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Richard J. Cote
Ram H. Datar *Editors*

Circulating Tumor Cells

Advances in Basic Science and Clinical Applications

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Metastasis is the process that defines cancer. Studies in understanding circulating tumor cells have been at the forefront of efforts to favorably impact the morbidity and mortality due to metastatic spread of the cancer. In the making of this book, an effort made possible by the world's leading investigators, it is important to remember and recognize those who we aim to cure. We dedicate this volume to those countless cancer sufferers across the world, and hope that this sharing of knowledge will move us closer to alleviating this emperor of maladies.

This book is dedicated to mentors and colleagues. Dr. Lloyd J. Old, Dr. Edward J. Beattie, and Dr. A. Munro Neville are entirely responsible for any contribution I may have made to this field. Dr. Clive R. Taylor allowed me to pursue these studies when most of the scientific community considered micrometastases and CTC to be of no consequence. And Dr. Ram H. Datar has been my longtime collaborator and friend. I am also deeply grateful to my family, Annie, Nick, Juliet, and Gracie, who have supported me throughout this endeavor and more.

Richard J. Cote, MD, FRCPath, FCAP

I would like to express my gratitude to Dr. Richard Cote for introducing me to the fascinating field of cancer metastasis! Dr. Cote has mentored me throughout as we collaboratively tackled the difficult clinical problem over many years and developed lasting associations with various thought leaders in the field, many of whom have actively contributed to this volume. I also take this opportunity to thank my parents and parents-in-law, wife Bharati, our son and daughter-in-law Nakul and Aditi, respectively, and our granddaughter Mallika, who all were persistent and unwavering in their support throughout.

Ram H. Datar, MPhil, PhD

Foreword

Metastatic spread of cancer is among the most leading causes of cancer-associated morbidity and mortality, and circulating tumor cells form the very basis of this process [1]. There was an emergent need for a volume that can serve as an authoritative compilation of information about circulating tumor cells (CTC) from thought leaders, which this volume has directly addressed.

Over the past decade, a variety of ultrasensitive assays have been developed to detect CTC in the peripheral blood and disseminated tumor cells (DTC) found in the bone marrow of cancer patients at the single-cell level. CTC can be distinguished and enriched from the surrounding leukocytes by either physical properties (e.g., density and size) or biological properties (e.g., expression of epithelial proteins such as EpCAM or cytokeratins). CTC/DTC are usually detected by immunostaining or RT-PCR assays, and more recently by the EPISPOT assay which measures the number of cells releasing/secreting tumor-associated marker proteins. Chapters 1–4 in this volume serve as a comprehensive survey of these detection technologies. The epithelial-mesenchymal transition (EMT) is an emerging important issue in CTC research. For example, at present, most assays rely on epithelial markers and may miss CTC undergoing EMT. New markers such as the actin bundling protein plastin-3 that are not downregulated during EMT and not expressed in normal blood cells might overcome this important limitation and, therefore, increase the sensitivity of CTC assays. Chapter 8 provides an overview of this important emerging area.

Interestingly, the bone marrow seems to be a common homing organ for cells derived from various epithelial tumors including breast and prostate cancer. However, a significant fraction of DTC remain over years in a "dormant" stage, and little is known about the conditions required for the persistence of dormancy or the escape from the dormant phase into the active phase of metastasis formation, concepts which are reviewed in Chaps. 5–7 of the present volume. Recent findings indicated that a subset of $EpCAM^{low}$, $CD44^{high}$, $CD47^{+}$, c-Met^{+} CTC obtained

from the peripheral blood of breast cancer patients might have an increased ability to colonize bone marrow in immunodeficient mice. However, it is unclear whether these CTC are metastasis-inducer cells because they were obtained from patients with advanced metastatic disease and extraordinarily high CTC counts. While further in-depth molecular analyses of CTC are expected to answer such questions, the current state of phenotypic and genotypic knowledge about CTC is nicely summarized in Chaps. 9 and 10 of this volume, while Chap. 10 addresses the need for functional characterization of CTC.

While bone marrow is an informative site for study, sampling of peripheral blood is much more convenient and less intrusive than collection of bone marrow samples, and many research groups are currently assessing the clinical utility of CTC for determining prognosis and monitoring of systemic therapy. In particular, monitoring of CTC during and after systemic adjuvant therapy (e.g., chemotherapy, hormonal therapy, antibody therapy) might provide unique information for the clinical management of the individual cancer patient and allow an early change in therapy years before the appearance of overt metastases signals incurability. There is an unmet need for biomarkers for real-time monitoring of the efficacy of systemic adjuvant therapy in individual patients. In particular, early changes in CTC counts might indicate success or failure of a particular therapy given to an individual patient. Chapters 11–14 and 16 have each covered distinct but critical issues pertaining to the relevance of CTC in monitoring therapeutic response.

Besides CTC, the analysis of ctDNA and circulating microRNAs may provide complementary information as "liquid biopsy." This information can be used as companion diagnostics to improve the stratification of therapies and to obtain insights into therapy-induced selection of cancer cells. CANCER-ID is a newly formed European consortium funded by the Innovative Medicines Initiative (IMI) with currently 33 partners from 13 countries aiming at the establishment of standard protocols for and clinical validation of blood-based biomarkers (www.cancer-id.eu/). It brings together experts from academic and clinical research, innovative Small-to-Medium sized Enterprises (SMEs), diagnostics companies, and the pharmaceutical industry, thus providing a unique setting for showing clinical utility of "liquid biopsies." Although systemic therapies are aimed to eliminate metastatic cells, the current stratification is usually performed on the primary tumors for practical reasons. However, there is increasing evidence that the phenotype and genotype of primary and metastatic cancer cells are discordant. Thus, the molecular analysis of CTC isolated from peripheral blood samples as "liquid biopsy" will reveal characteristics of metastatic cancer cells. This information can be used as companion diagnostics to improve the stratification of therapies and to obtain insights into therapy-induced selection of cancer cells. Chapter 15 in the present volume reviews the various technical and regulatory issues relevant to the use of CTC as companion diagnostics.

In conclusion, research on CTC opens a new avenue for detecting, understanding, and fighting early metastatic spread of tumor cells with important implications for future therapies. CTC have enormous potential as new biomarker and as the subject of basic research. Although CTC are already used in numerous clinical trials, their clinical utility is still under investigation. The present contributions by international experts in this book highlight the potential and current challenges of CTC research.

Hamburg, Germany Klaus Pantel

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Preface

Circulating tumor cells (CTC) were first observed and described as epithelial tumor cells found in blood over a century ago [1]. But reliable methods have been developed only recently to interrogate this rare population in blood. These methods are developed either utilizing unique antigen expression on CTC, such as the only FDA-cleared technology CellSearch™ [2], or utilizing their unique physical properties including size [3], density [4], electrical properties [5], etc. After an overview of the current status of our knowledge about CTC in the introductory chapter (**Chap. 1**), **Datar and coauthors** present details of affinity-based and non-affinity-based CTC capture technologies in **Chaps. 2** and **3**, respectively.

As more and more in-depth molecular and functional characterization of CTC studies has been carried out, the validity of CTC capture based on EpCAM expression is being questioned. CTC population is discovered to be heterogeneous and the gene expression levels vary from cell to cell even within the same patient sample [6]. To address this heterogeneity, an increasing number of studies have begun to look beyond CTC enumeration to elucidate the subpopulations among CTC. Details on molecular characterization of CTC that can help resolve this heterogeneity are described by **Lianidou and her colleagues** in **Chap. 4**. A potential subpopulation that is worth studying in CTC is the cancer stem cell population. Cancer stem cell population in CTC is further discussed by **Wicha and his colleagues** in **Chap. 5**.

