

Abstract

Despite significant progress in our understanding and treatment of metastatic cancer, nearly all metastatic cancers are incurable. In this Review, we use breast cancer as a model to highlight the limitations and inconsistencies of our existing treatment paradigms for metastatic disease. In turn, we offer a new theory of metastasis, termed "self-seeding. " The self-seeding paradigm, well validated in mathematical, experimental and animal models, challenges the notion that cancers cells that leave a primary tumor cell, unidirectionally seed metastases in regional lymph nodes and/or distant sites. In contrast, there is mounting evidence that circulating tumor cells can move multi-directionally, seeding not only distant sites but also their tumors of origin. Here, we show that the self-seeding model may answer many of the quandaries intrinsic to understanding how cancer spreads and ultimately kills. Indeed, redirecting our research and treatment efforts within the self-seeding model may offer new possibilities for eradicating metastatic cancer.

Contents

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1 Introduction

In the last 20 years, notable advances in the fight against cancer include the evolving fields of cancer genomics, improved imaging and detection techniques, and targeted, less toxic therapies. Despite these advances, cancer metastasis continues to undermine cancer survivability. And as such, improving the trajectory of cancer mortality necessitates profound change in our treatment paradigms. Historically, accepted theories of metastasis focus on the notion of a progressive, unidirectional pathway from a primary tumor to metastasis. As a consequence of increasing cell accumulation and genomic aberrancies, primary tumor cells acquire the ability to travel to distant organs, first proliferating microscopically and then forming gross metastases. Reflecting the continued mortality of many cancers, these prevailing theories are riddled with unanswered questions. Using breast cancer as a model, here we review select quandaries and contradictions inherent in prevailing theories of metastasis. We in turn offer a new paradigm, termed "*self-seeding,*" which offers an alternative roadmap for understanding metastasis. Self-seeding refers to the proven ability of peripatetic cancer cells to migrate multidirectionally—seeding not only to regional and distant sites in the body, but also returning to their original source: the tumor itself. Merging both biological and clinical observations, the clinical implications of self-seeding are significant, from helping to explain many current enigmas, but most importantly, to shedding light on new diagnostic and therapeutic advances.

2 Self-Seeding Model of Malignant Growth: The Biological Basis for Self-Seeding

The self-seeding model of malignant growth contests the idea that cancer cells which leave a primary tumor—often called circulating tumor cells or CTCs—unidirectionally seed metastases in regional (lymph nodes) or distant sites. The concept of

tumors self-seeding by CTCs was first published in 2009 after validation of the theory in diverse experimental models including colon and breast adenocarcinomas as well as melanomas [1, 2]. They demonstrated that CTCs can travel to and from distant and primary tumor sites. By this model, a large tumor may not only be a cause of distant seeding—the conventional concept—but also a result of self-seeding. In this sense, a large tumor grows from the "outside in" as opposed to from the "inside out." Kim et al. further demonstrated that the ability to seed is necessary but not sufficient to generate colonies in seeded sites; indeed, cells can lie dormant for decades in such sites without growing [1–3].

CTCs face many barriers for infiltrating and growing in distant organs. These include tight vascular capillary endothelial walls and an unfamiliar microenvironment. Thus, only the most adaptable and rare CTCs are successful in distant seeding of organs. However, CTCs re-entering the primary tumor itself face a leaky neovasculature and a fertile concentration of all the tissue-specific factors which initially permitted their circulatory exit [4]. Tumor-derived inflammatory cytokines, such as IL-6 and IL-8, act as CTC attractants. The self-seeding CTCs also express MMP1/collagenase-1, the actin cytoskeleton component fascin-1, and CXCL1 which promote accelerated tumor growth, angiogenesis, and the recruitment of myeloid cells into the stroma.

Using human cancer cells, it has been shown that the genetic toolkit for generating successful metastases appears to be site-specific, with unique signatures for lung, bone, and brain involvement [4–7]. The gene sets required for self-seeding, for example, the lung, brain, or bone overlap to some extent but are not identical [5–8]. The site-specific nature of metastases has been confirmed not only by in vivo experiments in mice using cell lines from human sources, but also by the analysis of recurrence-free survival curves in patients whose tumors have been classified by molecular signatures. Lastly, in support of the self-seeding experiments, there are increasing pathology reports of tumor-to-tumor metastases [9].

3 Mathematical Foundation of Self-Seeding

While the self-seeding model was born out of biological and clinical observations, it is buttressed by key mathematical concepts. We review the mathematical underpinnings of self-seeding in detail elsewhere, but we will briefly discuss certain evocative yet simple mathematical ideas [10]. It has been demonstrated experimentally and observed clinically that simple exponential or linear kinetics cannot explain the growth of a primary breast tumor [11]. For example, an average breast cancer takes roughly 2 years to grow from one cell to 10 billion cells. For that same tumor to grow by linear kinetics, it would take the tumor another 2 years to double in size. Were the tumor to grow by exponential kinetics, it would double in about 3 weeks. We know that neither scenario is uniformly true. Indeed, at varying time points, a tumor must grow by both linear and exponential kinetics [10].

Malignant growth is generally thought to be a result of mitosis, wherein one cell produces two. As such, at the nascence of a cancer's growth, the growth must be approximately exponential. However, as a cancer grows, it deviates from exponential kinetics, which in turn cannot be explained by mitosis. We now know that cancerous tumors must follow S-shaped curves intermediate between these two extremes, curves of the type described by Gompertz in 1825 [12, 13].

The self-seeding model accounts for an S-shaped Gompertzian growth curve. In the self-seeding model, CTCs are coming from the outside of any given mass which in turn suggests that a primary tumor is not one mass, but a conglomerate of contiguous masses. These contiguous masses grow as a function of surface area as opposed to volume. Since the stem-like cells are primarily on the surface (being defined here as the surface of each conglomerate) the ratio between the new cell production rate and the mass of the bulk of the tumor also drops as the tumor increases in size. Said differently, as the tumor increases in size, the ratio of its surface area to its volume decreases. This leads to a relative slowing of tumor growth, as is reflected in Gompertzian growth curves.

With an understanding of the biological and mathematical rationale behind the self-seeding theory, let us now evaluate the theory as it reconciles prevailing quandaries in clinical practice.

4 Prevailing Mysteries: Unpredictable Metastatic Pathways

4.1 Why do Some Patients Without Axillary Nodal Involvement Still Develop Systemic Metastases? And Why do Some Patients With Axillary Nodal Metastases not Develop Metastases Elsewhere, Even If Those Nodal Metastases are not Removed by Surgery or Irradiated?

At the end of the nineteenth century, William Halsted developed the basic concepts that underlie breast cancer surgery to this day. He asserted that the pathway of metastatic disease was predictably linear; cancer cells spread from the breast to the lymphatic system and then to the systemic circulation whereby they can seed distant organs. Consequently, surgically removing the whole breast surrounding the tumor as well as its attached ipsilateral axillary contents (radical mastectomy) would prevent metastatic disease [14]. And, as proof of his concept, radical mastectomies did and continue to cure many individuals of their breast cancer [15].

As further support of his surgical techniques, we now know that lymph node involvement portends a poorer prognosis than cancer-free lymph nodes [16]. Alternatively, if the first nodes draining lymphatic flow are without cancer cells, the rest of the axilla is nearly always free of cancer cells [17, 18]. This latter point underlies the basis for the practice of sentinel lymph node mapping.

Lastly, long-term experience continues to show that improved local control, such as with the addition of radiation therapy after breast conserving surgery, decreases the risk of local and distant recurrence [19]. The outcomes from the above-mentioned clinical practices—mastectomy, sentinel lymph node mapping, and improved local control—all seem to support a Halstedian view of malignant progression. Herein lies the conflict with his theory: some women with no axillary involvement may still develop distant metastases and some women with extensive axillary metastases may never develop distant disease.

In the face of the aforementioned paradox, Daniel Martin Shapiro, Bernard Fisher, Edwin Fisher and colleagues challenged Halsted's view of metastatic spread [20, 21]. They hypothesized that hematogenous as well as lymphatic pathways were necessary for metastatic spread. They posited and ultimately demonstrated that systemically targeted treatments such as select estrogen receptor modulators (SERMS) or chemotherapy were required to improve breast cancer survival [15]. Their early work provided the backbone supporting the use of adjuvant therapy, whereby chemotherapy and/or antihormonal treatments are given after the surgical removal of a primary tumor. In addition, several recent studies seemingly support their work. First, finding isolated tumor cells in axillary lymph nodes does not affect overall survival [22]. Second, patients undergoing breast conserving surgery and radiotherapy for small, hormone-responsive breast cancer and two or fewer involved axillary lymph nodes do not have increased rates of axillary recurrence if they do not have a complete axillary dissection. This is in spite of the fact that more than a quarter of those patients actually have residual axillary node metastases [23]. Third, two recent studies highlight the imperfect relationship between tumor size and lymph node status to clinical outcome. Specifically, Wo et al., demonstrate that in cases of extensive lymph node involvement, very small tumors may confer a more aggressive subtype than larger tumors with the same degree of lymph node involvement [24]. Work by Hernandez-Aya et al. indicates that in triple negative breasts cancers, the worse prognosis associated with lymph node involvement may not be greatly affected by the absolute number of positive lymph nodes [25].

Ultimately, it appears that both Halsted and Fisher's ideas are feasible. Halsted's idea of an anatomic pathway for metastatic spread is correct, but so is Fishers' idea that malignant spread does not require a linear anatomic pathway. How then does the self-seeding theory resolve these seemingly opposing views?

The self-seeding hypothesis helps to reconcile the friction between existing paradigms and our clinical observations. Halsted proposed that breast cancer cells need mechanical access to the axilla to seed it, which in turn supports the importance of sentinel lymph-node mapping [14, 15]. Alternatively, Fisher's theory implies that some breast cancer cells can colonize an axillary lymph node should it reach it, but others may in turn skip the lymph nodes altogether and instead spread hematogenously to distant sites.

In the self-seeding model, seeding is site-specific, with some gene sets targeting the lungs for example, whereas others may target the bone or brain. While these gene sets correlate, they are not identical [2]. For example, breast cancer cells can remain dormant in the bone in a non-mitotic state, manifesting as clinical relevant metastases even decades later [26]. As a result of the site-specific nature of self-seeding, finding isolated cancer cells in a distant organ does not always align with clinically meaningful metastatic behavior. And, the presence of unresected axillary nodal disease does not necessarily portend distant metastasis, but neither does its absence ensure the absence of distant spread [23]. Furthermore, in the context of radiation to a conserved breast for women with 1-2 + lymph nodes, it remains to be determined why surgical removal of additional lymph nodes does not improve local regional recurrence, yet radiation to the axilla may improve overall survival [22, 27]. Perhaps in the case of radiation to the axilla, CTCs can seed but not colonize an area of irradiation. The irradiated axilla may act as a poisoned sponge, attracting CTCs. But, in the face of inhabitable stroma, colonization is not feasible. In further support of this hypothesis, there is clinical trial evidence that radiation therapy to the breast after systemic therapy reduces systemic recurrences more than such radiation given before adjuvant chemotherapy [28].

Lastly, the recent research which indicates that a subset of small tumors may be highly aggressive despite their size may simply reflect relative seeding capacities [24, 25]. For example, a tumor may be a particularly poor self-seeder but an efficient distant seeder, as in the case of small, highly aggressive tumors. In some instances, a tumor may be such a poor self-seeder that it is occult while distant metastases abound. This latter scenario may explain adenocarcinomas of unknown origin or the often classic presentation of pancreatic adenocarcinoma.

4.2 Why is it That so Few Patients Present With Gross Metastatic Disease, Even When They May Have Large Untreated Tumors for a Long Time?

In the developed world, >5% of patients present with stage IV disease. Patients presenting with denovo stage IV disease have frequently had a primary tumor for years. The best observation of this phenomenon comes from records from the early to middle nineteenth century, before mastectomies were common. Patients often lived for years before they developed fatal metastatic disease [29]. We hypothesize that patients rarely present with stage IV disease because their CTCs are drawn back to the primary tumor, as attracted by the concentrations of chemoattractants that initially engendered growth in the breast [4]. In this instance, the primary breast tumor acts as a sponge, soaking up the returning CTCs and contributing to an enlarging locally advanced breast cancer. We await further definitive studies into how the resection of a primary breast tumor in the setting of metastatic disease accelerates or diminishes overall survival [30–32].

5 Molecular and Genetic Implications of Self-Seeding

5.1 Why is DCIS so Molecularly and Genetically Similar to Invasive Cancer?

Ductal carcinoma in situ (DCIS) lesions of the breast are categorized as pre-invasive lesions as they rarely grow to large sizes and neither invade the basement membrane of the breast duct nor metastasize. On the contrary, untreated invasive ductal cancers (in an otherwise healthy woman) are invariably fatal as a result of unchecked metastatic growth into vital organs. Given their phenotypic diversity, one would expect significant genomic diversity between DCIS and invasive breast cancer. Indeed, genetic analysis of invasive cancers demonstrate innumerable genetic aberrations; however, unexpectedly, DCIS also has similar molecular changes [33, 34]. We believe that DCIS and invasive cancer may share molecular similarities but have small differences in self-seeding capacity. DCIS lesions may lack self-seeding capacities and as such neither develop into large tumors nor metastasize. Because these differences may be qualitative and in yet unrecognized self-seeding genes, the molecular differences are subtle [34, 35]. Seemingly minor differences in self-seeding efficiency could dramatically alter the clinical trajectory of a breast cancer.

5.2 Why Does Sampling a Random Tiny Portion of a Tumor Reflect the Behavior of the Larger Tumor?

The stem cell hypothesis states that only very few cancer "stem-cells" or tumor initiating cells have the capacity for unchecked proliferation and metastasis to distant organs [36, 37]. At the same time, we now examine patterns of gene expression (such as with Oncotype^{TM} or Mammaprint^{TM}) to prognosticate chemotherapy benefit and survival [38–40]. Yet these genetic analyses are based on only a small piece of a tumor. For molecular profiling to be viable, select stem cells would have to be disbursed throughout the cancer mass such that any random sampling would capture them. How is this possible?

In the self-seeding model, the tumor mass is not one large uniform mass but rather an amalgam of smaller masses. Because the tumor is a conglomerate, with some parts growing from a stem-like seed or "self-metastasis," it is disorganized architecturally. On a molecular and genetic level, any random sampling of the tumor will contain the cells that represent the relative metastatic propensity of the whole tumor.

5.3 Why is Mammographic Breast Density a Risk Factor for Breast Cancer?

Breast density reflects the structural components of the breast, such as collagen, and is independent of mitotic rates [41]. One of the highest risks for the development of breast cancer is mammographic breast density [42]. Alternatively,

postmenopausal lobular involution is associated with a decrease in breast cancer risk. However, the lowered risk associated with lobular involution correlates weakly with mammographic breast density [43, 44]. Hence, it is unclear how structural components of the breast effects breast cancer risk.

In the self-seeding model, a dense breast provides more scaffolding (collagen matrix) for self-seeds. Each self-seeding tumor, forming multiple masses, in turn promotes a more perilous framework for the surface/volume ratio of a transformed cellular state. This would explain the correlation between breast cancer risk and breast density as well as the association with lobular involution and decreased risk. Supporting this idea, recent research links increased stromal collagen to mammary tumor formation and metastasis [45].

6 Clinical Applications of Self-Seeding

At present, the development of cancer drugs is predicated on animal models which demonstrate primary tumor shrinkage. As such, the clinical endpoints both in animal and ultimately human clinical trials is an antimitotic effect on a primary tumor. Anti-metastatic effects are not directly evaluated but rather presumed based on the reduction of primary tumor burden. We propose that a more viable drug development approach would be to screen for anti-seeding (anti-metastatic) activity in addition to anti-mitotic effects [2]. Many of our successful standard therapies may already interrupt both the anti-seeding and anti-mitotic processes. In the era of targeted therapy, the development of drugs as either anti-mitotic (causing shrinkage) or anti-metastatic (disrupting seeding) may allow for a more rational design of combination drug therapies. As IL-6, IL-8, MMP-1, and fascin-1 have already been identified in the laboratory and animal models, these are notable possibilities for future drug development [2].

In addition to focusing on anti-seeding drug development, attention should also focus on the seeds themselves, CTCs [46]. We know that CTCs must survive in the circulation, likely by switching from an aerobic to anaerobic metabolism. Drugs that perturb this switch may be successful in reducing metastatic burden [47]. Immunological manipulation may also augment both CTC attraction and attracted-CTC killing. We envision a situation whereby tumor ablation by interventional radiology or the use of drugs such as anti-CTLA4, could lead to CTC attraction and subsequent cytotoxicity [48].

Lastly, recent studies suggest that an irradiated site may attract CTCs but lead to an inhospitable stroma for CTC growth. We await the ongoing results of experiments to evaluate the effect and the timing of therapeutic radiation.

7 Conclusion

Reconciling the nomadic properties of CTCs, the imperfect relationship between tumor size and lymph node infiltration, and the importance of tumor surface, self-seeding offers a new paradigm for understanding previously opposing clinical and

biological observations. As opposed to a linear, unidirectional pathway from primary tumor to distant metastasis, cancer cells may now be viewed as fluid seeds variably driven by mitotic (primary tumor), seeding (metastasis), and self-seeding processes. In particular, as oncology moves toward increasing personalized care, the self-seeding model will require an understanding of this tenuous balance in each patient. We believe that the self-seeding paradigm will reshape our drug and clinical trial development, offering new genomic and clinical endpoints. Understanding the multidirectional course of metastasis from a biological as opposed to anatomical perspective will engender new advances in cancer prognosis and cure. And as such, we hope that the self-seeding model will redirect the current trajectory of cancer mortality toward a more promising horizon.

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Microenvironments Dictating Tumor Cell Dormancy

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Abstract

The mechanisms driving dormancy of disseminated tumor cells (DTCs) remain largely unknown. Here, we discuss experimental evidence and theoretical frameworks that support three potential scenarios contributing to tumor cell dormancy. The first scenario proposes that DTCs from invasive cancers activate stress signals in response to the dissemination process and/or a growth suppressive target organ microenvironment inducing dormancy. The second scenario asks whether therapy and/or micro-environmental stress conditions

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(e.g. hypoxia) acting on primary tumor cells carrying specific gene signatures prime new DTCs to enter dormancy in a matching target organ microenvironment that can also control the timing of DTC dormancy. The third and final scenario proposes that early dissemination contributes a population of DTCs that are unfit for immediate expansion and survive mostly in an arrested state well after primary tumor surgery, until genetic and/or epigenetic mechanisms activate their proliferation. We propose that DTC dormancy is ultimately a survival strategy that when targeted will eradicate dormant DTCs preventing metastasis. For these non-mutually exclusive scenarios we review experimental and clinical evidence in their support.

Contents

1 Introduction

Metastasis is responsible for the vast majority of cancer-related deaths. However, our understanding of this complex process is still vastly limited and so are our opportunities to prevent metastatic development. There are fundamental questions that remain mostly unanswered in this field: How does early dissemination start and what are the mechanisms? How does the tumor microenvironment aid this process? Are primary tumor niches responsible for programming DTCs to growth or quiesce at target organs? What role does the microenvironment of the metastatic niche play in determining the timing or extent of DTC dormancy? These questions have no or only partial answers.

The seed and soil theory of metastasis proposes that there is a match between the disseminated tumor cells (DTCs—the seeds) and the target organ (the soil) in which they can grow into overt lesions [1]. This is so because there is a relatively predictable pattern of target organ metastasis depending on the tissue origin of the primary tumor. While this is true, the timing of metastasis is difficult to predict because even in the matching sites, it can take a long time, sometimes decades, for metastases to develop [1]. It is thought that these long periods of clinical remission can be explained by minimal residual disease (i.e. DTCs) entering a non-productive or dormant state [1, 2].

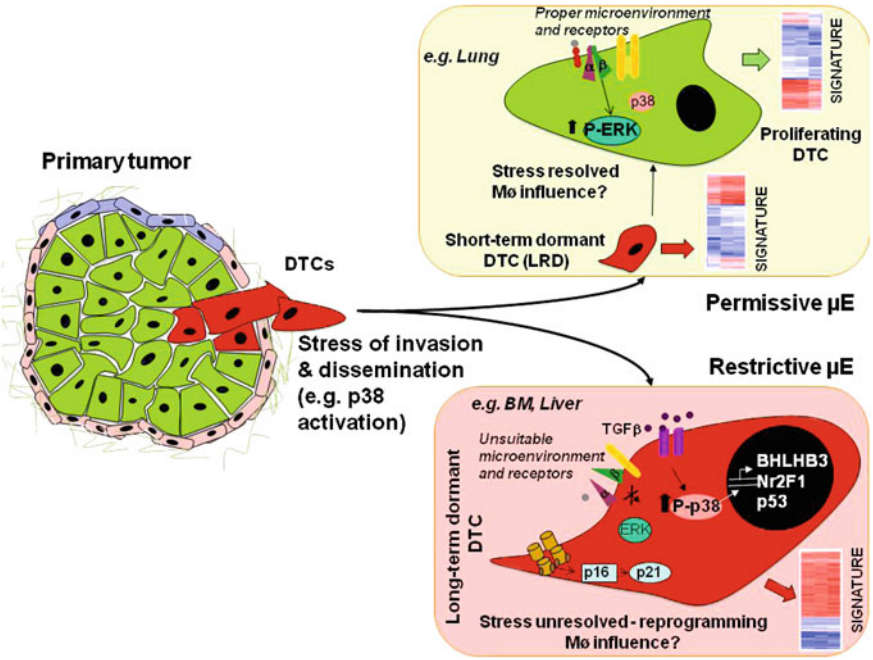

Fig. 1 Scenario 1 envisions that the target organ microenvironment (µE) has instructive signals that determine the fate of disseminated tumor cells (DTCs) that have already been influenced by primary tumor microenvironments and stress of dissemination. Upon arrival at the secondary sites, cells can encounter two different situations: In a permissive microenvironment, (e.g. lungs), DTCs undergo a transient phase of dormancy, but interactions with the favorable microenvironment and appropriate tumor cell surface receptors will allow DTCs to adapt, integrate growth-promoting signals, such as those derived from fibronectin to transduced by the urokinase receptor (uPAR—*red*)–$\alpha 5\beta 1$–integrin complex (*green* and *purple*) and the epidermal growth factor receptor (EGFR—*yellow*) which will result in activation of mitogenic signaling (activation of ERK, inactivation of p38) promoting DTCs proliferation. In the second situation DTCs will arrive to a restrictive microenvironment (e.g. bone marrow or liver), and either the loss of the surface receptors mentioned above will lead to inactivation of proliferative signals or interaction with growth-restrictive signals such as collagen-I results in stress signaling and activation of p38. This in turn leads to a prolonged dormancy. Activation of p38 could lead to the transcriptional induction of BHLHB3, NR2F1 and p53. Additionally, collagen-mediated activation of DDR2 can lead to subsequent p16- and p21-mediated tumor cell growth arrest. Furthermore, increased levels of TGFβ in the microenvironment (BM, for example) could also have a growth suppressive role. It is likely that other unidentified pathways are also involved in determining the DTC cell fate upon arrival at the secondary site. Overall, these signals might lead to specific gene expression profiles that could be derived from DTCs and used to classify patients (heat maps). Stromal cells such as machropages may also influence the choices between proliferation and dormancy, but these mechanisms have not been fully explored yet

In patients, DTCs that are not proliferating can be found in sites where they usually form secondary lesions or in sites where they never do [1]. Thus despite being able to disseminate these DTCs are “growth-suppressed” by certain organ microenvironments. Insight into these mechanisms might provide new pathways that if modulated could maintain DTCs dormant or eliminate them by blocking

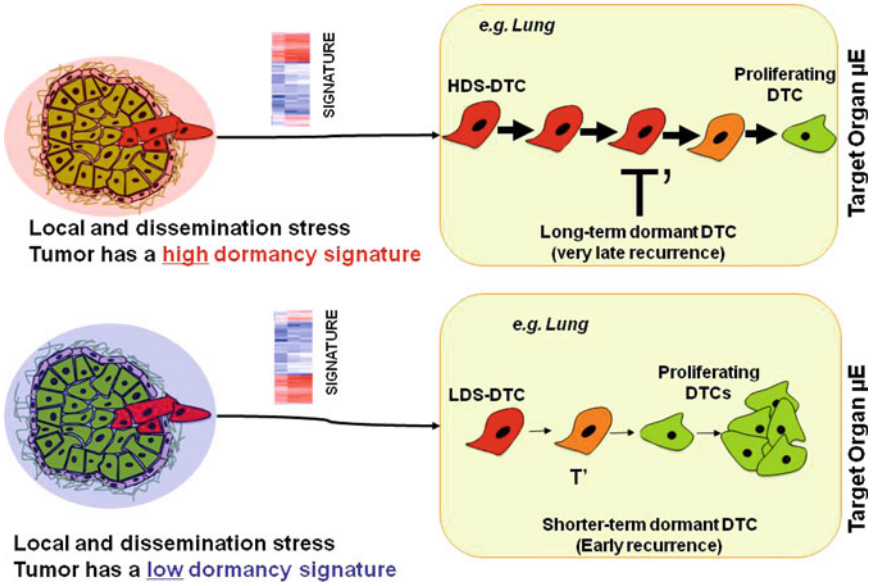

Fig. 2 Scenario 2 envisions that the primary tumor microenvironment (µE) (e.g. hypoxic, collagen dense) can influence the fate of DTCs. The presence of a high (*red shade—top*) or low (*blue shade—bottom*) dormancy score signatures encoded in the bulk of the tumor predisposes cells to enter prolonged dormancy (*large* T') or after a brief quiescence (*small* T') resume proliferation, respectively. *PT* primary tumor. *DTC* disseminated tumor cell. *HDS* high dormancy score. *LDS* low dormancy score

their survival pathways. This might also allow determining whether patients have dormant disease or not.

Several mechanisms are proposed to explain clinical dormancy. The lack of proliferation markers in surviving DTCs obtained from patients and experimental studies suggest that solitary DTC dormancy might be controlled by mechanisms of quiescence [1], a reversible growth arrest that can be brought about by different signals [3]. Angiogenic dormancy or immune system-mediated tumor mass dormancy might also be responsible for maintaining residual disease dormant [4, 5]. In this chapter we will review themes related to how solitary DTC fate is influenced by tumor-host interactions occurring in primary tumors and target organs. These two microenvironments are intimately interconnected by the biology of DTCs. Here, we will navigate three potential scenarios that might explain DTC dormancy. In the first, (Fig. 1) DTCs from invasive cancers activate stress signals in response to the dissemination process and/or a growth suppressive target organ microenvironment, inducing dormancy [1]. The second scenario (Fig. 2) proposes that therapy and/or microenvironmental stress conditions (e.g. hypoxia) acting on primary tumor cells carrying specific gene signatures prime new DTCs to enter dormancy [6, 7]. Here, specific primary tumor "stress microenvironments" might trigger long-term dormancy of DTCs. In the third scenario, lesions pathologically defined as

noninvasive carry cells able to undergo micro-invasion and disseminate. Here, although these DTCs were able to intravasate they are unfit for expansion in secondary sites but they survive mostly arrested and perhaps with occasional cell division they progress via epigenetic and genetic pathways to a fully metastatic cell able to grow in secondary sites. We propose that DTC dormancy is ultimately a survival strategy that when blocked will eradicate dormant DTCs.

2 Theoretical Considerations and Evidence for the Potential Scenarios of Tumor Dormancy

2.1 Scenario 1: The Target Organ Microenvironment as a Determinant of DTC Dormancy

Solitary DTCs in target organs can establish interactions with the extracellular matrix (ECM), immune cells as well as blood vessels in the stroma [8]. This and the distinct and predictable pattern of metastasis proposed by the seed and soil theory suggests that the target organ microenvironment can determine metastatic growth versus dormancy [1]. Studies on breast cancer cell lines specifically selected for vigorous growth in target organs via direct delivery to circulation identified gene expression programs that favor an organ-specific colonization [9]. On the contrary, some genes like the metastasis suppressor gene MKK4, through the activation of p38, mediates suppression of metastases [10] and this seems to respond to microenvironmental stress signals [11]. MKK4 belongs to a growing number of genes that selectively block growth at secondary sites and they include KISS1, MKK6, BHLHLB3/Sharp-1 and Nm23-H1 among others [11]. For a comprehensive review see [11]. These genes may inhibit metastasis by inducing DTC growth arrest [11]. That these genes do not suppress primary tumor growth but do suppress growth of DTCs at target organs further argues that these microenvironments provide a context where these genes now become functional.

In squamous carcinoma cells (HEp3) it was shown that reduced urokinase (uPA) receptor (uPAR) expression deactivated $\alpha 5\beta 1$-integrins and this made these cells incapable of binding efficiently to fibronectin (Fig. 1) [12]. This resulted in reduced FAK and EGFR signaling but also in p38 activation. Thus tumor cells that fail to establish appropriate interactions with the ECM may perceive this microenvironment to be growth restrictive and enter a quiescence state [1]. Other investigators have reproduced these findings showing that loss of $\beta 1$-integrin or FAK signaling in breast cancer models can also induce dormancy and that Src-MLKC signaling can prevent dormancy onset [1, 13]. In addition, an enriched collagen-I microenvironment in the lung can trigger intravenously delivered tumor cells to exit from dormancy as solitary cells [13]. On the other hand, environments rich in fibrillar collagen-I can induce quiescence of melanoma cells via activation of the discoidin domain receptor 2 and p15INK4b induction [14]. These results imply that stress signaling induced either by therapies or by a restrictive

(i.e. fibrotic or non-fibrotic target tissues depending on the tumor type) tissue microenvironment could activate dormancy (or its interruption) in DTCs.

In the HEp3 system activation of p38α/β while inhibiting ERK1/2 signaling, activates a stress adaptive response known as the unfolded protein response (UPR) [15–17]. These signals lead to an epigenetic reprogramming and induction of survival and quiescence of dormant HEp3 (D-HEp3) cells [18]. D-HEp3 cells inoculated in vivo enter a deep G_0–G_1 arrest characterized by p21, p27, p18 and p15 induction [15]. At least 3 transcription factors (TFs), p53, BHLHB3/41/Sharp1, NR2F1 were regulated by p38α/β and required for dormancy of tumor cells in vivo [15]. This program is also activated in dormant DTCs recovered from the bone marrow (BM) but it is reversed when tumor cells exit from dormancy or grow persistently in lungs (our unpublished results). Bone marrow derived dormant HEp3 cells displayed a low ERK/p38 signaling ratio and induction of BHLHB3/41/Sharp-1, NR2F1 and p53. Interestingly, metastasis suppressor genes (MSGs) like MKK4 and MKK6 are upstream activators of p38 [11], BHLHB3 is a target of p38 required for quiescence induction [15] (see below) and Nm23-H1 appears to function via the downregulation of EDG2 LPA receptor a strong activator of ERK1/2 [19]. Thus, it seems that different mechanisms might converge on the regulation of the ERK/p38 signaling ratio.

Can the target organ microenvironment where DTCs lodge activate these dormancy programs? In tumors like HNSCC and breast cancer bone metastasis occurs at a frequency of 10–30% [20–22]. However, the detection of BM DTCs is much higher (>50% of patients) [23, 24]. This suggests that not all DTCs go on to form metastasis and/or that a delay takes place. In mouse models of cancer (xenografts or transgenic), BM carcinosis or metastasis are rarely observed. For example, in MMTV-Neu transgenic mice, BM DTCs are readily detected but mice never develop bone metastasis [25] (see also Scenario 3). However, if the BM microenvironment is modified, via irradiation [25] or if p38 is systemically inhibited, now DTCs expand ([25] and our unpublished data). T-HEp3 squamous carcinoma cells spontaneously disseminate from primary tumors to lungs, lymph nodes (LN) [26], liver and BM, but only in lung and LN they develop overt metastasis [26, 27]. Instead, in BM, spleen and liver DTCs remain in small numbers (<100 DTCs/10^6 marrow cells). Importantly, systemic p38 inhibition drastically changed this behavior and after a 3-week treatment with p38 inhibitors now DTCs, micro and macro-metastasis, were found in places where they never grow including liver and spleen (our unpublished data). Thus, in certain organs restrictive signals mediated at least by p38α/β signaling can prevent occult DTCs from expanding.

The search for signaling mediators that might induce dormancy in the BM suggest that TGFβ, which is rich in the BM microenvironment [28–31], might be important in dictating DTC dormancy. Although tumors might depend on TGFβ to metastasize [6, 7], this ligand can, depending on the degree of progression of tumors, be a potent inhibitor of epithelial tumor cell proliferation [32, 33]. TGFβ is also required to maintain the quiescence of stem cells and progenitors in the BM [28–31]. Thus, some tumors may remain sensitive to TGFβ growth inhibition in microenvironments where this factor is present (i.e. BM) [34]. It is still unknown

whether during disease-free periods TGFβ maintains dormancy of DTCs. D-HEp3 cells express high levels of TGFβ2 mRNA and BHLHB3 is also induced by TGFβ2 (our unpublished results). Interestingly, BHLHB3 was also found to be upregulated by TGFβ and function as a metastasis suppressor in MDA-MB-231 breast cancer cells when the mutant p53 function present in these cells was eliminated [35]. Thus, enhanced paracrine/autocrine TGFβ signaling might contribute to dormancy in target organs if tumor cells have not subverted the pathways to stitch TGFβ signaling to be pro-growth and/or pro-invasion.

In melanoma progression a similar scenario develops. In early progressed melanoma TGFβ is anti-proliferative (tumor-suppressor), but in advanced melanoma it is pro-invasive [36–38]. How these two scenarios develop is not entirely clear [36–39]. It is possible that similar to the early dissemination in breast cancer (see Scenario 3) [40], melanoma might spread before the conversion from TGFβ-inhibitory phenotype to pro-invasive behavior is activated. Although counterintuitive, there is clinical evidence of early spread of uveal melanoma and, in a smaller proportion of patients, cutaneous melanoma thinner than 0.76 mm depth [41–43]. If true, then single cells arriving at distant sites, such as liver or BM [25] might remain in cell-cycle arrest due to high levels of TGFβ. Overall, these studies might identify therapeutic targets to induce or maintain dormancy or eradication of DTCs by targeting their survival signals or those provided by the microenvironment.

2.2 Scenario 2: Primary Tumor "Stress Microenvironments" Determine DTC Fate

In this section we review three lines of evidence on how the primary tumor might influence dormancy and progression toward metastasis. These include the gene signatures present in the primary tumors that predict the timing of metastasis development, the ability of tumor cells to return to the primary tumor to undergo further progression through self-seeding and the possibility that certain primary tumors may modulate the microenvironment of DTCs by instigating the mobilization of host cells (e.g. bone marrow derived cells) that can interact with and dictate the behavior of DTCs (Fig. 2).

Published data shows that gene signatures or even individual genes present in the primary tumors predict long-term metastatic relapse more than a decade later and in the absence of the primary tumor from which the signature derived [44, 45]. In some cases signatures from the surrounding microenvironment proximal to the tumor can also predict progression kinetics for patients [46]. In the case of hepatocellular carcinoma these signatures may inform about microenvironmental-favoring conditions for intrahepatic metastasis [47]. This suggests that a reciprocal influence of primary tumor and microenvironment in primary sites generates signatures that can dictate disease progression. Importantly, the majority of patients will undergo surgery and the deaths scored in Kaplan-Meyer curves are due to subsequent metastasis. One interpretation is that the gene signatures in the primary

tumor and the microenvironment determine the fate of the DTCs. Since metastasis, once diagnosed show homogeneous progression (~2 years in breast cancer for example [48]), these data suggest that the gene signatures in the primary tumor not only inform about overt lesion biology but most likely about DTC survival, dormancy or proliferation. One additional interpretation of these data is that many of the gene signatures that predict for longer metastasis-free periods when a gene or a signature is present or absent most likely are providing information about how those individual or groups of genes influence dormancy of DTCs. We found that the dormancy signature identified in dormant D-HEp3 cells [15] when highly represented in estrogen receptor positive breast cancer invasive primary tumors (high dormancy score, HDS) predicted for longer metastasis-free periods (Kim, Aguirre-Ghiso and Segall, unpublished results). In contrast, when this signature was underrepresented (low dormancy score, LDS) patients recurred with metastasis more frequently [45, 49] (Kim, Aguirre-Ghiso and Segall, unpublished results). This strongly suggested that (1) while the signature genes do not affect primary tumor growth they might induce slower progression to metastasis possibly through dormancy of DTCs; (2) conditions in the primary tumor could influence the expression of these dormancy genes and "stress microenvironments" induced by hypoxia or therapies might induce a dormancy signature. Modeling how the genes in these signatures influence DTC survival and quiescence or subsequent recruitment of blood vessels or interaction with the immune system might reveal how they regulate dormancy and minimal residual disease biology. Importantly, determining whether signatures derived from CTCs (i.e. recently intravasated tumor cells) are more or equally informative to the primary tumor signatures might further inform about the relevance of characterizing CTCs versus DTCs (i.e. CTCs that are already lodged and reside in target organs).

As mentioned above it has been proposed that CTCs might return to the primary tumor in a self-seeding process and this helps "breed" more aggressive variants that colonize target organs [50]. These studies showed that aggressive variants of MDA-MB-231 breast cancer cells (MDA-MB-231-LM2) were highly efficient in disseminating and cross-seeding contra lateral tumors. The less aggressive variants of different cancer cell lines were less efficient in the seeding self/cross-seeding process. These data suggest that development of a more aggressive metastatic progeny requires the ability of primary tumors to attract their own CTCs back and the ability of these tumor cells to efficiently re-colonize the primary tumor. Transcriptional profiling of the isolated CTC population showed that these selected cells have gene signatures resembling those of bone, brain and lung metastatic populations, suggesting that the re-seeding might prime CTCs to acquire these gene signatures. However, the above described model did not demonstrate that DTCs from target organs can indeed seed back to the primary tumors. It is possible that these events take place when multiple metastases co-exist (for additional discussion see [51]). It will be important to determine whether patients from early and advanced lesions CTCs indeed already carry these same signatures. It will also be important to establish why the aggressiveness brought about by self- or cross-seeding takes place in patients where the primary tumors were removed and that

develop metastasis years to decades after surgery of the primary tumor, which is required for the self-seeding process.

A third sub-scenario of primary tumor microenvironments influencing DTC fate includes the model of systemic tumor instigation. This hypothesis proposes that primary tumors can instigate the growth of otherwise-indolent tumor cells or micrometastases located at distant sites by mobilizing bone marrow cells (BMCs) into the stroma of the distant "indolent" lesions [52]. Although systems similar to the self-seeding model were used (MDA-MB-231 cells) the instigation model ruled out self- or cross-seeding, a discrepancy that remains unresolved. Nevertheless, McCallister and colleagues showed that instigating tumors secrete osteopontin that induces the expression of granulin by a specific subpopulation of hematopoietic cells in the host BM (Sca1+cKit–CD45+) [52]. These BMCs were activated and mobilized into the secondary sites where the responder tumors lay. There, they facilitated growth by inducing myofibroblast proliferation and thus creating a stroma supportive of tumor growth. These results suggest that growth and proliferation of poorly aggressive tumors (dormant DTCs and/or micrometastasis?) can be regulated on a systemic level by endocrine factors released by certain instigating tumors. However, like in the self-seeding model [50], it remains unclear how metastasis is instigated in the absence of a primary tumor and also the timing of these events in the experimental models does not explain why in patients that underwent surgery metastasis it take decades to develop.

Overall, these lines of evidence suggest that different mechanisms might "prime" tumor cells that exit the primary lesion to be productive and produce expanding metastasis with a predictable time lapse or non-productive [2]. The latter may be due to DTCs entering dormancy via quiescence or even if proliferative being more prone to be suppressed by the immune system or unable to recruit blood vessels (Fig. 2).

2.3 Scenario 3: Early Dissemination as a Determinant of DTC Dormancy

In this scenario we hypothesize that "pre-malignant" cells can readily undergo epithelial mesenchymal transition (EMT), making them invasive and facilitating early dissemination. However, we hypothesize that these early DTCs are not fully fit to initiate metastatic growth and thus undergo dormancy. For this to happen we propose that during these early progression stages the EMT is reversible and that upon arrival to the target organ, the stress signaling or suppressive signals from the microenvironment are reinstated to a level that prevents apoptosis but maintains quiescence of DTCs. We finally propose that micro-environmental, and epigenetic mechanisms that reverse the growth-restrictive signals and favor for example ERK1/2 activation [15] will allow early DTCs to grow and accumulate additional genetic alterations that eventually produce cells fit to initiate metastasis (Fig. 3).

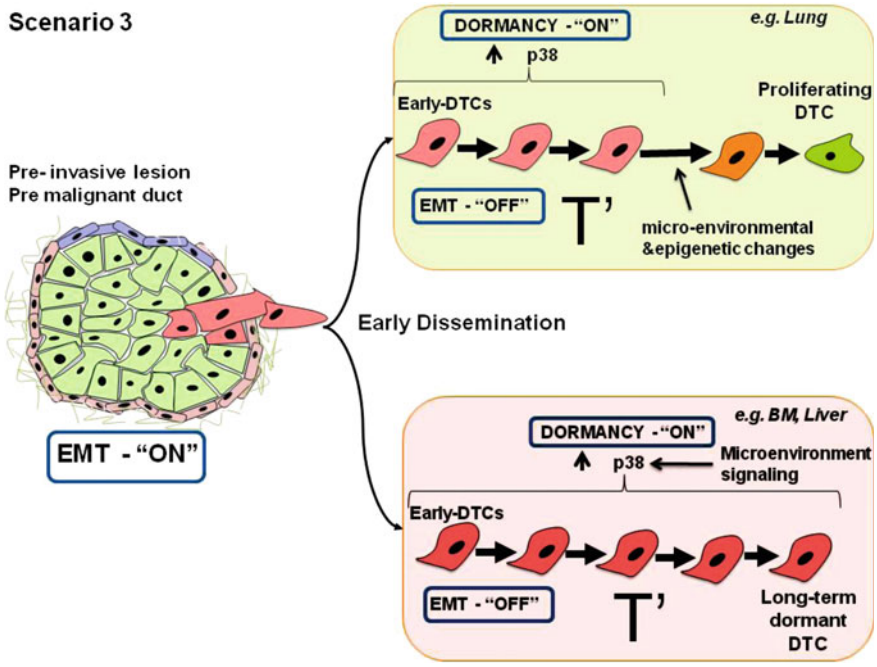

Fig. 3 Scenario 3 envisions that in "pre-malignant" cells, from pre-invasive lesions, p38 signaling is downregulated favoring an EMT and early dissemination to target organs (e.g., lung and bone marrow). However, these early DTCs are unfit to initiate metastatic growth, thus, once these early DTCs arrive to secondary organs a dormancy program is turned "ON" possibly mediated by plastic regulation of p38 that is restored by microenvironmental signals. Therefore, early DTCs will undergo a long dormancy phase before forming metastasis. This is deduced from the long time to metastasis development after primary lesion treatment. As described in Scenario 1, DTCs that lodge in favorable microenvironments, such as lungs, will be exposed to micro-environmental and epigenetic changes that will allow early DTCs to grow and accumulate additional genetic alterations that eventually produce cells fit to initiate metastasis. In contrast, DTCs that lodge in unfavorable microenvironments, such as BM or liver, will remain dormant. Early dissemination will produce long dormancy periods at any site because cells have to accumulate additional changes. But it is possible that in certain sites microenvironmental and epigenetic changes allow cells to initiate slow proliferation that then drives genomic instability when vigorous growth is initiated

Pre-malignant cells carrying specific genetic and epigenetic alterations are able to departure from early stage primary lesions and invade surrounding tissues. However, these accumulative modifications are not sufficient for these precursor cells to initiate proliferation enabling them to undergo a prolonged dormant state at secondary sites. Eventually, further subsequent genomic alterations will provide solitary dormant cells with proliferative capabilities and finally will resume metastatic growth [40]. What is the evidence supporting early dissemination? In breast cancer for example bone marrow (BM) DTCs are found in around 10–30% of breast cancer patients with noninvasive lesions (e.g., atypical ductal hyperplasia

(ADH) or ductal carcinoma in situ (DCIS)) [40–53]. Their presence in BM correlates with poor prognosis [54]. Modeling these findings in mice showed that DTCs were detected in BM of MMTV-ErbB2 mice with "pre-malignant" lesions [25]. Interestingly, electron microscopy analysis revealed the presence of pre-malignant MECs breaching the basement membrane and this correlated with upregulation of Twist mRNA levels [25]. This suggests that pre-malignant cells can perhaps undergo an EMT and locally invade the surrounding stroma and intravasate to later become the founders of metastasis in visceral organs.

Specific gene expression signatures that predict for early dissemination have not been reported. However, some studies hint at certain genes as being (or not) modulators of this process. For instance, p53 mutations are not needed for early dissemination in breast cancer [55]. These data suggest that DTCs might still be under the control of this tumor suppressor and therefore prone to entering apoptosis, senescence or quiescence; only the latter endpoint would allow for dormancy unless senescence is reversible [56]. But it remains unknown whether early DTCs formally enter the dormancy mechanisms described above to subsequently found distant metastases.

Other data point to GATA-3 as a negative regulator of early dissemination [57]. GATA-3 loss was found to facilitate early dissemination and metastasis in a model of mammary hyperplasia [57]. In this work GATA-3 is lost in early DTCs lodged in lungs [57]. Thus, detection of GATA-3 loss in ADH or DCIS lesions may serve as a "test" to predict early dissemination.

Can stress-signaling pathways influence early dissemination and early DTC fate? There is evidence that GATA-3 nuclear translocation is stimulated by p38 [58, 59] suggesting that by up-regulating GATA-3, p38 could block dissemination in the pre-malignant epithelium. Our focus on the role of p38α/β stress signaling in dissemination and dormancy revealed that its pharmacological inhibition accelerates early breast tumor progression by inducing anoikis resistance [60] and an EMT. Our data show that systemic p38α/β inhibition strongly stimulated early dissemination and accumulation of CK8/18+/ErbB2+ cells in the BM of MMTV-ErbB2 mice. This correlated with a dramatic downregulation of E-cadherin and increased nuclear accumulation of β-catenin and ERK1/2 activation—all genes regulated during an EMT—in the MECs of MMTV-Neu pre-malignant mammary glands. Our data suggest that p38α/β might have a gatekeeper function at the dissemination steps by blocking an EMT and early dissemination of pre-malignant cells.

However, if early DTCs enter dormancy the EMT must be reversible or it may not influence the quiescence of these DTCs upon arrival to the target organ. Also, p38α/β signaling must be reinstated to a level that maintains quiescence of DTCs. Alternatively the p38 signaling threshold to prevent an EMT is lower than that required to repress proliferation. If so only transient or marginal inhibition of p38 signaling might allow for EMT and dissemination while still functioning as a growth suppressor.

Several additional questions arise from this potential dormancy scenario: Is it possible that early DTCs share the same dormancy gene signature as those DTCs

released by invasive primary tumor mentioned in Scenario 2. Would this suggest that only early DTCs are responsible for recurrences? Why do larger tumors produce a similar DTC burden than smaller counterparts, but still have a worse prognosis? Are the same numbers of DTCs shed but their signatures are different? Which signals trigger the genetic switch responsible for metastatic growth? Do microenvironment (Scenario 1) and epigenetic signals precede genetic evolution of DTCs? Answers to these important questions will be critical for targeting and eliminating dormant DTCs before they can form lethal metastases.