CTC in circulation may assume one or more of several optional states: they could undergo elimination (by anoikis, apoptosis, necrosis, or immune attacks), successfully invade into secondary site, only to stay dormant or locked in mesenchymal states, or invade into a secondary site and metastasize by rapid proliferation. CTC dormancy studies are discussed in more detail in **Chap. 6** (**Allan and Chambers**) and **Chap. 7** (**Barkan and Chambers**). An important process involved in tumor metastasis that brings EpCAM-based CTC capture in question is the Epithelial-Mesenchymal Transition (EMT). In this process, tumor cells can potentially down-regulate their expression of epithelial markers including EpCAM and E-Cadherin and gain a mesenchymal and more invasive phenotype. In **Chap. 8**, **Thiery and colleagues** discuss more about this EMT process as a mechanism through which CTC establish distant metastasis.

An ability to perform phenotypic and genotypic studies of CTC is expected to lead to the development of assays with clinical applications to benefit cancer management. **Chapter 9** (**Dandachi and colleagues**) and Chap. **10** (**Magbanua and Park**) provide detailed discussions on phenotypic and genotypic analysis of CTC. In addition to molecular characterization of CTC, another interesting direction is the functional characterization of CTC employing technologies that enable viable CTC capture and culture. **Cote and colleagues** present a detailed review of functional characterization of CTC in **Chap. 11.**

With the emerging technologies to enumerate CTC from cancer patients, the clinical utilities of CTC have been investigated extensively in the past decade. One well-validated clinical application of CTC is their prognostic value at baseline. A detailed review on prognostic implications of CTC in breast cancer can be found in **Chap. 12** (**Smerage**). Although CTC have been well validated as prognostic markers for various cancer types, clinical applications for CTC as a surrogate endpoint, their use as a predictive marker to guide therapy, or use as an early detection marker are areas that are still largely unexplored and require large-scale clinical trials for validation. Although it is perhaps still a little early, it is not hard to envision CTC as powerful biomarkers as "liquid biopsy" which can provide valuable information via a minimally invasive blood draw. **Chapter 13** (**Cristofanilli**) and **Chap. 14** (**Polzer and Klein**) discuss in more detail the clinical applications of CTC. **Chapter 15** (**Huang and Lackner**) tackles a crucial concept for pharmaceutical industry, that of developing CTC assay as a companion diagnostic for either a pre-approved or an under-development anticancer drug. This chapter thus emphasizes an early and close partnership between the drug development sponsors and the CTC diagnostic companies to successfully navigate the regulatory landscape through phase II and III clinical studies, which in turn would allow for synchronized regulatory review of the drug and CTC assay. Finally, **Chap. 16** (**Kulkarni and Jeffrey**) summarizes the clinical applicability of CTC, while providing considerations for the future clinical trials.

As will be clearly evident, this volume is a result of a highly scholastic activity, with all of the contributors being thought leaders in the field, with decades of extensive contributions to the study of molecular biology of metastasis and the clinical applications of these critical findings.

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A volume with multiple chapters from busy and successful scientists and clinicians poses special challenges, and this one would not have been possible without the assistance and guidance of Ms. Patricia Malinowski and Ms. Gladys Garcia-Greenberg. Patricia ungrudgingly and efficiently handled the multiple communications to each of the contributing lead authors, who were nearly always in different time zones and often different continents! It is to her great credit and patience that all of our contributors expressed their admiration for her skills and help. Gladys, joining us towards the end of this undertaking, employed her outstanding linguistic skills in manuscript editing. We owe our being punctual to these two wonderful colleagues with excellent organizational and interpersonal skills, and we are sincerely grateful.

Finally, we would like to express our sincere appreciation to our loving families—Annie, Juliet, Nicholas, and Gracie Cote, and Bharati, Nakul, Aditi, and Mallika Datar—who uncomplainingly and patiently supported us throughout this endeavor.

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Dr. Cote obtained degrees in chemistry and biology at the University of California at Irvine and received his medical degree from the University of Chicago – Pritzker School of Medicine. He completed his residency at New York Hospital – Cornell University Medical College. His training included a clinical fellowship in pathology at Memorial Sloan-Kettering Cancer Center, a research fellowship in Human Tumor Immunology at Memorial Sloan-Kettering Cancer Center, and a fellowship in Molecular Pathology at New York University School of Medicine. Prior to joining the University of Miami in 2009, he was Professor, Department of Pathology; Professor, Department of Urology; Director, Genitourinary Cancer Program; Director, Laboratory of Immunology and Molecular Pathology; and Director of the USC Biomedical Nanoscience Initiative at the University of Southern California Keck School of Medicine/Norris Cancer Center in Los Angeles.

Dr. Cote's research is focused on the elucidation of cellular and molecular pathways of tumor progression and response to therapy. He has special interests in micrometastases detection and characterization and in the pathology of breast and genitourinary tumors. His laboratory is also focused on technology development, where he and his colleagues have developed immunohistochemical and molecular methods, such as antigen retrieval. Most recently, Dr. Cote and his colleagues at the University of Southern California, Caltech, and UC Berkeley have developed nanoscale technologies for cancer diagnostic applications, including bionanosensors for the detection of serum tumor markers, and technologies for the capture and

characterization of circulating tumor cells. As a result of these efforts, he established the Biomedical Nanoscience Program at USC and the Dr. John T. Macdonald.

Biomedical Nanotechnology Institute at the University of Miami (which recently received a $7.5 million naming gift from the Dr. John T. Macdonald Foundation), for the development of novel diagnostic platforms and targeted therapeutics. He is the recipient of over $43 million in peer-reviewed grant support and holds numerous patents for cancer-related and nanoscale technologies. He has led three of the largest clinical trials in breast, lung, and bladder cancer, which were based on discoveries from his research. Dr. Cote is the author of over 300 publications and participates on numerous scientific advisory boards for both academic- and industry-related institutions. He is a frequent lecturer and is the co-author of the standard textbooks *Immunomicroscopy: A Diagnostic Tool for the Surgical Pathologist* (now in its third edition) and *Modern Surgical Pathology* (now in its second edition).

He also serves as a member and advisor to a large number of national and international study groups, cancer programs and societies, including the National Cancer Institute. He is the founder of several technology-based companies; is listed in "US News and World report Top 1 % of Doctors", "Best Doctors in America", "America's Top Doctors", and "South Florida Super Doctors"; and is a Fellow of the Royal College of Pathology.

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Dr. Datar's areas of research focus include cancer molecular pathology and biomedical nanotechnology. Specifically, he has been involved in various aspects of detection and molecular characterization of occult metastases in cancers. For many years, he has also been involved in the development and application of micro- and nanoscale devices for biomedical diagnostic applications. Dr. Datar has played a pivotal role in the development of the microfilter device for capture and characterization of the circulating tumor cells (CTC), various nanotechnology sensor platforms to detect clinical biomarkers, and is among the inventors of these technologies with numerous awarded patents and in-process patent applications.

He has authored more than 70 original papers, and his publications have received more than 2200 citations. He is a reviewer for a number of international biomedical journals and has served as editor for Current Issues in Molecular Biology, Caister Academic Press, UK. He also serves on numerous scientific advisory boards, NIH Study Sections, and NSF Special Panels.

Part I
Technologies for CTC Identification

Chapter 1
Significance of Studying Circulating Tumor Cells

Ram H. Datar, Zheng Ao, and Richard J. Cote

Abstract Circulating Tumor Cells (CTC) are tumor cells released into blood. They are considered the pivotal component of the metastatic cascade and are being extensively studied only in the last decade or so. Understanding the biological and clinical impact of CTC is likely to reveal important information of the metastatic process and contribute to better management of cancer. We briefly discuss here the current clinical utility of CTC and their emerging clinical applications.

Keywords Current and emerging clinical applications of CTC • Epithelial–mesenchymal transition (EMT) • Circulating cancer stem cells • CTC genomics • CTC culture

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1.1 Introduction

Circulating Tumor Cells (CTC) are tumor cells released into blood. They are considered the pivotal component of the metastatic cascade and are being extensively studied only in the last decade or so. Understanding the biological and clinical impact of CTC is likely to reveal important information of the metastatic process and contribute to better management of cancer. We briefly discuss here the current clinical utility of CTC and their emerging clinical applications.

1.2 Current Clinical Applications

Ashworth first reported tumor cells in a patient's peripheral blood over one and a half century ago [1]. However, the study of these tumor cells has always been hampered by the rare existence of this cell population amid the excess of hematopoietic cells in blood. Various CTC isolation technologies have been developed only relatively recently, based on various principles such as affinity-based capture technologies including CellSearch™ [2], or non-affinity based technologies such as size based microfiltration [3], density-based gradient centrifugation [4], or electrical property-based dielectrophoresis (DEP) [5]. Most of the clinical data pertaining to clinical utility of CTC was collected utilizing CellSearch™, the only FDA-cleared technology for CTC enumeration for breast, colon, and prostate cancers.

1.2.1 CTC Enumeration at Baseline as Prognostic Marker

One well-validated clinical application of CTC is assessment of their prognostic value by CellSearch assay at pretreatment baseline. Patients with higher than 5 CTC per 7.5 mL blood were shown to have shorter progression-free survival and shorter overall survival in a study analyzing baseline CTC level in a cohort of 177 metastatic breast cancer patients in 2004 [2]. Subsequently, similar results were seen for metastatic prostate cancer in 2008 [6] and metastatic colorectal cancer in 2009 [7]. Several follow-up studies have confirmed the prognostic value of CTC, and as a result, CTC has been proposed in the new 2010 edition of the tumor-node-metastasis (TNM) cancer staging system manual as $cM_0(i+)$ [8], which is yet to be included in the clinical guidelines. The following paragraphs briefly discuss emerging clinical applications of CTC.

1.3 Emerging Clinical Applications

1.3.1 CTC as Surrogate Endpoint for Clinical Trials

One important potential clinical utility of CTC is their use as a surrogate endpoint for clinical trials. If approved to be informative, interrogating CTC with minimally invasive blood draws at follow-up time points to monitor treatment will greatly benefit the cancer management, and several clinical trials are designed to test the feasibility of this notion. For example, CTC enumeration analysis at follow-up visits for the same cohort of patients examined by Cristofanilli et al. above [2] showed CTC to be prognostic of progression-free survival and overall survival [9]. Similarly, another clinical trial in breast cancer also indicated that CTC as a surrogate endpoint is more reproducible and robust than radiographic response [10]. In prostate cancer, a clinical trial conducted in a cohort of 263 metastatic castration resistance prostate (mCRPC) cancer patients—the SWOG S0421 trial also indicated that, patients with rising CTCs at week 3 have significantly worse overall survival as compared with those with less or equal number of CTC at the week 3 follow-up visit [11]. In another study in mCRPC setting, CTC in combination with serum lactate dehydrogenase (LDH) level was shown to be a better surrogate for survival than PSA level [12]. Thus, so far, clinical trials attempting to interrogate CTC as a surrogate endpoint for clinical trials have shown some promising results, prompting further extensive follow-up studies.

1.3.2 CTC as Predictive Marker to Guide Treatment

As similarly encouraging evidence supports the value of CTC as prognostic markers in various cancers, an obvious question is: Can we use CTC to guide treatment selection? More specifically, can we use CTC measurement at baseline, or at follow-up time-points, to predict patient's response to treatment and thus guide therapy? An example is the SWOG S0500 clinical trial [13].

Results from the SWOG S0500 Phase III clinical trial (see Fig. 12.2, Jeffrey B. Smerage, for Trial Schema) were presented at the 2013 ASCO San Antonio Breast Cancer Conference. The trial was designed primarily to determine in a first line chemotherapy setting whether women with metastatic breast cancer and elevated CTCs by CellSearch assay (≥5 per 7.5 mL of whole blood) after 3 weeks of first-line chemotherapy derive increased benefit (overall survival and progression-free survival) from changing to an alternative chemotherapy regimen at the next cycle, instead of waiting for clinical evidence of progressive disease before changing to an alternative chemotherapy regimen. The trial was not designed to compare chemotherapies. The underlying hypothesis was that treatment decisions can be made based on CTC levels, with the belief that a significant number of patients resistant to their first line of therapy would respond to a second-line therapy.

Patients may benefit by switching early to a new line of therapy through avoiding the cumulative toxicities of ineffective therapy while spending more time on active therapy, thus improving quality of life and potentially tolerating future therapies better. Contrary to expectation, the patient data from randomized arms did not differ with respect to progression-free or overall survival. Given the very poor survival outcomes for this population, it was concluded that this population likely has a disease that is generally resistant to cytotoxic mechanisms. However, trial data did demonstrate a large, clinically significant, and statistically significant difference in prognosis for patients in whom the CTC remained elevated after one cycle of first-line chemotherapy. This is a population that should be considered for clinical trials of novel agents or novel treatment strategies early in the course of their disease.

In summary, the SWOG-S0500 trial validated the hypothesis that the group of patients with elevated CTC at baseline and 21 days after starting the first chemotherapy has a worse prognosis with regard to progression-free and overall survival, while low baseline CTC levels indicate a very good prognosis. The trial also showed that switching to a different chemotherapy sooner does not improve outcomes. For these patients, a clinical trial to investigate new targeted therapies should be considered, since chemotherapy is not effective in this population of patients.

To address the clinical utility of CTC in another direction, the ongoing METABREAST trial aims at identifying patients without the need for aggressive treatment if they have low CTC at baseline level. In this study, CTC were measured at baseline, and patients receive chemotherapy if they are detected with >5 CTC, otherwise they will receive endocrine therapy [14].

1.3.3 CTC as a Marker for Early Detection of Solid Tumors

In addition to investigating CTC as a surrogate endpoint and predictive marker, other studies focus on the possibility of using CTC for early detection for solid tumors. As reported in mouse model breast cancer research, tumor cells can “leave home early” [15] and establish metastasis without the necessity of experiencing the steps of transformation at primary sites [16]. Another study in pancreatic cancer transgenic mouse model revealed that CTC can enter blood stream even before tumor formation [17]. These observations encourage the notion that CTC could be used for early detection of cancer, as harbingers of impending malignancy. However, preliminary data from pilot clinical trials has stimulated some disputes. For example, a study probing for CTC in patients with benign colon diseases has detected CTC in 11.3 % of the 53 patients analyzed, which could be false-positive results [18]. Another potential problem of using CTC for early detection of cancer is the extremely low CTC count in early stage patients. The cut-off of CTC count in a nonmetastatic breast cancer setting by CellSearch is determined to be 1 per 7.5 mL blood draw, which, although it is prognostic [19], can be easily missed depending on the sampling of the blood and the analysis process. One solution to interrogate such a low level of CTC is to examine larger volume of blood. This can either be

achieved by an in vivo CTC capture probe—CellCollector® [20] or by taking advantage of a standard clinical procedure—leukapheresis [21] to harvest CTC from a much larger volume of blood. In general, emerging technologies with ability to interrogate larger volume of blood, or those with higher sensitivity to detect CTC in smaller blood volumes, might shed light on this clinical application of using CTC for early detection of cancer.