3 Conclusions and Perspectives

Our knowledge on how the biology and genetics of DTCs influence dormancy and progression is limited. Even less understood is how these tumor cells from pre-malignant or invasive tumors are influenced by the primary tumor microenvironment composition, by the therapies applied to patients and by the target organ. Specifically for the topic of this book it seems that characterization of DTCs would be the most promising because they carry the aggregate information about their origin (i.e. primary tumor microenvironment), the ones that survive therapy will carry the information about how treatment influenced their adaptation and/or selection and ultimately how the target organ microenvironment also influenced their adaptation and/or selection. CTCs, because they are shortlived in circulation [61] and thus are considered only recently intravasated cells, would only carry information similar to the primary tumor and acutely influenced by therapy. Thus comparing CTC and DTC gene profiles as well as genetics will provide crucial information about whether they provide similar, complementary or different information about dormancy phases and subsequent progression to overt disease.

A final element not fully discussed here due to space limitations is how modulating host stromal cells might influence DTC dormancy. For example, the specific interaction of DTCs with macrophages at these sites might influence the decision to enter or escape dormancy. This is of importance because as recently reviewed, different populations of macrophages present in the primary tumor and lungs dictate metastatic dissemination and growth. The Condeelis and Pollard labs showed that a signaling relay is established between macrophages (CD11b+, F4/80+, CSF-1R+, Ly6G−) and breast tumor cells where the macrophage produces EGF, which in turn stimulates the tumor cell to produce CSF1, an activator of macrophages [62]. Macrophage produced EGF also drives intravasation [63]. But lung macrophages, defined by the markers indicated above and also VEGFR1+ and CCR2+ aid mammary tumor cell seeding at disseminated sites [64]. It will be important to determine whether the exit of dormancy was accompanied by a cross talk with the macrophages as this would reveal that macrophages support the exit of quiescence. These studies might reveal two additional scenarios: (1) macrophages that activate the signaling relay are only associated with DTCs actively proliferating; (2) macrophages are associated with

dormant DTCs as well as with proliferative DTCs but the phenotype of these macrophages is different. These findings would perhaps identify novel "host-cell" targets to aid therapies aimed at the DTCs.

Overall the challenges presented by the problem of cancer dormancy are significant and studying DTCs and dormant disease is difficult. But the benefits of unraveling the inherent complexity of this step of metastasis biology should be of great impact for cancer patients.

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Part III
Technologies for Circulating Tumor Cell and Disseminated Tumor Cell Detection and Characterization

Immunomagnetic Separation Technologies

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Abstract

The largest difficulty one faces in the development of technology for detection of circulating tumor cells (CTCs) is whether or not tumor cells are present in the blood and at what frequency. Although the introduction of the validated CellSearch system for CTC enumeration has facilitated CTC research the question remains whether CTC are missed or whether the CTC that are reported are indeed clinically relevant. To fulfill the promise of CTC as a real-time liquid biopsy they will need to be present in the blood volume tested and need to be isolated without losing the ability to test the presence of treatment targets. To characterize a sufficiently large number of CTCs in the majority of cancer patients the volume of blood needed is simply too large to process without enrichment prior to detection. Here, we review the detection of CTCs by flow cytometry and fluorescence microscopy with and without immunomagnetic enrichment.

Contents

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1 Introduction

Reports of tumor cells present in blood of cancer patients have been around for a long time and have been associated in some of these reports with poor outcome [1–10]. The introduction of a validated method for CTC enumeration [11] and the results of multicenter prospective clinical systems conducted with this system [12–14] have led to a flurry of activities. This includes clinical studies confirming the earlier findings [15–20] or exploring new indications [21–23] as well as reports of alternative methods aiming to improve the sensitivity and specificity of CTC detection and characterization [24, 25]. The greatest challenge of CTC detection is to obtain a sufficient enrichment that permits detection of the CTC while minimizing losses. Low amounts of CTC need to be detected among erythrocytes ($4–6 \times 10^9$), platelets ($2–48 \times 10^8$) and leukocytes ($4–11 \times 10^6$) present in 1 mL of blood. Methods to reduce these cells are all accompanied with an increase of background of some kind. Components present in plasma and methods used for further enrichment can also give rise to a background that can reduce the ability to discern the CTC from all other compounds. Which method to use for cell enrichment depends on the detection method subsequent to the enrichment step and the application for which the CTCs are used after their detection. Another consideration for use of an enrichment technique is the volume of sample that can be processed in one measurement and the need for red blood cell lysis. Here, we review the use of flow cytometry to detect CTCs after erythrocyte lysis or immunomagnetic selection and compare this with detection by fluorescent microscopy and flow cytometer after immunomagnetic enrichment.

2 CTC Enrichment and Detection Methods

CTCs are rarely observed in the routine analysis of blood smears [1–9]. To increase the likelihood of detecting CTCs, enrichment either through depletion of undesired cells i.e. negative selection or selection of the desired cells i.e. positive selection is required. Volume reduction, CTC enrichment and keeping CTC loss to a minimum are important factors to be considered before contemplating avenues for CTC enumeration. The largest challenge for all enrichment methods is how to assess success, as the number or even presence of CTC in the original blood volume is not known. To aid in the development of CTC enrichment cells from tumor cell lines are used. Although high and reproducible recoveries of such cells are essential requirements for a successful enrichment, it is no guarantee that CTC also are successfully enriched. Density, size, antigen expression and rigidity of the cells used in the model systems can all be different from the CTC from a particular patient. Moreover, extreme heterogeneity of CTC within and between patients makes it extremely difficult to arrive at an optimal enrichment scheme for CTC. Combinations of both negative and positive selection are also feasible, in all cases one will have to take into consideration what detection method will be used. Different selection and detection methods are shown in Fig. 1.

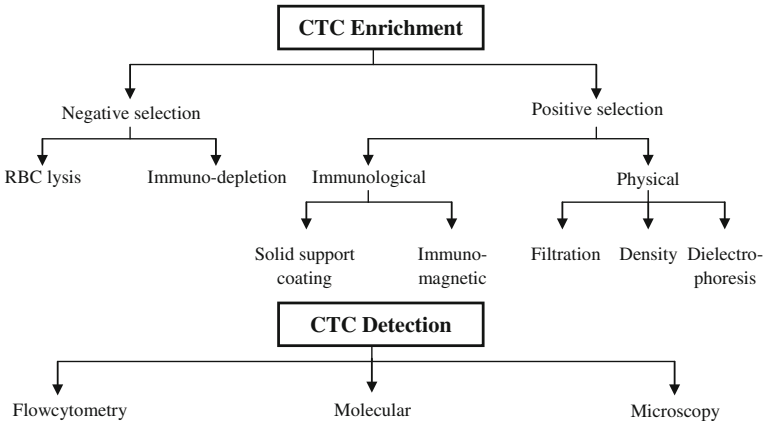

Fig. 1 Enrichment and detection methods for CTC enumeration

Negative selection can be achieved by red blood cell lysis, leaving only nucleated cells and platelets or depletion of leukocytes using magnetic beads or a solid support coated with leukocyte-specific antigens such as CD45 [26–30]. Depletion of leukocytes is mostly used in conjunction with erythrocyte lysis or density separation to reduce the volume and remove the erythrocytes to make it compatible with CTC detection. Detection of CTCs from erythrocyte lysed blood can be achieved by fluorescence microscopy [31] or flow cytometry [32, 33]. The abundance of leukocytes after erythrocyte lysis results in an excess of normal DNA or RNA that significantly inhibits the sensitivity of CTC detection by molecular detection with the polymerase chain reaction (PCR) of for example tumor-specific mutations or mRNA of cytokeratins. Density separation with or without leukocyte depletion is the most common strategy for CTC detection by molecular means [34–37]. The drawback of molecular detection is that although the presence of CTCs can be detected the actual number can only be estimated as the level of expression in an individual CTC is not known. The most traditional positive enrichment is based on differences of CTC in density using Ficoll or Percoll at specific densities [38–41]. More recent positive enrichments based on physical characteristics are utilizing differences in size [42, 43], rigidity [44] or dielectrical properties [45] of CTCs. Separation based on size use filters with pore sizes that pass erythrocytes and the majority of leukocytes but maintain CTCs that are larger in size or are more rigid. Differences in the movement of CTCs versus leukocytes are used for enrichment based on dielectrical properties [45]. All these methods will result in loss of CTCs as considerable overlaps lie with the leukocytes in the sample. The challenge is to keep the loss low, but still achieve an enrichment that is sufficient for the detection of the CTCs. The most common target for positive selection is the EpCAM antigen [46–49]. Antibodies directed against EpCAM are either coupled to ferrofluids [50–53], magnetic particles [54, 55] or a solid support [56, 57]. The disadvantage of positive selection methods targeting specific antigens is that they need to be expressed on the CTCs. Besides being present on the cell surface of CTC, the target

antigen will have to have no or very little expression on leukocytes. Intra-cytoplasmatic antigens have also been used for positive selection. Although no solid supports can be used ferrofluids coupled to antibodies directed against cytokeratins have been used successfully [58]. A combination of selection based on physical characteristics as well as immunological properties may address the shortcomings of both approaches. Flow cytometry [33, 59, 60] and fluorescent microscopy [11, 61, 62] are the most commonly used detection methods for CTC. Microscopy has the advantage that it enables better morphological characterization of the CTC. This, however, also increases the error of CTC classification as the actual morphological definition of a CTC is not known. Different definitions of CTC have led to enormous variations in the reported CTC counts and it is yet to be established what the best definition is. Optimization of the CTC definition based on the best relation with clinical outcome is a way to address this issue but requires the conduction of controlled clinical trials.

3 Detection of CTCs by Flow Cytometry without Prior Enrichment

The rigidity of CTCs as compared to other blood cells is not known although indications are that they can be fragile as processing of blood without stabilization is associated with degradation and loss of CTCs [63, 64]. Although enumeration and differentiation of the major leukocyte subsets in whole blood is feasible by flow cytometry detection of the more infrequent cell types is hampered by the enormous number of erythrocytes and platelets [65]. A frequently used method to eliminate this problem is erythrocyte lysis, which is generally used for immunophenotyping of leukocytes in blood. Although it is not expected that the lysing agents will affect the leukocytes more than the CTCs it may have a detrimental effect on the stability of the CTC undergoing apoptosis as well as the Tumor Micro Particles (TMP) that are present in much larger numbers in blood of patients with metastatic carcinomas as compared to the intact CTC [15, 66]. Alternative approaches for elimination of erythrocytes are density separations, the use of Ficoll or Percoll with a density of 1.077, which are the most frequently used for studying the mononuclear cell fraction. We studied the density of CTCs using a variety of different densities and CTC-related events were found in all the fractions after density separation (unpublished observations). To evaluate the frequency of CTCs in blood without the bias of an immuno pre-enrichment step we chose to use erythrocyte lysis, which enabled the investigation of the presence of CTCs in 100 μl of blood [32]. To illustrate the challenges for the analysis of larger blood volumes we analyzed ammonium chloride lysed blood from a healthy donor. Prior to flow cytometric analysis the blood was stained with the nucleic acid dye Hoechst 33342, the epithelial cell-specific monoclonal antibody EpCAM labeled with Phycoerythrin (PE) and the leukocyte-specific monoclonal antibody CD45 labeled with Allophycocyanin (APC). A typical example of a flow cytometric analysis is illustrated in Fig. 2.

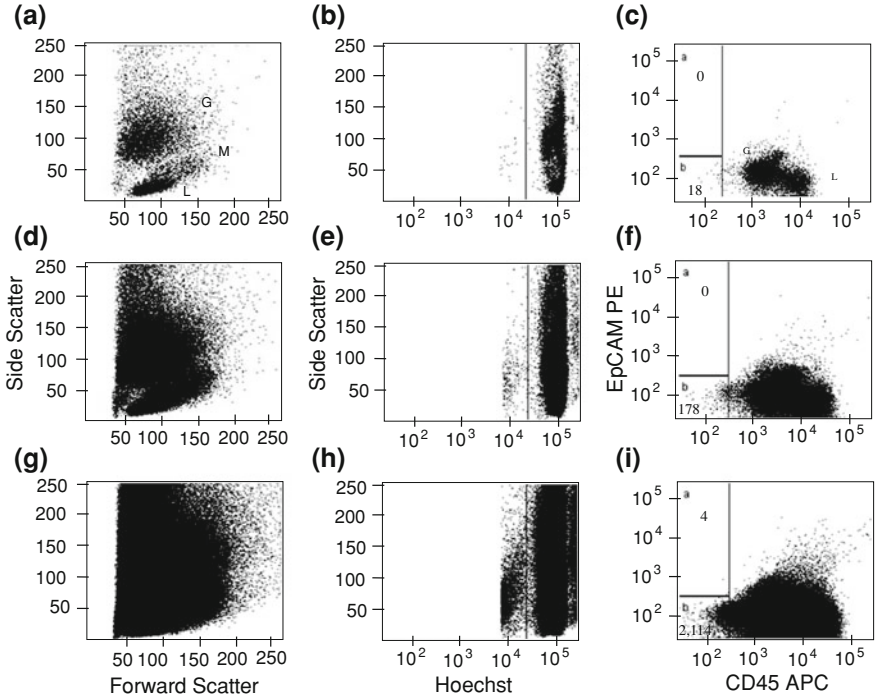

Fig. 2 Flow cytometric analysis for CTC detection. Ammonium-chloride lysed healthy donor blood sample is stained with Hoechst 33342 (5 μg/mL), EpCAM-PE (VU1D9 at 0.3 μg/mL) and CD45-APC (HI30 at 0.5 μg/mL). The sample is washed with PBS/Albumin (1%) to remove excess label and resuspended in PBS/Albumin (1%) prior to measurement on a FACS ARIA II flow cytometer

In the top three panels 10,000 Hoechst$^+$ events are displayed (2A–C), in the middle panels 100,000 events (2D–F) and in the bottom panels 1,000,000 Hoechst$^+$ events (2G–I). Whereas in panel a the position of the granulocytes (G), monocytes (M) and lymphocytes (L) can be clearly distinguished in the light scatter plot in panels d and g the clear discrimination is lost due to the accumulation of events. Panels b, e and h show the Hoechst 33342 staining versus the side scatter with a gate around the Hoechst signal to ensure recording of nucleated events only. With the increase in accumulation of events from panels b to e to h the size of the cluster of non-nucleated events is increasing and some of them appear in the nucleated events gate; likewise the cluster of nucleated events is increasing and some nucleated events will fall outside the gate. The scatter plots in panels c, f and i show the CD45-APC staining versus EpCAM-PE staining of all events that fall within the gate of panels b, e and h and pass the threshold on forward scatter. In panel c the population of granulocytes (G) and lymphocytes (L) can be discerned but this discrimination is no longer visible in panels f and i. CTC expressing EpCAM will fall in gate ***a*** and CTC not expressing EpCAM in gate ***b***. Gate C***a*** and F***a*** contain 0 events and I***a*** 4 events. The gate contains 18 events in C***b***, 178 events in F***b*** and 2,114 events in I***b***.

Detection of CTC expressing EpCAM is feasible with this approach although the emergence of 4 EPCAM+ CTCs in this healthy individual indicates that analysis of 1,000,000 nucleated events is at the border of what is feasible without decreasing the specificity. Analysis of 10,000 nucleated events already does not permit a reliable detection of EpCAM- CTC as events already are present in gate C***b***. Events in gate ***b*** will mainly consist of granulocytes which are the closest population to gate ***b;*** this gate will also contain circulating endothelial cells and nucleated red blood cells. A total of 1,000,000 nucleated cells represent approximately 200 μl of blood and 10,000 only 2 μl. To increase the sensitivity (larger blood volume) without sacrificing specificity the number of parameters acquired on the flow cytometer can be increased to enlarge the multidimensional space [65]. This increases the ability to discern smaller cell populations provided that the additional parameters identify the populations that are now present in the CTC gates. The cell concentration of the sample was 4.9×10^6/mL and the data acquisition performed on a FACS ARIA II (Becton–Dickinson, San Jose, CA, USA) at 3,700 events per second. Analysis at a higher speed resulted in a broadening of the coefficient of variation of the cell population, which leads to a decrease in the sensitivity of the measurements. If indeed the CTC gate can be kept clear of events by increasing the number of parameters one still has to realize that the analysis of 7.5 mL of blood as is performed with the CellSearch system after immunomagnetic enrichment will result in an acquisition time of $(7.5 \times \sim 5 \times 10^6$ leukocytes)/3,700 = 169 min. The cytometer has to be stable and the sample will have to be kept in good condition during this time which in practice will be difficult to achieve.

4 Immunomagnetic CTC Enrichment

The CellSearch system enriches CTCs from 7.5 mL of blood by incubation of the blood with ferrofluids labeled with monoclonal antibodies targeting the EpCAM antigen followed by magnetic separation and staining of the enriched suspension (Fig. 3). In the original studies [60, 67] it was noted that the quality of the blood samples degraded with time resulting in an increase of the background and a decrease in the number of detected CTCs. Before prospective clinical studies could be initiated a solution for the blood stability needed to be found. Addition of a cell preservative (Streck laboratories, Omaha, NE) immediately after blood draw addressed the stability issues and was implemented in the first multicenter prospective study [63, 64]. To simplify the procedure the preservative was combined with the anticoagulant EDTA and placed in a blood drawtube and used in all later studies (CellSave, Veridex). To avoid aggregation of ferrofluids by plasma components present in certain donors the blood is centrifuged before processing and the plasma is aspirated and discarded. A further complication noted in the early studies was the heterogeneity of EpCAM expression in patient samples and a decrease in tumor cell recovery in spiking experiments with cells from cell lines with decreasing EpCAM antigen densities. The variability of tumor cell recovery

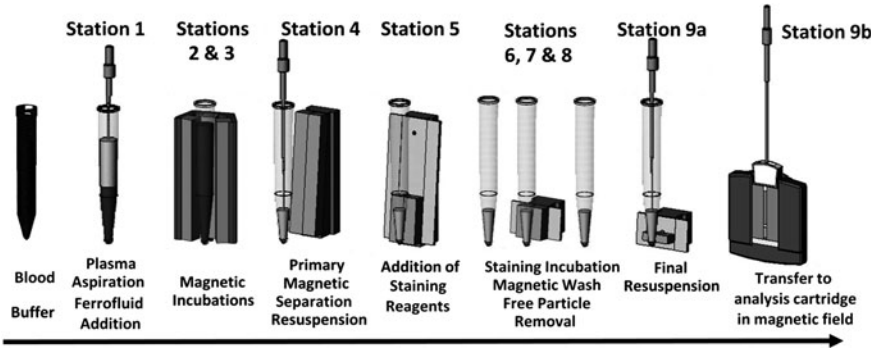

Fig. 3 Fully automated enrichment and staining of CTC from 7.5 mL blood samples by the CellTrack Autoprep. Eight samples can be placed on the system, which consist of nine stations. The samples reside at each station for 14 min. Before starting 7.5 mL of blood is transferred to a 15 mL conical CellTracks Autoprep tube and 6.5 mL of dilution buffer is added and after mixing by inversion the tube is placed in the centrifuge and spinned at 800 × g for 10 min. The tube is placed on the CellTracks Autoprep and moved to station 1 where the plasma is aspirated and EpCAM ferrofluid is added. Subsequently, the sample is moved to stations 2 and 3 at which the magnets are designed such that they can move back and forward to allow the ferrofluids to move through the sample thereby accelerating the incubation. At station 4 the first separation step takes place, the blood is discarded and the two slanted magnets permit the collect of the ferrofluids and ferrofluid labeled cells into a narrow line which facilitates the resuspension of the cells. In station 5 the staining reagents (CD45-APC, cytokeratin-PE and DAPI) are added. Additional staining reagents can be added here if desired. At stations 6, 7 and 8 the sample is incubated and free staining reagents are removed by a magnetic wash and after cell settling some of the free ferrofluids are removed. In station 9 the cells are resuspended and transferred to an analysis cartridge which is placed in the CellTracks Magnets

based on different levels of expression of the EpCAM antigen was significantly reduced by a controlled aggregation of the ferrofluids that resulted in multiple ferrofluids on the target cells after only a few EpCAM antigens were present [32, 68]. After immunomagnetic enrichment and volume reduction the cell suspension is stained with the nucleic acid DAPI, the Allophycocyanin (APC) labeled antibody CD45, and the Phycoerythrin labeled antibody cytokeratin 8/18 and cytokeratin 19. As cytokeratins are intracytoplasmatic a permeabilization reagent is added at the time of incubation. After an incubation period, magnetic wash steps remove unbound staining reagents. At the final step the cells are resuspended in a cartridge placed in the CellTracks Magnets (Veridex). This consists of two angled magnets that cause the ferrofluids and ferrofluid labeled cells to move to the analysis surface at the top of the cartridge [51]. The latter is used to avoid any cell loss that is frequently noted when using cytospins to present cells for analysis. In the first clinical study the sample preparation was performed in CellPrep an instrument that carried out the steps that were prone to human error. At a later time point the sample preparation was fully automated and performed by the CellTracks Autoprep.

The advantage of the CellTracks Autoprep system compared to other enrichment methods is that it is fully automated and not prone to human errors. The system is validated extensively and consistent recoveries are obtained after spiking of cells from tumor cell lines [11]. Still when no cells are detected one wonders whether they are missed or were not present in the first place. Tumor cells missing EpCAM and/or cytokeratin 8, 18 and 19 will not be detected by the system and whether or not all CTCs withstand the selection and staining procedure is not known. The antibodies on the ferrofluids and staining reagents used on the CellTracks Autoprep can easily be adapted as for example done for the detection of endothelial cells and melanoma cells [69, 70]. To improve the capture of CTC in carcinoma patients the question is which cell surface antigens to target to improve CTC capture and which antigens to target to improve cell identification. For example the use of EpCAM for both capture and detection can improve the yield as one no longer depends on the co-expression of the cytokeratins and the elimination of the cell permeabilization may reduce CTC loss. Cytokeratins are however expressed at a relatively high density so one will need to be assured that the detection of EpCAM antigens with antibodies does not cross react with the ones used for cell capture. A greater issue will be that alteration of the CTC immunophenotype implies that one will have to perform controlled clinical studies to establish a CTC threshold and determine whether or not the altered definition improves clinical utility.

5 Detection of Immunomagnetically Enriched CTCs by Flow Cytometry

Functional studies of CTCs are not feasible after enrichment of CTCs using the CellSearch Epithelial cell kit (Veridex) as cells are no longer viable with the use of CellSave tubes for blood collection and the permeabilization to detect the intra-cellular cytokeratins in this kit. Also the isolation of mRNA from CTCs is significantly reduced [71, 72]. Use of EDTA blood draw tubes in conjunction with the CellSearch Profile Kit (Veridex) can overcome this issue. This kit uses the identical immunomagnetic enrichment as the CellSearch Epithelial Cell kit but no staining and permeabilization reagents are included. For analysis of gene expression one however can only investigate those genes that are abundant in CTC and have a low or no expression in leukocytes [71, 72]. The cell suspension derived from the CellSearch profile kit can be stained and sorted by flow cytometry. To show an example of such analysis an EDTA anti-coagulated 7.5 mL blood sample from a healthy donor was enriched for CTC on the Celltracks Autoprep using the CellProfile kit. After enrichment the sample was stained with Hoechst 33342, CD45-APC and CD16-FITC. The latter recognized the FcRII expressed on neutrophils and natural killer cells. After an incubation period of 30 min at 37°C, the sample was diluted with 500 µl PBS/Albumin (1%) and the complete volume was measured on a FACS ARIA II (Becton–Dickinson,

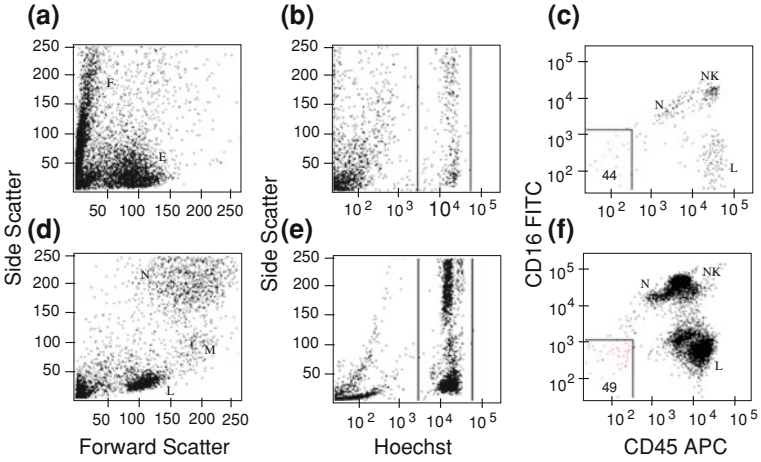

Fig. 4 Flow cytometric analysis of a blood sample from a healthy donor stained with Hoechst 33342, CD45-APC and CD16-FITC for CTC detection. Panels **a**, **b**, **c** show a sample immunomagnetically enriched from 7.5 mL of blood with the CellSearch Profile kit and panels, **d**, **e**, **f** show an ammonium chloride-lysed blood sample

San Jose, CA) flowcytometer as illustrated in Fig. 4, panels a–c. In the light scatter plot of panel a two major populations can be discerned; free ferrofluids (F) and erythrocytes (E). Some erythrocytes are still present after the enrichment procedure as no lysing or permeabilization reagents are contained in the CellProfile kit. The EpCAM ferrofluids are ~200 nm in diameter and one can discern them as a streak with increasing side scatter caused by the aggregation of the ferrofluids. In the scatter plot of panel b only 1,100 of the total of 30,000 events (3.7%) stain as nucleated cells and panel c shows the CD45-APC versus CD16-FITC staining of the Hoechst 33342 positive events. The positions of the lymphocytes (L), natural killer cells (NK) and neutrophils (N) are indicated in panel c. The gate shown in panel c contains 44 CD45-, CD16-, Hoechst+ events. CTCs will present themselves in this gate and from this analysis it is clear that there still is a background when employing this strategy to obtain viable CTCs that can be subjected to mRNA gene expression analysis. To illustrate the difference of this analysis with flow cytometric analysis without immunomagnetic enrichment, the same staining was conducted on the blood sample of a healthy donor after ammonium chloride lysis. In the light scatter plot of panel d the positions of the neutrophils (N), monocytes (M) and lymphocytes (L) are indicated. The Hoechst gate is shown in panel e and 10,000 Hoechst 33342 positive events are shown in panel f. In this panel the positions of lymphocytes (L), natural killer cells (NK), neutrophils (N) and monocytes (M) are shown. The gate where CTCs will present themselves contains 49 events. One however has to realize that the latter analysis only represents ~2μL of blood, whereas the enriched sample represents the analysis of 7.5 mL, a 3,750-fold increase in sample volume!

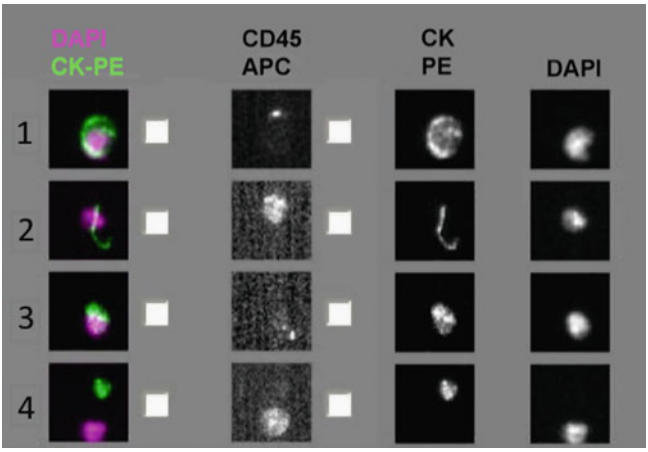

Fig. 5 Identification of CTC using the CellTracks Analyzer II. Thumbnail images of four events identified as DAPI +, CK + are shown in rows 1,2,3 and 4. The evenst in rows 1 and 3 are classified as a CTC. The event in row 2 represents a leukocyte with non-specific staining of PE debris adjacent to the cell. The event in row 4 represents a leukocyte and a tumor microparticle. The image analysis algorithm identified both events because of their proximity; still in this case they are clearly two separate events

6 Detection of Immunomagnetically Enriched CTCs by Microscopy

CTCs enriched and fluorescently labeled by the CellTracks Autoprep can be analyzed by fluorescent microscopy. To enable the viewing of all the cells in the sample the cartridge is placed inside the Magnets for analysis by the CellTracks Analyzer II. This is a computer controlled epi-fluorescence microscope with a mercury light source and a NA 0.45 10 × objective. The system first identifies the exact location of the analysis surface and focal plane of the sample cartridge. The images are taken with a high sensitivity monochrome 12-bit CCD camera and typically 175 locations are imaged to cover the entire surface. Each pixel represents a 650 nm × 650 nm area of the sample. At each location images are taken with filter cubes for the fluorochromes DAPI, FITC, PE and APC. After all images are acquired an image analysis algorithm is applied that automatically selects events with high contrast that appear in both the PE and DAPI channels. After all locations of PE+, DAPI+ events are determined events on the border of an image which touch an event on the adjacent image are combined into single objects to prevent double counting of CTC. For each event a thumbnail image is generated and presented to an operator for review. Each thumbnail consists of a false color overlay image showing PE (green) and DAPI (purple) signals, as well as monochrome images of APC, FITC, PE and DAPI signals as illustrated for four events from a patient sample in Fig. 5. All images are scaled from minimum to maximum

before presentation. Each row in Fig. 5 labeled 1–4 represents a DAPI+, PE+ event. The first column shows a thumbnail of an overlay of DAPI (purple) and Cytokeratin (green). The second column shows the CD45-APC staining, the third column the Cytokeratin-PE staining and the fourth column the DAPI staining. In rows 1 and 3 the event shows no staining for CD45-APC, with positive staining for cytokeratin-PE and DNA. The overlay pictures show morphologic intact cells which leads to the assignment of both events as CTC. The event in row 2 shows staining for CD45-APC and DNA, the morphology in the overlay picture is in agreement with that of a leukocyte. The PE staining that is partially overlapping the cell appears as a piece of debris that may be associated with aggregation of the PE reagents. Likewise are the features of the event in row 4 in agreement with that of a leukocyte. The PE staining is not associated with the leukocytes and the round morphology suggests that this might be a tumor microparticle [15, 66]. The fully automated scanning feature of the CellTracks Analyzer combined with the analysis algorithm certainly facilitates the identification of CTC. Still, in samples with many CTCs and CTC debris the review time can increase considerably. Linearity, precision and accuracy was extensively tested with cells of tumor cells spiked in blood as well as duplicate blood samples from patients with metastatic carcinomas [11]. The error when only few CTCs are detected is by definition large. To separate patients into those with and those without CTC the threshold was not set on the background observed in blood of healthy donors and patients with benign disease, but at a higher level $\geq$5 CTC for breast and prostate cancer and a threshold of $\geq$3 for colorectal cancer. Next to the limitation associated with the counting statistics at low numbers the differences in assignment of events as CTC by operators play an important role [73, 74]. Addition of an algorithm that can automatically classify these events can eliminate this source of error [75].

Treatment targets can also be assessed on CTC, some targets are however present at low antigen densities and cannot be detected with sufficient sensitivity using the reagent configuration of the CellSearch Epithelial Cell kit. The insulin growth factor 1 receptor (IGF1R) is an example of a low antigen receptor target. To enable the detection of IGF1R with the CellSearch system the cytokeratin PE is exchanged for cytokeratin FITC and opens the opportunity to use the most sensitive fluorescence dye Phycoerythrin for detection of the IGF1R [76, 77]. The drawback of this approach is that more cytokeratins will need to be present in a CTC before it can be detected with cytokeratin FITC and thus may result in a lower number of CTCs that will be detected. An example of detection of IGF1R on seven CTCs detected in a metastatic cancer patient is shown in Fig. 6. In this example four of the seven CTCs expressed IGF1R, which highlights the need for analysis on an individual cell basis as only in this manner one can assess the heterogeneity of treatment targets on tumor cells. In this case the results suggest that treatments targeting IGF1R only targets 57% of the tumor cells. In the thumbnail images in the first row another interesting observation can be made. The DAPI staining reveals the presence of a cluster of four cells; the cell on the top right corner is a CTC staining with Cytokeratin and expresses IGF1R, the cell on the top left corner is a leukocyte expressing IGF1R, the cell in the middle is

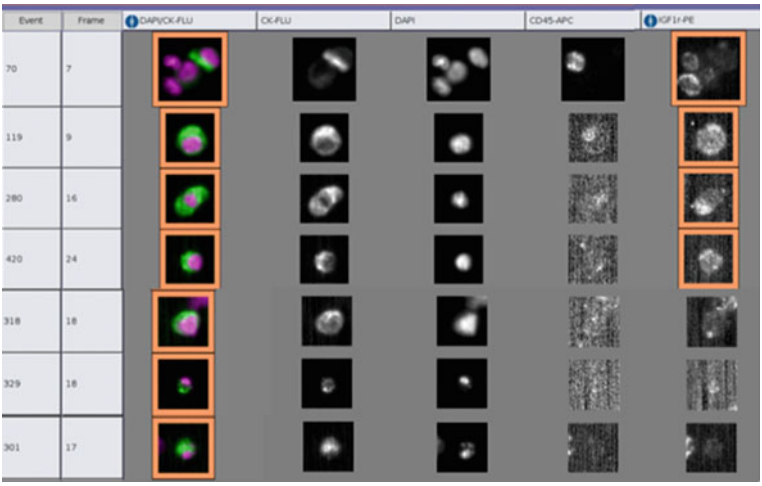

Fig. 6 Identification of IGF1-R expression on CTC using the CellTracks Analyzer II. Thumbnail images of seven DAPI+, CK+, CD45- CTCs from one patient are shown in rows. The event number and the frame in which the CTCs appear are indicated before the thumbnails. The last column shows the IGF-1R expression of the CTCs. Four of the seven CTCs express IGF1-R

a CTC dimly staining with both cytokeratin and IGF1R and the cell on the left bottom corner cannot be assigned to a category as it lacks the leukocyte antigen CD45 as well as cytokeratins. Still, it expresses the highest levels of IGF1R of all four cells. What the implications are of such observations remain to be determined and probably can only be addressed in controlled clinical studies.

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Microfluidic Technologies

Ali Asgar S. Bhagat and Chwee Teck Lim

Abstract

Presence of circulating tumor cells (CTCs) in blood is an important intermediate step in cancer metastasis, a mortal consequence of cancer. However, CTCs are extremely rare in blood with highly heterogeneous morphologies and molecular signatures, thus making their isolation technically very challenging. In the past decade, a flurry of new microfluidic-based technologies has emerged to address this compelling problem. This chapter highlights the current state of the art in microfluidic systems developed for CTCs separation and isolation. The techniques presented are broadly classified as physical- or affinity-based isolation depending on the separation principle. The performance of these techniques is evaluated based on accepted separation metrics including sensitivity, purity and processing/analysis time. Finally, further insights associated with realizing an integrated microfluidic CTC lab-on-chip system as an onco-diagnostic tool will be discussed.

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Contents

1 Introduction

Circulating tumor cells (CTCs) are cells of epithelial origin that escape from a primary tumor and journey through the vascular system to establish new lesions at distant organs/sites. This process, known as metastasis, accounts for ~90% of all cancer-related deaths [1] and clinical studies have revealed that presence of CTCs in blood is directly correlated with disease progression. Enumeration of CTCs can thus be used as potential biomarker to determine treatment efficacy and cancer management [2, 3]. This novel "liquid biopsy" approach for cancer diagnosis is less invasive and enables frequent clinical evaluation of cancer patient [4, 5]. However, as CTCs are extremely rare (~1 cell per billion blood cells), highly efficient and robust separation methods for achieving meaningful enrichment and isolation are required for their efficient enumeration. Currently, techniques relying on density gradient centrifugation, microfiltration and immunoselection have been extensively developed for CTC isolation [6–8]. In the past decade, isolation methods based on physical filtration and affinity capture have been reduced to the microscale abetted by advances made in microfluidics device development. In this chapter, we review the state of the art microfluidic approaches developed specifically for CTC isolation.

Microfluidics and nanofluidics offer an attractive solution for this application leveraging its numerous advantages to build a fully integrated system to process clinical blood samples [9–11]. Processing blood in microfluidic systems is challenging, due to the high volume fraction of cellular components in blood and the increased surface to volume ratio in microchannels leading to channel fouling due to increased cell-surface interactions. Despite this limitation, microfluidic approaches for CTCs separation and detection has emerged rapidly because of its small length scale (similar to the cellular scale), allowing better control of the microenvironment during blood separation. Microfluidic systems also allow retrieval of viable cells post sorting, a vital need geared toward the understanding of the cellular and molecular biology of these cells. The small dimensions lead to reduced sample and reagent volumes and allow the development of an integrated system with minimal human intervention reducing sample contamination and loss. The low power consumption due to the reduced size increases portability making microfluidic tools ideal candidates for point of care (POC) diagnostic in resource limited settings.

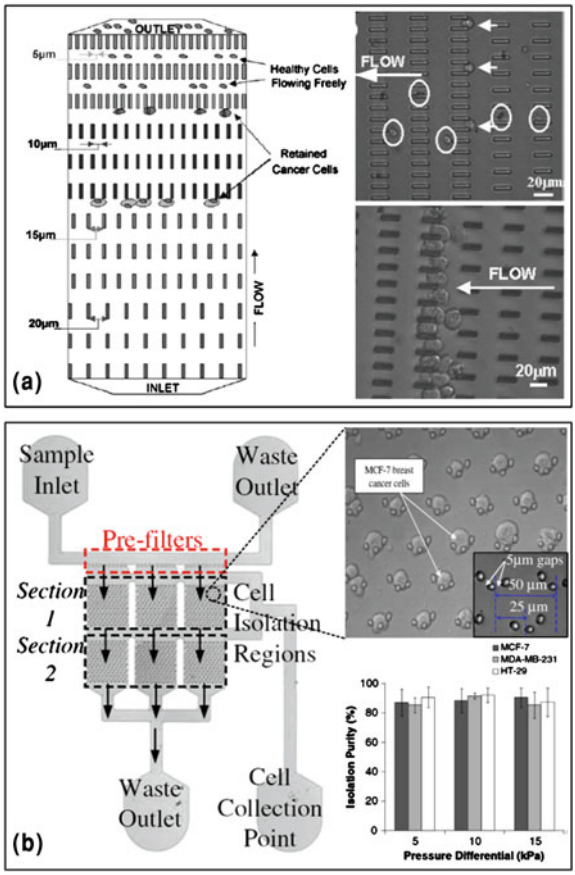

Fig. 1 **a** Schematic design of the arrayed micro-column device with varying channel gap widths. *Right* Microscope image demonstrating the isolation of BE(2)-M17 cells (*white arrows*) from blood cells (*white circles*). The neuroblastoma cells were retained at the 5 μm wide gaps while the blood cells could freely sieve through the columns. Figure reprinted from [15], copyright 2009, with permission from Elsevier. **b** Schematic illustration of a microfluidic device for CTCs isolation and enumeration. The design consists of six isolation regions incorporating arrays of crescent-shaped isolation wells to trap cancer cells. Separation results indicating ~80% purity for different cancer cells over a wide range of operating pressures is also shown. Reproduced from [16], copyright 2009, with kind permission from Springer Science + Business Media, LLC

The aforementioned advantages have led to an avalanche of development in microfluidics-based CTC isolation and retrieval methods. Here, we present a brief overview of a few popular techniques with discussion on the separation principles and performances including efficiency, enrichment and throughput. The techniques presented are broadly classified either under physical separation or immuno-based separation. Physical separation methods take advantage of the differences in

mechanical and physical properties, including size, density and deformability, of the CTCs as compared to other hematologic cells for isolation. Affinity or immuno-based separation methods rely on common epithelial surface markers to effectively isolate CTCs from blood. Examples of common surface markers include epithelial cell adhesion molecule (EpCAM), human epithelial antigen (HEA) and human epidermal growth factor receptor 2 (HER2). As our intent is to succinctly introduce the various microfluidic techniques, the readers are encouraged to read the original papers for further details.

2 Physical Separation Methods

Membrane-based microfiltration schemes are perhaps the most popular CTCs isolation methods relying on intrinsic physical differences between CTCs and blood cells [7, 12, 13]. Physical separation methods take advantage of the large differences in size (diameter) and deformability between CTCs and other hematologic cellular components (CTCs $\sim$16–20 μm; red blood cells (RBCs) $\sim$8 μm; leukocytes $\sim$10–15 μm) for separation [12, 14]. However, in microfluidics-based systems, the use of pillared structures is analogous to the membrane micropores and is frequently used for size and deformability-based cell sorting [15, 16]. The inter-pillar distance is the critical dimension in these systems and can be tailored to ensure that maximum CTCs are retained with little contamination from other mononuclear blood cells. Separation based on physical properties are appealing as they are label free, not requiring the use of biomarkers/antibodies, and thus more affordable.

One initial work in this area was reported by Mohamed and co-workers using an arrayed microcolumn structure in which the intercolumn gap progressively decreases from 20 to 5 μm [15]. The device thus has four segments with sieves of 20, 15, 10 and 5 μm gaps (Fig. 1a). CTCs spiked into blood are introduced into the microchannel inlet and are retained at the 10 and 5 μm gaps while all the hematologic cells pass through the sieve. The design was validated by performing tests with five neuroblastoma and three adult epithelial cell lines. The authors observed that a 5 μm gap was sufficient to retain all the cells from the eight different lineages tested, while allowing all other blood cells to successfully pass through.

Recently, Tan et al. introduced a novel CTC isolation biochip using an array of crescent-shaped isolation wells to retain the CTCs (Fig. 1b) [16]. The gap between the pillars was fixed at 5 μm to capture the larger and stiffer CTCs while allowing the more deformable red cells and leukocytes to escape. The placement and position of the crescent-shaped isolation traps were optimized using detailed computational models to ensure uniform flow profile and maximize capture efficiency. Using spiked cell lines in blood, the device exhibits superior performance capturing as low as 1 cell/ml and exhibiting an isolation efficiency of 80%. As CTCs from clinical samples have a highly heterogeneous size distribution and

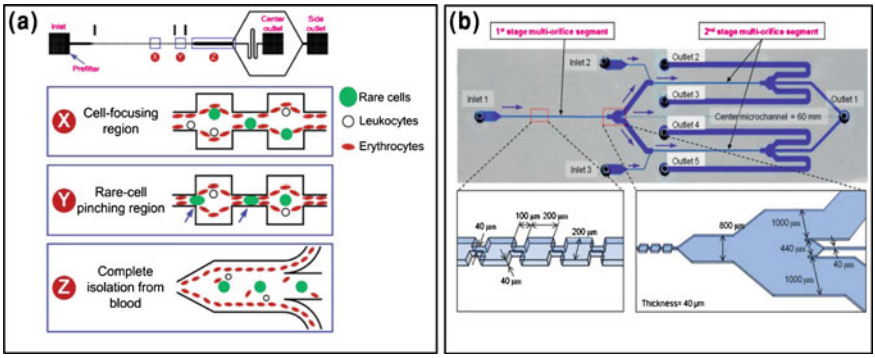

Fig. 2 **a** Schematic illustrating the isolation of the CTCs from blood using inertial lift forces. The device design consists of series of contraction–expansion regions and a three way bifurcating outlet. Designing high aspect ratio microchannels equilibrated all the cells along the two microchannel sidewalls in the cell-focusing region. A unique feature of the device is the rare-cell pinching region designed prior to the outlet to align the center of mass of the larger CTCs along the channel center. Thus, at the outlet the larger CTCs are collected at the center outlet while the smaller blood cells are filtered from the side outlets. Figure reproduced with permission from [19], copyright 2011, The Royal Society of Chemistry. **b** Schematic design of the multi-stage multi-orifice flow fractionation (MS-MOFF) using a cascaded configuration for increased isolation efficiency. Reproduced from [20], copyright 2009, with kind permission from The Royal Society of Chemistry

morphologies, the authors further validated their device performance with samples from patients with metastatic lung cancer [17, 18]. Processing only 2 ml of blood from clinical samples, the technique reports a 100% detection rate capturing an average of 50 cells with 83% purity.

Size-based CTC sorting using inertial lift forces has also been reported on the microscale [19, 20]. Inertial lift forces inherent to the cell motion can be used to precisely align/focus cells along the microchannel cross-section based on their size [21]. By using high aspect ratio microchannels, cells can be preferentially equilibrated along the longer microchannel walls due to the presence of a non-uniform shear gradient [22]. Bhagat et al. reported a microfluidic device design consisting of an array of contraction–expansion regions to align all the hematologic cells including CTCs along the two microchannel sidewalls under the influence of shear modulated inertial lift forces [19]. By designing a customized rare-cell pinching region (width $\sim$10 μm) prior to the microchannel outlet, the larger CTCs can be collected at the center outlet while the smaller blood cells are removed from the side outlets. Using spiked MCF-7 cells in blood, the authors reported $\sim$80% CTC recovery with 10^4-fold enrichment over leukocytes. Due to the high flowrates required for inertial focusing, cell separation throughputs of 10^8 cells/min were achievable. Higher recovery efficiency of $\sim$90% has been reported using similar device by employing a multi-stage cascade configuration by Sim and co-workers [20]. However, to minimize cell–cell interactions, this technique is limited to dilute samples requiring additional on-chip sample preparatory steps (Fig. 2).

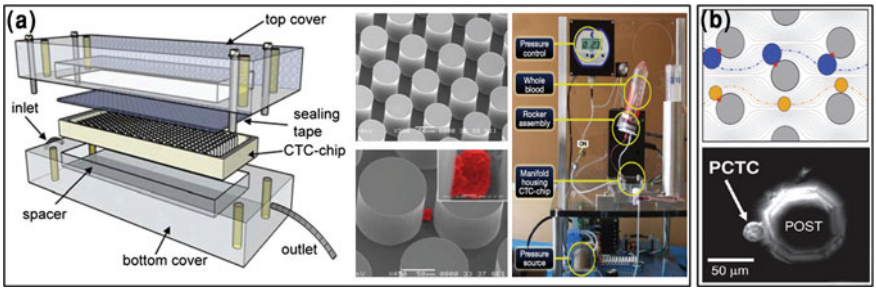

Fig. 3 **a** Schematic of the manifold assembly designed for CTC-chip testing. The chip layout consists of an antibody-conjugated micropost array to capture CTCs from whole blood. Inset shows an electron microscope image of a captured NCI-H1650 lung cancer cell between two functionalized microposts. The workstation setup including rocker for blood mixing and pressure controls for automated biochip testing is also shown. Reprinted with permission from Macmillan Publishers Ltd: Nature [26], copyright 2007. **b** Schematic illustrating the use of staggered obstacles (*gray circles*) to distort the flow streamlines, leading to increased wall interactions of the larger CTCs (*blue circles*) while the smaller blood cells (*yellow circles*) flow through the device with fewer interactions. The figure also shows a microscopic image showing a single prostrate tumor cell (PCTC) captured on the wall of a micropost. Reproduced from [28], copyright 2010, with kind permission from The Royal Society of Chemistry

3 Affinity-Based Separation Methods

Affinity-based separation are highly selective as they rely on certain specific molecules recognizable on the cells of interest within a population. Historically, fluorescence activated cell sorting (FACS) has been used extensively for rare-cell enumeration due to its extremely high throughput and sensitivity via fluorescently labeled antibodies. Although flow cytometry has been implemented on the microscale more than a decade ago, there have not been many CTC enumeration methods developed using FACS perhaps due to bulky and expensive supporting equipment required for laser scanning [23]. In microfluidic systems, affinity-based separations are achieved by binding the cells of interest to molecules immobilized on the walls/surface of the microchannels. The high surface to volume ratio of microchannels provides an ideal platform for such adhesion-based assays. The biomolecules (proteins/antibodies) are functionalized on the microchannel surfaces using covalent bonds or adsorption [24, 25].