In conclusion, although prognostic utility of CTC has been well validated for various cancers, their clinical application as a surrogate endpoint, as a predictive marker to guide therapy, or as an early diagnostic marker is still largely unexplored and will require large scale clinical trials for validation. Although it is still in development awaiting further validation, the future vision of a CTC test is to serve as a "liquid biopsy" that can provide clinicians comprehensive clinical information of the patient in a minimally invasive blood draw.

1.3.4 CTC as A Companion Diagnostic

As some of the technical hurdles around CTC enumeration and suitability of various CTC capture and analytic platforms for evaluation of biomarkers get resolved, there are ongoing efforts in parallel that address development of CTC assays as companion diagnostic to assess the efficacy, toxicity, and successful targeting of anticancer therapeutics in real time as they are being developed, both in preclinical studies as well as Phase I and II clinical trials. Needless to say, such use of CTC assays must stand the rigor of regulatory hurdles. In a 2012 publication, Punnoose and Lackner review these developments and 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 [22]. Chapter 15 of this volume also addresses this concept in detail.

1.4 New Directions for CTC Analysis

As CTC research evolves, it is noteworthy that almost all the clinical applications mentioned above have employed CellSearch™ system. Although it is reliable and powerful, CellSearch™ technology is built upon the principle that CTC can be captured via anti-EpCAM antibody. As exhaustive molecular and functional characterization of CTC studies have been carried out, the validity of CTC capture based on EpCAM expression has been questioned. CTC population has been shown to be heterogeneous and the gene expression levels among CTC vary even within the same patient sample [23]. To address this heterogeneity, an increasing number of studies have begun to look beyond CTC enumeration to CTC molecular characterization in order to elucidate the subpopulations within CTC.

1.4.1 Epithelial–Mesenchymal Transition (EMT) in CTC

One important process involved in tumor metastasis that calls EpCAM-based CTC capture in question is the Epithelial–Mesenchymal Transition (EMT). EMT phenomenon has been described as the process whereby tumor cells gradually transition from epithelial phenotype into mesenchymal phenotype during metastatic progression, ostensibly via downregulation of expression of epithelial markers (EpCAM, E-cadherin, cytokeratin, etc.) and upregulation of mesenchymal gene expression (e.g., vimentin), to achieve a more invasive phenotype [24]. EMT process has been extensively investigated in primary tumors but to a much lesser extent in CTCs. EpCAM-based technologies may tend to capture and enrich "epithelial" CTC, thus potentially missing the CTCs with mesenchymal phenotype that may be metastasis-initiating. A study by Lin et al. demonstrated that by employing a size-based isolation strategy without relying on EpCAM expression, CTC can be detected at higher sensitivity [25]. In addition, a study by Harouaka et al. has demonstrated that a mesenchymal phenotype CTC can be detected using size-based isolation technologies [26]. A 2013 study by Zhang et al. isolated viable breast cancer CTC using four target markers—$HER2^+/EGFR^+/HPSE^+/Notch1^+$, cultured the CTC and derived a population that metastasized to brain in a mouse model [27]; this specific population was EpCAM-negative, and would have been missed if EpCAM was employed as the sole target molecule for CTC capture. In contrast, it is likely that antigen-agnostic CTC capture methods (such as those based on cell size) or capture methods that exploit other target antigens (including epithelial and/or mesenchymal antigens) will likely provide more insights into this phenomenon. For example, various studies employing cell size-based CTC capture have reported mesenchymal-like CTCs expressing the mesenchymal marker Vimentin [26, 28]. Another study investigated EMT status of CTC captured from breast cancer patient samples using EpCAM, HER2 and EGFR as capture target antigens, and discovered that mesenchymal cells were highly enriched in the CTC population. The proportion of mesenchymal CTC increased during chemotherapy treatmentss [29]. A converse interesting notion barely examined in the context of cancer but worth studying in CTC is the concept of Mesenchymal–Epithelial Transition (MET), wherein mesenchymal CTC may revert back to an epithelial phenotype once at the secondary site, expressing cell attachment protein such as such as E-cadherin, thereby regaining ability to form proliferative epithelial growths in distant organ sites. In contrast, cells without this capability to revert back to epithelial status seem to be unable to initiate metastasis effectively [30]. This hypothesis could be the explanation for the observation that many EpCAM-based CTC capture technologies seem to be capturing CTC in an intermediate status that is neither epithelial nor mesenchymal but rather a transitional status, also referred to as epithelial-mesenchymal plasticity (EMP) [31]. Cells that have the EMP capability seem to be able to switch between epithelial and mesenchymal status and might be population of the utmost importance in circulation [14]. One of the many studies that supports this hypothesis is a clinical study looking at EMT status on CTC captured from metastatic breast cancer

and mCRPC patients, where 75 % CTCs were found to co-express Cytokeratin (epithelial), Vimentin (mesenchymal), and N-cadherin (mesenchymal), along with a stem cell marker CD133 expressed at a high frequency [32]. Although the association between CTC EMT status and clinical outcome is still unclear, such studies will be critical not only to choose an appropriate CTC capture technology (EpCAM versus non-EpCAM-based capture) but also to elucidate the biological nature of CTC and the clinical relevance of mesenchymal CTC subpopulations.

1.4.2 Cancer Stem Cell Subpopulation in CTC

Another potential phenomenon that is worth studying in CTC besides EMT is the existence of cancer stem cell subpopulation. It has been well demonstrated that the $CD44^{+}/CD24^{-/low}$ population can form tumor with much higher efficiency as compared with the other subpopulations in breast cancer [33]. It has been previously shown that disseminated tumor cells in bone marrow possess such putative stem-like phenotype ($CD44^{+}/CD24^{-/low}$) at a proportion that is significantly higher than that in the primary tumors [34]. It will be of interest to look for this subpopulation in breast cancer CTCs. A study in a pilot cohort of 30 breast cancer patients analyzed for CTC subpopulations found 35.2 % of the CTCs to be $CD44^{+}/CD24^{-/low}$, while another cohort was shown to contain 17.7 % CTCs that were $ALDH1^{+}/CD24^{-/low}$ [35]. A different group of researchers attempting to detect metastasis-initiating cells (MICs) using a xenograft assay demonstrated a subpopulation of CTC from luminal breast cancer patients that could initiate metastasis in mice, where they manifest a $EpCAM^{+}$, $CD44^{+}$, $CD47^{+}$, MET^{+} phenotype [36]. Thus, preliminary data has shown that there is a subpopulation of CTC, which possesses "stem-like" phenotype and can be responsible for metastasis initiation. Further studies interrogating these features in larger cohort of clinical trials and their correlation with patient clinical outcome can be informative and reveal more information about the "real culprit" CTC subpopulation that is responsible for metastasis, and the one that potentially could prove to be the valuable therapeutic target.

1.4.3 CTC in Clusters

While studies in subpopulations in CTC can be riveting, another interesting observation is the CTC clusters, also known as Circulating Tumor Microemboli (CTM). Their existence was first reported in Small Cell Lung Cancer (SCLC) patients using a size-based CTC isolation strategy. In this study, presence of CTM was shown to correlate with worse clinical outcome as an independent prognostic marker [37]. In addition, recent studies have revealed that CTC travel with other blood components as heterogeneous clusters including immune cells [38], macrophages [39], and platelets [40]. In addition, mouse model studies have shown tumor cells traveling

with stromal cells, potentially cancer associated fibroblasts as its own "soil" to establish distant metastasis [41], although these are to date not shown to exist in peripheral blood in human cancer patients. Study of CTC companion cells in circulation could reveal important information on metastasis initiation and expand the definition of "liquid biopsy" to include other cell types beyond CTC.