Perhaps the most noted example illustrating the use of affinity-based CTC isolation in microfluidic systems is the CTC-chip developed by Nagrath et al. [26]. Functionalizing anti-EpCAM antibodies on an array of microposts, the authors reported a $\sim$60% isolation efficiency with high purity. Tests with clinical samples further validated the performance of the CTC-chip with successful isolation of CTCs from peripheral blood in 115 out of 116 ($\sim$99%) cancer patients. The group further improved the isolation efficiency to 90% by designing a herringbone-chip capable of generating a vortex flow within the microchannels [27]. The vortex

helps to mix the blood cells during processing and significantly increases the interactions between the target CTCs and the EpCAM-coated chip surface, thereby leading to higher capture efficiency. A similar approach to increase the antibody-cell interaction using a staggered obstacle array functionalized with J591 monoclonal antibody was reported by Gleghorn et al. to capture prostate cancer CTCs from whole blood [28]. Recently, the use of magnetic beads conjugated with tumor-specific antibodies has also been demonstrated in microfluidics systems for isolating CTCs [29]. The throughput of these affinity-based systems is usually low (typically <1 ml/hr) due to low shear rates required to allow maximum interaction time between the CTCs and the capture surfaces for effective binding. The retrieval of viable bound CTCs for downstream molecular analysis is also challenging post isolation (Fig. 3).

Although, affinity-based assays are highly specific, the heterogeneous surface molecular signatures of CTCs arising from various cancers make it critical to accurately select ligands for efficient separation. Furthermore, highly heterogeneous expression levels of antigens within the same cancer types is also common; Sieuwerts et al. reported very low expression levels of EpCAM on various breast cancer cell lines [30]. To overcome this limitation, aptamers probes derived from cell-SELEX (Systematic Evolution of Ligands by EXponential enrichment) process have been demonstrated using microfluidic systems [31]. The cell-SELEX process generates highly specific aptamers in vitro, and thus prior information of the surface molecular signatures of CTCs from different cancer types is not required. The DNA aptamers are then functionalized onto microchannels walls for cell capture. The authors successfully demonstrate the proof-of-principle using three leukemia cell lines and their corresponding DNA-aptamers [31].

4 Conclusions and Future Outlook

We highlighted several microfluidic technologies that employ isolation principles involving either the physical separation or affinity-/immuno-based separation methods. These microfluidic approaches offer several advantages and benefits including reduced sample volume, faster processing time, high sensitivity and spatial resolution and portability. In fact, some of these technologies have successfully demonstrated that they can isolate and separate rare CTCs from clinical blood samples.

However, CTC separation is just the first crucial step toward a more comprehensive study and analysis of these cancer cells. What's next will be an integrated approach that can allow for a series of more complex processes to be performed on a microfluidic chip right after cell separation. These will involve manipulating and handling the isolated CTCs, providing a controllable microenvironment to allow for molecular characterization tests to be performed on these isolated CTCs and implementing high-resolution imaging techniques to analyze the biological functions or gene expression in these cells. The ultimate goal will be to design portable

diagnostic microfluidic CTC lab-on-chip systems that can integrate all the necessary complex processes onto the chip. Finally, these simple to use portable microfluidic systems will be useful to resource limited regions where clinical facilities and support are lacking.

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EPISPOT Assay: Detection of Viable DTCs/CTCs in Solid Tumor Patients

Catherine Alix-Panabières

Abstract

The enumeration and characterization of circulating tumor cells (CTCs) in the peripheral blood and disseminated tumor cells (DTCs) in bone marrow may provide important prognostic information and might help to monitor efficacy of therapy. Since current assays cannot distinguish between apoptotic and viable DTCs/CTCs, it is now possible to apply a novel ELISPOT assay (designated 'EPISPOT') that detects proteins secreted/released/shed from single epithelial cancer cells. Cells are cultured for a short time on a membrane coated with antibodies that capture the secreted/released/shed proteins which are subsequently detected by secondary antibodies labeled with fluorochromes. In breast cancer, we measured the release of cytokeratin-19 (CK19) and mucin-1 (MUC1) and demonstrated that many patients harbored viable DTCs, even in patients with apparently localized tumors (stage M_0: 54%). Preliminary clinical data showed that patients with DTC-releasing CK19 have an unfavorable outcome. We also studied CTCs or CK19-secreting cells in the peripheral blood

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of M1 breast cancer patients and showed that patients with CK19-SC had a worse clinical outcome. In prostate cancer, we used prostate-specific antigen (PSA) secretion as marker and found that a significant fraction of CTCs secreted fibroblast growth factor-2 (FGF2), a known stem cell growth factor. In conclusion, the EPISPOT assay offers a new opportunity to detect and characterize viable DTCs/CTCs in cancer patients and it can be extended to a multi-parameter analysis revealing a CTC/DTC protein fingerprint.

Contents

1 Introduction

Till date, immunocytochemistry and reverse-transcription-polymerase chain reaction (RT-PCR) are the main approaches used to detect CTCs/DTCs [1, 2]. A drawback of both approaches is that they are usually unable to distinguish between viable and apoptotic cells. Recently, a new technique that allows this important discrimination was introduced for CTC/DTC analyses [3–7]. This technique was designated EPISPOT (i.e., EPithelial ImmunoSPOT) and is based on the secretion, the shedding or active release of specific marker proteins, using an adaptation of the enzyme-linked immunospot (ELISPOT) technology.

2 Epispot Procedure

Briefly, the nitrocellulose membranes of the ELISPOT plates are coated with an antibody against a specific protein marker (Fig. 1). Then, cells are seeded in each well and cultured for 24–48 h. During this incubation step, the specific secreted proteins are directly captured on the antibody-coated membrane. Next, cells are washed off and the specific protein marker is detected by a second antibody conjugated with a fluorochrome. Immunospots are counted by video camera imaging and computer-assisted analysis (KS ELISPOT, Carl Zeiss Vision): one immunospot corresponds to the fingerprint of one viable marker protein-secreting cell. This assay is quantitative—the immunospots are counted—and qualitative—the proteins studied are well defined in the context of solid tumors, allowing the phenotypic characterization of the CTCs/DTCs.

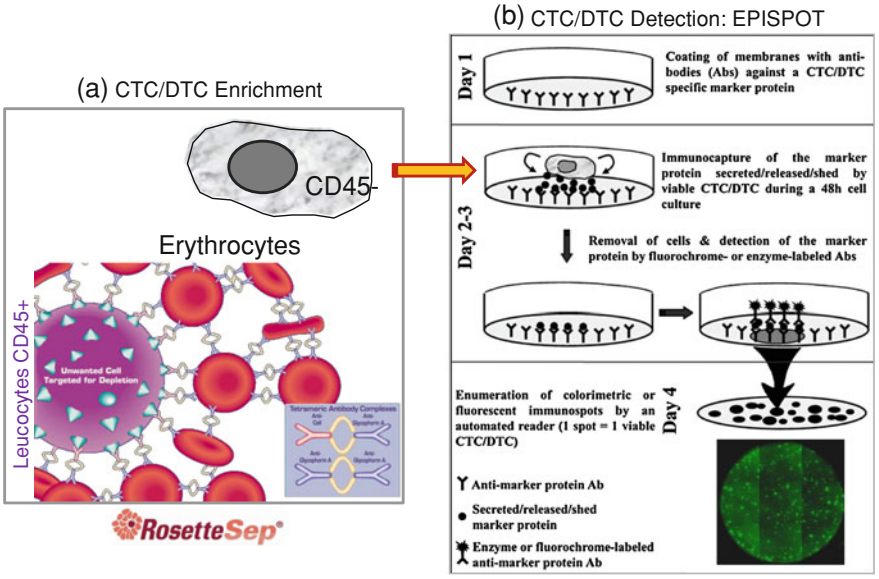

Fig. 1 CTCs/DTCs Enrichment method and EPISPOT assay procedure [2]. **a** $EpCAM^{-/+}$ tumor cells are enriched via a depletion of the $CD45^{+}$ hematopoietic cells. **b** Enriched CTCs/DTCs are then cultured in vitro during a short time in an appropriate enriched culture medium. Protein fingerprints of these CTCs/DTCs are finally counted under a microscope and correspond to viable secreting tumor cells

Dual fluorescent EPISPOT assay was quickly developed to target CTCs through the secretion of two different proteins. The optimization of triple fluorescent EPISPOT assay is still in progress.

3 CTC Enrichment Step

As CTCs occur at very low concentrations of one tumor cell in the background of millions of blood cells, a first step of enrichment is usually required. Being a detection method, the EPISPOT assay can be combined, in principle, to any kind of CTC enrichment step. It includes a large panel of technologies based on the different properties of CTCs that distinguish them from the surrounding normal hematopoietic cells: physical (size, density, electric charges and deformability) and/or biological properties (surface protein expression, viability and invasion capacity). Most of the current technologies are still based on epithelial cell adhesion molecule (EpCAM) expression; however, due to the assumption that an Epithelial-to-Mesenchymal transition may occur in particular during tumor cell dissemination, new emerging technologies also try to capture EpCAM-negative CTCs.

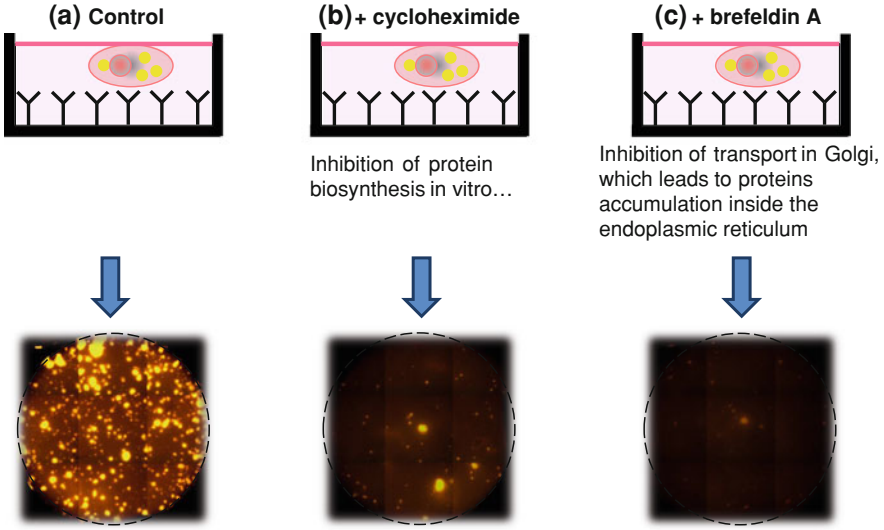

Fig. 2 EPISPOT assay and inhibition of protein secretion. As control, EPISPOT assay was performed with the enriched culture medium (**a**) hundreds of immunospots are observed. EPISPOT assay was also performed with cycloheximide (**b**) and brefeldin A (**c**) inhibition of immunospot formation

Until now, the EPISPOT assay was mostly combined with a depletion of CD45-positive cells using the RosetteSep system (StemCell Technology, Vancouver, Canada), avoiding direct contact with the target cells and allowing the study of EpCAM-negative CTC (Fig. 1).

4 Detection of Viable and not Apoptotic DTCs/CTCs

As previously shown, viable cells need to accumulate a sufficient amount of the released marker proteins during the in vitro cell culture of the EPISPOT assay to form immunospots and dying cells are therefore not detected using this assay [3, 8]. To be in the best conditions, it is important to notice that the cell culture medium is enriched with different growth factors, as it has already been described for the establishment of DTC cell lines [9].

Moreover, we performed additional drug experiments to show that only functional CTC/DTC are able to give rise to immunospots. Indeed, the addition of Brefeldin A or cycloheximide during the short-time cell culture step of the EPISPOT assay showed a clear inhibition of the immunospot formation (Fig. 2), confirming that only viable CTC/DTC are detected.

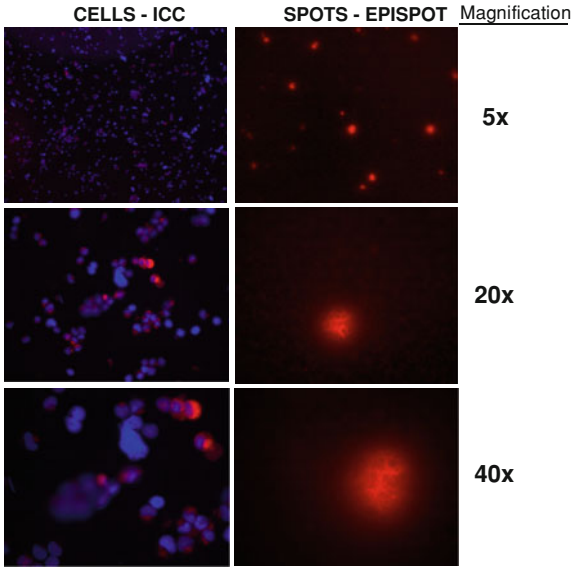

Fig. 3 Immunocytochemistry and EPISPOT assay. The sizes of the immunospots and the corresponding tumor cells are observed at the same magnification after immunocytochemistry (ICC) and EPISPOT assay, respectively

Another important observation is the size of the immunospots, significantly bigger than that of a single cell (Fig. 3). Thus, there is no doubt to distinguish a residual non-viable tumor cell on the membrane from a de novo immunospot established by continuous protein secretion of viable tumor cells cultured over 48 h.

5 Clinical and Translational Leads

In breast cancer, mucin-1 (MUC1) and cytokeratin-19 (CK19) were used as marker proteins [10]. MUC1 is a membrane-bound mucin overexpressed and aberrantly glycosylated in breast carcinoma cells. MUC1 is cleaved, shed and can be detected in the serum of cancer patients (tumor marker CA15-3) [11–13]. CK19, one of the three main keratins besides CK8 and CK18, is found in simple or stratified epithelium and in carcinomas, and it has been demonstrated to be abundantly expressed in primary epithelial tumors such as breast, colon, lung and hepatocellular cancer cells [14, 15], but not in normal mesenchymal hematopoietic cells.

MUC1-releasing cells (RC) were detected in blood of all advanced metastatic breast cancer patients analyzed, whereas such cells were not observed in healthy controls [3]. In fact, MUC1 was also weakly released by normal activated B lymphocytes; however, there was a significant difference between the release of

MUC1 by tumor and normal cells and we could define a clear cutoff for the size of the MUC1-immunospots obtained from tumor cells [3]. Thus, the enumeration of both MUC1- and CK19-RC allowed the detection of viable DTCs in bone marrow of 90 and 54% of breast cancer patients with and without overt distant metastasis, respectively [10]. These incidences are in the range of those obtained with sensitive RT-PCR-based techniques [16]. Interestingly, M_1-patients had significantly higher DTC counts than M_0-patients (Total nDTC: 3604 vs. 689; Median: 103 vs. 1; Mean: 180 vs. 19; Range: 0–813 vs. 0–262, for M_1 vs. M_0), but most of the DTC detected in M_0 patients showed the CK19+/MUC1- phenotype and may have stem-like properties [10, 17]. Most importantly, breast cancer patients with CK19-RC had an unfavorable clinical outcome due to the occurrence (M_0-patients) or progression (M_1-patients) of metastasis, whereas no clinical relevance was found with MUC1-RC [18]. However, these preliminary data need to be confirmed in a larger study.

In prostate cancer, prostate-specific antigen (PSA) was used as a marker protein [19]. We studied blood samples from prostate cancer patients with gross metastases (M_1). PSA- secreting cells (SC) were detected in the majority of M_1-prostate cancer patients (83.3%), whereas such SC were not observed in healthy controls or in patients with benign prostatic hyperplasia [10]. The EPISPOT assay also revealed viable CTCs in the peripheral blood of 65% of prostate cancer patients, even in the absence of overt metastases (stage M_0), but the number of PSA-SC in M_0-prostate cancer patients (median, 9; range, 2–197) was significantly lower ($P = 0.01$) than in M_1-prostate cancer patients (median, 29; range, 1–684), a finding that is in accordance with the different disease stages and total tumor burdens.

To better characterize CTCs in prostate cancer, we focused on fibroblast growth factor 2 (FGF2), a known stem cell growth factor also relevant for the in vitro growth of a breast micrometastatic cells [9]. We developed a dual fluorescent PSA/FGF2-EPISPOT assay to characterize PSA-SC for the secretion of FGF2 and applied it to blood samples from 19 patients with localized prostate cancer. PSA-SC were detected in 15 patients, and a subset of these SC also secreted FGF2, suggesting that a significant fraction of DTCs may secrete a factor potentially relevant to their outgrowth [10].

EPISPOT has been also applied to detect CTCs in patients with colon cancer, head and neck cancer and melanoma but the data are more preliminary.

6 Challenges and Future Directions

Distinguishing viable from apoptotic CTCs to detect and profile the most relevant metastasis-initiating CTCs is of utmost importance [7]. The EPISPOT assay is a new technique to detect viable CTC/DTC in cancer patients. Secreted, shed or released proteins are immunocaptured immediately on the membrane before being diluted in the supernatant. Thus, the EPISPOT assay has a sensitivity of up to four orders of magnitude higher compared with the quantification of these proteins in

the cell-free culture supernatants [3, 4]. However, a cell culture facility is required and the protein used to identify a CTC/DTC must be actively secreted, shed or released outside these cells. Moreover, it is crucial to be able to analyze the captured CTC at the molecular level and to compare their characteristics to those of the primary tumor and overt metastases.

Nowadays, there is a strong interest in developing micro-devices that can handle at least 10 times smaller sample volume than so far applied tests, thereby minimizing assay time and the use of expensive staining reagents. However, CTCs are extremely rare events and the analysis of large blood volume ($\geq$20 mL) might be preferable in particular in early-stage cancer patients with a small burden of CTC. Thus, technologies that can handle larger blood volumes still deserve special attention.

Our clinical data show that DTCs/CTCs in patients with cancer of the prostate or breast are viable and heterogeneous with regard to the secretion of relevant proteins. As many secreted proteins influence metastatic progression (e.g., growth factors and proteases) and as a large range of fluorochromes is available, it should permit the extension of this technique to a multi-parameter analysis and opens therefore also a new avenue in the understanding of the biology of metastatic cascade. The new EPISPOT technology may therefore reveal a unique fingerprint of single viable tumor cells and the subsequent molecular analysis of these tumor cells is in development.

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Advances in Optical Technologies for Rare Cell Detection and Characterization

Lou Dietz and Richard Bruce

Abstract

Scanning cytometry enables detection of circulating tumor cells without enrichment, minimizing potential loss of sensitivity due to variable expression of enrichment targets; however, some approaches lack specificity without imaging to identify false positives. High fidelity imaging enables identification of CTCs using morphological considerations and semi-quantitative measurement of biomarker expression for predicting targeted therapy but often lacks speed needed for the large number of mononuclear blood cells. A hybrid approach of first scanning a sample at high speed and high numerical aperture to locate CTCs followed by high resolution imaging of a small number of objects reduces the time needed for high resolution imaging without loss of detection sensitivity.

Contents

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1 Introduction

Circulating tumor cells (CTCs) are of considerable interest as a medium for providing an up-to-date assessment of disease status because they are accessible in peripheral blood by a repeatable, minimally invasive blood draw. They are attractive for both prognostic and predictive diagnostics, and both genetic [1] and protein biomarkers [2–4] have been observed on CTCs. Protein markers such as ER and HER2 have been observed to be discordant between CTCs and the primary tumor [2, 5] with levels that change during the course of therapy [6].

CTCs are generally identified by the presence of the epithelial marker, cytokeratin (CK), and the presence of an associated nucleus. Most but not all studies include the negative marker, CD45, as a control. Reports of CTC concentration in typical patients vary considerably, ranging from one to hundreds of CTCs per milliliter of blood. This extreme variation in typical CTC counts has remained largely unexplored, and few head-to-head tests have been performed to compare enumeration technologies and methods. Speculative explanations for this discrepancy include differences in detection methodology [1, 7, 8], patient populations, CTC identification criteria, or the strictness of staining such as the use of CD45 and morphological criteria to positively identify CTCs. It has been shown that a very wide range of identification criteria, including the enumeration of all CK-positive objects even when the said objects are clearly not viable cells, nevertheless possess prognostic value [9].

The phenotype, CK+CD45−, is requisite as CK+CD45+ cells have been shown to be not useful for prognosis [9]. The largest number of CK+CD45+ cells are generally eosinophils that can exhibit broad autofluorescence with many having CD45 expression. The problem is compounded by the transfer of CD45 to cells in contact with whole blood [10] so that weak CD45 can be observed on cells that by morphology are clearly not leukocytes. Differentiation of CTCs from autofluorescing false positives benefits by high fidelity imaging to identify low levels of CD45 expression and the use of CTC morphology such as size, shape and nuclear to cytoplasm ratio as identification criteria. No study has, to date, provided evidence that loose criteria identify objects which are useful for biomarker characterization. Intact, non-apoptotic CTCs may be necessary to accurately measure protein expression, while cell fragments containing nuclear material may prove adequate for some types of DNA analysis.

Because intact CTCs are often very rare in patient samples [11], a sample size of 5–10 ml whole blood, typically containing 25–100 million leukocytes, is desirable to increase the recovery of CTCs, and extremely high specificity is required to distinguish CTCs from this background of leukocytes. Consequently, enrichment is often used for CTC detection and carries the risk of loss of sensitivity due to the highly heterogeneous nature of CTCs. In this chapter, we will assess optical approaches for CTC detection that require no prior enrichment apart from red blood cell lysis. Such approaches enable high quality imaging for better visualization and quantification of biomarkers for predictive diagnostics as well as

better identification of CTCs, reducing false positives. Image fidelity is improved by a gentle labeling protocol and a planar substrate for optimal focusing.

2 Optical Detection Systems

2.1 Flow Cytometry

Flow cytometry (FC) is a powerful tool for research and clinical diagnostic applications, in which cells in liquid suspension are hydrodynamically focused into a single-file stream flowing past an optical detection system [12]. The detection system can use multiple lasers to excite fluorescence of molecular labels and multiple color channels for emission detection. The label emission levels for each cell can be classified by fluorescent intensity, or "gated", and individual cells falling within a specified range of one or more fluorescent labels can be sorted and collected for further use but at lower flow rates [12]. FC analyzes cells at a typical rate of 10^4 cells/s [13] though higher speeds are available with reduced sensitivity, and lower rates are usually used when sorting. FC can be exquisitely sensitive, using detection optics with numerical aperture (NA) greater than 1.0, to detect as few as 100 molecules of fluorophore per cell. The specificity, however, is insufficient for rare cell detection [14]. The one reported exception required preparation with 8 different fluorescent tags and reduced scan rates, 10^3 cells/s, to achieve the impressive specificity of 1–2 false positives per 10^7 mononuclear blood cells in samples with cancer cells spiked into normal blood [15]. Consequently, most approaches to cell detection employ morphological confirmation.

Imaging flow cytometry is a technique that combines the visual power of microscopy with FC by employing a precise method of electronically tracking moving cells, integrating the fluorescent image signal on a charge-coupled device detector that is clocked in synchrony with the flow stream [16, 17]. In this manner, multi-color fluorescent, brightfield, and darkfield images of each cell can be acquired, combining traditional FC analyses with microscopy-like imaging [18]. Imaging flow cytometry achieves sensitivity comparable to FC by integrating the signal for longer time periods. The imaging system is of high resolution, comparable to 40x microscopy [17] and allows evaluation of cell morphology and marker localization in addition to FC-gating analysis. With a typical scan rate of 250–1000 cells/s [19], the throughput is insufficient for rare cell detection applications without an enrichment strategy.

2.2 Laser Scanning Cytometry

Laser Scanning Cytometry (LSC) is another method of combining FC-gating with high resolution microscope imaging. The sample is prepared on a microscope slide, and individual target cells can be revisited for additional analysis or imaging.

A focused laser beam of 2.5–10 micron diameter is raster scanned across the slide, and fluorescence emission is imaged at moderately high spatial resolution, comparable to 4x–20x microscope magnification [20, 21]. The use of collection optics with a high numerical aperture and photomultiplier detection enables very sensitive detection comparable to FC. LSC instruments are available with multiple excitation lasers and photomultipliers, and four labels can be measured simultaneously. The high resolution imaging enables quantitative analysis of protein expression. The throughput of LSC, however, varies with instrument settings, and is inversely proportional to the spatial resolution. With 10 micron spot size (yielding spatial resolution roughly equivalent to 4x microscope magnification) and optimal cell density on the slide, throughput of about 100 cells/s can be achieved [20]. As with imaging flow cytometry, the throughput is insufficient for rare cell detection without sample pre-enrichment [22].

2.3 Automated Digital Microscopy

Automated digital microscopy (ADM) combines fluorescence microscopy platforms with robotic motion control systems to automate the imaging process. Microscopes with sophisticated automation systems are available from multiple vendors, allowing for automated control of every feature on the microscope. While detection sensitivity of fluorescence typically suffers compared to FC particularly at low magnification, microscope-based methods generally have adequate sensitivity for the types of stains used for rare cell detection.

Sensitivity of rare cell detection is reported to be greater than 90%, and specificity can also be better than 1 false positive in 10^6 nucleated blood cells depending on the staining strategy used [14]. However, detection requires long exposure times, and no reported scanning rate exceeds 1,000 cells/s [23, 24]. Due to the speed limitation of ADM, pre-enrichment strategies are required to achieve reasonable imaging times [25, 26].

2.4 Optical Enrichment Using Laser Scanning

A hybrid approach of using laser scanning to locate CTCs followed by ADM imaging can be used to circumvent limitations of existing imaging systems by first performing a low resolution, high speed scan to locate the CTCs followed by high resolution imaging with an ADM. Without enrichment, a large number of leukocytes (50 M) need to be scanned, which requires a slide area of about 130 cm^2 at an optimal density of 4,000 cells/mm^2. Large substrates are beneficial to minimize edge effects. For example a slide of 64 cm^2 (Paul Marienfeld GmbH, Bad Mergentheim) can accommodate 25 M leukocytes. Existing microarray laser scanners are designed for substrates no larger than standard microscope slides and use the same lensing system to scan and focus excitation light onto the sample and

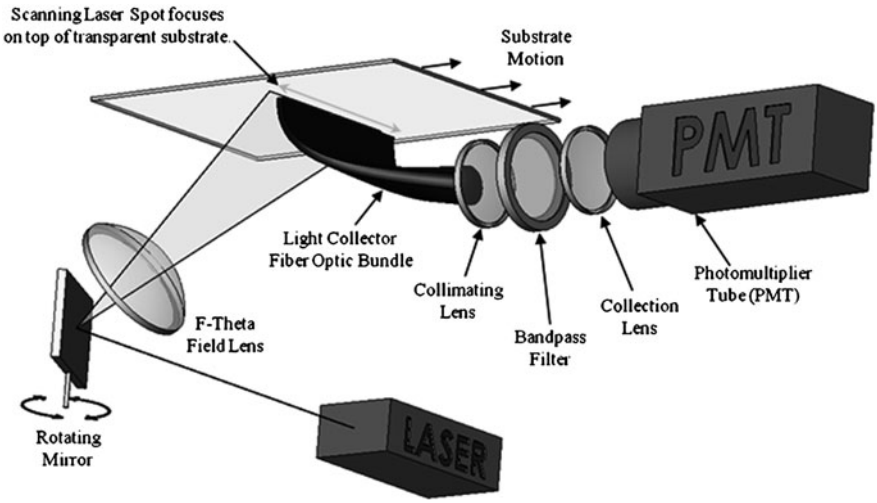

Fig. 1 The excitation laser is reflected from a rotating mirror and imaged through an F-theta lens to a focused spot (10 μm) that traverses the substrate with a constant speed (10 m/s). Emission is collected through the wide (76 mm) and narrow (1 mm) end of a bundle of optical fibers and is transmitted through a round (1 cm) aperture at the opposite end. The emitted light is collimated to $\pm 10^{\circ}$ for band pass filtering. This figure shows a filtering leg that collects 580 nm light. A second leg normal to the optical axis of the first leg that collects 525 nm light is not shown. The ratio of the emitted light at these 2 wavelengths is used to eliminate autofluorescence. Printed with the permission of IEEE

collect the fluorescent emission. This optical architecture requires either a lens that is larger than one dimension of the sample, with mechanical scanning in the orthogonal dimension [27] or requires scanning a small, light-weight lens across the sample [28, 29]. These architectures cannot be scaled to larger sample sizes without significant reduction in numerical aperture or scanning speed.

The use of fiber array scanning technology (FAST) enables a wide field of view without sacrificing either numerical aperture or speed [30]. Emission light is collected with a stationary fiber optic bundle that contains over 40,000 fifty-micron diameter fibers and is placed in close proximity to the substrate to maximize light capture. The numerical aperture of the glass bundle is 0.66. The scanning instrument employs laser printing optics to scan the laser at a high speed ($10\ ms^{-1}$) (Fig. 1). A single 488 nm laser is reflected from the faceted surfaces of a spinning polygon mirror and directed across the substrate. The laser reflects from one of 10 spinning facets enabling high scan speeds, and a $64\ cm^2$ substrate containing 25 M nucleated blood cells can be scanned in 60 s. Scanning is non-uniform but repeatable, allowing software compensation of nonuniformities. Testing with cultured HT29 cancer cells shows that detection sensitivity of the FAST cytometer is identical to that of automated digital microscopy. Resolution is limited to 10 μm by the diameter of the focused laser on the substrate. This

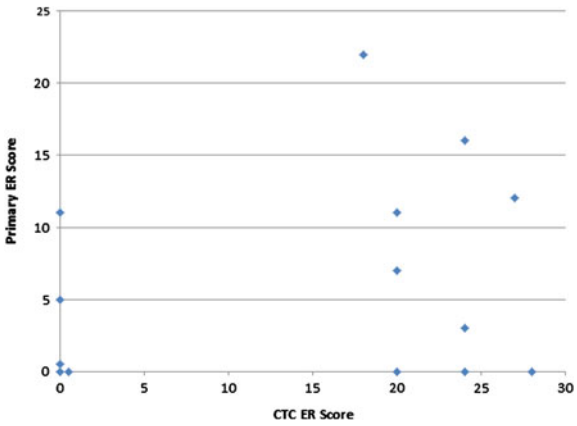

Fig. 2 Plot of ER score for the primary tumor versus ER score for CTCs for different patient samples. The CTC score is the average score for all CTCs in the sample times 10. The primary score is the percentage of expressing cells times the expression level divided by 10. ER scores for cells range from 0 to 3 to represent no, low, medium, and high expression levels. Six samples are discordant with the primary tumor using a cutoff of ≥1 as positive (n = 15). Two of these are actionable ER+CTC score and ER− primary score changing eligibility for hormone therapy

resolution is adequate for locating labeled objects but not for identifying them as CTCs [31]. The instrument accurately locates a relatively small number of objects to within 40 μm so that they can be relocated in an ADM for high resolution imaging, substantially reducing the number of exposures. The ADM images the multiple biomarkers (CK, CD45 and DAPI) needed for CTC identification as well as cancer biomarkers for predicting therapeutic efficacy.

The FAST scanner itself lacks inherent specificity, due to cell-scale resolution and detection of only a single fluorescent marker and without other measures will identify over 50,000 objects, the vast majority of which are autofluorescent debris or eosinophils. While these objects are easily eliminated with high resolution microscopy, imaging time would be prohibitive, so they are eliminated prior to imaging using a wavelength comparison technique. The technique involves measuring emissions at two different wavelengths, one at the target emission wavelength (580 nm) and the other at a wavelength intermediate (525 nm) between the target emission wavelength and the laser excitation (488 nm). Since labeled objects will have higher emission at the target emission, the ratio of emission in these two wavelengths is used to substantially eliminate autofluorescent particles. Filtering by 580 nm:525 nm ratio eliminates 99.8% of autofluorescent objects without loss of target CTCs. The typical specificity for a FAST scan is around 3×10^{-6}, resulting in 150 unfiltered CK-positive objects in a typical 10 ml sample containing 50 M leukocytes.

The FAST scanner has been used to study expression of cancer biomarkers (HER2, ER and ERCC1) in patients with advanced breast cancer using a multiplex assay [5]. HER2 expression was observed in 23% (n = 13) of patients who had a

HER2-negative primary tumor. These patients are of interest as they may respond to HER2 targeted therapy but are not eligible due to their primary phenotype. Likewise, ER expression was observed on CTCs in 40% (n = 5) of these patients whose primary was ER-negative (Fig. 2).

3 Discussion

Establishing medical value for CTC detection has focused on CTC count and change in CTC count to determine whether a patient with metastatic disease is responding to therapy [7, 32]. While some research has focused on the minimum acceptable criteria for clinical relevance in the enumeration of CTCs and CTC fragments, little attention has gone to determining proper prerequisites for biomarker quantification. For example CTC fragments containing nuclear material may provide useful DNA for mutation analysis, while mRNA and proteins are either lost or degraded.

Protein biomarkers are of particular interest in breast cancer because markers for ER and HER2 are widely used to predict efficacy of targeted therapies. A multiplexed assay that simultaneously evaluates two or more biomarkers would be desirable, in order to decrease processing and reagent costs. To ensure that the specific expression levels of the biomarkers are evaluated, only properly localized biomarker signal should be used. Such imaging places constraints on the sample preparation as well as the optical system.

Of the systems considered here, no single approach has the needed sensitivity, specificity, and speed to evaluate a large sample size without enrichment. Prescanning the sample can identify objects with adequate speed and sensitivity to enable automated microscopy for specificity and has been used with a multiplexed assay to evaluate three protein biomarkers in breast cancer patients.

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Size-Based Enrichment Technologies for CTC Detection and Characterization

Anthony Williams, Marija Balic, Ram Datar and Richard Cote

Abstract

The degree of metastatic outspread in malignant disease is one of the leading factors in determining the appropriate course treatment. Circulating tumor cells (CTCs) represent the population of cells that have acquired the means to gain access to the circulatory system, and the cell population ultimately responsible for the development of metastases at distant sites in the body. While promising as a biomarker for metastatic disease, the widespread study of CTCs has been limited by their rarity, as CTCs are reported to occur as infrequently as 1/mL of whole blood. In this text, we will discuss current and emerging technologies for the size-based enrichment of CTCs from whole blood, and compare some of the advantages and disadvantages of using a size-based approach to CTC enrichment versus affinity-based CTC enrichment platforms.

Contents

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1 Introduction

Metastatic disease accounts for 90% of cancer-related mortality, and is the most important determinant in clinical management of patients with cancer. The metastatic process is a highly complex set of events that involves the detachment of malignant cells from their primary site, invasion of peripheral tissues, and seeding of tumors at secondary sites. Metastasis occurs through local invasion of surrounding organs and tissues, spread through the lymphatic system, and entry into the circulatory system. The metastasis of tumors through circulating lymph and blood requires that tumor cells have acquired the ability to intravasate into vessel structures, survive in circulation, and extravasate from circulation at secondary sites to seed new tumors. Additionally, it has been shown that the metastatic process is inefficient; the overwhelming majority of tumor cells that break free from their primary sites will not have the ability to seed secondary tumors at distant sites. Therefore, it is logical to assume that for the successful formation of secondary tumors in lymph nodes and at sites distant from their origin, malignant cells must undergo a series of molecular changes at their primary site that allow them to migrate, as well as changes that make them capable of intravasation, anchorage-independent survival in circulation, extravasation, and colonization of tumors at the secondary sites.

Circulating tumor cells (CTCs) represent the population of cells that have acquired the means to gain access to the circulatory system, and the cell population ultimately responsible for the development of metastases at distant sites in the body. As a result, CTCs have emerged in recent years as a biomarker with strong potential to improve prognosis and diagnosis of recurrence, and the enumeration of CTCs with respect to progression free survival, overall survival, and therapeutic response has been widely reported in a number of malignancies [1–6]. In contrast to other sites where tumor cells may have disseminated, such as lymph nodes, bone marrow, and pleural effusions, assaying for CTCs requires only a simple, minimally invasive blood draw, providing a unique opportunity for repeated sampling in patients to monitor both metastatic disease as well as therapeutic response in real-time. Although promising as a biomarker, the evaluation of CTCs as a clinical assay has been hindered by their rarity; these cells occur as infrequently as 1 CTC/ml of whole blood. Thus, future efforts to investigate the role of CTCs in the metastatic process will be directly dependent on the development and easy availability of technologies for sensitive and efficient enrichment and isolation of CTCs. In this chapter, we will discuss traditional as well as emerging technologies developed for the size-based isolation of CTCs, and compare and contrast some of the advantages and disadvantages of size-based CTC isolation and enrichment with affinity-based CTC enrichment.

2 Size-Based Isolation of CTCs by Microfiltration

Size-based isolation of CTCs from whole blood is a technique that has been attempted since the 1960s, and has been revisited more recently. Utilizing the well-known characteristic that malignant cells are larger than surrounding normal blood

cells, CTCs are isolated by using filters fabricated with a defined pore size which allows for the passage of smaller blood cells to pass while capturing larger CTCs. Polycarbonate filters have been produced by particle track-etching, where a polycarbonate film is irradiated with ^{235}U fission fragments and small pores are later etched by incubation in warm sodium hydroxide [7]. More recently, Isolation by Size of Epithelial Tumor Cells (ISET), a size-based CTC isolation system has been developed, where a series of 12 polycarbonate filters are placed in parallel for CTC capture [8]. Briefly, whole blood samples are placed onto an automated device where they are drawn across the filter array by negative pressure. Later, the filters can be interrogated by IHC for identification and enumeration of CTCs, as well as other molecular techniques for CTC characterization. Pinzani and colleagues demonstrated using ISET the feasibility of laser capture microdissection (LCM) and qRT-PCR analysis of CTC post-capture, 27% of findings (12/44) of blood samples drawn pre-operatively from metastatic breast cancer patients positive for CTCs [9]. Additionally, evaluation of HER2 gene expression on CTCs isolated by LCM in 7 of the 44 patients analyzed showed strong correlation to HER2 gene amplification evaluated by FISH in corresponding primary tumors [9]. Hou et al. used ISET to investigate CTC in metastatic lung cancer, reporting an interesting finding that so-called circulating tumor emboli (CTM) recovered from blood may confer an anchorage-independent survival advantage through collective tumor cell migration, and could potentially contribute to the development of more aggressive disease and increased metastatic colony formation [10]. De Giorgi et al. investigated the presence of CTCs in cutaneous melanoma (currently unreported in any affinity-based CTC isolation platform), detecting CTCs in 29% of patients with primary invasive melanoma, 62.5% of patients with metastatic melanoma, and 0 in patients with in situ melanoma or from non-tumor, healthy blood donors [11]. CTCs detected by De Giorgi and colleagues in the same cohort showed strong correlation to tyrosinase expression evaluated by qRT-PCR [11]. As the ISET method for CTC analysis gains acceptance, others have begun pilot projects in various other malignant diseases [12]. While promising, however, pores deposited onto polycarbonate filters by particle track-etching are randomly dispersed, and often fused, creating larger than desired pore sizes, as displayed in Fig. 1. These confounding limitations can lead to low CTC recovery and filter clogging [13].

To alleviate these complications, our group has developed a novel membrane microfilter device for isolation of CTCs in blood by exploiting size differences between tumor and normal blood cells [13, 14]. The membrane microfilter device was designed with the intention to isolate tumor cells in a manner that provides a cheaper, better, and faster alternative to currently available methods of CTC enrichment. Our technology provides the opportunity to perform molecular characterization of CTCs beyond their enumeration, a critical step toward a better understanding of the mechanisms involved in their release, hematogenous spread, and colonization of organs at distant sites. Although based on similar principles for CTC enrichment, the fundamental differences between our novel technology and the ISET platform are (1) the material from which the filters are manufactured (i.e. parylene-C vs. polycarbonate), and (2) the manner in which the pores are deposited

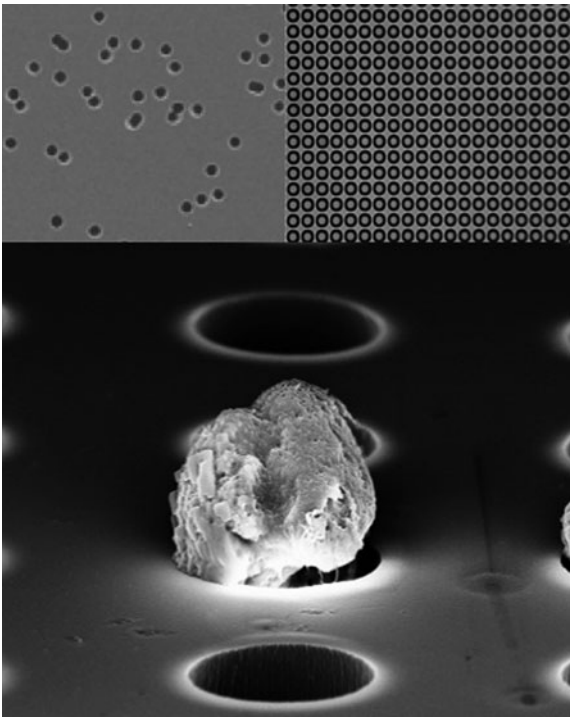

Fig. 1 Particle Track-Etching versus Parylene-Based Microfilters. *Upper left*; Polycarbonate filters have randomly and sparsely distributed pores with many of them fuse, resulting in larger openings that may contribute to the lower recovery rate published. *Upper right*; By contrast, our membrane has uniformly spaced pores, evenly distributed over a large filtering area. Below; SEM photo an MCF-7 breast cancer cell captured by the microfilter device in a model system

onto the membrane. Using parylene-C, microfilters are fabricated using a step-wise photolithography process. First, photoresist is spin-coated onto a silicon wafer. Next, parylene-C is conformally deposited to 10 μm thickness and patterned with oxygen plasma etching in reactive ion etching (RIE) technique, where either AZ9260 or Cr/Au is used as a mask layer. Finally, the whole film is released in acetone or photoresist stripper at 80° overnight. Within a 6 × 6 mm surface 40,000 pores are evenly distributed. Several different pore sizes and shapes were initially tested out. We concluded that the round pore shape with diameter 7–8 μm was ideal for capture and characterization of CTCs on the membrane. The final product of this process is a wafer with a set of 40–50 filters as illustrated in Fig. 2. The microfilter device is endowed with unique characteristics that make it ideal to enrich and isolate cells of interest from normal background cells in a size-based fashion.

In preparation for filtration, individual microfilters are removed from the wafer and placed into an acrylic housing device where it is sandwiched between two thin slabs of polydimethylsiloxane (PDMS), and clamped at each end to form an

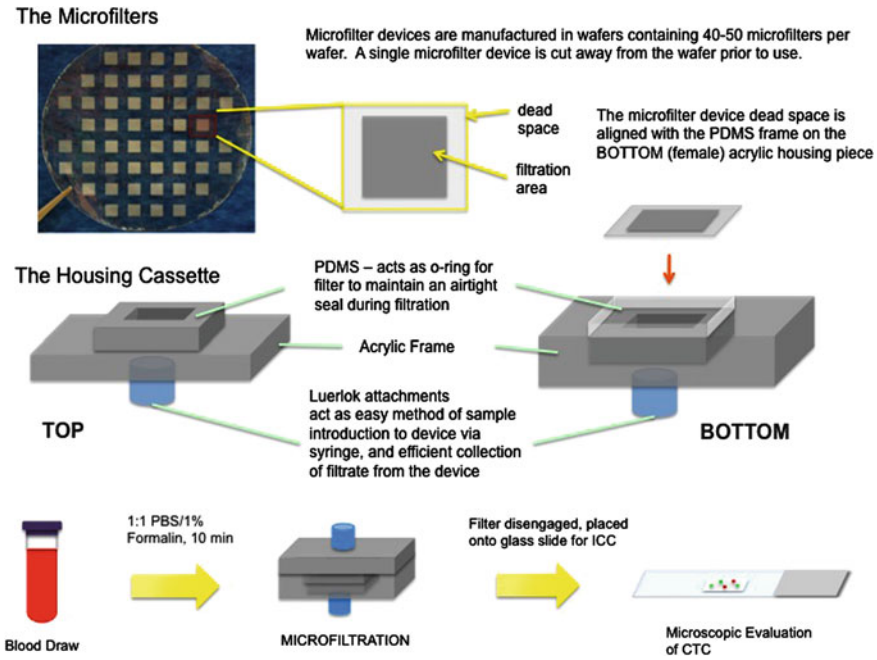

Fig. 2 The Membrane Microfilter Device. Microfilters are fabricated on wafers containing approximately 40–50 filters per wafer. Microfilters are cut away from the wafer and placed into an acrylic housing cassette clamped at both ends. Blood samples are diluted 1:1 in PBS and 1% formalin, and incubated for 10 min at room temperature. Following the brief incubation, the blood is raised into a syringe, fixed onto the luerlock, and passed through the microfilter with steady, low pressure. Following filtration, the filter is disengaged from the cassette and placed onto a glass slide for IHC and microscopic evaluation, as well as other molecular analyses

airtight seal (Fig. 2). Blood samples are collected into 10 ml tubes. Upon receipt, 7.5 ml whole blood samples are diluted 1:1 in 1 × PBS, and incubated in 1% formalin at RT for 10 min. Following partial fixation, blood is passed through the microfilter using a syringe fixed onto a luer lock with steady low pressure. In the case of the patient samples, the actual filtration takes under 2 min for the 15 ml final volume. Following microfiltration, the microfilter is disengaged from the device and placed onto a glass slide, and then subjected to immunofluorescence (IF), where a pan-cytokeratin/CD45 double staining protocol is used for positive selection of CTCs and negative selection of non-tumor blood cells, respectively. All cell analysis, including IF as well as other downstream molecular analyses (i.e. FISH, laser capture microdissection, DNA and RNA extraction), is performed directly on-membrane. We have evaluated the performance of the microfilter device in model systems and clinical samples, where in side-by-side comparison with the CellSearch platform, the microfilter device demonstrated superior sensitivity in both model systems and clinical blood samples [14]. Currently our efforts using the microfilter device are directed toward attempts at CTC

characterization in a phase III castration resistant prostate cancer clinical trial aimed at monitoring therapeutic response to two drugs, and gene expression profiling of CTC and corresponding metastatic breast cancer primary tumors from which they originated.

Although our microfiltration device is a potentially robust, powerful assay for CTC enrichment, it requires that samples be partially fixed in 1% formalin prior to filtration to preserve cell integrity and morphology. However, cross-linking introduced by formalin fixation eliminates the potential to perform functional studies on CTCs, such as subsequent cell culture or protein extraction and analysis, and could potentially diminish RNA quality for gene expression analysis. We have therefore begun developing the next generation microfilter platform based similarly on a size-based principle for CTC isolation that circumvents the requirement for fixation of blood samples, allowing capture of viable CTCs. In the viable cell capture design, we can precisely change the architecture of the filter by adding another membrane layer [15], or maintain a single-layer filter while changing the pore shape [16]. By adding a second membrane layer on top of the single membrane with a precisely controlled gap distance and pores that are slightly larger off-setting the pore positions of the lower membrane, small non-tumor blood cells are able to pass the top membrane, migrate laterally between the two membrane layers and pass the lower membrane [15]. Contrastingly, larger tumor cells are unable to migrate laterally between the two membranes and remain on the device. In model systems, we have demonstrated an ability to capture cells from normal healthy donor blood on the device, where they remain viable for at least 2 weeks [15]. A slightly different version of the device where the two membranes are separable, allowing for on-chip cell culture or mechanical release of captured cells onto other platforms (e.g. adherent culture flasks or matrigel), is also being developed. Using a 'slot' pore microfilter design, where 6×40 μm rectangular pores were prepared on single-layer parylene membranes, Lu et al. demonstrated an ability to capture cultured tumor cells in 1 mL of whole blood from a normal healthy donor with 90% capture efficiency and 90% cell viability by measuring telomerase activity by qRT-PCR [16].

3 Emerging Technologies for Size-Based Enrichment of CTCs

Even as microfiltration has been the primary tool for which size-based CTC enrichment technologies have been based upon, new emerging technologies have been developed more recently that capitalize upon size differences between CTCs and non-tumor blood cells. Bhagat et al. have developed a pinched flow coupled shear-modulated inertial microfluidic device for the isolation of CTCs, as well as other larger rare blood cells [17]. The microfluidic device is composed of a cell-focusing region, a rare cell-pinching region, and a collecting outlet in series. Due to shear-modulated inertial forces cells migrate along the channel sidewalls, and as they reach the pinching region, which has a diameter similar to that of a CTC, focuses larger cells with differential inertia along the axial center of the

microchannel while smaller non-tumor cells remain along the channel sidewalls. CTCs are then collected from the axial center, while smaller non-tumor cells are flowed through side outlet channels and removed. Using this device in model systems, Bhagat and colleagues demonstrated >80% recovery of cultured tumor cells spiked into blood from healthy donors, and a 10^4- and 10^5-fold enrichment of tumor cells above nucleated blood cells and red blood cells, respectively [17]. Gleghorn et al. have also developed a novel platform for CTC enrichment that is similar to the 'CTC-Chip' micropost array suggested by the Toner group [18]. However, through geometrically enhanced differential immunocapture (GEDI), the shape and the specific manner in which the tumor-specific antibody-functionalized microposts are arrayed on the their chip help to increase the collision frequency of larger CTCs while simultaneously decreasing the collision frequency of smaller non-tumor blood cells [19]. In a small set of 20 castration resistant prostate cancer blood samples, the GEDI microdevice was able to detect CTCs in 90% of cases [19].

4 Affinity-Based Versus Size-Based Methods for CTC Enrichment

Of the approaches commonly used for CTC detection and enrichment, affinity-based and size-based methods currently remain the only two that enrich CTCs in a sensitive and specific manner while also providing the opportunity for morphologic evaluation. The CellSearch platform is the most recognized affinity-based enrichment technology, and is currently the gold standard in CTC detection. But the CellSearch platform is limited in its ability to perform molecular characterization of CTC and is mostly restricted to enumeration. The CTC-Chip is an affinity-based platform that has the ability to perform molecular characterization of CTCs beyond only enumeration. However, the enrichment of CTCs using the CellSearch and CTC-Chip are based upon the expression of EpCAM. This limitation could provide for the escape of CTCs with little to no EpCAM expression from capture using these techniques, only isolating a homogenous sub-population of CTCs among a potentially heterogeneous population of metastatic cells with varying tumorigenic capacity. As our group has reviewed previously, it is unclear which sub-population of CTC the EpCAM positive fraction may belong to, including the cancer stem cell population in various malignancies [20]. Contrastingly, size-based approaches to CTC enrichment are tumor cell 'agnostic'; meaning that variation in the expression of EpCAM among different malignancies and different patients within the same malignancy has no effect on enrichment efficiency. Further, the ability to capture a heterogeneous population of CTCs with varying expression levels of different metastasis-associated markers may ultimately lead to more beneficial data defining the underlying mechanisms of the metastatic process. One of the primary concerns with the use of size to separate CTCs from non-tumor blood cells is the possibility that one may allow smaller, but still malignant cells to escape capture based on a defined cut off. Our group has investigated the potential for loss of smaller CTCs in model systems [13] and clinical blood samples (unpublished data), finding no significant loss of tumor cells in the

device flow-through. Additionally, the time required to process samples by microfiltration versus the affinity-based platforms herein is quite despairing. While the CellSearch platform is capable of processing up to 8 samples in about 3–4 h, our microfilter device and the ISET platform are capable of processing a 7.5 mL blood sample in 2–3 min [14]. Even with the microvortex-generating herringbone CTC-Chip (an updated version of the original CTC-Chip by Toner's group) recently described by Stott and colleagues, the maximal flow rate for ~80% capture efficiency was found to be 1.2 mL per hour [21]. Although the difference in sample processing time could be inconsequential in the research setting, the ability to process large sample sets quickly and efficiently could make size-based approaches to CTC enrichment more amenable for use in the clinical setting. Despite their potential limitations, the affinity-based technologies we have discussed have yielded the majority of clinically relevant data regarding CTCs and metastasis. As a result, the CellSearch platform remains the only commercially available FDA-approved technology to monitor therapeutic efficacy in patients with metastatic breast, prostate, and colorectal cancer [22]. By contrast, only one of the size-based technologies we have discussed, ISET, is commercially available, while many are in the early stages of development and require validation in large patient cohorts.

5 Conclusion

CTCs have become increasingly accepted as a biomarker with outstanding potential to improve therapeutic decision making for clinicians treating patients with cancer. Further, the molecular characterization of CTCs will help to reveal critical components of the mechanisms governing the metastatic process, and may ultimately lead to the development of novel targeted anti-cancer therapies and improvements in personalized patient management. Future efforts to investigate the role of CTCs in metastatic disease will be directly dependent on the development of sensitive, specific, and efficient technologies for their enrichment, a commodity that remains in high demand.

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Emerging Technologies for CTC Detection Based on Depletion of Normal Cells

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Abstract

Properly conducted, an enrichment step can improve selectivity, sensitivity, yield, and most importantly, significantly reduce the time needed to isolate rare circulating tumor cells (CTCs). The enrichment process can be broadly categorized as positive selection versus negative depletion, or in some cases, a combination of both. We have developed a negative depletion CTC enrichment strategy that relies on the removal of normal cells using immunomagnetic separation in the blood of cancer patients. This method is based on the combination of magnetic and fluid forces in an axial, laminar flow in long cylinders placed in quadrupole magnets. Using this technology, we have successfully isolated CTCs from patients with breast carcinoma and squamous cell carcinoma of the head and neck. In contrast to a positive selection methodology, this approach provides an unbiased characterization of these cells, including markers associated with epithelial mesenchymal transition.

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Contents

1 CTC Identification Relies on its Separation Strategy

With confirmation of cancer cells in the circulation over 50 years ago [1], one of the challenges has been developing technology with sufficient sensitivity and specificity to reliably examine the role of circulating tumor cells (CTCs) in cancer biology [2–5]. The prognostic and predictive relevance of CTCs as a validated biomarker has now been established by numerous studies in our institutions and by others [6–8]. Over the past several years cell separation technology has advanced substantially, and continues to evolve with research approaches which are of even greater sensitivity and suitable for rare CTC detection [9].

A milliliter of human blood contains an average of five billion RBCs, seven million WBCs, and 295 million platelets, and it is certainly a challenge to identify CTCs [10]. Given the generally accepted rarity of a CTC, (on the order of one cell per 1×10^6 nucleated cells) in the blood of patients with cancer, a general review of the literature will reveal a variety of techniques and procedures that either enrich and/or isolate, or merely detect and quantify CTCs. Older methods involving immunohistochemistry (IHC) with minimal pre-enrichment relied on direct human observation under the microscope of multiple slides [11]. This method was labor-intensive and time-consuming and had high false positivity. Newer approaches include the use of technologies, such as flow cytometry, in which CTCs are positively labeled with an antibody-fluorochrome conjugate, and molecular approaches, such as (reverse transcriptase-polymerase chain reaction) RT-PCR. However, for all three of these detection approaches, IHC, flow cytometry, and RT-PCR, it is highly advisable to use an enrichment step prior to the detection analysis [12].

2 Enrichment Methodologies

Properly conducted, the enrichment step can improve selectivity, sensitivity, yield, and most importantly, significantly reduce the time needed to perform the analysis. The enrichment process can be broadly categorized as positive selection versus negative depletion, or in some cases, a combination of both. An example of a positive selection system is the commercially available CellSearch™ System (Veridex LLC). The system is based on the enumeration of epithelial cells that are separated from blood by antibody-magnetic nanoparticle conjugates that target epithelial cell surface markers, EpCAM, and the subsequent identification of the CTCs with fluorescently labeled antibodies against cytokeratin (CK 8, 18, 19) and a fluorescent nuclear stain [8]. The CellSearch definition of a CTC is a nucleated cell lacking CD45 and expressing cytokeratins and EpCAM.

In negative depletion, what are believed to be normal hematopoeitic cells, such as CD45 positive cells, are targeted and subsequently removed, thereby enriching the blood cell suspension for the rare tumor cells. While less common than direct positive selection, such as with the use of the CellSearch system, a number of reports exist of the use of either red blood cell (RBC) lysis, or gradient separation to remove RBCs, followed by CD45 expressing cell removal, prior to analysis for potential CTCs. Both Iinuma et al. (2000) and Bilkenroth et al. (2001) used a Ficoll gradient to remove RBCs and targeted CD45 expressing cells with magnetic particles for further removal. They subsequently identified CTCs in these enriched peripheral blood samples from colorectal and renal carcinoma patients, respectively [13, 14]. Using a similar approach, Brakenhoff et al. (1999) and Partridge et al. (2003) identified disseminated tumor cells from the blood of head and neck of cancer patients [15, 16]. With respect to breast cancer, Tkaczuk et al., using an approach similar to the previously discussed approached, reported that negative depletion enrichment can isolate breast CTCs in all stages of breast cancer, including early stage breast cancer and that the number of CTCs correlated with disease outcomes and overall survival [17].

3 Advantages of CTC Pre-Enrichment by Depletion of Normal Cells (Negative Depletion)

Despite the success of the positive selection approach, there are significant limitations. Probably the greatest limitation is for a CTC to be detected, they must express the cell surface marker used to target the CTC. Commonly used positive selection technologies such as the CellSearch System and the CTC Chip [18] use antibodies targeting EpCAM (a commonly used epithelial cell surface marker).

However, increasing evidence suggests that not all tumors and not all CTCs express EpCAM. One study indicated that only 70% of 134 tumors with different histological types expressed EpCAM [19]. In another study, the number of cytokeratin-negative cells with aneusomy outnumbered cytokeratin positive cells [20]. In addition, cell surface epithelial markers can be lost in cell lines derived from disseminated tumor cells. For example, all micrometastatic breast cancer cell lines derived from the bone marrow displayed loss of epithelial cytokeratins (CK8, CK18, and CK19) and ectopic expression of vimentin commonly present in mesenchymal cells compared to tumor-derived breast cell lines [21]. The cells not expressing epithelial markers would be missed by positive selection techniques even though these cells may be the most clinically relevant indicators of a tumor's aggressive potential. This was experimentally shown by Sieuwerts et al. with breast cancer cell lines; the cancer cells with "normal like" phenotypes had the lowest recovery by CellSearch [22].

Depletion of normal cells prior to analysis (or negative depletion) has several other advantages including potential time/cost-efficiency and improved sample yield and purity allowing multiple biomarker analysis using immunocytochemistry [23] and RT-PCR [24]. Drawbacks to a negative selection methodology include the inability to obtain a high enough enrichment to be able to identify the "abnormal cells" or CTCs which exist in the specimen. Too many contaminating leukocytes may make it difficult to see the CTCs. Alternatively, a very high enrichment might result in the unintended loss of CTC, or other abnormal cells, with the removed normal cells. In addition, given that leukocytes are depleted using CD45 immunomagnetic separation, a CTC that expresses CD45 may be inadvertently removed from the sample, precluding its identification.

4 Depletion of Normal Cells Prior to Flow Cytometry or Other Optical Analyses

While a majority of the published studies on CTCs use a human observation of ICC to identify the cells, a number of studies use advanced electronic technology including flow cytometry and computer imaging. A purging of the sample of erythrocytes and PBMCs prior to FACS analysis is typically necessary in order to achieve the required high level of sensitivity as shown recently for a model of human breast carcinoma in athymic mice [25]. The mouse red blood cells were removed by lysis and the mouse PBMCs were removed by tagging with an anti pan-leukocyte antibody (anti CD45) attached to the magnetic bead followed by magnetic separation (EasySep kit from StemCell Technologies, Vancouver, BC, Canada). The limit of detection was one CTC in 10^5 mouse PBMCs based on a realistic metastatic tumor animal model and using a standard flow cytometer (four color XL-MCL from Beckman Coulter).

Rapid improvement in optical detection methods has opened the possibility of using laser scanning cytometry for CTC detection directly on blood smears on glass slides [26, 27]. A specialized system termed fiber-optic array scanning technology (FAST) has been tested on a model of metastatic colorectal tumor (HT29) spiked into whole blood from volunteer donors. The RBCs were removed by lysis and the remaining PBMC fraction was deposited on glass slides and stained for pan cytokeratin and cell nucleus markers. The combination of FAST screening followed by re-scanning of "hits" with a more conventional automated digital microscopy (ADM) resulted in average specificity of 1.5×1^{-5} and an average sensitivity of 98% at a scanning speed of 100 million PBMCs per hour (equivalent to approximately 5 mL whole blood per hour, excluding sample prep time).

Interestingly, the authors applied their technology to check for false negative results of CTC detection by a positive immunomagnetic CTC separation method based on expression of the epithelial cell adhesion molecular (EpCAM) marker. They reported two potential issues with the positive CTC enrichment when compared to FAST + ADM scan: (1) the positive immunomagnetic separation (using MACS microbeads and MiniMACS columns from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) leads up to 50% CTC losses in the process and (2) furthermore, the positive immunomagnetic separation is highly sensitive to EpCAM marker down-regulation. The EpCAM and HER2/neu marker down-regulation has been observed in clinical studies of metastatic cancers [27–30]. These would not be an issue with the negative CTC enrichment, proposed in this study, because it does not rely on expression of any particular CTC marker.

5 Magnetic Depletion Technologies

The enrichment for targeted cells by depletion of the unwanted cells is a general strategy beyond the search for CTCs, and specialized magnetic separation instrumentation and reagents are available commercially [31, 32]. We have recently developed a negative CTC enrichment strategy that relies on a combination of viscous flow that facilitates recovery of the unlabeled cells (CTC) and the magnetic force that traps the labeled cells (leukocytes) from whole blood samples obtained from cancer patients [24, 33, 34]. The method is based on the combination of magnetic and fluid forces in an axial, laminar flow in long cylinders placed in quadrupole magnets [35–46]. The combination of magnetic and viscous shear forces, using specifically designed geometries and magnetic fields, lowers the likelihood of non-specific CTC losses, below those encountered during magnetic separation from static suspensions [17]. The method combines advantages of using flow and the magnetic field to achieve high throughput (mL/min) and high enrichment rates (by as much as 10,000-fold, i.e., increasing the CTC frequency in the sample, e.g., from 1:100,000 to 1:10) [24].

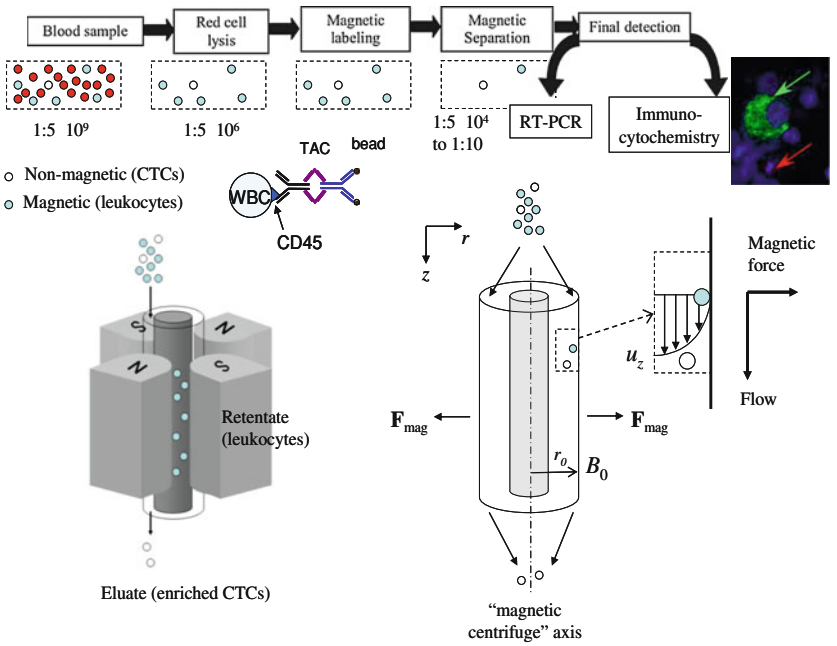

Fig. 1 CTC enrichment by magnetic depletion of normal cells. Top: Flow diagram of the experimental protocol indicating approximate number ratios of CTCs to normal cells. Bottom: Quadrupole magnet and annular shell separation channel for separation of non-magnetic CTCs from normal cells (tagged with tetrameric antibody complex, TAC) using magnetic and viscous shear flow forces. The radial magnetic forces act as a type of "magnetic centrifuge" on the magnetically tagged normal white blood cells (WBCs) retaining them inside the channel while the CTCs are washed out by the flowing fluid

6 Clinical Results With CTCs or Cancer Associated Cells Identified With Negative Depletion

6.1 Breast Cancer

Using our enrichment system presented in Fig. 1, in an ongoing study we have identified CTCs in all breast cancer stages and elevated CTCs pretreatment or after one cycle of treatment. This negative depletion yielded an average $\log_{10}$ depletion of nucleated cells of 2.74 and an overall, average $\log_{10}$ depletion of 5.2 (> 100,000 enrichment). CTCs were detected in both localized, non-metastatic, and metastatic breast cancer patients and staining for mesenchymal and stem cell markers was successful [47, 48]. No CTCs have been identified in healthy volunteers and in buffy coats purchased from the Red Cross.

Figure 2 is a set of photographs of microscopic images of an immunocytochemically stained, peripheral blood from stage I through IV breast cancer

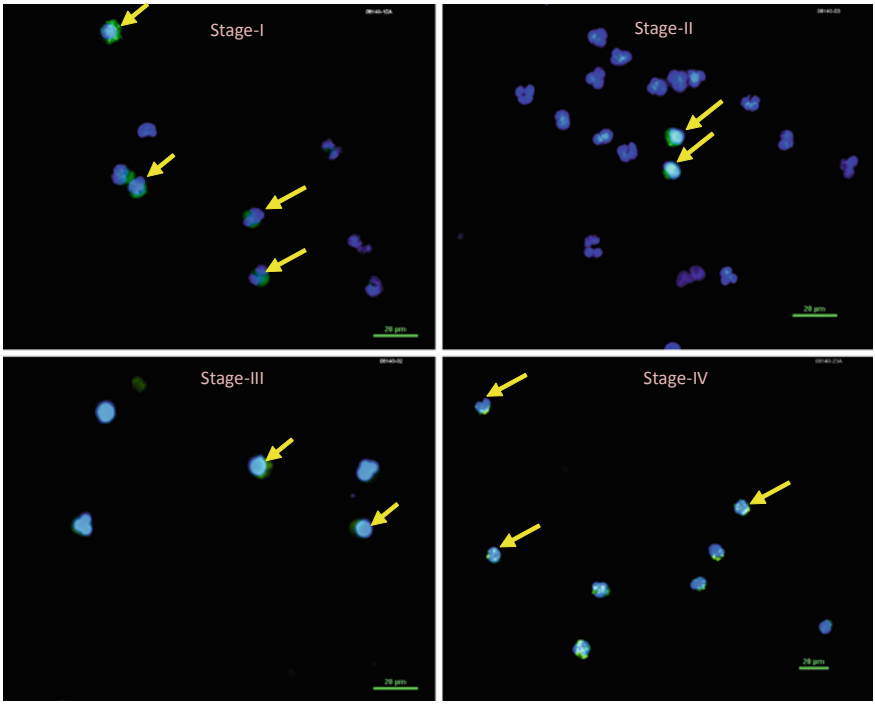

Fig. 2 CTCs in stages I–IV breast cancer obtained by magnetic CD45 + cell depletion as shown in Fig. 1. Nuclear staining with DAPI and cykeratin staining with pan cytokeratin 8/18/19 was used. Cells staining for both were counted as CTCs. The apparently normal volunteer donor blood was used as a control and showed no visible staining (results not shown)

patients. Nuclear staining with DAPI (blue color) and cytokeratin staining with pan cytokeratin 8/18/19 (green color) were used. Cells staining for both were counted as CTCs. The apparently normal volunteer donor blood was used as a control and showed no visible staining (results not shown).

An argument can be made that this approach does not conclusively prove that the cells that are positive for nuclei and cytokeratins are also negative for CD45, despite magnetic depletion for CD45. To address this concern Fig. 3 is presented, which is a set of representative, multiparameter flow analysis of a blood sample from a woman with metastatic triple negative breast cancer prior to therapy. The top row is pre-enrichment and the bottom row is post-enrichment by our magnetic CD45 + cell depletion approach. For this specific enrichment, a 3 $\log_{10}$ of the nucleated cells, based on hemocytometer counting, was obtained (i.e. a 1000-fold enrichment). The first column on the left-hand side of this figure is the ungated dot plot of the side scatter versus forward scatter is presented. A clear decrease in the location of a typical lymphocyte population in the dot plots is observed. The next column on the left is the CD45 stained population. Even after CD45 depletion, a population of CD45 is still present, even after the significant lymphocyte

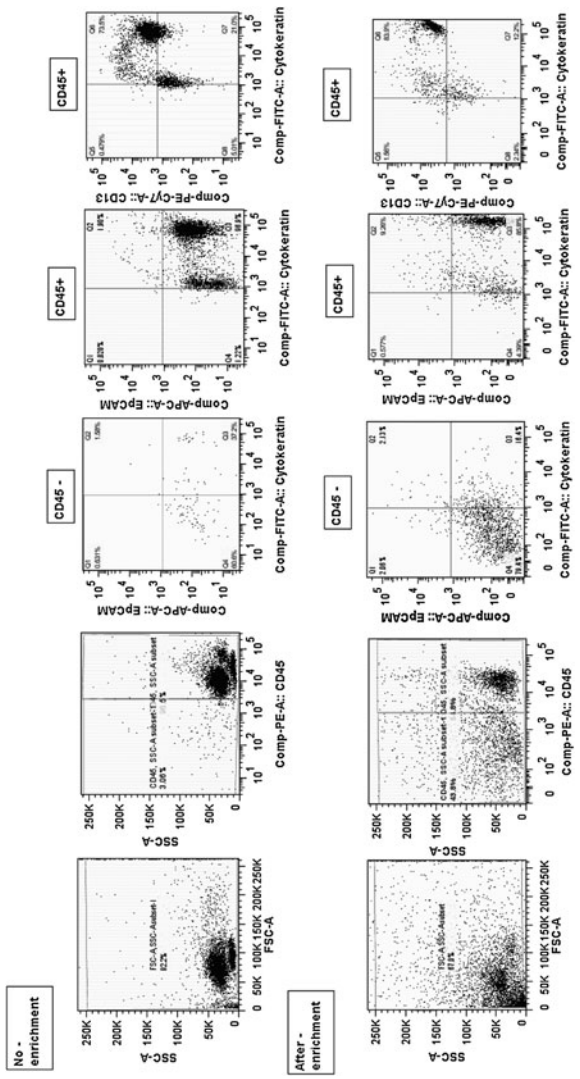

Fig. 3 Multiparameter flow analysis of a blood sample from a woman with metastatic triple negative breast cancer prior to therap. Top row is pre-enrichment and the bottom row is post-enrichment by magnetic CD45 + cell depletion. Note the presence of cytokeratin + and CD45 ± cells after enrichment. Also note the large number of CD45 + cells that are cytokeratin positive and all CD13 positive. Considering efficient depletion of lymphocytes the data show that the CD45 + cells in the depleted fraction are all CD13 positive and cytokeratin positive

depletion. The next column is further analysis of the CD45− population, gated in the second column. The CD45− population, after the magnetic enrichment, clearly has events that are positive for cytokeratin and mostly negative for EpCAM, although a number of EpCAM and cytokeratin positive events are present. Interesting, the CD45+ population, the fourth column from the left, has highly cytokeratin positive cells, mostly negative for EpCAM, but a noticeable number positive for both EpCAM and cytokeratin. Finally, all of these cytokerain positive cells are positive for CD13. CD13 can be expressed on granulocytes, monocytes, endothelial, and epithelial cells. These results, using this negative enrichment process, present a number of potentially interesting subpopulations for further

analysis, and also suggest that the absolute number of CTCs may not be the most relevant biomarkers as the majority of these cells are probably neither capable of establishing metastatic niches nor becoming dormant [49]. Further characterization of this subpopulations and molecular marker analysis may elucidate pathways for the development and progression of metastatic disease which can be used to develop novel targeted therapies.

7 Squamous Cell Carcinoma of the Head and Neck

In squamous cell carcinoma of the head and neck (SCCHN), another epithelial malignancy, there are limited studies on CTCs in the literature to date. Partridge et al. (2003) used a negative depletion methodology to identify disseminated tumor cells in SCCHN patients. The detection of disseminated tumor cells pre-operatively or intra-operatively indicated an increased risk of local/distant recurrence and decreased survival [16]. Using flow cytometry, Hristozova et al. (2011), reported that detection of CTCs (CD45-CK + EpCAM +) in inoperable SCCHN, correlated with a higher incidence of regional metastasis and that concurrent chemoradiotherapy reduced their frequency [50].

Our published early prospective clinical results of 48 patients with SCCHN with a mean follow-up of 19 months, showed a statistically significant worse disease-free survival in patients with CTC present in the blood taken at the time of surgical resection ($p = 0.01$), [51]. There was no correlation between the presence of CTC and tumor site, overall stage, nodal status, smoking or alcohol use, or the use of adjuvant therapy. On a number of samples, using Confocal microscopy with multimarker staining, we have found expression of other markers on the CTC from SCCHN patients, including EGFR, CD44, and vimentin. We have optimized our detection methodology to be able to obtain an overall average enrichment of 5.66 $\log_{10}$, and as high as >7 $\log_{10}$ total enrichment of CTCs in the blood of patients with SCCHN. Using our technique, we have identified 0 to over 3000 CTCs per mL of blood collected from SCCHN patients.

8 Epiethelial Mesenchymal Transition

The ectopic expression of vimentin, with the corresponding loss of cytokeratins is a proposed mechanism for the epithelial to mesenchymal transition (EMT). This process is hypothesized to be a marker for aggressiveness of tumor cells to establish metastatic sites [21]. Recent reports indicate that EMT potentially takes place during tumor cell invasion. Such a transition is characterized by the decrease in epithelial markers and the increase in mesenchymal markers [52–55]. Previously published studies show that more aggressive breast cell lines and tumors, such as basaloid subtype, [56, 57] have mesenchymal markers and increased stem cell markers [58–61]. It is possible that these cells have undergone EMT, a highly regulated process during which tumor cells lose epithelial characteristics and gain

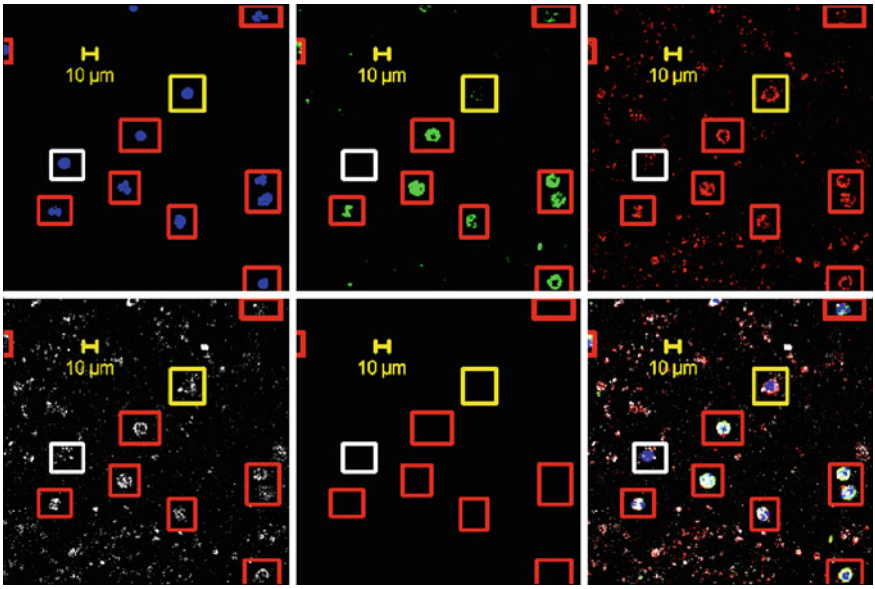

Fig. 4 Representative, confocal images from a negatively depleted blood sample from a patient with stage IV triple negative breast cancer. Markers shown are with DAPI/nuclei, FITC/cytokeratins, AF594/vimentin, and APC/CD44. In the composite, the cells in red boxes are positive for all four markers, while cells encircled with yellow boxes are negative for cytokeratins, yet positive for the other three markers. White boxes are cells negative for cytokeratins and vimentin, and CD44, positive for DAPI/nuclei

invasive mesenchymal and stem cell-like features [54, 62–64]. In contrast, less aggressive breast cancer lines, such as hormone receptor positive cells, have less mesenchymal features [65, 66]. Although conclusive evidence for EMT in vivo has not been established, there is emerging evidence for the role of EMT in generating mammary stem cells as a model for breast cancer invasiveness and metastases [58]. Positive selection technology, such as CellSearch has been shown to miss up to 98% of cells with high CD44 expression, and low EpCAM expression. Recently, CTCs with mesenchymal and stem cell markers, based on RT-PCR analysis of blood samples, was reported in the metastatic setting [67, 68]. However, the analysis was limited to a small set of markers by RT-PCR and not combined with the benefits of direct visualization with ICC.

Our work has shown that both CTCs from SCCHN and breast cancer express markers associated with EMT. Figure 4 is a representative set of confocal images from a negatively depleted blood sample from a patient with stage IV triple negative breast cancer. Markers shown are with DAPI/nuclei, FITC/cytokeratins, AF594/vimentin, and APC/CD44. In the composite, the cells in red boxes are positive for all four markers, while cells encircled with yellow boxes are negative for cytokeratins; yet positive for the other three markers. White boxes are cells negative for cytokeratins, vimentin, CD44, and positive for DAPI/nuclei.

Additional studies are needed to better understand the role of this subpopulation of CTCs in both women with localized, early stage, and metastatic breast cancer and to assess how these expression markers change with treatment. Investigating the up-regulation of mesenchymal markers in CTCs provides a valuable opportunity to understand mechanisms underlying metastasis which may lead to future novel therapies. The concept that CTC directly contributes to the metastatic process and undergoes EMT is an intriguing hypothesis. However, limitations in the current methods of CTC collection and phenotypic characterization with positive enrichment (EpCAM-based) technology have impaired efforts to test this clinically relevant hypothesis. A negative depletion method can help to eliminate the selection bias of CTCs, to provide an objective assessment of any atypical cells found in the blood.

9 Current Assumptions about "Normal Cell"

In addition to the currently accepted definition of a CTC (Cytokeratin and/or EpCAM positive nucleated cell that is CD45 negative), multiple groups are beginning to note other atypical cells in the blood of patients with cancer. These include CD45 positive cells that also have cytokeratin or EGFR. These cells have been called "double positives" by some groups including Toner et al. because they have both hematopoietic and epithelial markers. These findings raise the question: can a CD45 positive cell be a cancer cell? The exact origins of these cells are still under debate. The possibilities include the fusion of hematopoietic cells to circulating cancer cells, non-specific binding of CD45 antibodies to isolated cells, or most intriguingly cancer cells originating in the bone marrow with stem cell-like features. Additional studies are underway to help answer these questions.

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Molecular Assays for the Detection and Characterization of CTCs

Evi S. Lianidou and Athina Markou

Abstract

Molecular characterization for circulating tumor cells (CTCs) can be used to better understand the biology of metastasis, to improve patient management and help to identify novel targets for biological therapies aimed to prevent metastatic relapse. New areas of research are directed towards developing novel sensitive assays for CTC molecular characterization. Towards this direction, molecular detection technologies that take advantage of the extreme sensitivity and specificity of PCR, offer many advantages, such as high sensitivity, specificity, and significant flexibility in the clinical lab setting, in terms of high-throughput analysis, multiplexing, and quality control issues. Using molecular assays, a variety of molecular markers such as multiple gene expression, DNA methylation markers, DNA mutations, and miRNAs have been detected and quantified in CTCs in various cancer types, enabling their molecular characterization. Here, we present the main molecular detection technologies currently used for CTC analysis and molecular characterization.

Contents

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1 Introduction

Circulating tumor cell (CTC) analysis is a promising new diagnostic field toward the estimation of risk for metastatic relapse or metastatic progression [1–3]. Especially in breast cancer, CTC detection and enumeration have been established in several clinical studies, showing a correlation with decreased progression-free survival and overall survival in operable [4–9] and metastatic breast cancer [10].

Different analytical systems for CTC isolation and detection have been developed as immunocytochemistry and molecular assays, most including separation steps by size or biological characteristics such as expression of epithelial or cancer-specific markers. Recent technical advancements in CTC detection and characterization include multiplex quantitative reverse transcription-PCR (RT-qPCR) based methods, image-based approaches, and isolation technologies like microfilter and microchip devices [11]. New areas of research are directed toward developing novel sensitive assays for CTC molecular characterization. The molecular characterization of CTC can provide important information about the molecular and biologic nature of these cells. This is very important for the identification of therapeutic targets and resistance mechanisms in CTC as well as for the stratification of patients and real-time monitoring of systemic therapies [11, 12].

Here, we present the main molecular detection technologies currently used for CTC analysis and molecular characterization.

2 Molecular Detection Technologies for CTC Analysis

Molecular assays are based on the analysis of nucleic acids and take advantage of the extreme sensitivity and specificity of PCR. They are high-throughput and easy to perform since they are based on the isolation of total RNA from viable CTC, and subsequent RT-PCR amplification of tumor or epithelial specific targets. Especially, RT-qPCR assays can be *in silico* (through the use of specific software programs) designed, easily automated, and subjected to internal and external quality control systems [13].

The major advantages of molecular assays are the following: (a) they give information only for living cells since RNA is very sensitive, (b) a variety of molecular markers (gene expression, DNA methylation markers, DNA mutations, etc.) can be detected and quantified in CTC, enabling their molecular characterization, (c) offer extreme sensitivity; through this technology a small number of

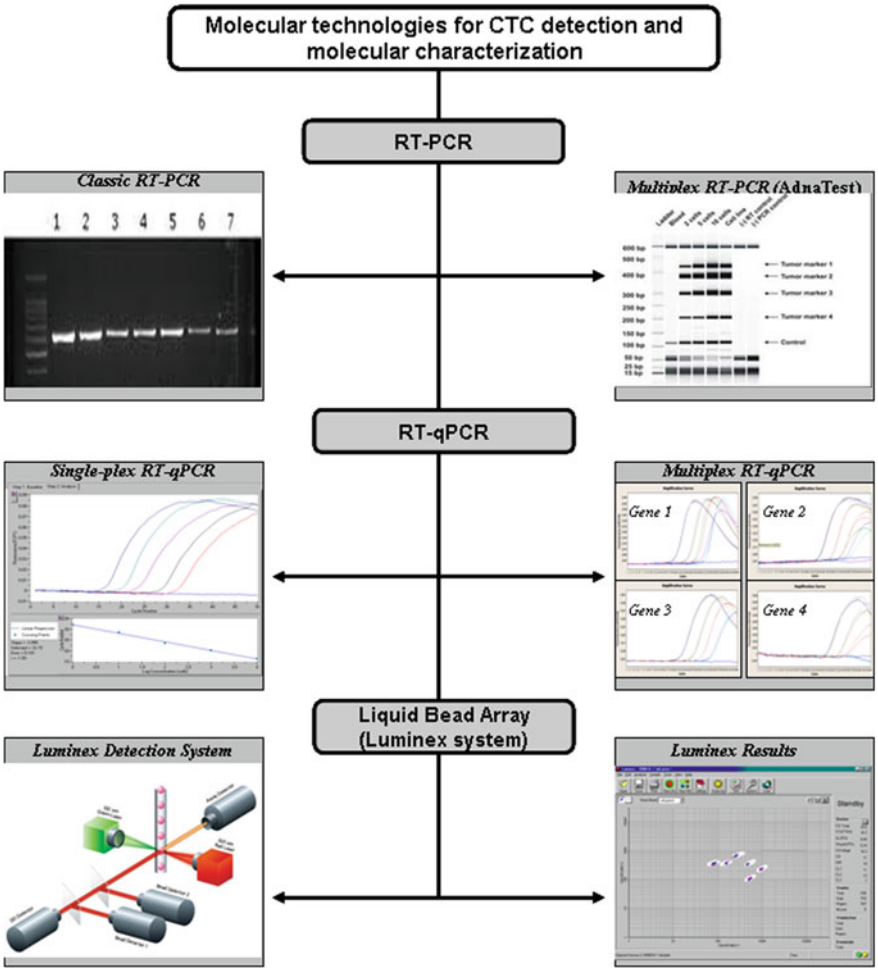

Fig. 1 Outline of the main approaches for CTC molecular detection technologies

CTCs can be detected through the highly sensitive detection of epithelial markers in the presence of millions of peripheral blood mononuclear cells, (d) offer a significant flexibility in the clinical lab setting, in terms of high-throughput analysis, multiplexing, (thus reducing the amount of sample required), time and cost of analysis and quality control issues. The main disadvantage of molecular assays by this approach is the fact that unless combined with imaging systems it is not possible to obtain any morphological information about CTC. Moreover, we cannot estimate accurately the number of CTCs present in a sample, since a different number of transcripts can be expressed from different cells. An outline of the main approaches for CTC molecular detection technologies is presented in Fig. 1.

2.1 RT-qPCR

RT-PCR methodology for the detection of micrometastases in patients with breast cancer has been based initially on the estimation of the number of *CK*-19 transcripts in blood and bone marrow samples [14]. Our group has developed for the first time a highly sensitive and specific real-time RT-qPCR assay for *CK*19-mRNA [15, 16] and evaluated its sensitivity, specificity and clinical potential for the molecular detection of occult carcinoma cells in peripheral blood of breast cancer patients [6–9]. In parallel, many different molecular assays based on RT-PCR, specifically designed for different gene transcripts in CTCs have been developed, such as mammaglobin [17], and EGFR [18–20]. By using a multimarker RT-PCR assay for CTC in early breast cancer, we have shown that *CK*-19, *Mammaglobin* and *HER*2- positive CTC are associated with shorter disease-free survival [7].

2.2 Multiplex RT-qPCR

Several mRNA markers have been already used for RT-PCR-based detection of CTCs. Quantification of these mRNAs is essential to distinguish normal expression in blood from that due to the presence of CTCs. Few markers provide adequate sensitivity individually, but combinations of markers may produce good sensitivity for CTC detection. An important limitation of most available methodologies for CTC analysis is the small number of gene targets that can be analyzed, due to the limited amount of available samples. However, identification of specific subtypes of CTC based on the expression of an increasing number of cancer important genes can provide information about the biology of metastasis and improve patient management. To be effective, the method used to identify CTC must detect all tumor cell types. However, the fact that CTCs are very rare and the amount of available samples is very limited presents a tremendous analytical and technical challenge [21, 22]. Multiplex RT-PCR assays for CTC analysis can overcome these problems.

By using RT-qPCR Obermayr et al. have shown that a panel of six genes was found superior to EpCAM and mammaglobin for the detection of CTC in breast cancer, and they may serve as potential markers for CTC derived from endometrial, cervical, and ovarian cancers as well [23]. Reinholz et al. have shown that molecular characterization of circulating epithelial cells using mammaglobin and B305D-C offers potential for early detection of invasive breast cancer [24].

By using the commercially available AdnaTest BreastCancer (AdnaGen AG, Germany) kit, (based on the enrichment of CTCs from peripheral blood of breast cancer patients followed by identification of CTC-associated marker transcripts by reverse transcription and multiplex PCR), Aktas et al., have detected EpCAM, MUC-1 and HER2 transcripts in CTC and found that a major proportion of CTC in metastatic breast cancer patients showed Epithelial Mesenchymal Transition (EMT) and tumor stem cell characteristics [25]. Interestingly, when the expression of the ER and PR was

assessed in CTCs by RT-PCR, the spread of CTCs was mostly found in triple-negative tumors and CTCs in general were mostly found to be triple-negative regardless of the ER, PR, and HER2 status of the primary tumor [26].

A quantitative gene expression profiling methodology based on RT-qPCR, specific and sensitive to detect one CTC was performed by using a set of genes with no or minor expression by leukocytes [27]. We have recently developed a highly sensitive and specific multiplexed quantitative RT-qPCR to detect the expression of six genes, (*CK*-19*, MAGE-A*3*, HER*-2*, TWIST*1*, hTERT* α+β+, and *mammaglobin)* and validated it in CTC of early and metastatic breast cancer patients [28].

2.3 Liquid Bead Array

A highly sensitive and specific multiplexed PCR-coupled liquid bead array to detect the expression of multiple genes in CTC has been recently developed [29]. By using this approach, six established CTC gene targets; *HER*-2, mammaglobin *(hMAM), CK*-19*, MAGE-A, TWIST*-1, and *PBGD* were simultaneously amplified and detected in the same reaction, in a very limited amount of CTC samples thereby saving precious samples and reducing the costs and time of analysis This novel assay forms the efficient basis for a multiplex approach to study the expression of up to 100 genes in CTC.

3 Molecular Characterization of CTCs

Molecular characterization for CTCs can be used to better understand the biology of metastasis, to improve patient management and help to identify novel targets for biological therapies aimed to prevent metastatic relapse. The role of CTCs in treatment failure and disease progression could be explained by their relation to biological processes, such as epithelial-to-mesenchymal transition and tumor dormancy [30]. Identifying metastatic stem cells through molecular characterization approaches in the CTC population might result in the development of new therapeutic concepts. CTCs are highly heterogeneous [1, 25–29, 31, 32] and this is highly important especially in the case that therapeutic targets are expressed in CTCs and not in the primary tumor. We present the main findings of the application of molecular detection technologies for the molecular characterization of CTC (summarized in Table 1).

3.1 Gene Expression Studies

CK-19. As already mentioned above, CK-19 is the most widely used epithelial marker in molecular assays for CTC detection and enumeration [6–9, 14–20].

Table 1 Molecular characterization of CTCs based on molecular detection technologies

Markers expressed in CTCs	Cancer type	Analytical methodology	Refs
Gene expression			
CK-19	Breast cancer/early: before adjuvant chemotherapy	Nested RT-PCR	[4]
CK-19	Breast cancer/early: after adjuvant chemotherapy	Nested RT-PCR	[5]
CK-19	Breast cancer/early: node negative patients	Real-time RT-qPCR	[6]
CK-19	Breast cancer/early: after adjuvant chemotherapy	Real-time RT-qPCR	[9]
CK-19, hMAM, HER-2	Breast cancer/early	Multimarker RT-PCR	[7]
HER-2	Breast cancer/ operable: after adjuvant chemotherapy	Nested RT-PCR	[40]
hMAM	Breast cancer/ operable	Nested RT-PCR	[17]
ER/PR	Breast cancer/early	RT-PCR	[26]
CK-19, HER-2, MAGE-A3, hMAM, TWIST-1, hTERT $a+\beta+$	Breast cancer/early and metastatic	Multiplex RT-qPCR	[28]
CK-19, HER-2, MAGE-A3, hMAM, TWIST-1	Breast cancer/early and metastatic	Liquid bead array	[29]
CK-19, HER-2	Breast cancer/patients prior chemotherapy	RT-PCR	[37]
EpCAM, MUC1, HER-2, ER, PR	Breast cancer/ metastatic	AdnaTest	[35]
DNA mutations			
EGFR	Non small cell lung cancer	DNA extraction from CTC, SARMS assay (Real-time PCR for mutation detection)	[45]
BRAF V600E	Melanoma	DNA extraction from CTC, peptide nucleic acid-clamping PCR assay	[46]
Androgen receptor	Castration resistant prostate cancer	DNA extraction from CTC	[47]
KRAS	Colorectal cancer	DNA extraction from CTC, digital PCR mutation detection assay	[48]
MiRNAs			
10 MiRNAS identified as differentially expressed in CTC	Breast cancer	Real-time RT-PCR	[49]

HER2. HER2 analysis in CTC may have clinical significance for HER2-targeted therapy. There is now a growing body of evidence that HER2 status can change during disease recurrence or progression in breast cancer patients [33]. Based on this, re-evaluation of HER2 status by assessment of HER2 expression on CTC is a strategy with potential clinical application [34–41]. Therapy-resistant CK-19 mRNA-positive cells in peripheral blood could be effectively targeted by trastuzumab administration [37]. Moreover, the detection of HER2 mRNA-positive CTC after the completion of adjuvant chemotherapy may provide clinically useful information concerning the efficacy of treatment and the prognosis of patients with operable breast cancer [40]. Changes of HER2 status in CTC compared with the primary tumor during treatment for advanced breast cancer have also been reported [40, 41]. Detection of HER2 mRNA-positive CTC after the completion of adjuvant chemotherapy may provide clinically useful information concerning the efficacy of treatment and the prognosis of patients with operable breast cancer [42].

ER/PR. The expression of estrogen receptor (ER) and progesterone receptor (PR) was assessed in CTC by RT-PCR by Fehm et al. [26]. According to their findings, the spread of CTCs was mostly found in triple-negative tumors and CTCs in general were mostly found to be triple-negative regardless of the ER, PR, and HER2 status of the primary tumor [26].

Mammaglobin. Mammaglobin expression has also been reported in CTCs and has been correlated with prognosis in patients with breast cancer [7, 17, 24].

EGFR. Payne et al. have shown that measurements of EGFR expression on circulating tumor cells are reproducible over time in metastatic breast cancer patients [18], while Liu et al. have shown that eradication of EGFR-positive circulating tumor cells and objective tumor response with lapatinib and capecitabine [19].

Cancer stem cell markers and EMT markers in CTCs. The expression of stemness and EMT markers in CTCs was associated with resistance to conventional anti-cancer therapies and treatment failure, highlighting the urgency of improving tools for detecting and eliminating minimal residual disease [43]. Although the relationships between EMT and CTCs remains largely unexplored, data validating the implication of EMT processes in CTC formation and animal models with transplantable human breast tumor cells to help characterizing EMT/CTC relationships have been recently reviewed. Indeed, through many different studies it has been shown that subsets of CTCs have a putative breast cancer stem-cell phenotype, and express EMT markers. The expression of cancer stem cell markers such as CD44, CD24, or ALDH1 by molecular assays [25] has also been shown in CTC.

3.1.1 DNA Methylation

In a recent study, Chimonidou et al. [44] analyzed DNA extracted from EpCAM-positive immunomagnetically selected CTC fraction for the presence of methylated and unmethylated *CST6*, *BRMS1*, and *SOX17* promoter sequences by methylation-specific PCR (MSP). According to this study, these tumor suppressor and metastasis

suppressor genes are epigenetically silenced in CTCs isolated from peripheral blood of breast cancer patients. These findings add a new dimension to the molecular characterization of CTC.

3.1.2 Mutations

Moreover, the molecular characterization of CTC may provide a strategy for noninvasive serial monitoring of tumor genotypes during treatment. Few studies till date have evaluated the presence of specific DNA mutations in CTC. Maheswaran et al. have captured highly purified CTC from the blood of patients with non-small-cell lung cancer (NSCLC) using a microfluidic device containing microposts coated with antibodies against epithelial cells and performed EGFR mutational analysis on DNA recovered from CTC using allele-specific PCR amplification. In this way they identified EGFR activating mutation in CTC for the first time [45]. Kitago et al. have tested for the expression of a melanoma-associated gene panel (MLANA, MAGEA3, and MITF) with RT-qPCR and for the presence of BRAFmt (a BRAF gene variant encoding the V600E mutant protein) in immunomagnetic beads—isolated CTCs from melanoma patients. By using a sensitive peptide nucleic acid-clamping PCR assay for BRAFmt analysis, they detected BRAFmt in 81% of the 21 assessed stage IV melanoma patients [46]. Moreover, Jiang Y et al. have recently detected coding mutations in the AR (androgen receptor) gene in CTC isolated from patients with castration-resistant prostate cancer, by using the CellSearch system for CTC isolation and subsequent molecular analysis in DNA isolated from CTC [47]. Punnoose et al. have shown that nucleic acids prepared from CTC captured using the CellSearch RUO profile kit were also amenable to biomarker assays including an RT-qPCR gene expression assay for breast cancer molecular subtype and a PCR assay for KRAS mutations [48].