1.4.4 Fate of CTC in Circulation

Another important question to be answered is the fate of CTC in circulation. It is reasonable to assume that there can be three potential fates for tumor cells in circulation. The first fate is that a given CTC will be "permanently non-productive" such that it will undergo either anoikis or apoptosis or necrosis, be eliminated by immune surveillance or simply remain unable to home to metastatic niche or unable to initiate the intravasation process. The second fate is that a CTC can be "temporarily non-productive," either successfully invading into secondary site and staying "dormant" (either stay in G0 phase of cell cycle or maintain an equilibrium of proliferative and apoptotic rates), or staying locked in mesenchymal status [30] and failing to colonize and form metastasis. The third fate is that the CTC is "productive," not only capable of invading into a secondary site but also forming metastasis by rapid proliferation. It is possible that CTC subpopulations are not committed to one certain fate. Thus, cells from "temporarily non-productive" fate can transform into "productive" fate and form metastasis after long-term dormancy under certain environmental cues or additional genetic mutations.

To interrogate the fate of CTCs, one study looked at expression of apoptotic marker (M-30) and proliferative marker (Ki-67) in breast cancer CTC, and the data supported the hypothesis that there were proliferative as well as apoptotic subpopulations of CTC in circulation. Apoptotic CTC were seen more in early stage breast cancer patients [42]. Another study looking at M-30 and Bcl-2 expression in CTC indicated that, surprisingly, apoptotic CTC with M-30 expression is associated with worse prognosis in patients with elevated CTC level, whereas patients with Bcl-2 CTC had better clinical outcomes in contrast to the notion that Bcl-2 will lead to anti-apoptotic effect on CTC and worse outcomes [43]. While the data on apoptotic CTC looks confounding, other groups have also examined proliferative subpopulations in CTC. Thus, a study investigating Ki-67$^+$ CTC concluded that proliferative CTC, independent of disease stage or treatment, is a rare population in circulation, and a fraction of non-proliferative CTC seem to be more chemoresistant [44].

Since data on apoptotic CTC remains elusive, and proliferative CTC seems to be a rare population in circulating, the key distinctive characteristics between the first "permanently non-productive" fate and the other two fates could lie in the homing to secondary site and initiation of extravasation process. Mechanisms of establishing micrometastasis at secondary sites can be a combination effect of physical trapping and chemical homing. Whereas physical trapping at secondary organ can be correlated to organ vasculature and tumor cell clustering (CTM lodging), chemical homing can be correlated with chemokines, micro-RNAs, and other tumor microen-

vironment signaling [45]. In 1889, Stephen Paget first brought up the notion that tumor cells form metastasis at secondary organs as "seeds" on congenial "soils" [46]. Later on, recent research has indicated that, certain "tumor tropism" signatures can be established to predict the potential secondary sites [47, 48]. Studying these signatures on CTC might reveal important traits that could shed light on the homing mechanisms of CTC to secondary organs.

It has been indicated that the half-life for CTC was 1–2 h. However, CTC can be detected in dormancy patients 8–22 years post mastectomy [49]. It is highly likely that CTC can be shed from micrometastasis and circulates in the blood, even seed back to the primary/metastatic lesions [50]. This indicates that, secondary organs, especially bones [51], can possibly serve as reservoirs for CTC, and thus will be critical to monitor for patients with metastatic dormancy.

1.4.5 Phenotypic, Genotypic Features of CTC for Clinical Applications

As we are looking into more and more in-depth biological characterization of CTC population, let us take a step back and ask the question: how can the phenotypic and genotypic studies of CTC feed back into clinical applications to benefit cancer management? An intuitive thinking will be assessing the therapeutic targets on CTC to monitor dynamic changes during treatment. One study looking at Androgen Receptor (AR) signaling in CTC from Castration-Resistant-Prostate-Cancer (CRPC) indicated that, "AR-on" signature was predominant in CTC population pretreatment. Post-treatment, a more heterogeneous population of CTC is observed. The increase of percentage of "AR-on" CTC population is correlated with worse outcome despite administration of abiraterone acetate therapy [52]. Another study looking at ALK-rearrangement status on CTC in non-small-cell lung cancer (NSCLC) patients indicated that ALK-rearranged CTCs were detected ALK-negative patients and ALK-rearranged CTCs harbored a unique pattern whereas primary tumor harbored more heterogeneous patterns [53]. This information can be useful in guiding therapies since ALK-rearrangement positive NSCLC patients do not benefit from EGFR Tyrosine-Kinase-inhibitor (TKI) and need to be treated with ALK inhibitor [54]. Another study conducted whole-exome sequencing for CTC in metastatic prostate cancer and compared it with that in the primary tumor and lymph node metastasis. This proof-of-principle study provides support for the notion that CTC can be used as a minimally invasive window to give us a peek at the mutational landscape of prostate cancer [55].

1.4.6 Functional Characterizations of CTC and CTC Culture

In addition to molecular characterization of CTC, another interesting direction is the functional characterization of CTC. Technologies that allow viable CTC capture enable such assays [56–59]. One study demonstrated development of oligoclonal

CTC culture from six metastatic luminal breast cancer patients, indicating that CTC culture can be potentially used for Next-Gen sequencing and, more importantly, for drug sensitivity screening [60]. Another study tested drug sensitivity on chip without establishing CTC culture in prostate cancer, where CTCs from docetaxel resistant CRPC patients do not respond to on-chip docetaxel and paclitaxel treatment [56]. A third study showed that xenograft assays of patient-derived CTC into immunocompromised mice can provide valuable prognosis information [36]. However, attempts to culture CTC are still at very early stage and have low efficiency. The CTC culture method reported by Yu et al. managed to establish 6 CTC culture from 36 patient samples attempted, while the xenograft assay only managed to establish CTC xenograft mice from patients pre-screened with more than 1000 CTCs per 7.5 mL blood. If CTC culture can be developed into a high-efficiency method and validated in larger cohorts to faithfully reflect patient treatment response without introducing culture-induced artifacts, it could be a powerful tool to guide therapy and greatly benefit cancer management.

1.5 Conclusions

The field of CTC analysis has grown exponentially in the past decade. As more and more researches have looked into the biological aspects of CTC, the field is going in divergence into two separate while equally important directions: studies focusing on clinical utility of CTC and studies focusing on biological nature of CTC. For clinical applications, the CTC assay needs to be standardized, reliable and robust. Thus, the clinical utility of CTC is still largely bound to be based on CTC enumeration since the CellSearch™ system is still the only FDA-cleared technology and it is so far only cleared for CTC enumeration, although a fourth channel on the device can be used for molecular characterization of CTC [61]. By utilizing CellSearch system, clinical trials, as described above, have looked extensively into clinical utility of CTC count beyond prognosis, and have begun to address various clinical applications including using CTC to monitor treatment efficacy, using CTC as surrogate endpoint for clinical trials and using CTC for early detection of solid tumor. Meanwhile, other studies are also investigating the biological features of CTC by attempting to answer questions about subpopulations of CTC (EMT, stem cell, clusters etc.), the fate of CTC, and how molecular and functional assays of CTC could benefit patients from the clinical perspective. These questions, if answered, can then feed back into the clinical applications, and expand or improve the clinical utility of CTC. Although it is a rapidly advancing field, there is still a lot to learn about CTCs. More ongoing research will likely be expanding the definition of "liquid biopsy," and we believe the ultimate goal will be developing a "universal test" that allows us to not only look at CTC counts but also phenotypic, genotypic, and functional features of CTC, as well as possibly other circulating blood-based biomarkers including associated cells, cell-free DNA and microRNA, etc., all studied in one or serial simple and minimally invasive blood draw(s).