3.1.3 miRNAS

Sieuwerts et al. used RT-PCR to study the expression of microRNAs (miRNAS) in CTC isolated with the EpCAM based CellSearch profile kit. They identified ten miRNAs that were more abundantly expressed in samples from patients with at least five CTCs in 7.5 ml of blood compared with samples from nine patients without detectable CTCs and healthy donors [49].

4 Quality Control in CTC Detection Systems: Comparison of Different Methodologies

The lack of standardization and harmonization of different technology hampers the implementation of CTC measurement in clinical routine practice. Clinical results of CTC analysis largely depend on the detection technology used. Despite the fact that most of these methods are highly specific and sensitive, there are not so far extensive studies especially designed to compare their efficacy when using the same clinical samples. This is an important issue for their clinical use, since especially in early disease, differences in analytical sensitivity between these methods plays a very critical role. Thus, standardization of micrometastatic cell detection and characterization is important for the

incorporation of CTCs into prospective clinical trials testing their clinical utility. Numerous single-institutional studies suggest that CTCs play an important role for risk stratification and monitoring of therapeutic efficacy. These findings need to be evaluated in trials to verify the principle of this concept in the clinical setting.

RT-qPCR based molecular methods can be used for routine clinical laboratory use since they can be standardized according to recently clearly described required quality issues such as Cq values, limit of detection, precision and accuracy, and recovery experiments [13]. A direct comparison of DTC detection rates in a large cohort of 385 patients using both standardized ICC and RT-PCR protocols has shown a significant correlation between ICC and RT-PCR ($P < 0.01$) and the results of both methods agreed in 73% of cases (280/385) [50]. Another recent study was designed to directly compare three techniques for detecting CTCs in blood samples taken from 76 patients with metastatic breast cancer (MBC) and from 20 healthy controls: the CellSearch CTC System, the AdnaTest Breast Cancer Select/Detect and a previously developed qRT-PCR assay for the detection of CK-19 and mammaglobin transcripts [51]. A substantial variation in the detection rates of CTCs in blood from breast cancer patients using three different techniques was observed. A higher rate of positive samples was observed using a combined qRT-PCR approach for CK-19 and mammaglobin, which suggests that this is currently the most sensitive technique for detecting CTCs. Standardization of the AdnaTest BreastCancer kit and direct comparison with other established breast cancer CTC enrichment and detection techniques is still lacking, but highly needed.

What is also very important is the fact that especially in early disease, CTCs are extremely rare as rare events follow the Poisson distribution [52]. To detect these cells occurring at these low frequencies reliably, a high assay efficiency and highly standardized preparation protocol are an absolute necessity. The limit of detection in the case of CTC is not limited by addition of extra CTC identifiers or instrument improvement but by the amount of blood that can be examined for the presence of CTC. This has to be taken into account seriously prior to starting any analysis, especially in the case of early disease [52].

Various studies address quality control issues in CTC, by comparing different methodologies, as outlined in a recent review [11].

5 Conclusions: Future Perspectives

Molecular characterization of CTCs will provide important information for identification of therapeutic targets and understanding resistance to therapies. Further research on the molecular characterization of CTCs will contribute to a better understanding of the biology of metastatic development in cancer patients. Toward this direction the combination of modern powerful technologies such as advanced imaging systems, molecular detection technologies, and next generation sequencing will enable the elucidation of molecular pathways in CTCs and lead to the design of novel molecular therapies specifically targeting CTCs.

The major advantages of molecular assays for CTC analysis are the following: (a) they give information only for living cells, (b) enable the analysis of a variety of molecular markers, such as gene expression, DNA methylation, DNA mutations in CTC, (c) offer extreme sensitivity, (d) offer a significant flexibility in the clinical lab setting, in terms of high-throughput analysis, multiplexing, (thus reducing the amount of sample required), time and cost of analysis, and quality control issues.

Molecular detection technologies enable the molecular characterization of CTC, and offer a significant flexibility in the clinical lab setting, in terms of high-throughput and cost-effective analysis, multiplexing, and quality control issues.

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Multiplex Molecular Analysis of CTCs

Anieta M. Sieuwerts and Stefanie S. Jeffrey

Abstract

Beyond enumeration, CTC characterization is expected to help guide therapeutic selection for personalized care of cancer patients. Different approaches may be used to simultaneously identify multiple CTC-specific markers for biological characterization; yet awareness of associated pitfalls is also important. We have focused this chapter on molecular profiling of CTCs following enrichment. We describe the MagSweeper technology that was specifically developed to isolate live and highly purified CTCs for pooled or single cell or pooled cell molecular analyses or for CTC growth in vitro or in vivo. However, most of what is discussed will apply to any multiplex analysis of CTCs, irrespective of the enrichment method.

Contents

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1 Introduction

Personalized cancer care depends on providing therapy that will be of most benefit and of least toxicity to an individual patient during their disease process. Chemotherapy is given to eradicate systemically shed tumor cells that not only travel to, but also possess the capability of surviving and replicating in distant sites, such as bone, lung, liver, and brain. However, primary breast tumors are heterogeneous, containing tumor cell populations that may differ in metastatic potential, in mechanisms governing metastatic homing to different organs [1–4], and in sensitivity to different chemotherapeutic agents [5]. Among CTCs, which are tumor cells that have migrated into and survived shear forces present within the circulatory system, there are likely subsets of circulating cells responsible for seeding and reseeding metastases. Thus, biological characterization of CTCs should impact both our understanding of metastatic disease progression and our choice of pharmaceutical agents at different times in the course of disease.

With limited number of CTCs present in a tube of blood, one of the challenges in the field is to assess as much information from as little material as possible. Thus, multiplex analyses offer great opportunities for discovering CTC biology. Given the tiny amount of material, such an approach may require some kind of pre-amplification. And for this, special care must be taken to ensure that after such an amplification step, the material still reflects the original composition. Moreover, vast numbers of neighboring leukocytes (10^6 white blood cells, WBCs, in 1 ml of blood) can potentially contaminate samples and confound molecular assays, and must be taken into account during molecular analyses.

2 CTC Enrichment Using the MagSweeper Technology

The MagSweeper was developed by our multidisciplinary team at Stanford to provide highly purified live CTCs [6] suitable for pooled or single cell analyses and for in vitro or in vivo investigations [7]. In brief, patient blood is drawn into a 10cc EDTA-coated tube to prevent coagulation, then labelled with magnetic beads functionalized with the human BerEP4 monoclonal antibody to epithelial cell adhesion molecule (EpCAM, also designated as tumor-associated calcium signal transducer 1, TACSTD1). Although most of our initial work was done with magnetic beads coated with EpCAM antibodies, labelling CTCs in blood with magnetic beads attached to other CTC cell surface antigens is feasible. Magnetic rods covered by plastic sheaths sweep through the labelled blood at a specified speed, generating a shear force that captures labelled cells while partially

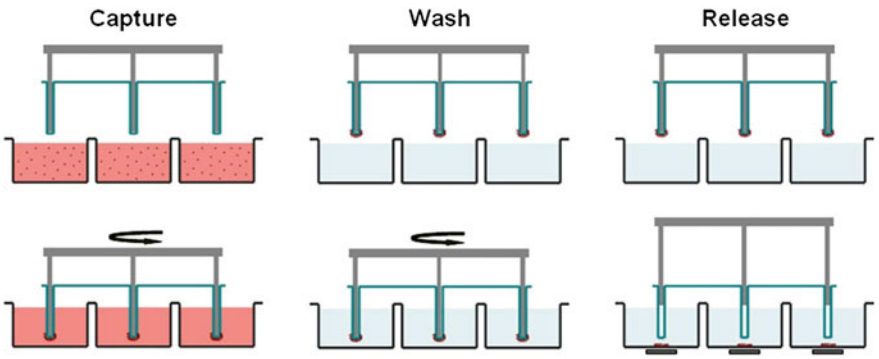

Fig. 1 Schematic showing the MagSweeper process of CTC capture, wash, and release using internal magnetic rods covered with plastic sheaths. The covered rods first sweep through a sample of immunomagnetically labelled unfractionated whole blood in concentric circular loops, then similarly sweep through a wash solution to remove loosely bound contaminating cells. In the capture solution, the magnetic rods are disengaged from their plastic sheaths and external magnets under the capture well facilitate release of CTCs and excess magnetic particles. An additional round of capture-wash-release eliminates the majority of remaining contaminant cells entrapped by excess magnetic particles. Permission from Proceedings National Academy of Sciences pending [6]

separating them from surrounding unlabeled leukocytes. The cells are then transferred to a washing well and finally released into a third well after the plastic sheath is disengaged from the magnetic rod (Fig. 1). A second capture/wash/release cycle produces highly purified cells that can be individually transferred via micromanipulation into a tube for single cell transcriptional analysis, mutation detection, or growth in culture.

3 Sensitive Nucleic Acid and Protein Isolation Techniques

If one aims to characterize multiple markers, it is imperative that all available material from the sample is isolated as purely as possible. This is even more important if it concerns isolation of material from just a few cells, as is often the case in the CTC field. Methods will therefore be preferable that allow separate analysis of genomic DNA, mRNA, microRNAs (miRNAs), and proteins.

New technologies with increased sensitivity are continuously being commercialized, so labs must remain up to date. But once an isolation method for the molecular compartment of interest has been chosen and lysates are used for downstream processing to characterize markers, either at the uniplex or multiplex level, it is sometimes difficult to switch to a different method. This is because any method will have inherent biases, either toward the nucleic acid or the protein side. Which procedure to follow may therefore depend upon the specific research question being studied.

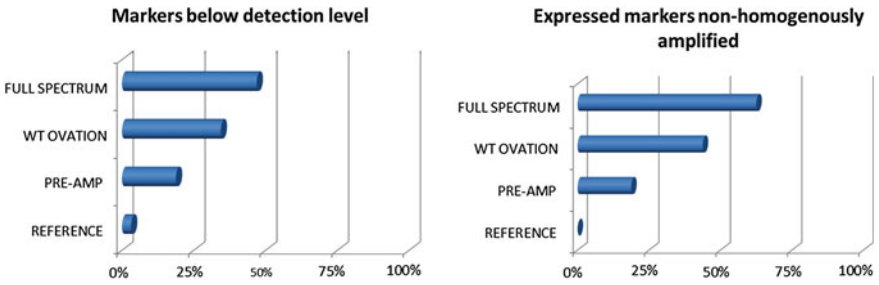

Fig. 2 Sensitivity and specificity of pre-amplification methods. Three different linear pre-amplification methods (TaqMan PreAmp from Applied BioSystems/Life technologies, suitable for multiplexing up to 100 gene expression targets; Whole Transcriptome Ovation RNA Amplification from NuGEN; Full spectrum RNA Amplification from System Bioscience) were utilized according to the manufacturer's instructions on RNA isolated from two epithelial breast cancer cells. The resulting pre-amplified cDNA preparations were analyzed by real-time PCR in a 20 µl reaction volume in an Mx3000P Real-Time PCR System (Agilent), and compared with expression levels measured in unamplified reference cDNA using TaqMan Gene Expression Assays in combination with TaqMan Universal PCR Master Mix No AmpErase UNG according to the manufacturer's instructions

4 Pre-amplification Methods

The next challenge is to find a method that will enable measurement of multiple markers in material isolated from as little as one cell in a linear and homogeneous (unbiased) manner. In view of the ultimate goal to characterize CTCs at the multiple marker level, any marker assay showing as a non-homogeneously amplified outlier in these tests cannot be used for further analysis because the data will not be truly representative of the original sample. Therefore, criteria require high sensitivity combined with a minimum number of non-homogeneously amplified marker assays.

An example of how to address this important issue is given in Fig. 2, which shows that, especially at the level of whole genome amplification techniques such as used by the WT Ovation and Full Spectrum RNA amplification methods, lack of sensitivity (Fig. 2, left panel), as well as homogeneous amplification (Fig. 2, right panel) might present a problem for certain markers [8].

Another challenge in any multiplex approach is to ensure that the multiplexing does not affect the efficiency and specificity of detection. One such example which forced us to change our strategy concerned the measurement of *hsa-miR*-22 located on chromosome 17 and which had attracted our attention based on its putative regulation of, amongst others, BCAR1 and ERBB3. For reasons still unknown, we were unable to get a quantification cycle (Cq) value for this particular microRNA if measured in a multiplex cDNA reaction that included 30 other microRNAs of our interest, while measurement of this particular microRNA

in a uniplex reaction resulted in very nice amplification curves with Cq values in the range of 20–30 and an efficiency close to 100%.

These data demonstrate that—besides reproducibility—reliability must be checked beforehand for each individual marker assay. For now it appears the chance of successful amplification is still higher for target-specific amplification methods such as the PreAmp method from Applied BioSystems/Life Technologies. The disadvantage is that these kinds of methods are restricted to a pre-selected set of markers. To include any new markers will require a new CTC enrichment and downstream processing procedure.

Fortunately, the field of single cell analysis is growing rapidly, and we therefore anticipate that unbiased whole genome amplification will soon—if not already is, such as for example, the PCR-based SMART^{TM} technology (Switching Mechanism At the 5′ end of RNA Transcript) from ClonTech—be possible at the one-cell level.

5 Estimating the Contribution by Remaining Leukocytes

Although most CTC enrichment systems allow capture of CTCs in cancer patient blood by selectively isolating, for example, EpCAM-positive cells followed by quantification of DAPI-and CK-8/18/19-positive cells, there may still be considerable quantities remaining of contaminating leukocytes (DAPI+/CD45+). For the MagSweeper device, contaminating leukocytes range between zero and one per CTC isolated [6]; however, other CTC systems may typically capture both CTCs and about 500–1,000 contaminating leukocytes after enrichment [8]. Thus leukocyte contamination, together with the fact that CTCs appear to occur in small numbers in humans (often fewer than five CTCs per 7.5 ml blood in metastatic breast cancer patients [9]), form pitfalls that cannot be ignored when one is interested in a multiplexed characterization specific for CTCs, no matter what system is used for CTC enrichment.

6 Selection of CTC-Specific Molecular Markers

Different approaches are possible to identify markers that are CTC specific. These include screening markers of interest against publicly available databases such as SAGE (http://cgap.nci.nih.gov/SAGE/AnatomicViewer). An example of this is shown in Table 1, where we illustrate the results of screening 20 putatively interesting gene markers for the expression levels measured in white blood cells, normal breast tissues, and breast cancer tissues. Based on the data presented in this table, one might decide to discard the lower eight markers based on their low expression in breast (cancer) tissue with probably no discrimination possible between leukocyte and breast tissue-derived expression levels. The upper eight markers on the other hand appear promising with levels differentially expressed between leukocytes (low) and breast cancer tissue (higher).

Table 1 Selection of CTC-specific markers

Gene Symbol	Database	Normal white blood cells [CD45+]	Normal breast	Breast cancer	Examples of differential range in breast subtypes	
KRT19	refseq_p5s				P1H12 DCIS	CD24+ META
MUC1	refseq_p5s				CD45+ IDC	CD24+ META
S100A16	refseq_p5s				CD24+ IDC	CD24+ META
CLDN3	refseq_p5s				CD24+ META	BerEP4+ DCIS
ERBB2	fl_p5s				CD45+ IDC	CD24+ IDC
SPDEF	mgc_p5s				CD45+ DCIS	CD24+ META
TACSTD1	refseq_p5s				CD24+ META	CD24+ IDC
AGR2	refseq_p5s				BerEP4+ IDC	CD24+ META
ESR1	refseq_sr				CD44+ META	CD24+ META
CCNE1	refseq_p5s				CD24+ META	CD45+ IDC
MELK	refseq_p5s				CD24+ META	CD44+ META
FGFR3	fl_p5s				CD45+ IDC	BerEP4+ IDC
FGFR4	refseq_p5s				CD45+ DCIS	BerEP4+ NB
PGR	refseq_sr				CD45+ IDC	BerEP4+ IDC
MKI67	fl_p5s				BerEP4+ IDC	CD24+ META
EGFR	fl_p5r				P1H12 DCIS	CD10+ DCIS
ERBB4	refseq_p5s				BerEP4+ IDC	BerEP4+ DCIS
FGFR2	fl_p5s				CD44+ META	CD24+ META
ALDH1A1	fl_p5s				CD44+ IDC	BerEP4+ NB
ALDH2	refseq_p5s				CD44+ META	BerEP4+ NB

Color Code										
Tags per 200,000	<2	<4	<8	<16	<32	<64	<128	<256	<512	>512

Despite these promising screening data for eight and possibly 12 out of the 20 markers, this unfortunately does not guarantee a successful CTC-specific multiplex analysis. For that, the actual levels need to be measured in a clinical cohort that consists of patients that presented themselves with and without CTCs, as well as a decent cohort of healthy blood donors without evidence of disease. Furthermore, it is at this stage important to screen these markers following well-defined standard operating protocols. Not only for the blood sampling, but also for the downstream processing and the performance of these markers in the actual multiplex protocol, which should then include all markers of interest.

Alternatively, one might consider skipping the in silico pre-screen and immediately perform analyses according to well-defined Standard Operating Procedures (SOPs) with all markers of interest included, and discarding markers that in the end do not fulfill the pre-defined criteria. But be aware that this approach still requires a sufficiently large enough control cohort and although more likely to identify additional markers, might in the end be the more expensive option.

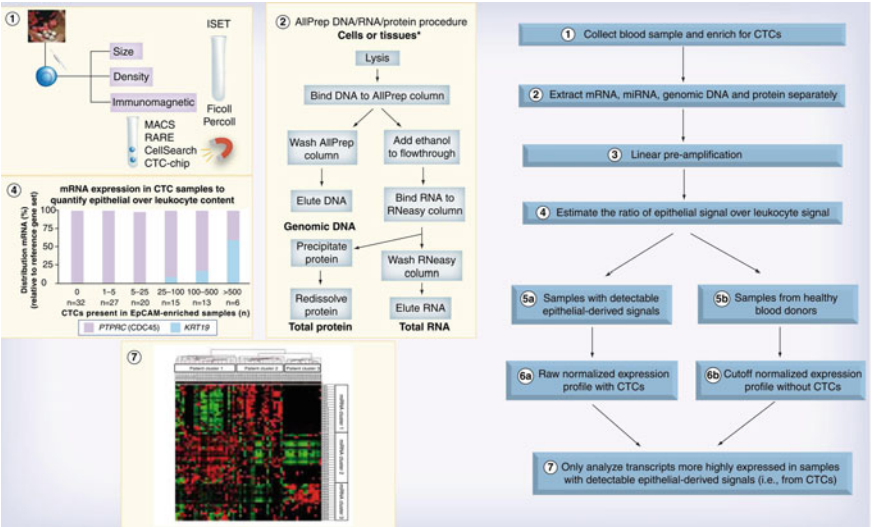

Fig. 3 Step-by-step schematic for reliable measurement of CTC-associated transcripts. After collecting blood samples in EDTA tubes to preserve RNA integrity and enrichment for CTCs (*1*), a sensitive isolation technique—preferably one that is able to isolate genomic DNA, mRNA, miRNA and protein in separate fractions (*2*, an example from qiagen.com)—and linear pre-amplification steps (*3*) are needed to enable detection of molecules in material from as little as one cell. Next, CTC- and leukocyte-specific signals are used to estimate the ratio of the tumor cell-specific signal over leukocyte-derived signal (*4*). Now, samples can be grouped into those with detectable epithelial-derived signals (*5a*) and those without detectable epithelial signals (*5b*), with the latter group comprising both patient samples without detectable epithelial signals and samples from healthy donors. Until consensus has been established on a robust reference miRNA set, normalizing on the mean expression of all expressed miRNAs in both groups (*6a* and *6b*) is probably the optimal method when multiple miRNA transcripts are measured at the same time. Finally, to ensure epithelial tumor cell-specific gene expression profiling of CTCs, levels measured in the samples without detectable epithelial signals (*6b*) are used as cut-off for the samples with detectable epithelial signals (*6a*) to calculate the remaining CTC-specific signals (*7*). Reproduced from Expert Review of Molecular Diagnostics 11(3), 259–275 (2011) [10] with permission of Expert Reviews Ltd

7 Data Analysis and Validation

In Fig. 3, we summarize the necessary steps to ensure epithelial tumor cell-specific transcript profiling of CTCs [10].

The data analysis may prove to be the most difficult part and greatly depends on the question being asked. If the interest is in identifying specific markers for use in monitoring prognosis or therapy response (i.e., if multiplex analysis was set up for screening purposes), a selection can be made from a subset of markers that were identified in the screening phase to be CTC specific and related to response. However, it is necessary that any such marker or set of markers be tested in an

independent cohort and according to a protocol that only includes the final selection of markers.

8 Examples of Multiplex and Other Molecular Analyses of CTCs

The amount of RNA in a single CTC is in the picogram range and currently insufficient for affordable and reproducible whole genome microarray analysis. Thus, we have been measuring the expression of up to 96 genes in single cells using a microfluidic qRT-PCR dynamic array from Fluidigm, finding different subpopulations of CTCs within one blood sample. Which genes are selected for expression measurement by the Fluidigm chip will impact the transcriptional profiles and the resulting phenotypic groupings of CTC subpopulations. Cells that express leukocyte-specific genes, such as *PTPRC* encoding for CD45, are eliminated from analyses.

We have also measured specific mutations—such as exon-specific *PIK3CA* mutations—in single CTCs isolated from cancer patient blood by the MagSweeper. We have found that within one tube of blood from a single blood draw, there may coexist diverse subpopulations of CTCs, some that are mutant or others that are wild-type. This is consistent with the findings of Dupont Jensen and colleagues [11] who have shown that within neighboring areas of an invasive breast cancer on a single hematoxylin and eosin slide were tumor cells with a *PIK3CA* exon 9 mutation, other tumor cells with a *PIK3CA* exon 20 mutation, and a third cluster of tumor cells that were wild-type. They also showed significant mutation discordance between primary tumors and distant metastases.

We have used the MagSweeper to isolate viable CTCs from a mouse xenograft model that contained profound regions of hypoxia and anoxia and produced lung metastases in all mice [7]. Using this model, we isolated CTCs from mouse blood and studied their response to hypoxia by growing the CTCs in culture under normoxic (room air, 21% oxygen) and hypoxic (<0.1% oxygen) conditions. We observed that CTCs had an altered response to hypoxia compared to parental tumor cells as well as distinctive expression and/or induction of anoxia-induced factors and target genes, including those involved in adaptation to nutrition deprivation (as might be present in tumor areas with poor blood supply) and the endoplasmic reticulum stress response. We observed that chronic hypoxia markedly increased colony formation in CTCs compared to parental tumor cells. Moreover, when CTCs were implanted into mouse mammary fat pads, the resulting CTC xenograft tumors showed a more aggressive phenotype, producing larger tumors that developed lung metastases twice as fast as xenografts generated from primary parental tumor cells. Together, the data showing that CTCs have a distinct response to hypoxia in vitro and greater aggression in vivo support the claim that CTCs have a different phenotype than the primary parental tumor cells from which they were derived. Our results suggest that CTCs may be selected for

Table 2A Examples of multiplexed analyses from the literature

Study	Enrichment method	Device name	Enrichment antigen	Molecular targets	Pre-amplification method	CTC-specific marker selection	Detection method	References
1	Flow Cytometry	FACSCalibur equipped with a second red-diode laser	CD45 + CD133 [negative selection], P1H12 and CD31 [positive selection for CEC/CEP]	CDH5, TEK, KDR, PROM1, ACTB	none	clinical relevance	flow cytometry, qRT-PCR	[18, 19]
2	Immuno-magnetically	CellSearch (Veridex)	EpCam	37 mRNA transcripts	WGA [MessageAmp Ambion]	cancer and CTC specific	qRT-PCR	[20]
3	Gradient density centrifugation + immuno-magnetically enrichment + culture of remaining viable single cells	EPISPOT assay	CD45 [negative selection] and CXCR4 + or -	MUC1, CATH-D (as secreted proteins), CXCR4 (for the sorting)	culture of viable cells	clinical relevance	colorimetric EPISPOT assay	[21]
4	Immuno-magnetically	?	EpCAM	PLAUR, ERBB2	none	clinical relevance	FISH	[22]
5	Micro-manipulation	N/A	none	whole genome	PCR-based global amplification	N/A	large-scale oligonucleotide array	[14]
6	Immuno-magnetically	in-house method with Dynabeads	EpCAM	KRT19, MUC1	none	CTC-specificity	RT-PCR	[23, 24]
7	None	N/A	N/A	KRT19, SCGB2A2, ERBB2, GAPDH	none	clinical relevance	real-time PCR, RT-PCR, IF	[25]
8	Immuno-magnetically	AdnaTest (AdnaGen)	EpCAM and MUC1	EpCAM, MUC1, ERBB2, ESR1, PGR, ACTB	none	clinical relevance	qRT-PCR	[26]

(continued)

Table 2A (continued)

Study	Enrichment method	Device name	Enrichment antigen	Molecular targets	Pre-amplification method	CTC-specific marker selection	Detection method	References
9	Immuno-magnetically	Microfluidic module (MIRACLE)	EpCAM and MUC1	20 mRNA transcripts	gene specific	literature-based and clinical relevance	electrochemical detection of MLPA fragments	[27]
10	Immuno-magnetically / CTC-chip	CellSearch (Veridex), and OncoCEE microchannel (Biocept Inc) and CELLective/On-Q-ity CTC-chips	EpCAM	12 mRNA and gene transcripts, KRAS mutation	yes, for KRAS	Subtype specific and clinical relevance	Taqman low density array (TLDA), IF, FISH, Taqman genotyping assay	[28]
11	Affinity-based	microfluidic "CTC-chip"	EpCAM	PSA, Ki-67, TMPRSS2-ERG fusion [negative marker: CD45)	WTA1	clinical relevance	CTCs: IF, RT-PCR, nucleotide sequencing	[29]
12	None	N/A	N/A	microRNA-10b, -34a, -141, -155 and-16	gene-specific	clinical relevance	qRT-PCR	[30]
13	Immuno-magnetically	CellSearch (Veridex)	EpCAM	55 mRNA and 10 microRNA transcripts	gene-specific	literature-based and clinical relevance	qRT-PCR	[31]
14	Gradient density centrifugation + immuno-magnetically	Ficoll-Paque PLUS (GE Healthcare BioSciences) + CELLection Epithelial Enrich (Invitrogen)	EpCAM	KRT19, ERBB2, MAGEA3, SCGB2A2, TWIST1, HMBS	none	clinical relevance	Liquid Bead Array Hybridization (Luminex)	[32]
15	Size filtration	ScreenCell MB	none	wild-type EGFR and mutations	gene-specific	clinical relevance	qRT-PCR, sequencing	[33]

(Continued)

Table 2B Examples of multiplexed analyses from the literature, (continued).

Study	Patient material	# Patients	mL blood	Negative controls**	Positive controls	Claimed sensitivity	Claimed specificity	Data analysis	Validation in independent cohort	References
1	cancer patients	84	100,000 cells/ blood sample	14 HBDs	cell lines	25 copies for CDH5	CDH5 solely in lympho-monocyte, not in granulocyte-like, cell population	standard calibration curve prepared from plasmids containing the respective target sequences relative to ACTB	no	[18, 19]
2	metastatic colorectal, prostate and breast cancer	30 31 13	7.5 mL	50 HBDs	74 metastatic cancer patients with at least 1 enumerated CTC [parallel study]	?	82%	standard calibration curve prepared from gene-specific RT-PCR products with known concentrations relative to RPS27A	no	[20]
3	metastatic breast cancer	22	10 mL	11 HBDs	cell lines	1 cell	100%	visual count of number of spots, defined by minimal size/area	no	[21]
4	advanced recurrent breast carcinoma	46	5 mL	leukocytes of same patient	cell lines	?	?	visual count over ref chromosome markers	no	[22]
5	none	0	?	?	cell lines	1 cell	N/A	GeneSpring software, normalized using Lowess and GAPDH	no	[14]
6	different stages of breast cancer	94	10 mL	28 patients with benign disease	cell line	?	100%	positive/negative at fixed threshold Cq	no	[23, 24]

(continued)

Table 2B (continued)

Study	Patient material	# Patients	mL blood	Negative controls**	Positive controls	Claimed sensitivity	Claimed specificity	Data analysis	Validation in independent cohort	References
7	stage I to III breast cancer	175	20 mL	31 HBDs	cell lines	1 CTC / 10^6 PMBC	97%	relative to MCF-7 equivalents/5 ug of total RNA in GAPDH positive cases	no	[25]
8	primary breast cancer	431	2 x 5 mL	106 HBDs [literature-based]	?	2 cells / mL blood	95%	evaluation of PCR fragments in actin-positive samples and use of cut-off concentration values [Agilent 2100 Bioanalyzer]	no	[26]
9	none	0	5–7.5 mL	none	(spiked) cell lines	2-5 MCF7 cells / 5 mL blood	?	standard calibration curve prepared from gene-specific RT-PCR products with known concentrations relative to ACTB	no	[27]
10	stage IV metastatic breast cancer and non-small-cell-lung cancer	38 BC [HER2] 34 LC [KRAS]	7.5 mL, extrapolated to 7.5 mL on CTC-chip	wild type DNA	cell lines	HER2 by IF: >3 CTCs KRAS Taqman genotyping assay: ≥10 mutant positive tumor cells Taqman gene expression (TLDA): ≥10 tumor cells	?	hierarchical clustering, visual count over ref chromosome markers, ratio mutant over wild-type	no	[28]
11	localized and metastatic prostate cancer	19 36	2–3 mL	17 HBDs and cell lines	cell lines	Detection rate in localized patients: 42%, in metastatic patients: 64%	100%	Automated image processing with subsequent visual evaluation	no	[29]
12	primary and metastatic breast cancer	89	400 uL serum	29 HBDs	cell lines	?	?	delta Cq over miR16	no	[30]

(continued)

Table 2B (continued)

Study	Patient material	# Patients	mL blood	Negative controls**	Positive controls	Claimed sensitivity	Claimed specificity	Data analysis	Validation in independent cohort	References
13	advanced recurrent breast carcinoma	50	7.5 mL	53 HBDs	cell lines	1 CTC / mL blood	93%	delta Cq ref genes and CTC-specific cut-offs	no	[31]
14	operable and metastatic breast cancer	84	20 mL	corresponding PMBC fractions and 17 HBDs	cell lines	1 cell	100%	fluorescence intensity per gene-specific bead in HMBS positive cases	no	[32]
15	none	0	3 mL	x? HBD	cell lines	2 cells /mL blood	~50 CD45+ events/mL blood	Cq values and ratio mutant over wild-type	no	[33]

by a combination of tumor hypoxia and nutritional deprivation and/or endoplasmic reticulum stress response [7]. Our study also shows that CTCs captured by the MagSweeper are indeed live cancer cells that produce metastasizing tumors in vivo.

9 Additional Examples of Multiplex Analysis of CTCs

In this last part of our whys, dos, and donts when setting up a multiplex molecular analysis for CTCs, we by no means intend to completely cover the large body of literature currently available on this subject. We have therefore decided to restrict the selection of articles to those published by the co-authors of this book. The examples depicted in Table 2 are merely to give an overview of how researchers started, learned, and with increased knowledge and novel equipment and molecular techniques available, have set out to get as much information as possible from CTCs.

10 Concluding Remarks

Tissues, and therefore CTCs, are rarely homogeneous. Therefore, any expression profile based on pooled CTCs will blend the true expression profiles of its constituent cells to identify the CTCs that are ultimately responsible for the development of a distant metastasis. Single cell analysis rather than analysis of cell populations may be more informative. Indeed, single cell methods have been developed for both microarrays [12–14] and, recently, RNA-Seq [15–17]. Although still too expensive and with questionable reproducibility for cost-effective and accurate clinical use, as whole genome single cell analysis technology develops further, these methods may become preferable for the analysis of small numbers of single cells, and may in particular be useful to study cells that are difficult to obtain in large numbers, such as CTCs.

While the field of multiplexed CTC analyses is now being explored extensively, validation and application to clinical context requires further study in independent clinical cohorts.

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Part IV
Other Blood-Based Biomarkers

Circulating DNA and Next-Generation Sequencing

Susanna Cooke and Peter Campbell

Abstract

Personalising cancer medicine depends upon the implementation of personalised diagnostics and therapeutics. Detailed genomic screening is likely to play a central role in this. As the range of drugs and other therapies for cancer continues to increase, there is an increasingly urgent need for sensitive and specific measures of disease burden to guide treatment regimens. The ability to quantify disease burden with high accuracy and sensitivity in patients with cancer would open many potential routes to personalising therapeutic choices. For example, the intensity of therapy could be guided by the amount of disease at diagnosis; monitoring the response of patients to drugs could allow extension of the period of treatment in responders or early changeover of therapy in nonresponders; and early prediction of recurrence could allow salvage therapy to be instituted before complications of relapse develop. The detection of tumour-specific rearrangements in DNA free in the serum or plasma may provide a substantial advance in the accuracy of monitoring disease burden in patients with solid tumours.

Contents

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1 Introduction

Monitoring disease burden using assays for tumour-specific somatic rearrangements in blood samples is routine in haematological malignancies, where rearrangements are recurrent between patients. Residual levels of BCR-ABL transcripts, as measured by real-time quantitative PCR, are prognostic of outcome following imatinib therapy in chronic myeloid leukaemia and subsequent rises in transcript levels provide early indication of relapse [1]. Until the advent of next-generation sequencing, however, mapping somatic changes in solid tumours, which tend to be unique, was too time-consuming and labour-intensive to be useful in a clinical context. With the falling costs and increasing throughput of next-generation sequencing technologies, we can now rapidly characterise the complex genomes of solid tumours, allowing the development of personalised assays for quantifying tumour-specific changes in blood samples in all cancer patients (Fig. 1). This paves the way for the clinical and diagnostic paradigms that have been established in haemato-oncology to be applied universally to cancer management.

2 Circulating DNA in Cancer

It is well established that cell-free DNA is present in the bloodstream of healthy individuals and that many pathological states, including cancer, can contribute to this load [2, 3]. However, the origins of elevated levels of circulating DNA (ctDNA) in cancer patients remain to be fully understood. While lysis of circulating tumour cells (CTCs) may contribute to the load of ctDNA, there are insufficient numbers of CTCs present to account for the levels of DNA found in blood. Apoptotic and necrotic release of DNA from both tumour and normal (bystander) cells are likely to be the main source of ctDNA, although active release of newly synthesised DNA from cells has also been proposed to contribute [4]. The majority of evidence for the origin of ctDNA comes from analysis of DNA size and fragmentation patterns. Apoptosis releases low molecular weight DNA with a laddering size pattern reflecting multiples of nucleosome size ($\sim$180 bp). Although necrotic DNA tends to be of high molecular weight, processing and subsequent release by macrophages can reduce it to $<$2 kb in size [5]. Both apoptotic and necrotic patterns have been reported in plasma DNA [6]. In addition, smaller ctDNA is enriched for tumour DNA fragments, suggesting that tumour ctDNA is more degraded than ctDNA from normal cells [7].

Total amount of ctDNA is not itself informative as a diagnostic or prognostic parameter in cancer patients, as it does not correlate well with disease stage, size or primary site and the DNA concentrations observed often fall within the range seen in healthy individuals [6, 8]. Levels of ctDNA are, however, consistently elevated in advanced metastatic disease [7–9]. For example, in a mixed tumour cohort, cases with known metastatic disease had an average DNA concentration of

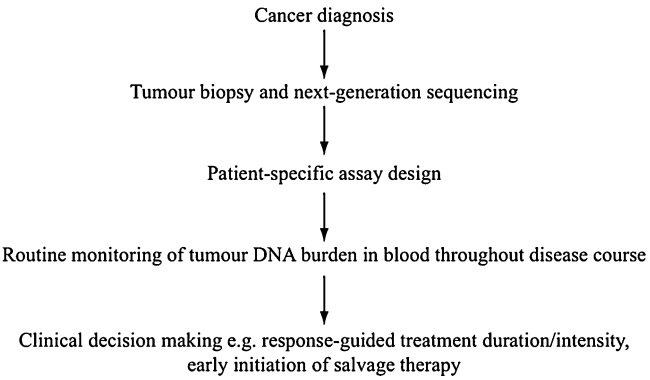

Fig. 1 Likely clinical workflow for the analysis of circulating DNA as a biomarker for tumour burden in cancer patients

209 ± 39 ng/ml in serum, while cases without metastatic disease had an average of 100 ± 30 ng/ml [8]. In colorectal cancer Dukes' stage D patients have a 12-fold higher level of DNA fragments in circulation than do patients with adenomas [7]. Although total quantity of ctDNA is not informative, preliminary data show that monitoring dynamic change in ctDNA levels over time, and in particular tumour-specific ctDNA, is a powerful approach for assessing tumour response to treatment and predicting relapse. In a study of advanced colorectal cancer, levels of circulating mutant cancer genes, including *APC* and *KRAS*, decreased by a median of 99% following complete resection but only halved following incomplete resection [10]. Furthermore, persistence of low levels of mutant ctDNA following surgery was correlated with eventual relapse, suggesting that ctDNA can be used to monitor minimal residual disease [10].

The earliest studies on tumour-specific sequences in ctDNA assayed point mutations in commonly mutated cancer genes, since these can be easily identified by targeted resequencing [11]. However, the large excess of normal DNA present in ctDNA limits the sensitivity of this approach. Tumour DNA may be <0.01% of total ctDNA in early stage disease [7] and minimal residual disease. Since normal as well as tumour ctDNA levels are elevated in advanced disease, even in stage IV colorectal cancer tumour-specific sequences are not a majority of total ctDNA present, although proportions as high as 27–37% have been reported [7, 12]. More recently, epigenetic changes, such as hypermethylation of tumour suppressor gene promoters, have been shown to be common in cancers and recurrent between tumours and these aberrant methylation patterns can be identified in the plasma and serum of cancer patients [13, 14]. In clinical sample sets detection of *SEPT9* hypermethylation in plasma as a candidate screening assay for colorectal cancer identified 70% of cases with a specificity of 90% [15] and this assay is now being developed commercially as a colorectal cancer biomarker. Use of methylation-specific primers that only amplify bisulphite converted DNA can partially overcome the problems of sensitivity caused by excess normal DNA. However,

careful optimisation of the bisulphite conversion of unmethylated cytosines is required to minimise loss of sensitivity due to either loss and degradation of DNA during bisulphite conversion or incomplete conversion. Since the development of next-generation sequencing technologies there has been growing interest in using somatic rearrangements as assay targets. Since somatic rearrangement junctions are unique to the tumour cells, they allow the development of highly sensitive and specific PCR assays that should be capable of detecting a single copy of a rearrangement in many millilitres of plasma, with minimal risk of false positives.

3 Next-Generation Sequencing of Solid Tumours

It has been known since the earliest cytogenetic studies that many solid tumours are characterised by abnormal chromosome rearrangements. However, there is extensive heterogeneity in the changes present between cases, and defining individual chromosome junctions at the resolution required to design specific PCR assays across them is extremely laborious using classical cytogenetic or microarray-based approaches. Since the release of next-generation sequencing platforms, including 454 (Roche) [16], Illumina [17] and SOLiD (ABI) [18], it has become possible to characterise the genomic landscape of a cancer, including rearrangements, point mutations and small insertions/deletions (indels), in a matter of days to weeks [19]. Massively parallel paired-end sequencing generates short sequence reads (50–100 bp) from each end of millions of small DNA fragments ($\sim$500 bp) of a tumour genome and aligns them to the reference human genome sequence. Read pairs that map back with inconsistent positions between the pair are then used to infer underlying somatic rearrangements [19]. The breakpoint resolution achieved using this approach is sufficient for direct design of PCR assays to tumour-specific junctions.

The rapidly falling price of next-generation sequencing means that analysis of tumour genomes using this approach could soon be readily accessible in a clinical setting. The only requirements for personalised assay development are that tumour DNA, either from biopsy or surgical resection, is available for sequencing and that at least one somatically acquired rearrangement is present in the tumour genome. Once designed, patient-specific PCR assays can be used throughout disease course to assess tumour burden through quantification of tumour-specific DNA in the bloodstream. This approach is therefore applicable to virtually all cancers, although further work is needed to optimise sequencing from limited amounts of starting material and from FFPE material, in order to improve clinical utility.

4 Tumour-Specific Rearrangements in ctDNA

To date, somatic rearrangement assays in plasma have been used in a small number of patients to track decrease in tumour load following surgery and chemotherapy and to monitor minimal residual disease prior to relapse. In a single colorectal cancer case, digital PCR quantification of tumour-derived DNA in plasma taken at six timepoints during treatment showed that levels decreased from 37% of total ctDNA to 14% within a day of surgical removal of the primary tumour. Further decrease was seen following chemotherapy but mutant ctDNA levels remained at 0.3% even after 137 days, consistent with the presence of residual disease observed as metastatic lesions in the liver [12]. In a case of osteosarcoma, minimal residual disease could be detected by identification of low levels of tumour-specific rearrangements in plasma throughout first- and second-line chemotherapy [20]. Increasing levels of tumour ctDNA 14 months after diagnosis corresponded with localised progression, and continued increase during salvage chemotherapy indicated subsequent relapse with widespread metastases [20]. These early data suggest that tumour ctDNA could prove to be an extremely sensitive biomarker.

There are several reasons why ctDNA-based biomarkers may prove more effective than current measures of cancer response and progression. The use of tumour-specific rearrangements allows high sensitivity. Single copies of tumour DNA can be detected in DNA extracted from 2 ml of serum, which could contain several 100 ng of contaminating normal DNA [20]. Persistence of tumour ctDNA has been reported even when disease is clinically undetectable following therapy [10, 20], supporting an important role for ctDNA in assessing minimal residual disease. In contrast, radiological imaging only detects lesions once they reach >0.5 cm in size. CtDNA is more sensitive than the protein biomarker carcino-embryonic antigen in colorectal cancer, showing more marked changes in response to tumour resection and greater capacity for predicting relapse [10]. Early data suggest that tumour ctDNA levels are extremely responsive to tumour status, allowing real-time assessment of disease. For example, spikes in ctDNA levels have been reported following surgery as DNA is released from tissue damaged during surgery. However, by 24 h post surgery there is a significant decrease in tumour ctDNA due to decreased tumour burden [10]. The dynamic nature of ctDNA is likely due to the rapid turnover rates of DNA in the circulation. Based on the clearance rate of foetal DNA in maternal circulation, it has been estimated that an advanced-stage tumour weighing approximately 100 g releases more than 3% of its DNA into the circulation each day, while an early stage tumour releases around 0.15% of its DNA [7]. Although tumour ctDNA levels during chemo-therapy have not yet been assessed, if they are as responsive as levels following surgery and during relapse they will have a significant advantage over current imaging-based assessments of tumour response, which rely on reductions in tumour size that may only become visible many weeks into a chemotherapy regimen.

Although initial data show promise for ctDNA use as a biomarker, there are still many questions that need to be addressed before translation into clinical practice. One potential caveat of personalised ctDNA biomarkers is the presence of clonal heterogeneity within cancers. Subclone-specific changes may be below the threshold of detectability and relapse following a long disease-free interval may be associated with significant clonal evolution, requiring new assay development. The optimal methods of ctDNA isolation and quantitation needed in order to establish a high sensitivity and specificity assay suitable for use by routine molecular diagnostic laboratories in the clinical setting remain to be defined. There are conflicting reports in the literature over whether plasma or serum is a better material from which to extract ctDNA. In general, plasma is considered to be more informative because serum tends to be more heavily contaminated with normal DNA due to lysis of lymphocytes during clotting. However, some studies argue that rapid processing of serum avoids the release of lymphocyte DNA while still providing higher ctDNA quantities than plasma for tumour DNA detection [21]. Choice of extraction method for ctDNA is critical due to the size characteristics of tumour DNA in the circulation. One of the most commonly used extraction kits, the QIAamp DNA Blood Kit (Qiagen), is not optimised for retrieval of small fragments and samples processed using this technique underestimate ctDNA levels leading to loss of sensitivity [22]. Overcoming these challenges should allow ctDNA to be used as a sensitive and accurate indicator of tumour burden, leading to many healthcare advances in cancer management.

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Circulating MicroRNAs as Noninvasive Biomarkers in Breast Cancer

Maria Angelica Cortez, James William Welsh and George Adrian Calin

Abstract

MicroRNAs (miRNAs) are master regulators of gene expression. By degrading or blocking translation of messenger RNA targets, these non-coding RNAs can modulate the expression of more than half the protein-coding genes in mammalian genomes. MiRNAs play important regulatory roles in a variety of cellular functions and in several diseases, including cancer. Aberrant miRNA expression has been well characterized in cancer, with implications for progression and prognosis. Recently, the discovery of miRNAs in body fluids, such as serum and plasma, opens up the possibility of using them as noninvasive biomarkers of disease and therapy response. In this chapter, we discuss the use of circulating miRNAs as biomarkers of disease and therapy response and as diagnostic and prognostic markers in breast cancer. We also discuss the main issues related to establishing circulating miRNAs as biomarkers in cancer.

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Contents

1 Introduction

MicroRNAs (miRNAs) are short (~22-nucleotide) non-coding RNAs that play important roles in post-transcriptional gene silencing of target messenger RNAs (mRNAs) [1]. MiRNAs are involved virtually in all biologic processes, including cell proliferation and apoptosis, development, differentiation, metabolism, immunity, neuronal patterning, stress response, aging, and cell-cycle control [1–5]. MiRNAs are strongly conserved among distantly related invertebrates, vertebrates, and plants [6], and more than 1,400 have been identified in humans [7]. It has been estimated that more than 50% of protein-coding genes are regulated by miRNAs in mammalian genomes [8, 9]. MiRNAs negatively regulate gene expression through mRNA cleavage in cases of perfect complementarity to the 3'-UTR of the target mRNA or through translational repression in cases of partial complementarity [10–12]. However, the results of recent studies demonstrate that miRNAs can also target the 5'-UTR of a target mRNA, both open reading frames and promoter regions [13, 14]. Recent studies have demonstrated that the open reading frames of many repeat-rich genes contain strikingly large numbers of particular miRNA target sites [15, 16].

Because a single miRNA can target hundreds of mRNAs, aberrant miRNA expression is capable of disrupting the expression of several mRNAs and proteins and is involved in the initiation of many diseases, such as cancer [17]. The first evidence of miRNAs' involvement in cancer was found in a study on *miR*-15*a* and *miR*-16*a*, which are located on chromosome region 13q14, a region that is deleted in more than half of all B-cell chronic lymphocytic leukemia patients [18, 19]. Since then, several studies have detected aberrations in miRNA expression in virtually all cancer types [20–25]. In breast cancer, Iorio et al. [26] identified 29 miRNAs differentially expressed in 76 breast tumor and 34 normal tissue specimens. In addition, *miR*-30 expression is associated with biopathologic features such as estrogen receptor (ER) and progesterone receptor (PR) expression, and *miR*-213 and *miR*-203 expression is related to tumor stage. Mattie et al. [27] identified unique sets of miRNAs associated with breast cancers currently defined by their HER2/neu or ER/PR status.

With the exception of leukemias, for which malignant cells are easily available, tissues for profiling solid cancers are obtained either by biopsy or surgery. Therefore, studies that demonstrate the diagnostic and prognostic usefulness of circulating miRNAs in body fluids, such as serum and plasma, are of high interest.

MiRNAs have also been detected in other body fluids, such as tears, breast milk, bronchial lavage, colostrum, and seminal, amniotic, pleural, peritoneal, and cerebrospinal fluids [28]. Specific compositions and concentrations are found in each body fluid type analyzed. These findings might be useful if a correlation exists between specific miRNA levels in body fluids and various disease states.

In this chapter, we discuss detecting circulating miRNAs in serum and plasma and their applicability as diagnostic and prognostic markers in breast cancer.

2 Circulating MicroRNAs in Breast Cancer

The first study that measured miRNA levels in serum was conducted by Lawrie et al. [29], who found that sera levels of *miR*-21 were associated with relapse-free survival in patients with diffuse large B-cell lymphoma. Since then, several studies have assessed the potential use of serum or plasma miRNAs as biomarkers in different types of cancers, such as prostate cancer [30], lung cancer, colorectal cancer [31, 32], ovarian cancer [33], renal cell carcinoma [34], squamous cell carcinoma of the tongue [35], and glioblastoma [36].

In breast cancer, some studies have assessed the use of circulating miRNAs as biomarkers to differentiate normal from diseased states and monitor response to therapy. In one of the first studies, Zhu et al. [37] demonstrated that PR-positive tumors had higher *miR*-155 expression levels than did negative tumors in serum specimens from 21 women with and without breast cancer. In another study, Heneghan et al. [38] identified cancer-specific miRNAs that were significantly altered in the circulation of 148 breast cancer patients and that increased systemic *miR*-195 levels in breast cancer patients were reflected in breast tumors. Furthermore, the authors found that circulating levels of *miR*-195 and *let*-7*a* decreased in cancer patients after tumor resection and that specific circulating miRNAs were correlated with certain clinicopathologic variables, namely nodal status and ER status.

In one study, serum miRNA levels were highly correlated with breast tumor tissue types. *miR*-21, *miR*-106*a*, and *miR*-155 were significantly overexpressed in tumor specimens compared with in normal controls, whereas *miR*-126, *miR*-199*a*, and *miR*-335 were significantly underexpressed. Furthermore, the relative expression levels of *miR*-21, *miR*-126, *miR*-155, *miR*-199*a*, and *miR*-335 were closely associated with breast cancer histologic tumor grades and sex hormone receptor expression status [39]. Asaga et al. [40] demonstrated that circulating *miR*-21 concentrations could be used to distinguish breast cancer patients from healthy women and further distinguish patients with distant metastases from those with locoregional disease. Expression levels of circulating *miR*-10*b*, *miR*-34*a*, and *miR*-155 were used to discriminate 59 breast cancer patients from 29 healthy individuals [41]. Zhao et al. [42] identified 26 differentially expressed miRNAs in 20 breast cancer patients compared with 20 healthy donors. In this study, *let*-7*c* and *miR*-589 were significantly downregulated and upregulated in breast cancer patients, respectively. Using a deep sequencing technique, Wu et al. [43] found

significantly higher miR-29a and miR-21 in the serum of breast cancer patients compared to controls. Another avenue of current research is the identification of miRNAs in circulating tumor cells (CTCs) in the peripheral blood. Sieuwerts et al. [44] identified 10 miRNAs that were more abundantly expressed in 32 patients with CTCs than in 9 patients with no detectable CTCs and healthy blood donors. Technical issues regarding CTC isolation must be addressed to establish CTCs' relevance in clinical use, but they represent a promising approach because they are shed from the primary tumor or its metastases.

Some studies have identified differentially expressed circulating miRNAs in breast cancer patients and controls; few cases of overlapping expression have been reported. The main reason for these disparities is the lack of a standardized and robust method, with universal parameters, for detecting tumor-specific miRNA in body fluids. Nonetheless, studies of the circulating miRNAs associated with breast cancer have been limited to date. Therefore, more extensive studies are needed to establish circulating miRNA as noninvasive biomarkers and predictors of therapy response. Furthermore, not only technical aspects compromise the establishment of circulating miRNAs as biomarkers in breast cancer and also in other types of cancer; to date the source of circulating miRNAs is not clear. Therefore, more studies are necessary to clarify whether circulating miRNAs detected in body fluids are tumor-specific or the product of dead cells, either from tumor or healthy tissues.

In the following section, we discuss the main findings regarding the origin of circulating miRNAs, as well as recently published data that may explain the stability of circulating miRNAs in the bloodstream.

3 Stability and Origin of Circulating MicroRNAs

The potential of circulating miRNAs as cancer biomarkers relies mainly on their high stability and their capacity to reflect tumor status and predict therapy response. Many studies have systematically demonstrated that circulating miRNAs remain stable after being subjected to severe conditions that would normally degrade RNAs, such as boiling, low or high pH levels, extended storage, and ten freeze–thaw cycles [45, 46]. This remarkable stability is partly explained by miRNAs' association with protein complexes and the presence of these small RNAs in circulating microvesicles called exosomes.

Recently, Arroyo et al. [47] found that most circulating miRNAs in plasma are cofractionated with Argonaute2 (Ago2), suggesting that circulating Ago2 complexes are the mechanisms responsible for plasma miRNA stability. Ago2 is part of an RNA-induced silencing complex and is the key effector protein of miRNA-mediated silencing. The results of Arroyo et al. study suggest that vesicle-associated versus Ago2 complex-associated miRNAs originate from different cell types and reflect cell type-specific miRNA expression or release mechanisms. However, only 10% of circulating miRNAs were vesicle associated in plasma. These findings were confirmed by Turchinovich et al. [48], who also demonstrated that extracellular miRNA is ultra filtrated with Ago2 and that most miRNA in

plasma and cell culture remains in the supernatant after ultracentrifugation at 110,000 g, indicating a non-vesicular origin for extracellular miRNA. Furthermore, non-miRNA species such as U6 RNA, RNU24, RNU43, RNU44, RNU48, RNU6B, and mRNAs, which are not associated with Ago proteins, are absent in the extracellular environment or present in only low amounts. Other proteins may be associated with circulating miRNAs because some were present in the supernatant after Ago2 immunoprecipitation [47]. Recent findings demonstrate that the RNA-binding protein nucleophosmin 1 plays a role in the exportation, packaging, and protection of extracellular miRNAs [36]. Another mechanism that may involve the nSMase2 pathway was discovered, demonstrating that high-density lipoprotein transports circulating miRNAs and can alter gene expression by transferring miRNAs to recipient cells [49]. Nevertheless, miRNAs may also be involved in autocrine and paracrine miRNA signaling through exosomes. Of note, studies that have demonstrated that most circulating miRNAs are exosome-free only used samples from healthy donors or culture media [47, 48]. The two populations of circulating miRNAs (i.e., extracellular and exosomal) were not assessed or compared between healthy donor and cancer patients in these studies. Importantly, the results indicate that cancer patients have elevated levels of tumor-derived exosomes in plasma compared with those in healthy donors [50]. Furthermore, exosomes containing miRNAs were found not only in blood [51] but also in other body fluid types, such as saliva [52]. In Fig. 1, we depicted a hypothetical way in which circulating miRNAs are generated in the bloodstream. Nonetheless, further studies are necessary to explain the origin of these small RNAs in body fluids.

Interestingly, one group of researchers demonstrated the existence of tumor-derived exosomes [53] and an miRNA signature for circulating ovarian cancer exosomes [54]. This miRNA signature was significantly correlated with primary tumor miRNA expression in cancer patients compared with in benign disease patients and was not identified in normal controls. A similarity between miRNA signatures in circulating exosomal miRNAs and originating tumor cells was also found in lung adenocarcinoma [55], with a significant difference in exosomal miRNA levels between cancer patients and controls. Therefore, exosomes may be a newly discovered mechanism by which donor cells can communicate and influence the gene expression of recipient cells. More studies are needed to elucidate its importance in cancer progression [56]. Indeed, one study confirmed these findings and demonstrated that exosomes released by glioblastoma cells containing mRNA, miRNAs, and angiogenic proteins, such as epidermal growth factor receptor vIII, are taken up by normal recipient cells, such as brain microvascular endothelial cells [50]. Another study showed that exosomal miRNAs are associated with maintenance of dormant breast cancer cells in bone marrow stroma, which is related to recurrence and poor prognosis in breast cancer. In this study, Lim et al. [57] found that miRNAs play a role in breast cancer cell quiescence by demonstrating their passage through gap junctional intercellular communication and stroma-derived exosomes between breast cancer quiescent cells and bone marrow stroma.

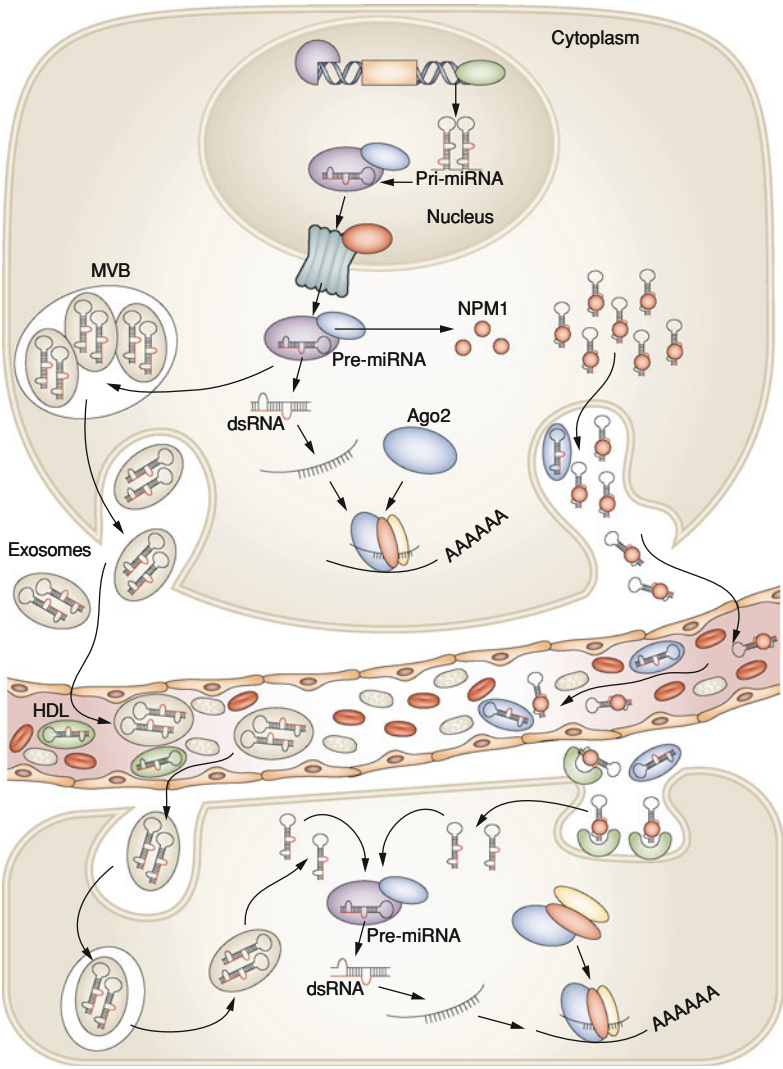

Fig. 1 After being transcribed in the nucleus, pre-miRNA molecules can be processed further by dicer in the cytoplasm. In addition, based on recent findings [36, 47, 49] there are at least two ways that pre-miRNAs can be packaged and transported using exosomes and MVBs or other (not fully explored) pathways together with RNA-binding proteins. After fusion with the plasma membrane, MVBs release exosomes into the circulating compartments and bloodstream. Likewise, pre-miRNA inside the donor cell can be stably exported in conjunction with RNA-binding proteins, such as NPM1, and Ago2, or by HDL. Circulating miRNAs enter the bloodstream and are taken up by the recipient cells by endocytosis or, hypothetically, by binding to receptors present at the recipient cellular membrane capable of recognizing RNA-binding proteins. More studies are necessary to elucidate how miRNAs are loaded into exosomes and how they can be internalized by recipient cells. Exosomal miRNAs are processed by the same machinery used in miRNA biogenesis and thus have widespread consequences within the cell by inhibiting the expression of target protein-coding genes. *MVBs* multivesicular bodies, *NPM*1, nucleophosmin 1, *Ago*2 Argonaute2, *HDL* high-density lipoprotein. Figure modified with permission from Cortez etal. [58]

Because Ago2/miRNA complexes are extremely stable and found in the cellular cytoplasm, some researchers have hypothesized that extracellular circulating miRNAs originate from dead cells [41, 48]. Indeed, miRNAs in body fluids can originate from apoptotic and necrotic cells of tumors and other sources, such as blood cells, the liver, the lungs, the kidneys, and other organs in which extensive contact between cells and the blood plasma occurs [48]. This hypothesis suggests that caution should be applied in using extracellular circulating miRNAs as biomarkers because cancer-specific miRNAs can be masked by circulating miRNAs from healthy tissues. It is necessary to clarify whether differential expression between tumors and normal tissues is affected solely by the tumor or by the affected organ or system. The real origin of circulating miRNAs, the mechanisms by which miRNAs are generated in the bloodstream, and the biologic effects of these molecules at distant sites are unknown and require further study.

4 Conclusions

Successful breast cancer treatment relies on early disease detection. Because aberrant miRNA expression is an early event in tumorigenesis, circulating miRNAs may represent noninvasive biomarkers in breast cancer. Nonetheless, studies in large populations are needed and some aspects of experimental reliability must be assessed before circulating miRNAs can be used as biomarkers. Likewise, given that most current approaches to cancer screening are invasive and unable to detect early stage disease, it is important to determine when tumor-related circulating miRNAs are detectable in the bloodstream during disease evolution. Moreover, important issues need to be addressed to establish circulating miRNAs as biomarkers for cancer.

First, larger prospective clinical trials are needed to validate these results because most published studies have small sample sizes and lack long-term outcome data. Second, because common upregulated miRNAs in body fluids are shared by several cancer types, especially those with common origins, further studies are necessary to establish a well-characterized panel of miRNAs specific to each tumor type. The use of known biomarkers as cancer antigens, along with miRNAs, can also increase cancer detection specificity and sensitivity. Third, more studies are necessary to determine which circulating miRNAs indicate early or advanced cancer stage, response to treatment, and patient outcome. Fourth, a robust method for tumor-specific miRNA detection in body fluids with universal parameters is needed. Finally, to use miRNAs as biomarkers in cancer, it is important to determine the source of tumor-specific miRNAs in body fluids and establish a signature capable of differentiating diseased from healthy states.

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Circulating Endothelial Cells and Circulating Endothelial Progenitors

Patrizia Mancuso, Angelica Calleri and Francesco Bertolini

Abstract

The roles of circulating endothelial cells (CECs) and circulating endothelial progenitors (CEPs) are currently being investigated in several diseases including cancer and metastases development. Preclinical and clinical data suggest that CEC enumeration might be useful to identify patients who might benefit from anti-angiogenic treatments while CEPs seem to have a "catalytic" role in different steps of cancer progression and recurrence after therapy. The definition of CEC and CEP phenotypes and the standardization of CEC and CEP enumeration procedures are highly warranted to use these cells as biomarkers in clinical trials in oncology, and to compare results from different studies.

Contents

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1 Introduction

Although the endothelial cell turnover at the vessel level has always been thought to be very slow compared with other tissue, cells with endothelial morphology were found to circulate in the blood more than 35 years ago [1]. In the following years, the endothelial nature of these cells was confirmed by immunohistochemistry (IHC) studies, and their enumeration by means of positive enrichment, IHC, or flow cytometry (FC) indicated that circulating endothelial cells (CECs) are increased in a variety of human pathologies, including coronary artery and autoimmune disease, diabetes, and cancer [2–7].

The evaluation of CEC kinetic in tumor-bearing mice and in cancer patients treated with anti-angiogenic drugs confirmed that these cells are interesting surrogate biomarkers of angiogenesis and of the preclinical and clinical activity of anti-angiogenic drugs [7–15].

Characterization of the endothelial phenotype showed that some CECs had a phenotype compatible with terminally differentiated endothelial cells (EC), in some cases being apoptotic or necrotic and thus most likely derived from the turnover of vessel walls. Some other cells expressed progenitor-associated antigens in addition to endothelial antigens, and are currently considered circulating endothelial progenitor (CEPs) candidates [2–22].

2 CEC and CEP Phenotypes

The two most frequently used techniques to enumerate and isolate CECs and CEPs are immunoistochemistry (IHC) and flow-cytometry (FC) [2–15].

According to IHC enumeration, CECs are large cells present in a frequency of 10–100/ml in healthy subjects [5, 6]. According to FC, events with an endothelial phenotype show in most cases small dimensions and are counted with a frequency of 10–10,000/ml [7–14]. Antigenic promiscuity between CECs and platelets has been overcome by FC procedures where DNA staining reagents have allowed the count and the sorting of platelet-depleted, DNA-containing cells with an EC phenotype (DNA+CD45−CD31+CD146+) [7]. Studies that have used transmission electron microscopy (TEM), confirmed that these sorted CECs are of endothelial nature by virtue of the presence of EC-specific Weibel-Palade bodies and of RNA transcripts for the EC-specific gene VE-cadherin [9]. TEM studies also offered an explanation of the controversies about CEC frequency in the blood. The majority of sorted CECs, in fact, were found to be apoptotic or necrotic cellular fragments, most likely lost at count after the cell processing involved in IHC enumeration. Along with apoptotic CEC, however, TEM showed the presence of small, viable, and lymphoid-like cells that are compatible with a progenitor cell morphology.

TEM will be of help to dissect the functions of candidate CEC and CEP subpopulations. Both these cell families, in fact, encompass subpopulations with different roles. Multiparametric FC has shown that among DNA+, CD45−,

CD31+, CD146+ CECs there are some expressing other EC-related antigens such as CD143, CD144, VEGFR1, VEGFR2, VEGFR3, along with activation antigens such as CD105 (endoglin).

Although most studies to date have looked at detection of CEPs using conventional FC procedures, there has been a lack of consensus regarding the definition of these cells using surface expression of specific antigens. Monoclonal antibodies most widely used to identify CEPs are CD34, CD133, and VEGFR-2, in different combinations [9, 15–17].

However, CD34 and VEGFR2 antigens are expressed also by mature CECs, and the use of the CD133 antigen for CEP identification [18, 19] has led to the sorting of cells that not all laboratories were able to differentiate in vitro and in vivo along the endothelial lineage [20, 21]. Recently, Case and his group have proposed a protocol for CEP detection by FC where CEPs are defined as CD45−CD34^{bright}CD133−VEGFR2−. These cells were capable of in vivo vessel formation [22]. Further studies are necessary to understand the role of this cell population in cancer and in cancer patients treated with anti-angiogenic drugs.

3 CEC Kinetic and Cancer Treatment

Despite different results in CEC absolute quantification, both FC and IHC studies have indicated that in some types of cancer patients CEC numbers and viability are increased when compared to healthy controls [7, 23]. This is probably due to the angiogenic switch associated with cancer growth and the related production of angiogenic growth factors such as VEGF, bFGF, HGF, and possibly others by cancer cells and/or various host cells [10]. The recent and unexpected finding of an autocrine loop in ECs [24] is of particular interest, because it might be that the increase of viable CECs in the blood of cancer patients mirrors an aberrant vascular turnover/remodeling associated with high local levels of VEGF produced by cancer cells.

Following the preclinical evidence that CEC count can be used as a surrogate biomarker for angiogenesis and anti-angiogenic drug activity by means of determining the optimal biological dosage of anti-angiogenic drugs [11, 25], CEC number and viability have also been measured in different clinical trials involving cancer patients treated with various anti-angiogenic therapies [12–14, 26–28]. An increase in the number of apoptotic CECs after 60 days of therapy was associated with prolonged progression-free survival and overall survival in metastatic breast cancer patients treated with a doublet low-dose metronomic (anti-angiogenic) chemotherapy regimen [12]. When the humanized anti-VEGF antibody, bevacizumab, was added to the metronomic chemotherapy for the treatment of metastatic breast cancer, patients who showed a clinical response in a phase II clinical trial (as well as a larger population of patients who had a clinical benefit from the treatment) had significantly greater baseline levels of viable CECs than patients who did not respond to therapy. Moreover, the number of apoptotic CECs before the beginning of therapy was associated with prolonged progression-free survival [28].

In patients with renal cancer treated with the small molecule anti-angiogenic agent, sunitinib, changes in CECs differed between the patients with clinical benefit and those with progressive disease [29]. Taken together, these data suggest that the investigation of CEC number and viability by FC has potential for the stratification of cancer patients who are more likely to benefit from anti-angiogenic treatments [10, 30, 31]. Prospective randomized clinical trials are necessary to confirm these results.

4 CEP Role in Cancer Growth and Metastasis Development

Prior to 1997, the predominant concept behind blood vessel formation in adults was believed to be angiogenesis, i.e., vessel generation from mature ECs. In 1997, Asahara et al. [16] reported in Science that they had purified a population of putative CEPs from human peripheral blood displaying properties of ECs in vitro and in ischemia animal models. These authors proposed the bone marrow (BM) as a source of CEPs that differentiate to bona fide ECs during postnatal vascular growth. The concept of CEPs was for over a decade widely considered an essential mechanism leading to neoangiogenesis and tumor growth promotion [32, 33].

Since the first description of CEPs, their identification and role in tumor vasculature has often been debated [34, 35]. Understanding whether or not all types of tumor rely—at least in part—on CEP-dependent vessel generation has been elusive, primarily because the relative contribution of CEP-derived vessels was found to be extremely variable in different preclinical models of cancer [32, 36]. Clinical studies in patients who received a gender-mismatched bone marrow transplant before cancer recurrence [37] indicated that CEP-derived vessels were indeed present, albeit at a low frequency (on average, 5% of all vessels).

Benezra's group reconciled these apparently conflicting data by demonstrating that the recruitment of CEPs into tumor vasculature depends on the tumor grade and also by showing that CEPs are key contributors in the first steps of tumor vascularization in small tumors. However, following the establishment of cancer vessels, their relative contribution to neoplastic angiogenesis is quantitatively less relevant as these cells become progressively diluted with the division of differentiated endothelial cells [38].

Angiogenesis-mediated progression from micro- to lethal macro-metastasis is a leading cause of death in cancer patients. Using preclinical models of pulmonary metastasis, the Mittal laboratory reported that tumors induce Id1 expression in CEPs. Id1 suppression after metastatic colonization blocked CEP mobilization, caused angiogenesis inhibition, impaired pulmonary macrometastases, and increased survival of tumor-bearing animals [34]. In addition, a new perspective has recently emerged regarding what could be a critical role for CEPs in tumor angiogenesis, namely, following acute types of cytotoxic therapy. For example, Shaked et al. [33] found that treatment of tumor-bearing mice with

vascular disrupting agents (VDAs), i.e., drugs which target the established but abnormal tumor vasculature, causing a rapid shutdown of blood flow followed by extensive tumor hypoxia and necrosis, leads to an acute mobilization of CEPs, which subsequently is home to the viable tumor rim that usually remains after such therapy, and drives 'rebound revascularization' and tumor regrowth/recovery following VDA therapy. In another study, we and others [39] found that certain chemotherapy drugs (taxanes in particular) administered at maximum tolerated doses (MTDs) can also induce a rapid CEP mobilization, most likely generated—at least in part—by the modulation of circulating SDF-1 levels. Prevention of the CEP spike by concurrent treatment with targeted anti-angiogenic drugs, e.g. treatment with anti-VEGFR-2 or anti-VEGF monoclonal antibodies, or by genetic manipulation strategies (e.g., undertaking treatment of tumors in Id mutant mice), or by the use of anti-SDF-1 neutralizing antibodies, resulted in enhanced antitumor activity of the administered cytotoxic chemotherapeutic drug. These findings raise the possibility that therapeutic strategies, which aim to reduce CEP mobilization, might enhance the efficacy of certain cytotoxic anti-cancer therapies and—at the same time—reduce the risk of cancer metastases. These results point to a sudden and "catalytic" function for CEPs, which may be a consequence of the aforementioned cytotoxic drugs not only being able to induce rapid mobilization of CEPs from the bone marrow, but also because the agents can damage the tumor vasculature, thereby creating the need and favorable circumstances for their physical incorporation into damaged vessels as part of a rapid host repair response. Indeed, cardiovascular researchers have investigated the hypotheses that rapid mobilization of CEPs following damage to blood vessels caused by pathologic events as stroke or infarcts represents such an adaptive (reactive) host repair process. Interestingly, when certain chemotherapy drugs are administered at much lower doses in a frequent repetition fashion (i.e., "metronomic" chemotherapy), the acute CEP mobilization response seen with MTDs is not only avoided, but such cells are actually targeted [25, 40]. This may be one of the mechanisms by which low-dose metronomic chemotherapy can cause an anti-angiogenic effect [41].

5 Conclusions

Based on past observations, it is possible assign to CECs and CEPs two different roles and two separate fields of clinical investigation.

Some CEPs (along with other hematopoietic cells) appear to have a transient "catalytic" but critical role in promoting angiogenesis during tumor growth, in stimulating growth of micro- to macro-metastases, and in 'rebound' revascularization after certain therapies are stopped. These cells are potentially promising targets for anti-cancer therapies and for adjuvant therapeutic strategies in patients at risk for cancer relapse.

CECs in most cases are apoptotic or necrotic cells, being released into the circulation as a consequence of vascular turnover, and thus they would not represent a "druggable" target for anti-cancer therapies. On the other hand, CEC presence in the blood seems less pulsating (i.e., more stable) than CEPs and there is increasing evidence of their potential as surrogate biomarkers of cancer angiogenesis and of anti-angiogenic drug activity.

Because several different types of blood cells with proangiogenic properties are implicated in cancer development and progression, further studies are required to determine the exact role that each cell plays in this process.

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Part V
Disseminated Tumor Cells and Circulating Tumor Cells in Breast Cancer Clinical Research and Practice

DTCs in Breast Cancer: Clinical Research and Practice

W. Janni, Brigitte Rack, N. Kasprowicz, C. Scholz and P. Hepp

Abstract

Minimal residual disease (MRD), i.e., isolated tumor cells (ITC) in bone marrow, may be the source of potentially fatal overt distant metastases in solid tumors even years after primary treatment. MRD can be detected by immunohistochemical methods using antibodies directed against cytokeratins, cell-surface markers, or molecular PCR-based techniques. Among solid tumors, the clinical relevance of MRD has been most extensively studied in breast cancer patients. The highest level of evidence for the prognostic impact of MRD in primary breast cancer was reached by a pooled analysis comprising more than 4,000 patients, showing poor outcome in patients with MRD at primary therapy. Yet, clinical application of MRD detection is hampered by the lack of a standardized detection assay. Moreover, clinical trial results demonstrating the benefit of a therapeutic interference derived from bone marrow status are still missing. Recent results suggest that in addition to its prognostic impact, MRD can be used for therapy monitoring or as a potential therapeutic target after phenotyping of the tumor cells. Persisting MRD after primary treatment may lead to an indication for extended adjuvant therapy. In a pooled analysis bone marrow aspirates of 726 patients from academic breast cancer units in Oslo (n=356), Munich (n=228), and Tuebingen (n=142) were analyzed during recurrence-free follow-up at a mean interval of 31.7 months after primary diagnosis of breast cancer pT1-4, pN0-3 pM0. Persistent ITC was detected in 15.4% of the patients (n=112). The Kaplan–Meier estimate for mean

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distant relapse-free survival estimate was 163.6 months in patients with negative and 105.2 months in patients with positive BM status. Patients without evidence of persistent ITC had a significantly longer overall survival (165.6), than patients with positive bone marrow status (103.3 months, $p < .0001$). Given these inspiring results on ITC in the bone marrow, several trials currently analyze the prognostic relecance of circulating tumor cells (CTC) in peripheral blood in the adjuvant setting. Persisting MRD after primary treatment may lead to an indication for extended adjuvant therapy. However, until clinical consequences of MRD detection in solid tumors and particularly in breast cancer have been validated, the detection of isolated tumor cells in bone marrow should be performed mainly in clinical trials.

At the time of diagnosis most breast cancer patients show no clinical signs of distant disease. Nevertheless, even patients with no lymph node involvement and disease limited to the breast are at risk for metastatic disease even years after primary diagnosis. Therefore the perception of breast cancer as a locally determined disease has changed in the past decades toward a systemic illness. One possible explanation for metastasis in early stage breast cancer is the shedding of single tumor cells into the bloodstream, which then reside in other compartments until clinically overt metastasis occurs. One of the best-explored compartments where disseminated tumor cells (DTCs) have been found is the bone marrow. In a large pooled analysis conducted by Braun et al., taking into account data from over 4,500 patients, 31% were DTC-positive in bone marrow aspirations at the time of diagnosis [1]. In this analysis the evidence for the prognostic value of these tumor cells was striking, with DTCs being an independent predictor of a poor outcome.

There are several trials employing different techniques for detection of bone marrow DTCs which were able to show a worse prognosis for DTC postive patients at primary diagnosis as well as during the course of the disease [2, 3]. To account for the systemic part of the disease chemotherapy and targeted therapies play an ever more important role in the concept of breast cancer treatment. Yet, some tumor cells seem to "survive" systemic treatment. One possible explanation for this observation is the theory of DTCs as dormant cells which evade systemic treatment by not proliferating and therefore escaping the mechanism of action of most therapeutic agents [4, 5]. In line with this hypothesis is the finding that the rate of DTCs that expresses the proliferation marker Ki-67 is small. Given that Ki-67 is absent in G0 and early G1 phase of the cell cycle most DTC seem to rest in these inactive cell cycle phases at the time of primary diagnosis [6–8]. However, when DTC are examined with regard to their potential to proliferate in cell culture with media containing appropriate growth factors, they seem very well capable of escaping their proliferative dormancy [9, 10]. Therefore, it seems that the microenvironment surrounding DTCs in bone marrow can impede metastatic growth at least to the point where some DTCs overcome "dormancy control" and start to develop to overt metastases [11]. The exact mechanisms leading to this change in behavior are still not fully understood. Presumably some kind of

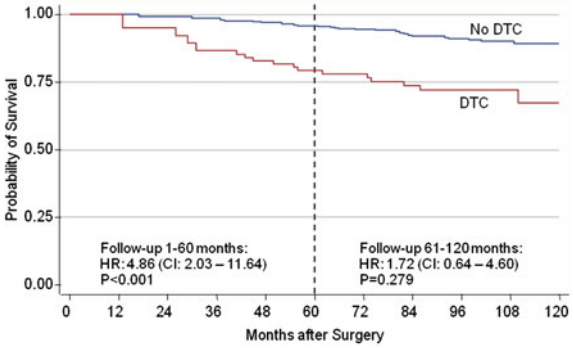

Fig. 1 Overall survival of patients with and without evidence of DTC during follow-up of breast cancer

selection driven by the microenvironment surrounding the DTC takes place which eventually leads to a proliferating cell clone [12].

DTCs can be found in up to 40% of stage I–III breast cancer patients [1, 11]. Interestingly, there is evidence that DTC are present already in patients with ductal carcinoma in situ (DCIS), a nonobligatory precursor of invasive breast cancer. The prevalence of DTCs in patients with DCIS at the time of primary diagnosis was calculated to be 21% in a proof-of-principle study with 19 patients with DCIS [13]. Despite the limited number of patients included, these data support the hypothesis of very early tumor dissemination.

When detected at the time of primary diagnosis, disseminated tumor cells can either persist or vanish in repeated bone marrow aspirations after initial treatment. Persistence of DTCs in the bone marrow is reported in around 16% of patients with previously detected DTCs [2]. Their presence at the time of primary diagnosis or failure to vanish during the course of the disease are independent of other known prognostic factors such as age, tumor size, presence of lymph node metastasis, histological grading or hormone receptor status. In this pooled analysis, persistent bone marrow DTCs were even the strongest independent factors negatively influencing disease-free and overall survival. Evaluation of the prognostic significance of DTCs in 676 patients during relapse-free follow-up showed that DTC-positive patients had a significantly worse outcome with respect to disease-free survival, distant disease-free survival, cancer-specific survival and overall survival during the first 5 years of follow-up, but not beyond [2] (Fig. 1).

In patients with high-risk breast cancer (inflammatory or >4 positive lymph nodes), one study investigated the significance of DTCs to predict the efficacy of chemotherapy [14]. Patients with persistent or newly positive DTC after chemotherapy had significantly worse relapse—and overall survival rates than patients who were DTC-negative after chemotherapy. A recent study focused on triple receptor-negative patients, which are considered to be especially responsive to neoadjuvant chemotherapy [15]. In this study there was only a non-significant

trend toward a higher rate of pathological complete responses (pCR) after neoadjuvant chemotherapy in comparison to luminal A or B breast cancer patients. However, patients with triple-negative breast cancer who did achieve pCR, had also been cleared of DTCs after NAC, whereas DTC were still present in 29–38% in the luminal A or B or HER2 group with pCR. Although this study is underpowered and presents only preliminary data it suggests a role of DTCs in late recurrence of luminal subtypes, in which NAC does not seem to be particularly efficient at eradicating DTC.

There seems to be an association between the presence of DTCs and overt metastases, but not with locoregional relapse. While only 17–21% of patients with locoregional recurrence have disseminated tumor cells in the bone marrow, the prevalence of DTCs in patients with distant metastases is 73–79% [16, 17]. An amount 2.5 tumor cells/1 million bone marrow cells, seem to be an independent risk factor for decreased overall survival in the setting of recurrent disease, independent if the patients had visceral or bone metastases [17]. Perhaps not surprisingly, patients with skeletal metastases were more likely to have an increased number of DTCs in the bone marrow.

At the time of primary diagnosis, established prognostic factors are utilized to determine adjuvant treatment. However, after primary treatment, reliable tools or markers to estimate the pending risk for recurrence are lacking. Thus, the detection of DTCs in the bone marrow might be a new approach to determine individualized therapeutic strategies.

Nonetheless, there are only few data so far to support standardized evaluation of bone marrow aspirates and hence intervention with altered therapeutic strategies. Beside its role as a therapeutic agent in patients with bone metastases, a new light is shed on bisphosphonates in respect to DTCs. It is hypothesized that bisphosphonates or newly developed drugs such as RANK-ligand inhibitors interfere in the interaction of tumor cells with osteoclasts and hence alter the DTC microenvironment in the bone. Therefore, bisphosphonates target tumor cells independently of the cell cycle—in contrast to chemotherapeutic agents. A recent study showed that the concurrent administration of zoledronic acid and neoadjuvant chemotherapy led to a reduction of detectable DTCs after 3 months and an increased number of persistent DTC-negative patients in contrast to patients who received neoadjuvant chemotherapy without additional zoledronic acid [18]. Another pilot study with a small number of patients revealed a better clinical outcome of patients with positive DTCs receiving zolendronic acid additionally to adjuvant therapy than those without bisphosphonate treatment [19].

In addition to bisphosphonates, it has to be further evaluated whether endocrine or targeted therapies are able to affect tumor cells accordingly. In breast cancer patients with HER2-neu overexpression, two agents are part of the standard treatment regimen: trastuzumab, an anti-HER2-neu monoclonal antibody and lapatinib, a tyrosine kinase inhibitor. The application of one of these agents depends on the detection of HER2-neu in the primary tumor or the overt metastasis respectively. Interestingly, patients with a HER2-negative primary tumor happen to have HER-2-positive disseminated or circulating tumor cells [18, 20]. One

explanation for this phenomenon might be the acquisition of HER2-neu amplification as a result of metastatic progression [18, 21]. As the overexpression of HER2-neu on disseminated tumor cells represents a poorer prognosis [22], current research focuses on whether additional patients benefit from HER2-directed therapies in case of HER2-negative primary tumor, but positive disseminated or circulating tumor cells. The German Detect-III-Trial will be launched in early 2012 and will address this issue.

In conclusion, disseminated tumor cells in the bone marrow represent an appealing target to provide prognostic information as well as a tool to monitor and target anticancer therapy.

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CTCs in Primary Breast Cancer (I)

Brigitte Rack, Ulrich Andergassen, Wolfgang Janni and Julia Neugebauer

Abstract

The prognostic and predictive value of circulating tumor cells (CTCs) in primary breast cancer patients is subject of several recent publications. In the context of neoadjuvant chemotherapy CTCs were detected in 22–23% of patients before and in 10–17% after systemic treatment. These findings did not correlate with primary tumor characteristics or tumor response rates. One major trial evaluated the prognostic value of CTCs in 2.026 primary breast cancer patients after tumor resection but before adjuvant chemotherapy. The prevalence of CTCs was 22%. In multivariate analysis, the presence of CTCs before treatment was shown to be an independent predictor for both disease-free (hazard ratio; HR 1.88) and overall survival (HR 1.91). Results demonstrate that not only the mere presence but also the quantity of CTCs is associated with worse outcome. The risk for recurrence or tumor-related death increased with higher numbers of CTCs detected ($\geq$5 CTCs: HR 4.04 for DFS and 3.05 for OAS; $p < 0.05$). In subsequent analyses of smaller subgroups within this trial, using a cutoff for positivity of >1 CTC, 10% of patients with the detection of CTCs before chemotherapy remained CTC-positive after completion of chemotherapy. Eight percentage of initially negative patients showed CTCs immediately after chemotherapy. Early data demonstrate that persisting CTCs after cytostatic treatment correlate with a decreased disease-free survival

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($p = 0.0623$). Increasing evidence confirms the prognostic relevance of CTCs in primary breast cancer. CTC detection could help to identify patients with increased risk for relapse. Present trials will show whether CTCs can also be used as a valid tool for treatment monitoring or direct treatment target.

Contents

1 Introduction

The prevalence of circulating tumor cells in peripheral blood (CTCs) of metastatic breast cancer patients has been evaluated by several groups. The presence of CTCs was shown to correlate with poor progression-free and overall survival and reflect treatment response [2, 4–6]. The prognostic and predictive value of CTCs in primary breast cancer patients is still under discussion. Several techniques, including immunocytochemistry and molecular approaches have been evaluated.

2 CTC Detection by Immunocytochemistry

CTC isolation and detection by immunocytochemical approaches primarily used EpCAM and cytokeratin antibodies. Most data available for immunocytochemical approaches are based on the use of the CellSearch system (Veridex, USA), which has been described previously. This semi-automated system is well standardized and FDA approved for the detection of CTCs in metastatic breast cancer. In brief, cells are isolated by immunomagnetic labeling using Anti-Epcam antibodies. Fluorescent anti-cytokeratin 8/18/19 and anti-CD45 antibodies are added to identify epithelial cells and leukocytes. An additional antibody, e.g., Her2, can be used for further phenotyping. After immunomagnetic enrichment and labeling of the cells, detection and enumeration of CTCs is carried out with the help of a semi-automated microscope. The cell cut-off in metastatic breast cancer, approved by the US Food and Drug Administration for the *CellSearch* System is >5 CTCs [4, 6, 14].

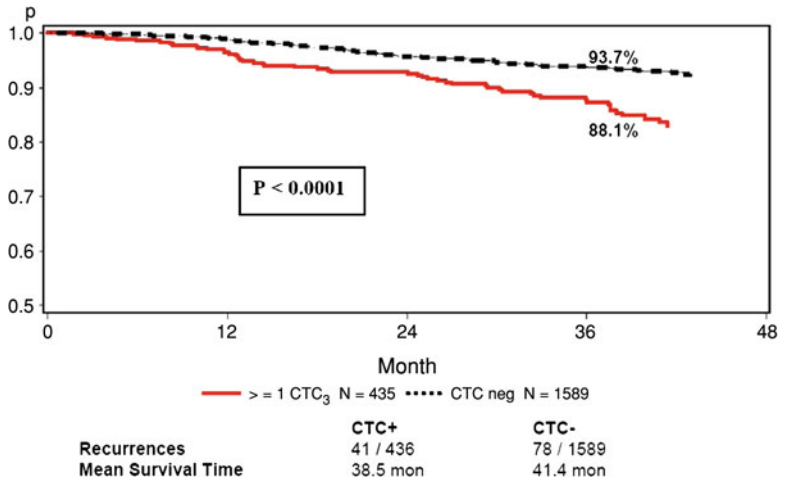

Fig. 1 Disease-free survival by CTCs in before chemotherapy

3 Prevalence and Prognostic Relevance of CTCs in the Context of Neoadjuvant Treatment

Several groups have collected blood samples before and after neoadjuvant chemotherapy in patients with large operable and locally advanced breast cancer and used the CellSearch system for the evaluation of CTCs. Pierga et al. investigated pre- and postneoadjuvant samples from 118 patients [9]. At least 1 CTC was found in 23% of the patients before systemic treatment. After neoadjuvant chemotherapy tumor cell positivity rates were 17%. While the persistence of CTCs at the end of neoadjuvant chemotherapy was not correlated with primary tumor response, the presence of CTCs was an independent prognostic factor for shorter distant metastasis-free survival ($p = 0.017$).

Riethdorf et al. evaluated blood samples of 287 patients from the German multicenter GeparQuattro trial [15]. The clinical study consisted of epirubicin/cyclophosphamide treatment prior to randomization to docetaxel alone, docetaxel in combination with capecitabine or docetaxel followed by capecitabine and additional trastuzumab treatment for patients with HER2- positive tumors. The prevalence for $\geq$1 tumor cell before neoadjuvant chemotherapy was 22%, whereas after chemotherapy 10% of the patients had persistant CTCs ($p = 0.002$). A total of 15% initially CTC-positive cases were CTC-negative after chemotherapy, whereas 8% of cases were CTC-positive after chemotherapy, although no CTCs could be found before chemotherapy. Similar to the findings of Pierga et al., CTC detection did not correlate with primary tumor characteristics and there was no association observed between tumor response to neoadjuvant chemotherapy and CTC detection. Survival data are not yet published for this trial. An additional

immunocytochemical Her2-phenotyping in a subgroup of patients was performed and showed HER2-overexpressing CTCs in 14 of 58 CTC-positive patients (24%), including eight patients with HER2-negative primary. These trials demonstrate the feasibility of CTC detection with immunocytochemical methods in primary breast cancer patients.

4 Prognostic Value of CTCs Before Adjuvant Chemotherapy

Using the CellSearch system the prognostic value of CTCs was evaluated in a large cohort of 2.026 primary breast cancer patients with node-positive or high risk node-negative disease within the German multicenter SUCCESS trial. In contrast to the previously described neoadjuvant studies, a larger blood volume was entered into the analysis (23 ml compared to 7.5 ml). In 22% of patients ($n = 435$) CTCs were detected after the resection of the tumor but before the start of systemic treatment (median 1.3, range 1–827), whereas 78% were found to be CTC-negative [10]. A total of 12% had 1 CTC and 4% had 2 CTCs in the blood sample. In 2% of patients 3 to 4 CTCs were found and 3% were diagnosed with 5 or more CTCs. This prevalence is concordant with results of previous studies using immunocytochemical detection methods. Throughout smaller cohorts of early breast cancer patients detection rates varied between 18 and 30% [1, 3, 15]. CTC-positive patients were significantly more frequently node-positive ($p < 0.001$), but no correlation to other clinico-pathological parameters, such as tumor size, grading and hormone receptor status could be found.

After a median follow-up of 35 months (range 0–54) the presence of CTCs before systemic treatment predicted poor disease-free (DFS) ($p < 0.0001$), distant disease-free survival ($p < 0.001$) and overall survival (OAS) ($p = 0.0002$). In multivariate analysis, detection of CTCs before treatment was confirmed as independent predictor for both disease-free (hazard ratio; HR 1.88) and overall survival (HR 1.91) next to tumor size, grading, lymph node involvement and hormone receptor status (p for all <0.05) (Fig. 1).

Not only the mere presence but also the increased number of CTCs is associated with poorer prognosis. The outcome was worst in patients with 5 CTCs or more with a fourfold increased risk for recurrence and a threefold increased risk for death (HR 4.04 for DFS and 3.05 for OAS; $p < 0.05$). These results are consistent with published data of single institution studies using different technical approaches proving CTCs to be a predictor of reduced disease-free and overall survival [7, 8, 16, 17].

In a subgroup analysis according to CTC counts >0, >1 and ≥ 5 CTCs, disease-free and overall survival were significantly reduced in all subgroups compared to patients with no evidence of CTC in their blood samples. The cut-off of one cell for positivity therefore seems justifiable.

5 CTC Evaluation Immediately After Chemotherapy and During Recurrence-Free Follow-Up

From the above-mentioned SUCCESS trial, also follow-up measurements after completion of chemotherapy and after 2 years of follow-up are available. In a considerable number of clinically recurrence-free breast cancer patients CTCs could be detected after the completion of adjuvant chemotherapy. A subgroup of 1,500 patients was analyzed for the persistence of CTCs after completion of adjuvant systemic treatment [11]. Cut-off for positivity for this analysis was more than one CTC. In 10% of the patients ($n = 143$) more than 1 CTC was detected before the start of systemic treatment (mean 14, range 2–827). After completion of chemotherapy, 9% of patients ($n = 130$) presented with >1 CTC in peripheral blood. Of those patients, who were initially CTC-positive, 10% remained positive ($n = 15$), whereas of those patients without CTC at time of diagnosis, 8% returned with a positive test ($n = 115$, $p = 0.42$) after chemotherapy [12]. Preliminary results demonstrate a prognostic relevance of persisting CTCs after cytostatic treatment with a reduced disease-free survival ($p = 0.0623$). Patients with a persistent positive CTC count both before and after chemotherapy had a highly increased risk for relapse ($p < 0.0001$).

In a subgroup of 579 patients a follow-up blood sample is available 2 years after completion of adjuvant chemotherapy. At this time point CTCs could be detected in 10% of patients (≥1 CTC), whereas more than 5 CTCs were found in 1.2% of patients, showing a long-time persistence of CTCs in peripheral blood [13].

6 Conclusions and Therapeutic Implications

Most trials using immunocytochemical techniques report a prevalence of CTCs in 20–40% of patients. While the prognostic relevance of CTCs in metastatic breast cancer has been confirmed by multiple trials, data in early disease are limited by small sample sizes or short follow-ups. Despite these weaknesses, however, available data demonstrates the presence of CTCs before and after the administration of chemotherapy to be an independent prognostic factor for poor clinical outcome. Furthermore, the persistence of CTCs has been shown for several years after the completion of primary surgical and cytostatic treatment. Therefore, CTCs could be used to identify patients with increased risk for recurrence both at primary diagnosis and during follow-up, and become a valuable tool for treatment monitoring. Additional phenotyping of CTCs could be the basis for more individualized treatment approaches. The German Detect-III-Trial, e.g., will evaluate the benefit from HER2-directed therapies in the case of HER2-negative primary tumor, but Her2-positive CTCs. Whether the implementation of CTC detection and phenotyping in our patient care will help to improve the prognosis of breast cancer patients has to be evaluated in prospective clinical trials.

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CTCs in Primary Breast Cancer (II)

E. Saloustros and D. Mavroudis

Abstract

CTCs can be detected by real-time RT-PCR for CK19 mRNA in the blood of early breast cancer patients before the start and after the completion of adjuvant chemotherapy and during adjuvant hormonal therapy and the follow-up. Patients with CK19 mRNA-positive cells both before and after chemotherapy have the worst prognosis with shorter disease-free and overall survival. The same is true for patients who have detectable CK19 mRNA-positive cells despite adjuvant tamoxifen while persistent detection during the follow-up predicts for late disease relapse. Thus CTC monitoring offers the opportunity to evaluate the efficacy of adjuvant therapy and identify those patients who are more likely to benefit from secondary adjuvant treatments.