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Chapter 2
Affinity-Based Enrichment of Circulating Tumor Cells

Zheng Ao, Richard J. Cote, and Ram H. Datar

Abstract Study of CTC in cancer has always been hampered by its rare existence in blood. In this chapter, we discuss one of first principles employed to capture CTC from cancer patients' peripheral blood—the affinity-based enrichment of CTC. We briefly discuss the different technologies utilizing antibodies to capture CTC based on specific antigen expression. Then we address the downstream molecular and functional characterization of CTC by utilizing these technologies. We also discuss the limitations of affinity-based CTC enrichment.

Keywords Circulating tumor cells • Affinity-based cell capture • Molecular characterization of CTC • In vivo CTC culture

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2.1 Background

Circulating Tumor Cells (CTCs) are tumor cells found in the peripheral blood of cancer patients, and the presence of these cells are considered the "real culprits" of metastatic disease that accounts for 90 % of all cancer-related deaths. Several studies were conducted in various types of cancers (breast, lung, colon, prostate, melanoma, etc.), presenting CTCs have proven prognosis value in these cancer types [1–3]. CTCs have also been shown to be valuable to predict drug-targeted therapy response in lung cancer patients [4]. As a result, there is increased interest in CTC research. In order to study CTCs these cells must be isolated and this can be challenging. In this chapter, we highlight one of the most widely used CTC-isolation techniques—affinity-based CTC capture. We discuss the principles behind the techniques, as well as explore emerging technologies employing these techniques. Lastly, we discuss the value and limitations of these various techniques.

2.2 Affinity-Based Enrichment of CTCs

As a rare population of cells in circulation, CTCs are difficult to study without the use of powerful technology that sets these cells apart from the surrounding and overwhelming population of leukocytes, red blood cells and other blood components. Among the various CTC enumeration technologies currently available, the use of the affinity-based isolation technique is the most widely adopted. These technologies utilize a unique antigen expression pattern on CTCs. The rationale behind the use of this type of CTC enumeration technology is based on unique antigen(s) expressed by CTC depending on their tissue of origin that are not shared with other normal components circulating in the blood. One of the most common examples among these is the use of the Epithelial Cell Adhesion Molecule (EpCAM) for CTC capture in epithelial cancer types. EpCAM is a transmembrane glycoprotein that is being expressed in cancers of epithelial origin, and not in normal components in circulation. Thus, antibodies against EpCAM can be used to selectively capture epithelial CTCs from peripheral blood. Here, we discuss several types of affinity-based technologies utilizing EpCAM or other tumor specific antigens in the capture of CTC cells (CellSearch and MagSweeper), as well as technologies that combine affinity-based capture with other physical parameters such as cell size (CTC-Chip and GEDI).

2.2.1 CellSearch

To date, CellSearch is the gold standard in the CTC field. It is the first and still the only technology that is cleared by US Food and Drug Administration (FDA) for clinical CTC testing in metastatic prostate, breast, and colorectal cancers. Utilizing the

principle of magnetic field-based cell sorting, CellSearch captures CTCs by labeling them with ferrofluid nanoparticles functionalized with anti-EpCAM antibodies. These cells are then isolated via a magnetic field. Briefly, a blood sample is mixed with buffer and centrifuged, followed by aspiration of the plasma and buffer. Ferrofluid nanoparticles are then added to enrich CTCs with EpCAM expression. Post CTC enumeration, cells are labeled with Cytokeratin (Cytokeratin 8,18,19-phycoerythrin), which are epithelial markers to identify CTC; CD45 (CD45-allophycocyanin), which is a leukocyte marker for negative identification and DAPI, which labels nucleated cells. Results of harvested cells are then reviewed and CTCs are identified based on their staining profile (Cytokeratin positive, DAPI positive and CD45 negative) and morphology features [5].

The clinical utility of the CellSearch platform has been validated in various studies. The first milestone study in metastatic breast cancer patients indicates that, based on a cutoff of 5 CTCs detected by CellSearch system per 7.5 mL blood sample, metastatic breast cancer patients can be stratified to predict their disease outcome (Progression Survival and Overall Survival) [1]. This has also been validated in studies of other disease types such as prostate and colorectal cancer with a cutoff set at 5 and 3 CTCs respectively [2, 3].

2.2.2 MagSweeper

MagSweeper is an automatic immunomagnetic separation technology for CTC enumeration. Blood samples are pre-diluted with a buffer and then incubated with magnetic-bead coated with anti-EpCAM antibodies to mark epithelial CTCs. Samples are then subjected to MagSweeper enumeration, during which magnetic rods covered with a plastic sheath are placed to sweep the sample well. After CTCs are captured on the rods, they are washed with a buffer and collected by external magnets that are located at the bottom of the well [6].

2.2.3 CTC-Chip

CTC-Chip technology utilizes microposts functionalized with anti-EpCAM antibodies to capture CTCs. On the first generation of the CTC-Chip, 78,000 microposts were distributed on a 970-mm^2 surface and were functionalized with anti-EpCAM antibodies. Blood samples were processed through the CTC-Chip at a flow rate of 1–2 mL/h and captured CTCs were then identified via Cytokeratin positive, DAPI positive and CD45 negative staining. Although CTC-Chip technology is effective in capturing and analyzing CTCs, the volume that can be processed is limited to 2–3 mL [7]. This could be a major drawback if applied in clinical settings, as the standard blood sample collected is 7.5 mL. Reducing the sample volume could affect the sensitivity of the CTC capture. Thus, a second generation of the

CTC-Chip, HB-Chip, was designed with a Herringbone structure to disrupt the flow of blood and increase CTC collision with antibody-functionalized walls on the chip. This new design not only increases the volume of blood samples that can be processed using the chip, but also enables the capture of CTC clusters [8] that have shown to be clinically significant when analyzed by other CTC enrichment platforms [9]. The third generation of the CTC-Chip, CTC-iChip, utilizes a combination of hydrodynamic size-based cell sorting to deplete small cells in blood such as red blood cells and platelets. The inertial focusing microfluidics positions the cells into a single cell line and then either enriches the affinity-based labeled EpCAM positive CTCs or depletes the CD45 positive leukocytes with a magnetic field. This third generation of CTC-iChip not only allows for positive affinity-based enumeration of CTCs by magnetic activated cell sorting, but also allows for negative depletion of leucocytes for antigen agnostic CTC enumeration [10].

The clinical utility of the CTC-Chip still awaits large-scale clinical trial examination. A pilot study has indicated that by employing the CTC-Chip to analyze EGFR mutation in CTCs harvested from lung cancer patients, the responses for targeted therapies can be potentially predicted [4].

2.2.4 GEDI

Geometrically enhanced differential immunocapture (GEDI) is a technology that captures prostate circulating tumor cells (PCTCs) based on their expression of prostate membrane-specific antigen (PSMA). The geometry of the device is designed to allow large prostate CTC's to collide onto obstacles, while smaller, non-target cells are displaced onto streamlines that do not collide with the microposts. This combination of affinity and size-based enrichment results in better capture efficiency and purity [11].

Worth noting, by switching the antibody used to functionalize the GEDI device, it is possible to achieve capture of CTCs from breast and gastric cancers using anti-HER2 antibody [12], pancreatic CTCs using anti-EpCAM and MUC1 antibodies [13], and mouse epidermal stem cells using anti-CD34 antibody [14].

2.3 Emerging Technologies for Affinity-Based Enrichment of CTCs

As a fast expanding community, CTC research has gained more and more attention in recent years. Thus, as previously described affinity-based CTC capture platforms are improving and a boom of fast-developing technologies are emerging. A few examples of these are discussed below:

2.3.1 NanoVelcro

NanoVelcro is a chip to capture CTCs based on their EpCAM expression. It is a device that is comprised of two parts; a patterned silicon nanowire substrate coated with anti-EpCAM antibodies and a polydimethylsiloxane (PDMS) chaotic mixer to generate flows that increase the collision between CTCs and capture substrates. Post CTC capture processes, CK/CD45/DAPI staining are executed to identify CTCs on the chip. A small, pilot cohort study of prostate cancer patients, utilizing the NanoVelcro chip, showed promising results for clinical applications [15]. Additionally, by grafting the NanoVelcro Chip with poly(*N*-isopropylacrylamide) (PIPAAm), a thermo- responsive polymer, captured tumor cells can be released via a temperature switch from 37 to 4 °C and cultured as demonstrated in model systems [16].

2.3.2 Graphene Oxide Nanosheets

This technology utilizes flower-shaped gold patterns to absorb graphene oxide (GO) and then functionalize the GO nanosheets with anti-EpCAM antibodies to capture CTCs in a PDMS chamber, and identify CTCs stained with CK/CD45/DAPI. The chamber-like platform also favors on-chip cell culture, tested with model systems where cultured tumor cells are spiked into blood, retrieved by GO nanosheets and cultured on chip [17].

2.3.3 VerIFAST

VerIFAST is a technology based on immiscible phase filtration (IPF) in which target cells are incubated with paramagnetic particles (PMPs), coated with EpCAM antibodies for capture and pulled with magnetic force into sequential chambers to achieve staining for EpCAM, Ki67, and Hoechst nuclear staining. The sieve chamber design in this technology can allow for an on-chip capture and staining workflow that minimizes substantial cell loss [18].

2.3.4 Immuno-microbubbles

This technology uses perfluorocarbon gas filled microbubbles conjugated with anti-EpCAM antibodies to capture CTCs. After CTCs are captured by the microbubbles, the layer of bubbles can be separated by centrifugation. The captured cell population is then subject to pan-CK/CD45/Hoechst staining for CTC identification [19].

2.3.5 GILUPI CellCollector

This emerging technology addresses the increasing need for in vivo CTC collection. This device collects CTCs by a functionalized and structured medical wire with anti-EpCAM antibodies that is inserted into the cubital vein of cancer patients for 30 min. This in vivo collection process, via this device, can analyze CTCs from 1.5 to 3 L of blood as compared with 7.5 mL blood analyzed by other ex vivo CTC assays [20].

2.4 Molecular and Functional Analysis of Circulating Tumor Cells Downstream of Affinity-Based CTC Enrichment

There has been a recent surge in the CTC capturing technology industry. Molecular and functional characterization of CTCs is becoming "hot-beds" for researchers seeking better utilization of CTCs in the clinical setting. Although the identification and quantification of CTCs alone can be effective in prediction of disease outcome [1–3], molecular and functional characterizations of CTCs are likely to increase the specificity of the CTC assay, leading to personalized targeted therapies.

Results from a recent clinical trial, SWOG 0500, indicates that chemotherapy treatment decisions based on elevated CTC numbers after a first cycle of chemotherapy does not benefit metastatic breast cancer patients [21]. Although the clinical utility of CTC numbers alone in determining treatment options needs further validation, these results exemplify the importance of, and demand for, CTC characterization. Here, we briefly discuss the characterization of CTCs enabled by the technologies we discussed above.

2.4.1 Gene Expression Analysis to Characterize CTC Post Affinity-Based Enrichment

Gene expression analysis is important in the prognosis and treatment of cancer as validated in primary tumor gene expression profiling experiments [22–24]. It is very probable that CTC gene expression analysis will more than likely provide critically necessary information for the management of diseases. It will also provide a critical research tool that will enhance the study of metastatic processes, tumor dormancy, tumor tropism, etc. Current attempts are trying to interrogate CTC gene expression profile at different levels.

Gene expression analysis at the mRNA level can be achieved on chip via RNA in situ hybridization (RNA ISH) as demonstrated by HB-CTC Chip to analyze human pancreatic CTC expression of *Wnt2* [25].

Off-chip mRNA analysis for products of tumor-specific genes can be analyzed by qRT-PCR or RNA-seq methods from pooled CTCs as demonstrated by data

obtained from CTC enrichment methods such as GEDI, GO nanosheets, and HB-CTC Chip [11, 17, 25]. However, due to contaminant leucocytes and other blood components captured nonspecifically by these affinity-based CTC enrichment methods, performing high-throughput mRNA analysis on both tumor and non-tumor-specific genes requires further isolation of pure population of CTCs in the post-capture process. This can be achieved by either the micropipetting of single cells (feasible by CTC-iChip technology) [10] or by microfluidics enabled by a single-cell analysis platform like Fluidigm (feasible by using MagSweeper technology) [26]. These single-cell analysis assays can be implemented to address issues raised regarding CTC heterogeneity that could be critical in the prognosis and treatment of cancer.

CTC gene expression profiles can also be interrogated at the protein level. This interrogation is already seen in most affinity-based CTC capture techniques. As an example, immunofluorescence (IF) staining is being employed to help distinguish CTCs from the background of leukocytes and other blood components. By adding an extra antibody or additional marker(s), it can be helpful in the qualification/quantification of protein marker expression levels when enabled by multi-spectrum fluorescence microscopy. CellSearch is a good example of technology that provides a user-defined channel tool that can be used in the characterization of CTCs [27]. There are several ongoing clinical trials are currently utilizing this feature to characterize CTCs for their Her2/Ki-67 expression and using the findings to determine the endocrine therapy used in metastatic breast cancer patients [28]. Multiplex immunofluorescence staining can also be used in the detection of fusion protein products such as TMPRSS2ERG fusion protein found in Castration-Resistant Prostate Cancer patients as demonstrated by the GEDI platform [11].

2.4.2 Genomic Analysis of CTC Post Affinity-Based Enrichment

CTC genomics is another important aspect in the management of cancer. Analyzing mutations and chromosome rearrangements, as well as lineage tracing using Next Gen Sequencing (NGS) in CTCs, can provide valuable information in treatment selection and personalized therapy. It can also provide important biological-based information regarding the metastasis. DNA fluorescence in situ hybridization (FISH) is a common methodology utilized to analyze DNA point mutation and chromosomal rearrangement on chip. As demonstrated by HB-CTC Chip, FISH for Androgen Receptor (AR) gene locus can be performed on CTCs for copy number analysis [8]. Another efficient method for DNA mutation detection is PCR. This has been shown to be very successful in the detection of EGFR mutation from pooled CTCs isolated with CTC-Chip in lung cancer patients as described above [4]. DNA mutation detection by PCR has also proven to be an effective in the detection of KRAS and other gene mutations from CTC isolated by GILUPI CellCollector [20]. DNA mutation and chromosome translocations can also be detected on CTCs by RNA-ISH/IF techniques that can detect the mRNA or protein product of the mutation/fusion DNA [8, 11].

Next-Gen Sequencing is also being applied to analyze CTCs isolated by affinity-based methods. With a broader spectrum of CTC genomics, it can provide valuable information in treatment options, as well as insight in tumor evolution and the resistance development process. This is exemplified in a publication authored by Jens et al. addressing whole-exome sequencing of CTCs isolated from metastatic prostate cancer patients. Using a combination of automated cell picking with MagSweeper CTC isolation technology, they were able to achieve single cell whole-exome sequencing [29]. Xiaohui Ni et al. also achieved whole-exome sequencing using the CellSearch Platform to isolate CTCs from lung cancer adenoma patients. In this study, whole-exome sequencing was performed to analyze the copy number variation (CNV), and the patterns observed in the CTCs were found to be representative of metastatic tumors [30].