Contents

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1 Introduction

Circulating tumor cells (CTCs) are those cells present in the blood that possess antigenic and/or genetic characteristics of a specific tumor type [1]. Different markers have been used for the detection of CTCs based on their expression on epithelial cells (epithelial-specific markers) or in breast tissue (breast tissue-specific markers). Cytokeratin-19 (CK-19) is among the most well-studied markers [2]. It is stably and abundantly expressed on epithelial breast tumors but not on mesenchymal hemopoietic cells and has been successfully used for the detection of breast cancer cells in the bone marrow, lymph nodes and peripheral blood.

2 CTC Detection by RT-PCR for CK19 mRNA

In addition to the appropriate marker selection, the unambiguous identification and characterization of CTCs requires extremely sensitive and specific detection methods. Most of the presented data on the prognostic value of CK19 mRNA-positive cells in women with early breast cancer are based on a quantitative real-time RT-PCR assay which was developed and validated using the LightCycler™ system as previously described (graphically presented in Fig. 1) [3]. Briefly, PBMCs were isolated from 20 ml of peripheral blood, by centrifugation through Ficoll-Hypaque, RNA was extracted and cDNA was synthesized. According to the analytical detection limit of this assay, the presence of $\geq$0.6 MCF-7 equivalents/5 μg of total RNA was considered a positive result. Using the above cut-off, only two of 89 healthy female donors were positive for CK19 mRNA detection (2.2%), while none of nine women with benign breast disease had positive blood samples.

3 Prognostic Value Before Adjuvant Chemotherapy

Using this methodology the prognostic value of CTCs' detection was first reported in a group of 167 patients with axillary node-negative operable breast cancer [4]. The detection rate of CK19-positive cells, after surgical excision of the primary tumor and before the administration of any adjuvant therapy was 21.6%. There was no correlation between CTCs' detection and known pathologic and clinical prognostic factors with the exception of *HER2* amplification (score 2+ or 3+ by immunohistochemistry) ($p = 0.033$). Multivariate analysis revealed that CTCs' detection was associated with early metastatic relapse ($p < 0.001$) and disease-related death ($p = 0.008$). These results indicate that dissemination of tumor cells via the hematogenous route could be an early event in the course of the disease, occurring even before the lymphatic spread. These findings were verified in an expanded cohort of 444 women with stage I–III breast cancer (36.7% of whom

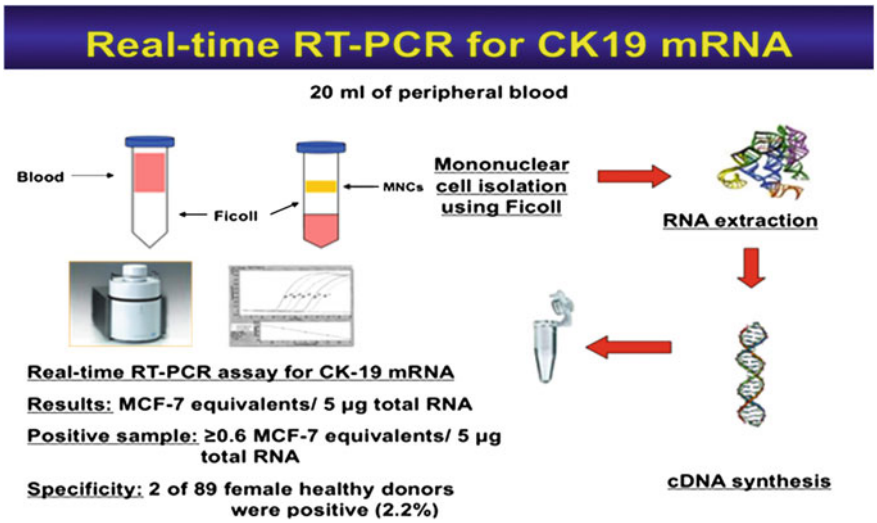

Fig. 1 Graphic representation of the real-time RT-PCR assay used for the detection of CK19 mRNA-positive cells

were node-negative) [5]. CK19 mRNA-positive patients before the start of adjuvant chemotherapy experienced shorter progression-free (PFS) ($p < 0.001$) and overall survival (OS) ($p < 0.001$).

4 Prognostic Value After Adjuvant Chemotherapy

The effect of adjuvant chemotherapy and the prognostic significance of CTCs' detection after the completion of adjuvant chemotherapy have also been reported [6]. In 437 patients blood was analyzed for CK19 mRNA detection both before the administration and after the completion of adjuvant chemotherapy. A total of 179 patients (41.0%) were CK19 mRNA-positive before chemotherapy. One out of two of these patients (51%) became CK19 mRNA-negative after the completion of adjuvant chemotherapy. At the same time, 22% of the patients with initially undetectable CTCs became CK19 mRNA-positive despite treatment administration. These results indicate that CTCs are often resistant to standard adjuvant chemotherapy. Significantly decreased DFS and OS were reported for the post-chemotherapy CK19 mRNA-positive patients ($p = 0.001$). Moreover, detection of CK19 mRNA-positive cells both before and after adjuvant chemotherapy was associated with the worst clinical outcome and emerged as an independent factor for decreased DFS ($p = 0.001$) and OS ($p = 0.003$) in multivariate analysis. The authors concluded that the detection of CTCs after the completion of adjuvant chemotherapy is an independent risk factor indicating the presence of chemotherapy-resistant residual disease.

5 Prognostic Value in Molecular Subtypes

Based on the heterogeneity of breast cancer [7] and using the same methodology it has been demonstrated that CK19 mRNA detection in the blood predicts the prognosis in clinically relevant subgroups of early stage breast cancer patients [5]. A total of 444 patients were analyzed for CK19 mRNA detection after primary surgery and before the initiation of adjuvant chemotherapy and 181 (40.8%) were found to be CTC-positive. Among them, 109 (41.9%) of 260 patients with estrogen receptor (ER)-positive tumors were CTC-positive, 71 (40.6%) of 175 with ER-negative tumors, 27 (35%) of 77 with triple-negative tumors, 35 (39.8%) of 88 with HER2-positive tumors, and 82 (44.1%) of 186 patients with ER-positive/HER2-negative tumors. After a median follow-up of 53.5 months, overall patients with detectable CTCs experienced significantly reduced DFS and OS. Nevertheless, this was mainly observed in patients with ER-negative, HER2-positive or triple-negative tumors. On the other hand, despite the presence of CTCs, patients with ER-positive or ER-positive/HER2-negative tumors did not have any significant difference in clinical outcome. In multivariate analysis, the interaction between CTCs and ER status was the strongest independent prognostic factor for reduced DFS (hazard ratio [HR], 3.808; 95% CI, 2.415 to 6.003; $p < 0.001$) and OS (HR, 4.172; 95% CI, 2.477 to 9.161; $p < 0.001$). This study demonstrated for the first time that CTCs' detection by a sensitive quantitative PCR assay had a different prognostic value among the molecular subtypes of early breast cancer.

6 Prognostic Value During Hormonotherapy and Follow-Up

The prognostic role of CTCs' monitoring is not limited to the peri-chemotherapy period. The unfavorable clinical outcome of patients with detectable CTCs during the administration of adjuvant hormonal treatment and subsequent follow-up was shown in two recent studies. A total of 119 patients with hormone receptor-positive breast cancer treated with adjuvant tamoxifen were tested for CK19 mRNA detection in their blood [8]. Twenty-two patients (18.5%) were CTC-positive after the completion of adjuvant chemotherapy and before the initiation of tamoxifen [8]. The majority of them (68.2%) were also resistant to adjuvant tamoxifen administration (persistently CTC-positive: 12.6% of the 119 patients). Sixty-eight patients (57.1%) remained CTC-negative throughout the follow-up period (persistently CTC-negative: 57.1% of the 119 patients). Failure of tamoxifen to eradicate CTCs was an independent prognostic factor for short DFS and OS (HR = 22.318, $p < 0.001$ and HR = 13.954, $p < 0.001$, respectively).

More recently the clinical relevance of CTCs' detection at different time points during the follow-up period after the completion of adjuvant chemotherapy was evaluated in patients with operable breast cancer for its prognostic value on late disease relapse and death [9]. Blood was analyzed for CK19

mRNA detection from 312 women with operable breast cancer who had not experienced disease relapse during the first 2 years of follow-up. The first sample was obtained 3 months after the completion of adjuvant chemotherapy and subsequent samples every 6 months thereafter for a 5-year follow-up period. Eighty patients (25.6%) remained CTC-negative throughout the 5-year period. A change in CTCs' status was observed in 133 patients (42.6%); 64 patients (20.5%) with initially CK-19 mRNA-positive CTCs during the first 24 months turned CTC-negative afterwards while 69 (22.1%) who were initially CTC-negative became CTC-positive. Ninety-nine patients (31.7%) remained persistently CK19 mRNA-positive. After a median follow-up period of 107 months (range: 38–161 months), the persistently CTC-positive patients with either hormonal receptor-positive or -negative tumors had a higher risk of late disease relapse compared to the persistently CTC-negative patients (36.4 vs. 11.2%, $p < 0.001$). Multivariate analysis also revealed that persistently CTC-positive patients had a shorter disease-free ($p = 0.001$) and overall survival ($p = 0.001$). It was concluded that the persistent detection of CK19 mRNA-positive CTCs during the first 5 years of follow-up is associated with an increased risk of late disease relapse and death for patients with operable breast cancer, indicating the presence of chemotherapy- and hormonotherapy-resistant residual disease. This prognostic evaluation and monitoring of the CTC status could be useful when deciding on subsequent adjuvant systemic therapy.

7 Conclusions

Although all these studies on the detection of CK19 mRNA-positive CTCs in patients with early stage breast cancer were conducted by a single research group and therefore need confirmation by other investigators, they do suggest a potentially significant clinical utility. Patients with CK19 mRNA-positive CTCs before and/or after adjuvant chemotherapy or during hormonotherapy and follow-up experience a significantly shorter survival than CTC-negative patients. Hence, the detection of CTCs in a patient with early breast cancer may indicate the presence of micrometastasis and an aggressive biological behavior of the primary tumor. Besides the prognostic information, the detection and monitoring of these cells may also be used for evaluating the efficacy of adjuvant systemic therapy. However, there are no prospective data as yet to show that CTC detection can be used to modify the treatment strategy and thus prolong survival or improve quality of life for breast cancer patients. Prospective clinical studies are now ongoing or planned to evaluate whether eradication of CTCs in the blood is correlated with improved clinical outcome in the adjuvant setting. If these studies confirm a significant clinical benefit with CTC detection, then CTCs' monitoring will become part of the routine breast cancer patient care. Furthermore, the markers expressed and the signaling pathways activated on CTCs may guide the individualized use of targeted therapies in the future.

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CTCs in Metastatic Breast Cancer

Antonio Giordano and Massimo Cristofanilli

Abstract

Circulating tumor cells (CTCs), enumerated by the Food and Drugs Administration-cleared CellSearch® system, are an independent prognostic factor of progression-free survival (PFS) and overall survival (OS) in metastatic breast cancer (MBC) patients. Several published papers demonstrated the poor prognosis for MBC patients who presented basal CTC count ≥ 5 in 7.5 mL of blood. Therefore, the enumeration of CTCs during treatment for MBC provides a tool with the ability to predict progression of disease earlier than standard timing of anatomical assessment using conventional radiological tests. Randomized clinical trials are ongoing to demonstrate whether CTCs detected by CellSearch® may help to guide treatments in MBC patients and improve prognosis. Moreover, the ability to perform molecular characterization of CTCs might identify a new druggable target in MBC patients. For example, the RT-PCR-based approach AdnaTest BreastCancerSelect™ showed a high

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discordance rate in receptor expression between the primary tumors and CTCs. Theoretically, the phenotypic analysis of CTCs can represent a "liquid" biopsy of breast tumor that is able to identify a new potential target against the metastatic disease.

Contents

1 Introduction

The natural history of breast cancer suggests that the disease has the capability to develop distant recurrence to specific organs and become a lethal disease. To leave the primary site and to soil in the metastatic niche, cancer cells need to disseminate through the blood and/or lymph. The "seed and soil" theory, postulated by Paget in 1889 [1] and revived fully by Hart in 1980 [2], represents the milestone for the study of CTCs in metastatic breast cancer (MBC) patients. CTCs might represent the seed necessary for cancer dissemination and the cells responsible to initiate the metastatic process. In 1869, Thomas Ashworth reported that, "*Cells identical with those of the cancer itself being seen in the blood may tend to throw some light upon the mode of origin of multiple tumours existing in the same person.*"[3] Only recent advances in detection methods have enabled their reproducible identification and further characterization.

Although CTCs provide a link between the primary tumor and metastatic sites, the factors involved with CTC survival in the blood circulation and eventual metastasis are not well understood. So far, much of what is known on CTCs in MBC patients simply regards numbering and prognostic value. Recently, advances in technology have facilitated the detection of even very small numbers of CTCs in the peripheral blood of MBC patients. Research is currently focused on specifically identifying these CTC subsets and characterizing them at the molecular level to ultimately provide a tool that would allow tailoring of treatment on an individual basis. Moreover, the biology of the metastatic process leads researchers to study the plasticity properties of tumor cells that leave the primary tumor, invade the blood stream and travel to the specific distant organs with a developed metastatic niche. The epithelial-to-mesenchymal transition (EMT) seems to be strictly involved in CTC plasticity. In the following paragraphs we will describe the utility and results of CellSearch® for CTC identification in MBC patients, and other different techniques of CTC molecular characterization.

Table 1 CellSearch® detection rate, PFS, and OS in MBC

Study	No. of patient studied	Line of treatment	Patients with ≥5CTCs (%)	Median follow-up	PFS (months) ≥5 v <5 CTCs	OS (months) ≥5 v <5 CTCs
Cristofanilli [4]	177	Any	49.2	9.7[a]	2.7 v 7	10.1 v > 18
Botteri [11]	80	Any	61.3	28	NA	~7 (HR)
Maestro [9]	192	Any	49	Na	NA	NA
Bidard [12]	65	Any	53.8	8.8	2.5 (HR, ns)	NA
Liu [5]	68	Any	35	13.3	3.2 *v* 5.1 (ns)	NA
Nakamura [6]	118	Any	37.3	NA	NA	3.1 (HR)
Pierga [8]	264	1st	44	14.9	8.2 v 9.6 v 19.9[b]	20 v NR
Giuliano [13]	235	1st	40	18	7 v 12	21.9 v 40.1
Giordano [14]	516	Any	39.9	11.7	7.3 v 11.4	18.7 v 30.4

NA not applicable, *ns* not significant, *NR* not yet reached
[a] minimum follow-up
[b] ≥ 5 v 1–4 v 0 CTC

2 CellSearch® System for CTC Identification in MBC

Since the blood stream is composed of an abundance of cells such as leukocytes (WBC) and erythrocytes (RBC), it is challenging to identify CTCs. CTC identification has been based mostly on the detection of epithelial cell markers, such as the EpCAM adhesion molecule, intracellular cytokeratin expression, and nuclei presence (4′,6-diamidino-2-phenylindole positivity), in patient blood and on the contemporary exclusion of normal blood cells, such as WBC markers, e.g., CD45. The CellSearch® system (Veridex Corporation, Warren, NJ, USA) is the only Food and Drugs Administration-cleared CTC detection system and has the most robust clinical data with reproducible results across different laboratories. Cristofanilli [4] showed for the first time in 2004 that the number of CTCs detected by the CellSearch® system before starting a new line of treatment is an independent predictor of progression-free survival (PFS) and overall survival (OS) in patients with MBC. This prognostic utility was independently confirmed by different groups [5–9]. Table 1 summarizes all clinical trial in MBC patients using CellSearch® CTC detection system. Patients with ≥5 CTCs per 7.5 ml of peripheral blood have significantly inferior PFS (median PFS, 2.7–8.2 months) and OS (median OS, 10.1–21.9 months) compared to patients with <5 CTCs (median PFS, 7–12 months; median OS, 18–40.1 months). Moreover, CTC counts at the first follow-up visit showed similar prognostic value [10].

Superior survival among patients with <5 CTCs per 7.5 ml was observed regardless of histology, hormone-receptor status and Her2 status, and whether the patient had recurrent or de novo metastatic disease [4, 15]. The prognostic value was independent of the line of therapy (that is first-line versus second-line or more), site of metastasis (for example visceral versus soft tissue and/or bone) and subtype of disease (for example basal versus luminal). This prognostic impact was demonstrated to be superior to tumor burden as measured by Swenerton score or by serum tumor markers, suggesting a special biological value of CTCs [16]. Recently, it was shown that, in MBC patients, CTCs at the first follow-up could accurately predict prognosis beyond the functional response assessed by FDG-PET and/or CT scan [17]. As reported in the last American Society of Clinical Oncology (ASCO) recommendation for the use of tumor markers in breast cancer, the measurement of CTCs should not be used for initial diagnosis of breast cancer or influence any treatment decisions in patients with breast cancer. Similarly, the use of CellSearch® in patients with MBC cannot be recommended until further validation confirms the clinical value of this test [18]. The clinical utility of these findings are now being prospectively addressed in a randomized trial led by the Southwest Oncology group (www.cancer.gov/clinicaltrials/SWOGS0500). The primary objective of this trial is to determine whether women with MBC and elevated CTCs (≥5 per 7.5 mL of whole blood) after 3 weeks of first-line chemotherapy derived an increased OS from changing to an alternative chemotherapy regimen at the next course rather than waiting for clinical evidence of progressive disease. On the same basis, the CirCe01 trial will assess the use of CTC detection in third-line MBC patients (http://clinicaltrialsfeeds.org/clinical-trials/show/NCT01349842). These two pivotal studies will conclude whether the measurement of CTCs will help guide therapy in cancer patients, which would be one of the most important topics for implementing CTC counts into clinical practice [19]. However, in a recent publication [13] we showed that women with high baseline CTC counts received very little survival benefit from first-line endocrine treatment, even if they were appropriate candidates for this therapy based on the hormone-receptor status of their primary or metastatic tumor (3.5 months versus 14.1 of PFS in patients with ≥5 CTCs and <5 CTCs respectively, $P = .001$). Moreover, we speculated that with CTC count it is possible to identify a group of patients with worse prognoses (≥5 CTCs) who benefited greatly from more aggressive treatments, including combination chemotherapy and mono-chemotherapy plus bevacizumab (both compared to single agent chemotherapy). Therefore, with the advent of targeted and/or biological therapy, the type of treatment directly affects tumor cells (trastuzumab) or modifies the tumor microenvironment (bevacizumab) and thus could influence processes such as intravasation, extravasation or clearance of CTCs. We and other authors suggested a limited prognostic value of CTCs in MBC patients treated with anti-angiogenetic therapy [8, 12, 20] or in patients pretreated with anti-Her2 therapy [13, 21]. Beyond the mere numeration of CTCs by the CellSearch® system, the molecular characterization and predictive value of detected CTCs can be the most effective utility for predicting the progression of disease early on and identifying a new druggable target in MBC patients.

3 Clinical Implication of CTC Molecular Characterization

Beyond the enumeration of CTCs, their molecular characterization provides a key to demonstrate their cellular origin from primary and metastatic tumor deposits, and it may also provide clues to their evolution during the course of cancer treatment [22]. Aktas et al. studied the ER, PR and HER2 expressions on CTCs from 87 MBC patients [23]. Blood samples were analyzed for CTCs with the AdnaTest BreastCancer™ (AdnaGen AG, Langenhagen, Germany), which enables the immunomagnetic enrichment of tumor cells via epithelial and tumor-associated antigens. The authors showed that in 48/62 (77%) patients with ER-positive tumors, CTCs were ER-negative and that 46/53 (87%) patients with PR-positive tumors did not express PR on CTCs. Primary tumors and CTCs displayed a concordant ER and PR status in only 41% ($p = 0.260$) and 45% ($p = 0.274$) of cases, respectively. Moreover, regarding the immunohistochemical subtype, they demonstrated that most of CTCs were triple-negative (39 of 86 patients 45%) or HER2-positive (27 of 86 patients, 32%). The remaining 23% of CTCs (20 of 86) were ER and/or PR-positive with positive or negative HER2 status. Clinical trials in which treatment is chosen on the bases of CTC markers are now ongoing (http://clinicaltrials.gov/ct2/show/NCT00694252, and http://clinicaltrials.gov/ct2/show/NCT00820924).

Therefore, the most recent technologies are being developed with the possibility of studying CTC molecular profiling (Table 2). So far, the major CTC detection assays use an EpCAM-based enrichment method (CellSearch®, AdnaTest) and/or cytokeratin expression (CK RT-PCR detection) as the first step to isolate tumor cancer cells. Around 30–35% of MBC patients did not show any CTCs in the blood, and current technologies are likely missing a substantial number of CTCs. These no-detectable CTCs might have an aggressive phenotype such as an EMT associated with stem cell behavior. Several papers have shown that some CTCs detected in breast cancer patients have EMT features [27, 37, 38], and with the development of EMT phenotype and reversal of the epithelial characteristics, the expression of adhesion molecules and cytokeratins becomes downregulated [28, 37, 39, 40]. In a recent oral communication at the last ASCO annual meeting, we showed that CTCs with EMT characteristics in a subgroup of 30 HER2-positive MBC patients were detected independently of epithelial cell enrichment, suggesting that most of the CTCs may be in partial EMT [41]. This evidence supports the idea that the biologic characteristics, along with the number of CTCs, need to be carefully taken into account in future analyses. Therefore, the commonly used EpCAM-based enrichment method could lack in identifying the aggressive and stem-like CTCs.

In conclusion, CTCs counted by CellSearch® might be useful in distinguishing different prognostic and predictive MBC patient groups. The American SWOG-S0500 and the European CirCe01 trials will address the clinical value of CTCs versus the conventional clinical and radiological evaluation. Therefore, molecular characterization could better describe the heterogeneity of CTCs and their relation

Table 2 Main studies on CTC molecular profiling in MBC

Study	CTC detection methods	CTC Molecular profiling methods	CTC findings
Aktas [23]	AdnaTest BreastCancerSelect	RT-PCR	Most of the CTCs were ER/PR- despite the presence of an ER/PR+ primary tumor
Tewes [24]	AdnaTest BreastCancerSelect	RT-PCR	86% EpCAM+; 86% MUC1+; 32% HER2+; 35% ER+; 12% PR+
Somlo [25]	Miltenyi CD45 microbeads, immunostaining	Fiber-optic array scanning technology (FAST)	Discordance rates of 40 and 23%, respectively for ER and HER2 expression in primary tumors and CTCs
de Cremoux [26]	CELLection beads Dynabeads®	RT-PCR	Significant correlation between the presence of MUC1+ cells and advanced stage
Kallergi [27]	CELLection beads Dynabeads®	Immunostaining	High rate of CTCs expressing Twist and Vimentin, suggestive of an EMT, are identified in patients with MBC
Gradilone [28, 29]	CELLection beads Dynabeads®	PCR	The presence of mesenchymal markers on CTC more accurately predicted worse prognosis than the expression of cytokeratins alone predicted
Markou [30]	CELLection beads Dynabeads®	PCR-coupled liquid bead array	KRT19, ERBB2, MAGEA3, SCGB2A2, and TWIST1 overexpressed in CTCs of MBC patients
Flores [31]	CellSearch profile kit	FISH	Significant discordance exists between the HER2 amplification of patient CTCs and that of the primary and metastatic tumor
Sieuwerts [32]	CellSearch profile kit	qRT-PCR of mRNA and miRNAs	55 mRNAs and 10 miRNAs were expressed more abundantly in samples from 32 patients with at least 5 CTCs
Armstrong [33]	CellSearch profile kit	Immunostaining	Over 75% of CTCs from women with MBC co-express cytokeratin, vimentin, and N-cadherin
Hayashi [34]	CellSearch epithelial kit	Immunostaining and FISH	Discordance rate of 13.6% for HER2 expression in primary tumors and CTCs
Punnoose [35]	OncoCEE microchannel Biocept, On-Q-ity's CTC-Chip	Immunostaining, qRT-PCR and FISH	High concordance rate for HER2 expression in primary tumors and CTCs
Königsberg [36]	OncoQuick® plus	Immunoflourescence	EpCAM independent enrichment technologies seem to be superior to detect the entire CTC population

to therapeutic intervention. New advanced technologies will allow us to define the CTC nature and identify new druggable markers of the metastatic process.

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HER2-Positive DTCs/CTCs in Breast Cancer

Andreas D. Hartkopf, Malgorzata Banys and Tanja Fehm

Abstract

The presence of circulating tumor cells (CTCs) in the blood as well as disseminated tumor cells (DTCs) in the bone marrow of breast cancer patients is associated with a worsened prognosis in the primary as well as in the metastatic situation. Next to their detection, evaluation of human epidermal growth factor receptor (HER2) expression is a valuable feature of CTCs/DTCs. As the HER2 status may change during disease progression CTCs/DTCs might (1) characterize the phenotype of minimal residual disease in the adjuvant setting and (2) serve as a "real time biopsy" of metastatic breast cancer. Phenotyping of CTCs/DTCs will thus help to understand mechanism of resistance to HER2-directed therapy. Moreover, patients that are likely to benefit from HER2-directed therapy despite a HER2-negative primary tumor might be identified.

Contents

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1 Introduction

The Human epidermal growth factor receptor (HER2) is a 185-kDa tyrosine kinase receptor that is encoded by a proto-oncogene located on chromosome 17q21. It is over-expressed by approximately 20–25% of all primary invasive breast cancers and associated with aggressive tumor behavior. Women suffering from HER2-positive breast cancer are at increased risk of disease progression and death. As compared with their HER2 negative counterparts, HER2 positive tumors are more frequent in younger women, more often nodal positive and more likely to be resistant to cytotoxic and endocrine therapy [1, 2]. HER2 positive tumors are, however, eligible for treatment with the humanized monoclonal antibody trastuzumab, which is indicated in the metastatic as well as in the primary situation [3–6]. Other agents that specifically target HER2 positive breast cancer are pertuzumab and lapatinib [7, 8].

Patients are selected for HER2 directed therapy based on immunohistochemical detection of HER2 over-expression as well as on gene-based fluorescence in situ hybridization (FISH). As the current gold standard, evaluation of HER2 over-expression and/or gene amplification is made on the primary tumor [9]. This strategy implicates that the primary tumor is representative for the entire tumor burden and that its initial phenotype will not change during the course of disease. However, a discrepancy between the antigenic profile of the primary tumor and distant metastases was observed in 7–20% of the cases (Table 1). HER2 gene amplification can be acquired during breast cancer progression and could be a potential target for HER2-directed therapy [10]. As determination of the HER2 status at primary diagnosis was not a standard procedure until trastuzumab found its way into the clinical routine, breast cancer patients currently suffering from a relapse may have an unknown HER2 status. Abandonment of HER2 targeted therapy could thus result in unintentional under-treatment.

After surgical resection of the primary tumor, single tumor cells in secondary sites represent an attractive surrogate for minimal residual disease (MRD) and might help to identify patients in need for additional systemic therapy [21]. Using highly sensitive immunocytochemical and molecular assays, disseminated tumor cells (DTCs) can be detected in the bone marrow (BM) or in the peripheral blood of breast cancer patients. DTCs in the BM of breast cancer patients are an independent predictor of poor prognosis [22–29]. The detection of circulating tumor cells (CTCs) in the peripheral blood of metastatic breast cancer patients is associated with a worsened prognosis and may be predictive for response to treatment [30, 31]. Next to their detection, major advantages in characterization of DTCs and CTCs by phenotyping and genotyping have been achieved in the past years [32]. This will not only help to identify biological mechanisms of early dissemination but might also serve as a predictor for systemic therapy. In the following chapter we will therefore discuss a valuable feature of DTCs and CTCs: the presence or absence of the HER2 proto-oncogene. This may be of important clinical relevance,

Table 1 The comparison of HER2 status of primary tumor and distant metastasis in metastatic breast cancer patients

Author	N	HER2 pos. primary tumor (%)	Method	HER2 pos. metastasis (%)	Concordance (%)
Zidan et al. [11]	58	14 (24)	IF, FISH	20 (35)	86
Edgerton et al. [12]	93	3 (20)	IF, FISH	5 (33)	74–84[a]
Tanner et al. [13]	44	13 (30)	FISH	13 (30)	100
Gancberg et al. [14]	100	13 (13)	IF	19 (19)	94
	84	21 (25)	FISH	20 (24)	93
Niehans et al. [15]	30		IF		97
Masood et al. [16]	56		IF		98
Shimizu et al. [17]	21		IF		100
Dowsett et al. [18]	39		IF		92
Luftner et al. [19]	80		IF		82
Regitnig et al. [20]	31		IF, FISH		74

IF immunofluorescence, *FISH* fluorescence in situ hybridization
[a] Depending on methodology (*IF* vs. *FISH*)

as the detection of HER2 positive DTCs/CTCs could contribute to an adequate selection of patients eligible for HER2 targeted therapy.

2 Evaluation of the HER2 Status of DTCs/CTCs

Different methods to assess the HER2 status of single tumor cells have been described. As illustrated in Fig. 1, the phenotype can be evaluated by using immunological double staining techniques. For this purpose, Meng et al. established an immunofluorescence staining score (1+, 2+, 3+) [10]. Further, HER2 over-expression can be characterized by FISH analysis using a centromeric probe for chromosome 17 and a locus-specific probe for HER2. When determining the HER2 status of the primary tumor, FISH analysis has proven to be more predictive of a favorable response to trastuzumab than did immunohistochemistry [33]. With regard to CTCs a high concordance between the two techniques could be observed [10, 34, 35]. This is of great importance as the relocation of CTCs for FISH analysis may be difficult to perform in large clinical trials [36]. Meng et al. suggested that at least 10 CTCs are necessary for an optimal evaluation of the HER2 status [10]. However, fewer CTCs may be sufficient with respect to the question whether a patient might benefit from HER2 directed therapy. In our previous study we defined a case as HER2 positive when a minimum of 5 CTCs were detectable

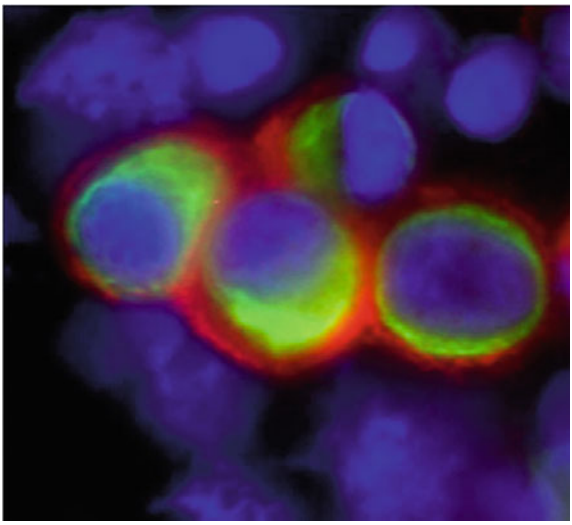

Fig. 1 Cluster of cytokeratin (CK)-positive and HER2-positive disseminated tumor cells (double immunofluorescence staining). Tumor cells were stained with an anti-CK-fluorescein isothiocyanate (*green*) and anti-HER2 detected by a secondary Texas Red labeled goat anti-rabbit (*red*) antibodies. Nuclei are stained blue with DAPI (× 40 oil immersion objective)

and at least one CTC had an immunostaining score of 3+ [36]. In a neoadjuvant trial, Riethdorf et al. used a cut-off level of at least one HER2 positive CTC/7.5 ml blood [35]. The number of CTCs is, however, much lower in the non-metastatic situation.

Another method to characterize DTCs/CTCs is to use PCR-based assays. Evaluating three tumor-associated transcripts by multiplex RT-PCR (GA 73.3, MUC1 and HER2), we recently compared the molecular *AdnaTest Breast Cancer*™ with the immunocytochemistry-based *CellSeach*™ assay [36]. There was no correlation between these two methods with regard to the HER2 status of CTCs. As an explanation *CellSeach*™ assay evaluates the HER2 status of individual tumor cells and thus represents the heterogeneity of disease. *AdnaTest Breast Cancer*™, however, determines the average HER2 expression of all tumor cells.

3 Clinical Impact of HER2-Positive DTCs/CTCs in Primary Breast Cancer

As MRD is the target of all adjuvant treatment strategies the need to determine expression profiles of residual tumor cells is becoming increasingly important. Several aspects of the HER2 status of DTCs/CTCs must be considered. Firstly, tumor cells in secondary sites reflect only a subclone of cancer cells from primary tumor. This selected subpopulation of tumor cells frequently features factors

Table 2 The comparison of HER2 status of primary tumor and DTCs/CTCs in primary breast cancer patients

Author	N	HER2 pos. primary tumor (%)	Method	HER2 pos. DTCs/CTCs (%)	Concordance (%)
Braun et al. [41]	24	7 (29)	DTCs at the time of initial diagnosis, IF	15 (63)	58
Becker et al. [50]	105	26 (25)	DTCs at the time of initial diagnosis, IF	22 (21)	77
Solomayer et al. [43]	45	14 (29)	DTCs at the time of initial diagnosis, IF	20 (44)	63
Krawczyk et al. [44]	31	5 (6)	DTCs at the time of initial diagnosis, IF	8 (26)	64
Jückstock et al. [51]	129	34 (26)	DTCs after adjuvant therapy, IF	49 (38)	68
Krawczyk et al. [44]	14	1 (7)	DTCs after adjuvant therapy, IF	5 (36)	71
Wulfing et al. [52]	27	3 (11)	CTCs at the time of initial diagnosis, IF	14 (52)	48
Fehm et al. [53]	58	22 (38)	CTCs at the time of initial diagnosis, RT-PCR	9 (16)	53
Riethdorf et al. [35]	58	21 (36)	CTCs in neoadjuvant disease, IF	14 (24)	60
Apostolaki et al. [54]	216	24 (11)	CTCs before adjuvant therapy, RT-PCR	53 (25)	68
Ignatiadis et al. [55]	101	19 (19)	CTCs during 5-year follow up, IF	8 (8)	71

CTCs circulating tumor cells of the peripheral blood, *DTCs* disseminated tumor cells of the bone marrow, *IF* immunofluorescence, *FISH* fluorescence in situ hybridization, *RT-PCR* reverse transcriptase polymerase chain reaction

linked with poor clinical outcome, e.g., negative hormone receptor status and up-regulation of urokinase-type plasminogen activator receptor [37, 38]. Moreover, due to their highly extravasative potential, HER2 positive cells benefit from a growth and survival advantage and are therefore more likely to persist in secondary microenvironments such as the bone marrow or the peripheral blood [39]. As a result, the HER2 status of DTCs/CTCs does not necessarily reflect the HER2 status of the primary tumor [11, 14, 40–44] (Table 2). However, the indication for HER2 targeted treatment (trastuzumab, pertuzumab or lapatinib) depends exclusively on the HER2 status of the primary tumor. Consequently, patients with HER2 positive DTCs/CTCs but HER2 negative tumors are not eligible for this treatment regime. According to previous studies, HER2 directed therapy is able to eliminate HER2 positive DTCs/CTCs [10, 45–47]. In an interventional clinical

trial provided by Jückstock et al., trastuzumab was able to eradicate HER2 positive CTCs in seven out of twelve patients in a post-adjuvant setting [48]. This could explain why some patients benefit from trastuzumab therapy despite a HER2 negative primary tumor [49].

Furthermore, to be able to disseminate from the primary tumor, persist in distant sites of the body and finally initiate metastatic growth, MRD needs to permanently evolve. Mutations of p53, accumulation of genomic imbalances, secretion of growth factors and HER2-mediated signaling are factors associated with tumor relapse [10, 21]. The characterization of DTCs/CTCs might help to investigate these and other mechanisms underlying disease progression. Recently, we observed an increase in the prevalence of HER2-positive DTCs in patients who remained DTC positive following systemic therapy [44]. The acquisition of genomic aberrations linked to more aggressive tumor behavior, such as HER2 amplification, may suggest disease progression and plays a crucial role in the metastatic cascade [10]. This underlines the importance of re-evaluation and re-characterization of DTCs/CTCs during the course of disease [56]. In contrast to tissue evaluation—a single event—monitoring of MRD provides the opportunity of real-time insight into cancer progression.

DTCs/CTCs may have a different chemosensitivity than the primary tumor [35, 57, 58]. Therefore, targeted therapy seems to be an ideal candidate to specifically hit MRD. Apostolaki et al. used a molecular assay to investigate the prevalence of HER2 positive CTCs before as well as after the administration of adjuvant chemotherapy and found that in 70% of the cases adjuvant systemic therapy was unable to eradicate HER2 positive CTCs [40]. The prevalence of HER2 positivity is higher in CTCs than in the primary tumor and HER2 positive CTCs are associated with a worsened prognosis [40, 41, 59, 60]. Bozionellou et al. investigated trastuzumab therapy in 30 primary breast cancer patients with chemotherapy resistant CTCs and/or DTCs as revealed by a PCR-based assay [47]. Eighty three percent of the patients were positive for HER2 mRNA expression. After trastuzumab administration 67–93% of the patients became CTC/DTC negative. Nonetheless, elimination of minimal residual disease may not have a direct influence on clinical outcome. Whether the indication for HER2 targeted treatment in an adjuvant setting should be extended to patients with HER2 positive MRD regardless of primary tumor status remains to be evaluated.

4 Clinical Impact of HER2 Positive DTCs/CTCs in Metastatic Breast Cancer

Tumor disease progression and the development of distant metastasis is a highly selective process and only a minority of tumor cells will fulfill the requirements to initiate metastatic growth. Dissemination of tumor cells is thus accompanied by genetic changes and women with HER2 negative primary tumor may have HER2

positive metastases or vice versa [11, 14, 20, 61]. Furthermore, systemic therapies may influence the prevalence of a given tumor clone over the others and HER2 targeted therapy may exert selective pressure on HER2 positive tumor cells. In a patient that has not yet gained resistance to HER2 targeted therapy it is reasonable to assume that HER2 positive metastases will respond to trastuzumab irrespective of the HER2 status that was found in the primary tumor. Generally, assessment of HER2 is performed in the primary tumor even if metastases appear several years later. Tissue sampling from distant metastatic sites is associated with increased morbidity and is thus not routinely performed. However, the HER2 status may change during the course of disease and also vary among different sites of metastasis [14] (Table 1).

In contrast to tissue sampling from solid metastasis, CTCs represent an attractive alternative to noninvasively re-evaluate the phenotype of the "total tumor load". Conducted by a simple blood test, CTCs might serve as a "real-time biopsy" for metastatic breast cancer patients and thus enable more individualized and optimized anti-metastatic therapies [21]. Meng et al. treated four patients with initially HER2 negative breast cancer but HER2 positive CTCs with trastuzumab-containing therapy: one patient had a rapid and complete remission; in two patients a partial response was observed [10]. Another interesting approach was proposed by Bernhard et al.: HER2-specific T-lymphocytes were transferred to a patient with metastatic HER2 positive breast cancer. This experimental therapy was able to eliminate HER2 positive cancer cells from the BM, but did not penetrate into solid metastases [62]. Based on these results clinical trials for metastatic breast cancer patients need to be designed that correlate clinical responses of HER2 targeted therapy to HER2 status of CTCs rather than to HER2 status of the primary tumor.

To investigate the HER2 status of CTCs in advanced breast cancer as compared to that of the primary tumor, a prospective multicenter trial was established by the DETECT study group (www.detect-study.de) [36]. Using the FDA-approved *CellSearch*® assay with a cut-off level of $\geq$5 CTCs/7.5 ml blood as a threshold, 122 out of 245 patients (50%) were considered as CTC positive. Of these, 122 CTC positive cases 50 (41%) patients had at least one CTC/7.5 ml blood that showed strong immunostaining for HER2. When the HER2 status of CTCs was compared to the HER2 status of the primary tumor, non-concordant results were found in 36% of the cases: HER2 positive CTCs were found in 25 out of 76 patients (33%) with initially HER2 negative primary tumors and 13 of 31 initially HER2 positive patients (42%) had HER2 negative CTCs. This study is in line with data presented by numerous other authors (Table 3) and was conducted in preparation for a prospective multicenter trial that aims to compare standard therapy alone versus standard therapy plus lapatinib in patients with initially HER2 negative metastatic breast cancer but HER2 positive circulating tumor cells (DETECT III).

Table 3 The comparison of HER2 status of primary tumor and DTCs/CTCs in advanced breast cancer patients

Author	N	HER2 pos. primary tumor (%)	Method	HER2 pos. DTCs/ CTCs (%)	Concordance (%)
Braun et al. [34]	40	12 (30)	CTCs, IF/FISH	15 (37)	68
Becker et al. [42]	15	3 (20)	CTCs, IF/FISH or RT-PCR	5 (33)	40
Tewes et al. [63]	22	5 (23)	CTCs, RT-PCR		36
Meng et al. [64]	52	–	CTCs, IF/FISH	–	87
Fehm et al. [36]	107	31 (29)	CTCs, IF	43 (40)	64
	79	22 (56)	CTCs, RT-PCR	37 (47)	48
Ignatiadis et al. [55]	39	6 (7)	CTCs, IF	14 (36)	54
Pestrin et al. [34]	40	12 (30)	CTCs, IF/FISH	15 (37)	68
Meng et al. [10]	33	15 (46)	CTCs, IF/FISH	11 (33)	88

CTCs circulating tumor cells of the peripheral blood, *DTCs* disseminated tumor cells of the bone marrow, *IF* immunofluorescence, *FISH* fluorescence in situ hypridization, *RT-PCR* reverse transcriptase polymerase chain reaction

5 Conclusion and Perspectives

The HER2 status of DTCs/CTCs may differ from that of the primary tumor. Preliminary data demonstrated efficiency of trastuzumab in patients with HER2 positive CTCs. Large prospective trials should investigate whether women suffering from HER2 negative breast cancer but with HER2 positive DTCs/CTCs would benefit from HER2 targeted therapy in the adjuvant as well as in the metastatic situation. As different DTCs/CTCs among one BM/blood sample may be heterogeneous with respect to their HER2 status prospective trials should also evaluate appropriate criteria to determine HER2 positivity of a patient.

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DTCs/CTCs in Breast Cancer: Five Decades Later

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Abstract

Since circulating tumor cells were first reported in 1955, the field has seen major advances in their detection and has established their prognostic impact. Here we review the current evidence for the prognostic and predictive value of circulating tumor cells in metastatic breast cancer. We then evaluate the role of CTCs and DTCs in early stage breast cancer. The weight of the evidence supports the role of CTCs and DTCs as prognostic indicators, however their role in therapy prediction remains unclear. Ongoing trials may provide answers and newer detection methods which improve sensitivity and specificity may have greater impact. At this point, the data does not support incorporation into clinical practice for early breast cancer patients.

Contents

Although 90% of invasive breast cancers appear limited to the breast and regional lymph nodes at diagnosis, occult micrometastases are common. Up to 70% of these tumors will recur without systemic therapy. While tumor size and nodal status are the best predictors of systemic recurrence, accumulating evidence

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suggests that analysis of blood and bone marrow for micrometastases may give additional prognostic information. Despite enormous controversy and frustration, the field of disseminated cancer cells has crept forward and approaches utility in clinical practice. With five decades of research on the topic, the outstanding question is not whether disseminated tumor cells (DTCs) or circulating tumor cells (CTCs) truly exist, but what do they tell us about the biology of the disease, and how best to apply this knowledge to the treatment of our patients.

The first observation by Engell in 1955 that neoplastic cells could be found in the circulation of cancer patients opened the door to this field [1]. Following this report, early studies on micrometastatic disease did not show consistent results, however, accumulated evidence suggests that epithelial cells detected in the blood and bone marrow of breast cancer patients are independent prognostic factors of disease-free and overall survival [2–7]. There are countless methods used to detect disseminated tumor cells, ranging from immunohistochemical to molecular methods, however, few have come into the market for commercial use and fewer still with FDA approval. Prospective trials are ongoing in an attempt to validate the use of such methods.

1 Metastatic Breast Cancer

The majority of research on CTCs has been conducted in the setting of metastatic breast cancer. In 2004, Cristofanilli et al. reported the utility of CTCs measured by the Veridex CellSearch system, the only FDA-approved methodology, in a prospective trial of 177 patients with measurable metastatic disease. CTCs were measured at baseline and the first visit after starting therapy, an average of 3–4 weeks later. The authors found that patients with CTC levels greater than or equal to 5/7.5 mL of whole blood had a decreased median progression-free survival compared to those with fewer than five cells/7.5 mL (2.7 months versus 7.0 months; $p < 0.001$) as well as a decrease in overall survival (10.1 months versus >18 months; $p < 0.001$). An unplanned subset analysis, however, did raise the issue of predictive accuracy not being consistent across all subgroups—specifically the number of CTCs before hormonal therapy was not associated with overall survival. Of great interest was the observation that the presence of greater than 5 CTCs at the first time point, whether or not the patient had greater or less than five cells/7.5 mL at baseline, suggested ineffectiveness of therapy [8]. However, this trial could not answer whether a switch in therapies at an earlier time point would improve survival. A follow-up study found that the association with PFS and OS based on CTC detection was significant at multiple time points after the initiation of therapy, ranging from 3 to 20 weeks [9]. The authors reported that a change in CTC levels from detectable to nondetectable was predictive of response to therapy, whereas change in CTC levels from nondetectable to detectable was predictive of a lack of treatment efficacy.

A subsequent series of studies were conducted to validate the predictive value of CTCs. These studies have variable outcomes. Bidard et al. studied 67 patients with metastatic breast cancer who were treated with first line chemotherapy combined with bevacizumab and found that using a cutoff of 5 CTC/7.5 mL was not predictive

of tumor response or time to disease progression at baseline, however, a level of 3 CTC/7.5 mL at baseline was predictive of these endpoints. By 6 weeks following treatment initiation CTC count was not associated with time to progression or tumor response regardless of cutoff, suggesting that many time points and cut-points had been examined in an attempt to establish significance. The authors suggested that perhaps bevacizumab modifies the predictive value of CTC due to impaired tumor cells intravasation through vessel endothelium, although this seems highly speculative [10]. Giordano et al. similarly did not find a predictive value for CTC in patients with HER-2 positive disease who received HER-2 targeted therapy. However, the authors reported that CTC strongly predicted survival in HER-2 negative metastatic breast cancer and the prognostic value was independent of subtype and disease site in this patient population [11]. This was especially true in hormone receptor positive disease and bone involvement, in contrast to the data from the Cristofanilli trial. These studies point out the variability in detection of CTCs by tumor subtype and suggest that methodology must be improved to make this biomarker a useful measurement. Furthermore, as none of these trials was randomized, the predictive value of switching therapy based on CTCs cannot be determined. The ongoing SWOG 0500 trial is specifically designed to assess the clinical utility of using CTCs to change therapy early in the course of treatment and should more definitively answer the question, in the context of the Veridex methodology.

HER-2/neu detection on CTCs in metastatic breast cancer is less studied, and there is no standardized method for determining HER-2 status in CTCs. Nunes et al. used a semiquantitative RT-PCR for cytokeratin 19 combined with immunoselection (to remove background caused by contaminating mononuclear cells), to predict response and progression in a group of HER-2 positive patients. When compared to circulating levels of the HER-2 extracellular domain, as a more established tumor marker, a close correlation was seen and the prognostic and predictive role of CTCs using this method was supported [12]. A question that arises when looking at HER-2 in CTCs is how to interpret discordance between the HER-2 status of the primary tumor and CTCs. A small study by Reuben et al. measured receptor expression in primary and metastatic tumors and CTCs in 20 women with metastatic breast cancer. Expression of ER, PR and HER-2 was similar in primary and metastatic tumors—16 (80%) were ER-positive, 11 (55%) were PR-positive and 3(15%) were HER-2 positive. However, only three patients expressed ER, none expressed PR and 11 expressed HER-2 in their CTCs. Two patients with HER-2 negative primary tumors expressed HER-2 in the CTCs and had response to trastuzumab-containing regimens [13]. This study suggests that CTCs might be used as a 'virtual biopsy' to determine sensitivity to trastuzumab treatment, however, it should be noted that the regimens in this study contained chemotherapy in addition to trastuzumab. Fehm et al. performed a larger study where they investigated HER2 status in CTCs, using both the Veridex CellSearch and AdnaTest. They also found discordance, where 32% of patients with HER-2 negative primary tumors had HER-2 positive CTCs using CellSearch and 49% with AdnaTest. When considering patients with CTCs on both tests, only 50% of patients had concordance in HER-2 positivity [14]. It has been shown that HER-2 is frequently expressed in the peripheral blood cells of healthy individuals,

suggesting that false positive results should be considered. You et al. found HER-2 positivity in CTCs from metastatic breast cancer patients with HER-2 negative tumors using immunoselection for Epcam and RT-PCR for HER-2 mRNA. Questioning this result, the authors used positive immunoselection for subpopulations of mononuclear cells from normal blood and identified the NK/granulocyte fraction as the source of HER-2 positive cells. They further presented a method of negative immunoselection to remove background signal and thereby improve specificity of testing for HER-2 positive cells [15]. To evaluate the potential role of HER-2 targeted agents in the setting of a HER-2 tumor negative/CTC positive clinical scenario, there is now an ongoing clinical trial of lapatinib in advanced breast cancer patients with HER-2 non-amplified primary tumors and HER-2 positive or EGFR-positive CTCs (clinical trials.gov identifier NCT00820924). Such prospective testing will be necessary to understand if there will be a benefit to use CTCs in the setting of treatment with HER-2 directed therapy.

CTCs are absent in up to 50% of patients with metastatic breast cancer, and it has been suggested that this in part may be secondary to lack of sensitivity in the methods of CTC detection. Mego et al., using the Veridex CellSearch system, reported that undetectable CTC status correlated with the presence of brain metastasis and inversely correlated with bone metastasis. Overall, lack of circulating CTCs was associated with superior survival, suggesting that CTCs are a marker of disease burden. The authors also hypothesized that current detection methods may miss cells that are undergoing the epithelial to mesenchymal transition (EMT) as the usual capture and visualization methods use EPCAM and cytokeratin [16]. RT-PCR has been used in other studies to detect levels of EMT inducing transcription factors—such as TWIST 1, SNAIL 1, SLUG, ZEB1 and FOXC2. However, a study by Mego et al. found no association between the overexpression of any EMT-associated transcription factors and the positivity of CTCs. It should be noted that this study evaluated a small subset of patients undergoing neoadjuvant chemotherapy and may have underrepresented poor prognostic subgroups more likely to express EMT markers [17]. In contrast, Aktas et al. reported an increase in prevalence of mesenchymal markers on CTCs in metastatic breast cancer. They analyzed blood samples from 39 women with metastatic breast cancer for the three EMT markers—Twist1, Akt2, PI3Kα, and found the majority of patients (62%) who had CTCs detected using the AdnaTest also expressed these EMT markers, as did 7% of cases where CTCs could not be detected [18]. Most recently, Gradilone et al. also found that presence of mesenchymal markers on CTCs more accurately predicted worse prognosis than the expression of cytokeratins alone [19]. These studies suggest that this most aggressive/invasive cell population may be overlooked by targeting epithelial antigens as CTCs may lose epithelial antigens during EMT. The study by Raimondi et al. further supports this contention, where CTCs were isolated using CELLection Dynabeads^{TM} coated with an EpCam antibody. In this study, 34% of CTCs that were negative for epithelial markers (CK/CD45), expressed markers of epithelial-mesenchymal transition (vimentin and fibronectin) [20]. The concept of variable expression of antigens on CTCs is clearly of value but requires further study before these results can be incorporated into clinical trials or clinical practice for metastatic breast cancer.

Advances are being made to increase sensitivity in the methods of CTC detection. One such method utilizes a microfluidic CTC chip coated with an anti-EpCAM antibody to capture a high yield of cells, with a median of 50 CTCs/mL [21, 22]. This method also offers the advantage of obtaining CTCs from whole blood directly, without preprocessing of samples and as a result viable cells can be obtained, in contrast to the non-viable cells isolated using CellSearch. An enhanced version—the herringbone (HB) chip utilizes microfluidic flow patterns to increase efficiency of cell capture [23]. Another microfluidic system aims to detect cells that do not express EpCAM or CK, such as those undergoing the EMT, utilizing in situ fluorescent labeling of capture antibodies to obtain higher detection of CTC's than anti-EpCAM [24].

2 Early Stage Breast Cancer

Unlike metastatic breast cancer, where studies have established >5 CTC/7.5 mL of blood as a positive cutoff point using CellSearch, no clear guidelines exist for definition of CTC positivity in early stage breast cancer. Some studies, such as the GeparQUATTRO study, have used >1 CTC/7.5 mL as a cutoff, given that CTCs with a threshold of 1 CTC/7.5 mL were not detected in healthy individuals [25, 26]. With this cutoff, they detected low numbers of CTCs in patients at baseline prior to receiving neoadjuvant chemotherapy (46 of 213 patients), however, a decrease in CTCs during treatment was not found to correlate with clinical characteristics or response of the primary lesion to treatment. The authors also found discordance between HER-2 status in the CTCs and the primary tumor, again suggesting that HER-2 negative primary tumors might benefit from trastuzumab treatment if HER-2 positive CTCs were detected [27]. This observation should be evaluated in larger studies, such as the aforementioned metastatic trial.

In 2000, Braun et al. published a study in the New England Journal of Medicine, where occult cytokeratin positive metastatic cells in the bone marrow of non-metastatic breast cancer patients correlated with occurrence of distant metastasis and death from cancer-related causes ($P < 0.001$). The presence of DTC in bone marrow was an independent prognostic indicator of the risk of death from cancer (relative risk, 4.17), after adjustment for the use of systemic adjuvant chemotherapy [28]. The prognostic significance of DTCs in early stages of the disease was studied by another group who found the presence of DTCs, measured at a median of 37 months after diagnosis of breast cancer, to be an indicator of reduced disease-free survival, distant disease-free survival, cancer-specific survival and overall survival during the first 5 years after diagnosis [29].

The correlation between CTCs and DTCs is less clear in early stage breast cancer. Krishnamurthy, et al. studied T1 and T2 tumors and found no correlation between the occurrence of CTCs and DTCs in either group. They also reported the lack of correlation between detection of these cells and clinicopathologic markers of disease (lymph node involvement, hormone receptor or HER-2 status, or histologic grade) [30]. In contrast, others have found that when looking at cohorts of non-metastatic patients at varying risk for relapse, DTC and CTC are seen

in a significant proportion of patients with high risk of recurrence. Specifically, 70% of those in a high-risk group (greater than three positive lymph nodes) had detectable CTC over the course of follow-up testing, as compared to 29% of those in a low-risk group (node-negative, T1 size) [31]. The authors suggest that those patients who remain CTC-negative might be those few who will not relapse. Other studies point out that given the short half-life of CTCs in comparison to DTCs (only 1–24 h), genetic material from apoptotic cells can be useful to measure. LOH of such cell-free DNA was found to be a correlate of disease progression [32].

As bone marrow aspiration has not been routinely used as a diagnostic tool for early stage breast cancer in many countries, CTCs have been the focus of study in the recent literature. Whether or not CTCs act as a direct correlate for DTCs, CTCs may provide prognostic information about themselves both before treatment and after adjuvant chemotherapy. The SUCCESS trial, presented in abstract form in 2010, showed CTCs to be of prognostic relevance in the adjuvant treatment setting and a useful treatment monitoring tool. In this trial, more than 1 CTC before treatment was a significant prognostic factor for DFS and OS and persistence of >1 CTC after chemotherapy was predictive of worse outcome. More than 5 CTCs were indicators of poor DFS and OS at all time points [33]. In a study of CTCs, Bidard et al. studied 115 early breast cancer patients using a cutoff of >1 CTC/7.5 mL of blood using CellSearch, and found that CTC positivity before chemotherapy was an independent prognostic factor for both distant disease-free survival and overall survival, but CTC detection after chemotherapy was not predictive [34].

Other groups have reported the use of reverse transcriptase polymerase chain reaction for CK-19 mRNA to successfully detect patients at higher risk of relapse after adjuvant therapy [35]. A recent study by Xenidis et al. demonstrated that the detection of CK-19 mRNA-positive CTCs after adjuvant chemotherapy was an independent factor associated with reduced disease-free survival ($P < 0.001$) and overall survival ($P = 0.003$), indicating the presence of chemotherapy resistant residual disease [36]. Sandri et al. studied CTC before and after breast surgery and found that approximately 30% of patients with localized breast cancer had CTC before and after surgery using the Cellsearch System. However, this study showed no correlation with clinicopathologic characteristics and 40% of patients had discordant results between CTC detection and tumor extirpation i.e., equal numbers changed from positive to negative and the reverse. These results suggest a lack of specificity for tumor burden using this methodology in early stage disease [37]. In the setting of preoperative therapy, both the GeparQuattro study and the study by Pierga et al. failed to show a correlation between CTC and primary tumor response to chemotherapy. These studies suggest that, as of yet, CTC detection in the perioperative period does not provide useful information using current technologies.

It is likely that CTCs and DTCs will find a future use in the adjuvant setting as most studies suggest correlation with outcome. However, additional prognostic markers have not been particularly useful in the adjuvant setting and significant advances in prediction of treatment response will be needed. With improved sensitivity and specificity of methodology, one can envision the use of CTC as a ‘virtual biopsy’, allowing assessment of treatment benefit in real-time. Ongoing,

prospective trials evaluating the utility of CTCs in the neoadjuvant and adjuvant settings should improve our understanding of their role, particularly with improved methodologies.

3 Recommendations

Circulating tumor cells have shown prognostic value for outcome of metastatic breast cancer. However, the value of this biomarker in treatment response has not been adequately assessed. We await the results of the SWOG 0500 trial to help answer this question as a randomized trial of switching early or not is the only way to adequately assess the value of CTCs in the metastatic setting. In early stage breast cancer, CTCs and DTCs appear to have prognostic value. In some settings, this may aid physicians and patients in clinical decision-making, but should be limited to settings where assay validation has been performed. While these biomarkers may one day prove useful as monitoring tool during neoadjuvant or adjuvant therapy, current data does not support this utility. In addition, the measurement of HER-2 on CTC/DTC biospecimens is not yet ready for incorporation into clinical practice, as specificity of these measures is still in doubt. Newer detection methods are on the horizon and show great promise. The weight of evidence suggests that detection of CTC/DTCs has the potential for enormous impact, and is likely to play an important role in the future.

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Part VI

Drug and Circulating Tumor Cell Co-development

Challenges in Drug and Biomarker Co-Development

Sheila E. Taube and Tracy Lively

Abstract

Co-development of drugs and biomarkers should be considered when the biomarker is intricately related to the use of the drug. There are risks and benefits to co-development and these need to be considered carefully early in the process. The current chapter attempts to delineate when it is appropriate to plan for co-development and to discuss a range of issues. Challenges include the determination of the type of assay (laboratory-developed test vs. reference laboratory vs. kit), the designs of trials for evaluation of clinical utility, and the regulatory pathway. Successful co-development requires planning very early in the process and assembling the appropriate multi-disciplinary team.

Contents

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1 Introduction

Biomarkers can be used in a variety of ways, and the intended use of the biomarker will generally determine the development pathway. Circulating Tumor Cells (CTCs) are no exception to this principle. The settings that might benefit from the use of a biomarker include among others detection of disease, baseline measurements to allow subsequent assessment of efficacy of treatment, estimation of aggressiveness of disease, presence of a target for a specific therapeutic, or detection of disease recurrence. Development of biomarkers for a setting that does not relate to specific therapeutic agents does not require or lend itself to co-development of the biomarker with an agent. Settings where the biomarker is intricately related to a specific agent should lead to consideration of co-development.

Biomarkers are often grouped into categories which include diagnostic, prognostic, predictive, pharmacodynamic and monitoring. For the purpose of this discussion, we will use the following definitions:

- Diagnostic—markers that aid in identification of the nature of the disease. In oncology this usually means determining the organ of origin of the lesion, the cell type and whether the lesion is benign or malignant.
- Prognostic—markers that help to estimate the likely course of a patient's disease in the context of standard care.
- Predictive—markers that indicate the likelihood of response to a specific agent or class of agents. For example, breast cancers that express estrogen or progesterone receptors are more likely to respond to hormonal therapy than those that do not. Predictive markers are usually measured on baseline samples before starting a course of therapy.
- Pharmacodynamic—markers that indicate whether a drug is modulating its intended target in the appropriate tissue. Pharmacodynamic measures are important during agent development, but the markers that might be useful for assessing pharmacodynamic behavior may not be useful as predictors of patient response or benefit.
- Monitoring—markers that are used following treatment to assess the efficacy of the treatment or to detect recurrence of the cancer.

Some further distinctions are important. Markers that can be measured prior to reaching a survival endpoint of a trial, often called interim markers or endpoints, have been proposed as surrogate markers or endpoints. A common example in clinical trials is the use of a time-to-event endpoint such as progression-free survival as a surrogate for overall survival, the actual endpoint of interest. Another example of a possible surrogate is residual disease, which might be measured using CTCs. It is of critical importance to understand that a surrogate marker must

be shown to be an accurate predictor of the true endpoint. There has been significant discussion of this issue, and in the United States the FDA has generally only accepted surrogate markers, such as progression-free survival, for accelerated approval [1, 2]. The European Medicines Agency has a comparable program and progression-free survival has been acceptable as an endpoint for indications such as breast cancer [3, 4].

2 When Should Co-Development Be Considered?

Using the above definitions, the type of markers that would most likely be considered for co-development would be predictive markers. The benefit of co-development depends on the nature of the therapeutic and the availability of an appropriate biomarker/assay [5]. If a therapeutic agent is expected to show a detectable level of benefit in an unselected population, then co-development of an assay would add unnecessary complexity. On the other hand, if the therapeutic agent is likely to work only in patients whose tumors have particular characteristics, such as mutations in a specific gene or pathway, it may not be possible to demonstrate the drug's effectiveness in a clinical trial without enriching the population for subjects likely to respond. Under these circumstances, the regulatory agencies may require that the agent be used only in the patients whose tumors demonstrate these characteristics, and it can be to the manufacturer's advantage to consider co-development of a marker/assay with the agent so that the assay and agent combination can be reviewed concurrently. Co-development must start early in order to accomplish this.

3 General Issues to Be Considered in Co-Development

The question of whether to restrict trial eligibility to a subset of patients selected on the basis of a biomarker assay is a key decision point in the development of an agent. Focusing on a subset of likely responders could potentially reduce the size of the trials required to demonstrate benefit, shorten development timelines and limit the number of patients exposed to an agent which would be ineffective for them. However, the decision to restrict eligibility also poses serious risks. From that point on the drug and the assay are locked together, and it becomes difficult or impossible to evaluate the performance of the assay as a predictor. Patients without the marker are not treated, so it is not possible to determine the rate of false negatives, i.e., the number of patients who tested negative but who have the marker and might have responded to the agent, and therefore to know how well the assay distinguishes responders from non-responders. If the assay provides a continuous read-out, the cut point used to establish trial eligibility may later be questioned, but data to resolve the issue may be lacking. The form of the assay used in the clinical trial may become a requirement for regulatory approval of the drug, so it may be difficult to make adjustments or improvements to the assay

afterward. For all of these reasons it is problematic to shift to development in marker-selected populations too early. In phase II trials the usual preferred strategy is to use marker assays as stratification factors.

Predictive markers can be used in different clinical settings, and these settings have implications for the trial designs used to evaluate the utility of the markers. The two clinical settings most often addressed are measurement of a marker prior to therapy to determine the choice of agents and measurement during or after therapy to assess effectiveness of the agents.

Markers used prior to therapy often include prognostic indicators. Since markers can be both predictive and prognostic [6, 7], it is important to identify truly predictive markers that can be closely associated with a given therapy. These are the only markers that are likely to be useful during the development of a therapeutic agent and needed for approval if an enrichment design was used in the registration trial. A randomized clinical trial with a control arm in which the marker is measured in both sets of patients is required to determine the extent to which a marker is predictive for a given therapy, as opposed to prognostic.

Markers used during or after therapy are designed as indicators of response. Traditionally such markers have been focused on detecting residual disease or the emergence of drug-resistant tumor cells. Serum markers like carcinoembryonic antigen, CA-125 or prostate-specific antigen have been used in this setting and are generally considered measures of tumor burden. Much of the literature on CTCs to date has focused on the change in numbers of tumor cells as a measure of tumor burden, which could result either from treatment that did not fully eradicate existing disease or the presence of drug-resistant tumor cells. Enumeration of CTCs may provide information that is independent of imaging, since there is often only a modest association between CTC counts and estimates of tumor burden from imaging [8]. As discussed in earlier chapters of this book and in the companion chapter on co-development, new approaches including the molecular characterization of CTCs may allow more informative measurements to distinguish between residual tumor and emergence of resistant cells as well as alternative treatments that might be considered.

There are more examples of failures and/or problems than of successes in the history of marker-agent co-development, often resulting from the difficulty in performing evaluation of the agent and the marker in parallel.

The success in the case of trastuzumab and HER2 resulted from the significant understanding of the basic biology prior to developing the agent [9–13]. Restricting eligibility to patients with HER2-amplified tumors in the pivotal phase III trials was necessary to achieve a clear-cut demonstration of benefit. However, there were downstream problems that resulted from less than adequate coordination between the development of the HER2 assay and the clinical studies of the agent [14]. The HER2 assay that was used in the pivotal trial was a research tool, not a fully developed in vitro diagnostic device suitable for FDA clearance and marketing, and specimens from the trial were not collected. Therefore, it was necessary to perform a “bridge study” to demonstrate adequate concordance between the clinical trial assay and the IVD submitted for clearance.

Much of the clinical development of inhibitors of the epidermal growth factor receptor (EGFR) was done in unselected populations. Assumptions were made early during the development that overexpression of the receptor would predict effectiveness of anti-EGFR antibodies, and this led to a focus on tumor types known to frequently express EGFR. However, expression of EGFR (as measured by immunohistochemistry) did not prove to predict response [15, 16], and the picture became even more complicated as small molecule inhibitors were introduced. EGFR mutations have been shown to predict response to erlotinib and gefitinib in non-small cell lung cancer [17, 18]. It is still not clear what the correct markers are that will accurately predict response to EGFR inhibitors in colon cancer. KRAS mutations have been shown to be associated with lack of response to cetuximab. In contrast, the nature of bRAF mutations that predict response to bRAF inhibitors was known on the basis of strong preclinical data, and this accelerated the development of biomarker assays.

For anti-angiogenesis agents such as bevacizumab, the search for predictive markers has been quite elusive [19]. These agents exert their effects on the vasculature rather than the tumor cells, and there seems to be a complex interaction between patient genotype, angiogenesis and tumor biology. Bevacizumab was not developed in enriched populations and is approved for several indications where clinical trials showed a benefit in all comers [20]. However, the benefit of its use for post-operative treatment of early stage breast cancer may be limited to a subset of patients, and a predictive marker assay would be of considerable value.

4 Assay Development in the Context of Co-Development: Planning for Success

All biomarker assays that are ultimately cleared or approved by regulators for use in the care of patients must meet certain criteria of analytic validity, clinical validity and clinical utility [21]. In the context of co-development, clinical utility is ultimately demonstrated in phase III clinical trials and is determined by the effectiveness of the treatment: administration of the agent as indicated by the results of the assay. The clinical validity of the assay is determined by the strength of the association between the result of the assay and the effect of the agent. This association is best measured in trials that stratify, but do not select, patients on the basis of the assay [22]. It is critical to note that neither of these can be assessed reliably until the assay meets adequate standards of analytic reproducibility, sensitivity and specificity.

Candidate predictive markers are often identified in the course of preclinical research or pharmacodynamic studies during early clinical trials. Marker assays devised in the research laboratory require considerable modification before they are suitable for use as stratification or selection factors in clinical trials. Assays for which the result will be reported to the patient and/or the physician must by U.S. law be performed in a laboratory certified under the Clinical Laboratory Improvement Amendments (CLIA). The CLIA regulations specify that the assay

be carried out according to well-documented standard operating procedures with attention to such matters as the quality control of reagents and documentation of run-to-run reproducibility. The CLIA regulations apply even when the assay is investigational and is performed as part of a clinical trial. In some instances there may also be a requirement for an Investigational Device Exemption (IDE) from the FDA [23].

Successful co-development requires early consideration of the need to move an assay into a CLIA-certified laboratory setting, since the development of standard operating procedures, calibrators and controls takes time and effort. The investment in developing a clinical-grade assay may not bear fruit if the results of subsequent trials of the drug are disappointing. But failure to make this investment may result in delay at the point where the assay is needed to stratify or select patients for a trial. There is no clear way to avoid either of these risks.

Assay development can be facilitated by the availability of appropriate sets of archival specimens. These specimens have to be similar to the specimens that will be tested in the clinical setting, both in terms of the similarity to the disease type and stage of interest and in how they have been collected and stored. For the early stages of assay development, when basic analytical performance is being evaluated and attempts are made to optimize the assay and standardize the procedures, little demographic or clinical data about the patients from whom the specimens derive is required. However, when clinical validation is being performed, more information is needed and the availability of specimens with data may be more limited and limiting. To establish the relationship with clinical outcome, it is often necessary or preferred to have specimens from patients who participated in clinical trials where the specific treatments are known and the outcome data is more complete and reliable. For many technologies, fairly standardized preparation of specimens, particularly formalin fixation and paraffin embedding of tumor tissue is acceptable. On the other hand, most of the CTC technologies require collection and special handling of blood. Often the blood must be processed and the assay performed within a relatively short period of time (hours to days) (see earlier chapters). Therefore, the availability of useful archival specimen sets for assay development is expected to be quite limited.

The decision regarding whether the assay should be a test that is performed by a single laboratory as an in-house laboratory-developed test (LDT) or should be developed into a kit for manufacture and sale has implications for the way the assay is to be designed and therefore should be carefully considered early in the process. The most easily controlled path is to have the test performed by a single reference lab; the regulatory hurdles are more straightforward, the test reproducibility has to be proven in only a single setting, and there can be greater control over the variables post sample collection and submission. However, there must be a laboratory that is willing and able to develop and perform the test.

LDTs have been shown to be subject to considerable inter-laboratory variability. The HER2 and hormone receptor assays for breast cancer provide well studied examples of the problems that can arise when individual laboratories that

are CLIA-certified develop and perform their own tests [24–27]. The same sample may be considered positive when measured in lab A and negative in lab B resulting in opposite treatment recommendations. Therefore, if co-development of an agent and an assay is planned, and an LDT will be used in more than one laboratory in multi-center clinical trials it is critical to control and document the inter-laboratory variation in test results so that the outcome of the trial will be interpretable. (For an account of how the inter-laboratory reproducibility of the CellSearch device was established, see Allard et al. [28]) And if an LDT is used in the studies leading to approval of the agent, it is important to consider how the test is to be disseminated into the community and how training and proficiency assessment programs will be developed.

Development of a kit has the potential advantage of ensuring the quality and consistency of a disseminated test but the disadvantage of requiring significant resources for development, manufacture and clinical studies. Kits require standardized reagents, assessment of shelf-life and proof of consistency in performance both within a single lab and across several labs.

Whichever development path is chosen for the assay, clinical studies will have to be performed with both the assay and the agent. Multi-institutional studies are likely, and there will need to be clear standard operating procedures set out for specimen collection and an ongoing assessment of adherence to the procedures and the quality of the samples. If any processing steps are required at the collection sites, a plan to assess the comparability of the processing from site to site is needed.

The coordination of the assay development and its evaluation with the plans for the clinical trials of the agent is a major challenge in co-development. Early planning with a clear understanding of the scientific, technical and regulatory requirements is essential for success.

5 Regulatory Challenges in Co-Development

Differences in the regulatory requirements for drugs and in vitro devices (marker assays) affect efforts to co-develop predictive assays and drugs. If developed separately, review of drugs and in vitro devices takes place in different centers of the FDA and the European regulatory agency. Significant data about the performance of an assay is required by the Center for Devices and Radiological Health in order to assess its safety and effectiveness [21]. This includes data on reproducibility, accuracy, precision, sensitivity and specificity. False positives and false negatives affect the safety of a test, and therefore, data from patients both with and without the marker is required. This is problematic when an assay is being co-developed and is being used to select patients with the marker for treatment with the agent although use of stratification designs in phase II trials can potentially provide informative data.

There are efforts by the FDA to better integrate the review and approval of companion assays with their targeted agents, but there are many complexities [29]. Generally, an attempt is made to have the assay reviewed alongside the drug or biologic. In addition to differences in the basic regulatory structures that apply to agents and assays, the requisite expertise for the assay review usually is missing from the agent review panels. Assays are classified based on their complexity and risk, and the review of products that fall within different risk classes usually requires different types of data. For example, a high risk device requires a pre-market approval (PMA) which depends on clinical data in addition to the data regarding the assay's analytical performance [30]. A class II assay generally goes through a substantial equivalence review (known as 510 (k) for the section of the regulations that applies) which requires mainly analytical performance data and results in clearance to market the product [21].

Another complexity in the regulations is that tests that are developed and performed in the same laboratory and not sold as a kit (LDTs) have historically only been regulated under CLIA although the FDA does have discretion in this area. CLIA regulation focuses on intra-laboratory reproducibility and record keeping and does not address clinical validity (how well the test relates to the clinical outcome of interest, e.g., response to therapy, survival, etc.) or clinical utility (whether the results of the test provide information that can contribute to and improve current optimal management of the patient's disease) [31, 32].

Whichever development path is relevant for a given assay, significant time and effort are required to develop the assay to a point that will allow proper evaluation for clinical utility or in the case of a targeted agent, for its ability to predict response to the agent of interest. The history of assays that prove not to be of value provides serious disincentives to manufacturers of agents for the co-development of a predictive assay. In addition, the reimbursement for assays in the clinical setting is very much lower than that for new drugs. The large teams with different areas of expertise that are required to successfully co-develop an agent and an assay add to the upfront expense and affect the profit margin for the test. All these issues must be considered before embarking on co-development efforts.

6 Conclusions

Development of drugs, biologic agents and predictive tests are all challenging in their own rights. With new targeted agents, it appears to be increasingly apparent that predictive assays will be needed. There are considerable challenges to the development of these assays and particularly to co-development. A series of recommendations were developed following a workshop co-sponsored by the US NCI and the FDA [5] to address the challenges of co-development. These recommendations point out issues that need to be considered when planning co-development. There were no simple solutions that could be recommended to solve problems identified at the time, and it is fair to say that the situation has not

changed much in the last 2 years. Resources for development of assays are still inadequate and the misalignment of incentives for the development of an assay versus a drug is still a problem.

Despite the obstacles and challenges cited, the increased recognition of the need for and potential importance of predictive assays is leading to discussions among stakeholders about how to address the problems. In the meantime, a clear recognition of the challenges, teamwork and advance planning are all necessary elements of a successful co-development effort.

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Challenges and Opportunities in the Use of CTCs for Companion Diagnostic Development

Elizabeth A. Punnoose and Mark R. Lackner

Abstract

Circulating tumor cells offer promise as a surrogate source of cancer cells that can be obtained in real time and may provide opportunities to evaluate predictive biomarkers that can guide treatment decisions. In this review, we consider some of the technical hurdles around CTC numbers and suitability of various CTC capture and analysis platforms for biomarker evaluation. In addition, we consider the potential regulatory hurdles to development of CTC-based diagnostics. Finally, we suggest a path for co-development of anticancer therapeutics with CTC-based diagnostics that could enable clinical validation and qualification of CTC-based assays as companion diagnostics.

Contents

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1 Introduction

Cells with morphological properties similar to neoplastic cells have been recognized to circulate in the blood of cancer patients for over 140 years [1]. Recent data has suggested that these cells exhibit hallmark characteristics of transformed cells. These findings have supported the notion that such circulating tumor cells (CTCs) are shed from tumor masses into the peripheral bloodstream [2, 3], though the relationship between CTCs and other cells that remain within the tumor proper remains somewhat unclear [4, 5]. In patients with late stage metastatic breast cancer (MBC), it has been demonstrated that the detection of elevated levels of CTCs at any time prior to, or during, the course of therapy is an independent predictor of disease progression and mortality [6–8]. As such, CTC levels constitute a prognostic biomarker. Other recent studies have shown that they may also have utility as a surrogate endpoint of anti-tumor activity in early phase clinical trials [9, 10]. CTCs can also be detected in patients with early stage breast cancer and elevated levels have been reported to be associated with poor prognosis, although these patients generally have fewer detectable CTCs than patients with more advanced breast cancer [11, 12].

The primary focus of this review is whether CTCs may provide additional value to biomarker studies. A key question is whether the molecular characteristics of CTCs can be used in predictive diagnostic assays for molecularly targeted therapeutics. As discussed in detail in the accompanying chapter by Taube and Lively, a predictive diagnostic can be defined as a test that can be performed prior to treatment to indicate the likelihood of response to a particular therapeutic or class of therapeutics. Following prospective clinical qualification with a technically and analytically validated assay, a predictive diagnostic can gain regulatory approval as a companion in vitro diagnostic (IVD) test intended to be used in conjunction with a therapeutic agent in order to identify patients likely to benefit or in whom the agent is expected to have little or no effect [13]. Examples include HercepTest™ and pharmDx™ tests used to determine HER2 protein and DNA copy number, respectively, as the basis for treatment with agents such as Herceptin® (trastuzumab) and Tykerb® (lapatinib) [14]. Current companion diagnostic assays rely predominantly on archival tissue that is collected at time of diagnosis through biopsy or surgical resection. Such tissue may not be representative of a patient's current disease, particularly after multiple lines of therapy. As such, there is a pressing need for tissues that are more representative of disease at the time a patient is undergoing therapy. This chapter will consider a number of the technical and biological challenges that must be overcome if CTCs are to be successfully used as a source of cancer cells for biomarker analyses.

2 On the Relevance of CTCs as a Source of Representative Cancer Tissue for Predictive Dx

There are many reasons to suppose that diagnostic approaches that rely on archival tissue could be inadequate and result in inaccurate results. Consider for example prostate cancer, which can have a long indolent period of 10–15 years that can be

managed through hormonal therapies, prior to progressing to late stage castrate resistant prostate cancer. Recent studies have shown that metastatic tumor samples can be quite different from the original primary tumor and acquire additional alterations. For example, large-scale genomic profiling of primary and metastatic prostate cancers revealed much more widespread activation of PI3K, PTEN, Rb and RAS/RAF signaling in metastatic tissues compared to primary tumors [15]. In some cases, upregulation of these signaling pathways may be an escape or evolutionary mechanism whereby tumors evade chemo- or hormonal therapies [16], again suggesting a need to obtain representative tissue at the time new therapies are being considered.

Even in cases where metastatic tissue can be obtained, it is not clear that tissue from a single site is representative of the majority of the metastatic lesions, and instances of substantial heterogeneity have been reported [17]. Similar considerations apply to other solid tumor types including breast cancer, which can also have long indolent periods punctuated by treatment that could cause changes in the molecular portrait of a patient's metastatic disease. Recent analyses have indicated that biomarker status can, in some instances, be discordant between primary and metastatic breast cancer. A notable example is mutations in the PIK3CA oncogene that occur with greater frequency in metastatic compared to primary lesions [18].

It seems clear that making treatment decisions based on diagnostic assays conducted on primary tumor tissue is a suboptimal solution to personalized medicine strategies. However, collecting metastatic tissue via biopsies imposes risks, anxiety and inconvenience to patients and is perceived to be a barrier in enrolling and conducting clinical trials [19]. For all of these reasons, characterization of predictive biomarkers in CTCs holds tremendous promise to potentially provide a real-time snapshot of the molecular makeup of a patient's cancer prior to administration of therapy, essentially providing surrogate tissue from a "liquid biopsy."

3 Technologies for Molecular Characterization of CTCs

Development of new and robust technologies for the capture and characterization of CTCs will aid biomarker analyses and is an area of active investigation. A recent tally indicates that up to 30 devices are currently in development ranging from mature concept to prototype device to commercially available instrument. The Veridex CellSearch® platform is FDA approved for prognostic purposes based on CTC enumeration in colorectal, breast and prostate cancer [20], but to date no platform or instrument is approved for a companion diagnostic application. Without a gold standard, it is difficult to compare different technologies and to determine their true sensitivity and specificity to detect CTCs. Adding to this complexity is the heterogeneity observed in CTCs both in expression of phenotypic markers such as EpCAM and cytokeratin [3, 21, 22] as well as in size and

morphology [23–25]. This complexity makes a "one size fits-all" platform for capturing all challenging CTCs. The ideal platform would isolate the majority of this rare cell population at high sensitivity, in an intact state, across multiple tumor types and with high purity from the surrounding blood cells. In addition, the ideal technology would lend itself to multiple types of downstream molecular analysis. These include the major methodologies used for biomarker assessment: (1) immunofluorescence (IF) or immunohistochemistry (IHC) to evaluate protein expression, (2) fluorescence in situ hybridization (FISH) to score cytogenetic lesions, (3) mRNA expression analysis by quantitative polymerase chain reaction (qPCR) and (4) mutation profiling and copy number analysis using DNA. These applications have distinct requirements in terms of yield and purity, presenting a formidable technological challenge for a single platform. It may thus be that distinct platforms with strengths in a particular type of application are required, at least initially. An example of an application with a requirement for high yield is cell-based assays (IF, IHC, FISH), which typically require deposition of CTCs with intact morphology for high resolution, multi-color microscopy. Yield is more important than purity for this type of assay, since contaminating surrounding blood cells can be excluded for analysis by using phenotypic criteria to define CTCs (Cytokeratin+, DAPI+, CD45−). However, sufficient numbers of CTCs are required to evaluate potential heterogeneity in the biomarker and to minimize errors due to false positives and negatives. For example, when comparing concordance between HER2 status in archival tumor with HER2 status in CTCs as measured by an IF assay on CellSearch, we found that a minimum of 3 CTCs were required to minimize type I and II errors [3]. Similarly, in a study evaluating ERG rearrangements and PTEN loss by FISH in CTCs from CRPC patients using CellSearch, results from a minimum of four individual CTCs were required to overcome the underlying false positive rate of assay [26]. Based on these experiments, we propose a minimum requirement of $\geq$5 CTCs for cell-based assays, albeit this number will have to be empirically determined for each assay during the validation stage of biomarker development. However, even a minimum cutoff of $\geq$5 CTCs poses a significant challenge in the number of patients that will meet these criteria and be suitable for biomarker analyses. For example, even in prostate cancer, the tumor type with the highest reported CTC counts, only 41% of prostate cancer patients have $\geq$5 CTCs [27]. This concern may be somewhat mitigated in patient populations with late stage disease, since we and others have found that CTC counts can be higher in more advanced disease when evaluating patients who have progressed beyond frontline therapy [3, 26, 28]. For example, in CRPC patients who have progressed on docetaxel treatment, $\sim$70% had >5 CTCs using the CellSearch platform [9]. This underscores the importance of having a step in the assay validation process to be using blood samples from the target patient population of interest, and also suggests that proof-of-concept studies with molecular characterization of CTCs on the CellSearch platform may currently be best suited to patient populations with advanced disease.

Clearly, it would also be advantageous to be able to capture higher numbers of CTCs for biomarker analyses in order to meet the suggested criteria of ≥5 CTCs per patient, and there are emerging platforms with surfaces amenable to high-resolution imaging and higher reported CTC counts. These include the fiber-optic array scanning technology from EPIC Sciences [25], which uses immunostain and morphological features determined by automated scanning on a histology slide to distinguish CTCs from WBCs. The isolation by size of epithelial tumor cell (ISET) method from Rarecell Inc also holds some promise in this regard, as a recent report identified 41% of patients with morphologically malignant circulating non-hematological cells using standard cytopathology staining and microscopic analysis in patients with resectable non-small cell lung cancer [29]. These platforms need to be evaluated in side-by-side comparisons with CellSearch on matched patient blood samples to determine the utility and feasibility of use for cell-based assays.

In contrast to cell-based assays where CTC purity is not as critical, molecular assays on CTC-derived DNA or RNA typically require high purity (upwards of 50%). Such purity is required to overcome the background "noise" from contaminating blood cells that are included in the initial cell lysate preparation step. Impressive levels of purity (~50%) as well as high capture rates (median, 50 CTCs per mL) have been reported for the microfluidic CTC-chip platform [30]. In a study evaluating EGFR mutations in metastatic lung cancer, they observed the expected EGFR mutation in 12 of 13 patients [31], suggesting sufficient purity for this type of downstream molecular analysis. However, maintaining this level of purity through scale-up and standardization process has been a challenge, with increased variability observed in CTC purity ranging from 50 to 0.1% [32]. At the lower end, this level of purity is similar to what is observed on CellSearch and the commercial CTC-chip technologies from Cellective and Biocept [3].

An architecturally distinct next generation microfluidic CTC-chip, the herringbone (HB)-chip, was developed in order to overcome some of the shortcomings of the original three-dimensional micropost-based platform [33]. Using the HB-chip, the Haber and Toner labs demonstrated the ability to detect the TMPRSS2-ERG fusion transcript in RNA extracted from CTCs in metastatic prostate cancer [34]. It will be interesting to determine the utility of this new design for other molecular assays.

Another approach to circumvent the purity requirements for molecular assays is to use laser capture microscopy (LCM) to further purify CTCs from enriched CTC preparations. LCM has been used to purify CTCs captured on the Cellective microfluidic CTC-chip and transcripts derived from CTCs showed strong concordance to those from primary and metastatic tissues from an orthotopic xenograft model [35]. Another technology that has reported >50% purity as assessed by cell line spike-in experiments is the MagSweeper, an immunomagnetic separation technology that enriches target cells while eliminating cells unbound to magnetic particles [36]. This device has been coupled with Illumina's genomic platforms to analyze CTCs at the single cell level by RNA sequencing (RNA-Seq).

Technologies suitable for cell-based assays:

Technology	Surface	Capture method	Assays
CellSearch, Veridex LLC	CTCs held planar by magnetic field	Immunomagnetic capture by EpCAM	IF, FISH
EPIC sciences	Blood cells are visualized on microscope slide	No selection. CTC identified by morphology and immunophenotype	IF, FISH
ISET, Rarecells Inc.	Filter based membrane	CTC captured by size	IHC, IF, FISH

Technologies suitable for molecular assays:

Technology	Purity (%)	Capture method	Assays
Microfluidic CTC-chip	0.1–50	EpCAM or other antibodies	Mutation assays, RNA expression
HB-chip (2nd generation microfluidic CTC-chip)	14	PSA or other antibodies	Mutation assays, RNA expression
Cellective CTC-chip + LCM	0.1 further enriched by LCM	EpCAM	Gene expression (RNA)
MagSweeper + Illumina genomic analysis	51	EpCAM	RNA-Seq

4 HER2 Diagnostics in CTCs

Demonstrating that the molecular phenotype in CTCs accurately represents the molecular characteristics of the tumor cell is an important step in qualifying CTCs for molecular biomarker detection and patient selection. A test case investigated by several labs has been to compare HER2 status in CTCs from breast cancer patients to that in matched tumor tissue. HER2 is a gold standard for biomarker validation because it is a well-characterized marker where the metrics for HER2 positivity have been tested and correlated with response to trastuzumab treatment. The neoadjuvant GeparQuattro study showed that while CTC numbers are low in early stage breast cancer patients, it is possible to quantitate HER2 levels in these CTCs [37]. Furthermore, this study identified a number of patients with HER2-negative primary tumors who had HER2-positive CTCs, and suggested that HER2 biomarker analyses in CTCs might be helpful for stratification and monitoring of HER2-directed therapies [37].

We recently evaluated HER2 status by IF in CTCs from 29 patients with advanced metastatic breast cancer whose HER2 primary tumor status was known and found a high degree of concordance at 89% [3]. Other reports have shown lower concordance in the range of 50–70%, with HER2-positive CTCs observed in patients where the primary tumor was HER2-negative, as well as HER2-negative CTCs in patients with HER2+ primary tumors [38–41]. These results suggest the

possibility that HER2 status can change over time, though further study is required to rule out testing errors as a source of the differences. Prospective clinical studies will be required to qualify HER2 status in CTCs as a relevant predictive biomarker that can be used in real-time assessments. Several such studies are underway to determine whether positive HER2 status in CTCs are indicative of benefit to HER2 targeted therapies. These include Phase II study of the HER2 targeted inhibitor lapatinib in breast cancer patients with HER2 non-amplified primary tumors and HER2-positive CTCs (NCT00820924 clinicaltrials.gov), and a Phase II study evaluating the effects of trastuzumab in combination with the chemotherapeutic agent vinorelbine in patients that are positive for HER2. The latter study also includes patients whose original breast cancer was negative but whose CTCs are positive for HER2 (NCT01185509 clinicaltrials.gov). The outcome of these and other studies will inform the clinical utility of molecular analysis in CTCs for predictive biomarker analysis and patient selection.

5 Applications of Predictive Biomarker Analyses in CTCs to Treatment of Metastatic and Early Stage Disease

Metastasis of solid tumor malignancies to distant organs almost always results in patient mortality and accumulating evidence suggests that the process of metastasis may be mediated by CTCs in peripheral circulation or by disseminated tumor cells (DTCs) that can be found in bone marrow [42]. Early stage breast cancer without obvious local or distant metastases is often cured by surgical intervention, though a subset of these patients relapse due to metastatic disease which is likely attributable to minimal residual disease in the form of micrometastatic lesions, DTCs and/or CTCs [43]. This phenomenon is the basis for adjuvant therapy wherein patients are treated with systemic chemo- or targeted therapies following surgical resection of primary tumors in order to eradicate the remaining residual cancer cells. A key limitation of this approach is that treatment is almost always made without knowledge of the molecular makeup of the residual cancer cells, and could surely be improved if treatment could be tailored based on the molecular characteristics of CTCs or DTCs from an individual patient. Incorporation of CTC biomarker evaluations in patients in the adjuvant setting may be initially more challenging, since these patients not only have fewer CTCs, but also generally have better survival prospects and longer time to clinical events that prolongs drug development timelines. Based on this, it may be prudent to focus initial efforts on validating CTC predictive biomarker assays in metastatic patients. Success in this setting could then be followed by application to early stage breast cancer, analogous to the development of novel therapeutics, which are typically validated in the metastatic setting prior to testing in the adjuvant setting.

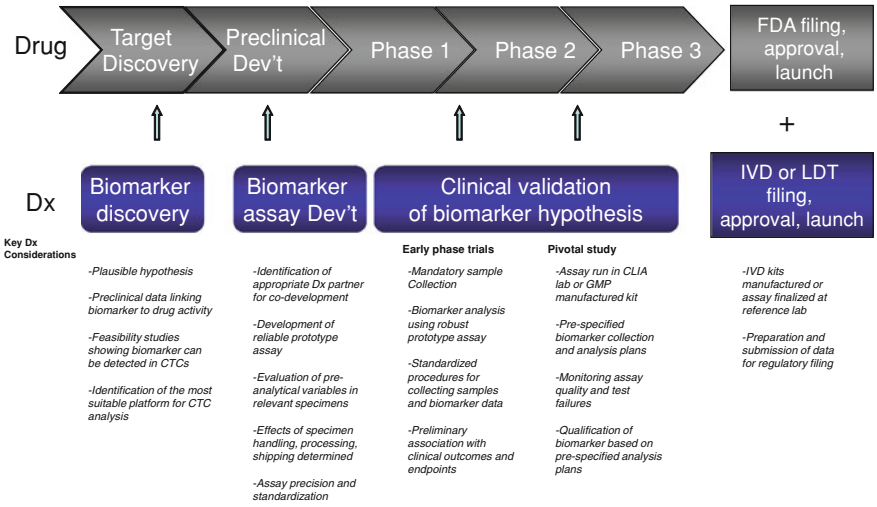

Fig. 1 Proposed drug-CTC diagnostic co-development paradigm

6 The Path Forward

The current drug-diagnostic co-development paradigm requires early identification of predictive biomarkers to allow prospective validation in clinical trials, ultimately leading to joint regulatory filings on the drug and diagnostic test [44]. For CTC-based diagnostics to conform to these expectations, a number of challenges will need to be overcome. First, indications need to be identified where the majority of patients have CTCs in sufficient numbers to allow molecular characterization of the biomarker of interest. Second, the platform most suitable to performing the assays needs to be determined. Third, in the USA, all predictive biomarker tests used for patient management must be run under appropriate laboratory conditions. Specifically, assays must comply with the Clinical Laboratory Improvement Amendments (CLIA) act of 1988 [45, 46], or the guidelines of the office of in vitro diagnostics (OIVD) of the FDA. This creates a challenging regulatory path for new and unproven diagnostic technologies [46]. Below, and in the accompanying Fig. 1, we propose a stepwise process to address technical and regulatory development hurdles and clinically validate CTC-based predictive diagnostic tests.

As discussed in the accompanying chapter, a first step in biomarker development is the creation of a robust prototype assay that is technically and analytically validated and can be applied to early phase clinical trials. Important considerations in this process include detailed specification of the technical protocol and defined assay validation criteria [44]. This encompasses both pre-analytical parameters such as effects of specimen handling, processing, shipping and storage, as well as post-analytical parameters such as establishing inter-and intra-assay precision, linearity and standardization [47]. While this is feasible for tumor tissues that can

be fixed and stored embedded in paraffin, it imposes special challenges in the area of CTCs, since current technologies require fresh blood collection and processing within a time frame of 72–96 h [3]. A possible path forward here would be for the drug sponsor to work closely with clinical investigators on pre-analytical validation prior to initiating clinical studies. Steps here would be to prospectively procure blood samples with basic clinical data and appropriate pre-analytical variables, and use these samples for analytic validation of the prototype diagnostic. It should also be possible and may be desirable to include cell line controls representing differing biomarker status in "spike-in" experiments as a basic quality control for inter-experiment variability and to calibrate results between runs. The next step in the process is clinical qualification, the process of linking biomarker status with clinical outcomes or endpoints in a trial appropriately designed to test the biomarker hypothesis. A robust prototype assay that met the above validation criteria would ideally be used at this point in biomarker qualification, initially in proof-of-concept studies but culminating in prospective analysis in a pivotal study.

The next consideration is whether the assay should be a laboratory developed test (LDT) run at a single reference lab, or a kit that can be widely distributed and run in community laboratories. As discussed by Taube and Lively, both paths have pluses and minuses that can impact the overall chances of success, though the regulatory and analytical hurdles may be easier to achieve in a single laboratory setting under the LDT model. Given that numerous CTC capture platforms are in development and a clear winner has yet to emerge that is superior for all forms of molecular characterization, a practical solution may be for the drug developing entity to form an early partnership with a lab that offers a technology suitable for the biomarker question of interest. The early phase of the partnership could consist of the preclinical validation studies described above, with a plan to coordinate clinical evaluation of the drug and diagnostic in appropriately designed early phase clinical studies. At the same time, the diagnostic company could take steps to obtain FDA clearance of the CTC platform or instrument for biomarker testing, and could plan the path with the drug sponsor for biomarker data collection and analysis in pivotal studies, leading to a joint regulatory filing for approval of the drug and CTC-based diagnostic.

7 Conclusions

Molecular characterization of CTCs holds significant promise to aid in companion diagnostics development by providing a representative source of tumor material from a minimally invasive procedure. For this promise to be fully realized, both additional advances in technology as well as careful consideration of drug and CTC diagnostic co-development will be required.

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