2.4.3 Functional Characterization of CTC Enabled by Affinity-Based Enrichment

An emerging topic in CTC research is functional characterization of CTCs. This requires techniques that maintain cell viability in samples during the CTC capture process. To achieve optimal CTC viability, additional requirements for the handling, preserving, labeling, and shear pressure control should be implemented. It is worth noting that the CellSearch platform does not allow for viable CTC capture under harsh conditions. Some affinity-based platforms that allow for viable CTC capture are the CTC-iChip [10], GEDI [11], NanoVelcro chip [16], and Graphene Oxide nanosheets [17]. As a result, technologies that allow for viable CTC capture are driving CTC culture technology development and ex vivo drug treatment assays [11, 31] (Fig. 2.1).

	Gene Expression Analysis	Genomic Analysis	Functional Characterization
CellSearch	Third marker immunofluorescence [28]	Copy Number Variation Analysis [31]	-
CTC-Chip	-	PCR [4]	-
HB-Chip	RNA ISH/ Pooled Cells RNA-Seq [25]	FISH/ Fusion transcript by RT-PCR [7]	-
CTC-iChip	Single Cell qRT-PCR [9]	-	CTC Culture/ Ex-vivo drug treatment [32]
MagSweeper	Single Cell qRT-PCR [27]	Whole Exome Sequencing [30]	-
GEDI	Pooled Cells RNA-seq [11]	Fusion protein by immunofluorescence [11]	Viable CTC Capture/ Ex-vivo drug treatment [11]
NanoVelcro	-	-	Viable CTC capture/ Culture in model system [16]
GO nanosheets	Pooled Cells qRT-PCR [17]	-	Viable CTC capture/ Culture in model system [17]
GILIPI CellCollector	-	PCR [20]	Viable CTC capture [20]

Fig. 2.1 Summary of molecular and functional analysis of circulating tumor cells downstream of affinity-based CTC enrichment

2.5 Limitations and Emerging Applications of Affinity-Based Enrichment of CTCs

2.5.1 Limitations

Although affinity-based enrichment of CTCs has shown to be versatile and effective, the fact remains, that limitations related to this type of technology still exist. A major concern for affinity-based capture of CTCs is its utilization of antigen expression. Although EpCAM has shown to be an effective marker for CTC capture and is being used extensively in affinity-based CTC capture platforms, an increasing number of studies are also revealing its limitations. These limitations have been identified that CTCs with no or low EpCAM expression will be overlooked when EpCAM is used as the capture target. This was the case in a study featuring characterization of CTCs with potential for creating brain metastasis, this sub-group of CTCs were found to be EpCAM negative [32]. Another study conducted in metastatic breast cancer patients demonstrated how by combining of Cytokeratin and EpCAM expression for CTC detection, the sensitivity of CTC detection can be increased as compared with using solely EpCAM expression for CTC detection [33]. This could be due to the heterogeneity of CTC populations and the hypothesis that tumor cells undergo Epithelial–Mesenchymal Transition (EMT) and shed epithelial phenotypes such as EpCAM expression in order to increase invasion and metastatic capabilities [34]. It is important to note that the quality of the antibody and the position of the epitope that an antibody recognizes can also affect capture efficiency [35].

2.5.2 Emerging Applications for Affinity-Based Enrichment of CTCs

Different strategies were attempted in order to overcome the potential bias introduced by EpCAM-based CTC capture. One strategy sought to increase the sensitivity of affinity-based CTC enrichment by using comprehensive panels to capture CTCs like using Cytokeratin in combination with EpCAM [33] or using a combination panel of EGFR, HPSE, ALDH1 [29]. Another strategy called for the negative depletion of non-tumor cells using an affinity-based isolation method instead of positively captures cells expressing certain markers. The ^{neg}CTC-iChip is a notable example for this type of technology. By combining depletion of non-tumor cells using anti-CD45 and anti-CD15 antibodies with hydrodynamic cell-sorting based on cell size, the ^{neg}CTC-iChip mode is able to isolate CTCs without biasing CTC capture with their antigen expression profiles.

2.6 Summary

Affinity-based enrichment serves as the first and most widely accepted principle employed in the isolation of CTCs. CellSearch technology, which utilizes this principle, is considered the gold standard in CTC research. Novel and evolving technologies seeking to increase capture efficiency are introducing state-of-the-art microfluidic designs. Numerous application potentials are being investigated to characterize CTCs captured with affinity-based principles at DNA, RNA and protein levels, as well as functional characterization of CTCs. Nevertheless, affinity-based CTC capture also faces some limitations with potential loss of sensitivity as a result of CTC heterogeneity, EMT process, and capture antibody efficiency. To overcome these limitations, technologies have been exploring various approaches to capture CTC that include the combination of panels of targets for CTC capture, combination of affinity-based capture with other principles such as size-based capture, and switching from positive capture to negative depletion of non-tumor cells in the sample.

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Chapter 3
Size-Based and Non-Affinity Based Microfluidic Devices for Circulating Tumor Cell Enrichment and Characterization

Zheng Ao, Kamran Moradi, Richard J. Cote, and Ram H. Datar

Abstract Circulating Tumor Cells (CTCs) are tumor cells found in cancer patients' peripheral blood. Enumeration of CTCs can provide prognosis information for cancer management (Cristofanilli et al., N Engl J Med 351(8):781–791, 2004; Cohen et al., J Clin Oncol 26(19):3213–3221, 2008; de Bono et al., Clin Cancer Res, 14(19):6302–6309, 2008; Poveda et al., Gynecol Oncol, 122(3):567–572, 2011).

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However, the technical hurdle for studying CTCs is their rare presence in blood, thus, isolating them is a non-trivial task. Two major categories of technologies have been developed in the past to isolate CTCs based on their biological expression of antigens (affinity-based capture) or based on their physical properties (non-affinity based capture). This chapter dedicates itself to the non-affinity based method for CTC capture. CTCs, as tumor cells, are inherently distinct from normal blood components. The chapter touches on the how these differences are reflected in their gene expression profiles, as well as their physical properties. We discuss how researchers utilized the unique biomechanical and electrical properties of CTCs to isolate them from enormous numbers of erythrocytes and leukocytes present in peripheral blood. We begin the chapter with technologies utilizing biomechanical properties (cell density, size, deformability) to isolate CTCs and then move on to discuss the development of dielectrophoresis (DEP) based CTC isolation, based on their distinct electrical properties.

Keywords Circulating tumor cells • Microfluidics • Non-affinity based cell isolation • Dielectrophoresis

3.1 Background

Circulating tumor cells (CTC) are tumor cell found in cancer patient's peripheral blood, which hold significant clinical value. Enumeration of CTC can provide prognosis information for disease management, as validated by CellSearch system in several disease settings, including breast cancer, colorectal cancer, prostate cancer, and ovarian cancer [1–4]. CellSearch system, as discussed in the previous chapter, although effective, can lead to neglect of certain subpopulations of CTC [5]. And this concern is shared among other affinity based CTC capture platforms, as affinity based CTC capture relies on expression of certain antigen on tumor cells, which can vary due to the heterogeneity observed among CTCs [6]. In contrast, non-affinity based techniques isolate CTC based on their physical properties. These antigen agnostic capture platforms may address the concerns of neglect of certain CTC subpopulations and give us a more comprehensive picture of CTC.

Current existing non-affinity based CTC capture platforms can be briefly categorized into two major types: capture platforms based on mechanical properties and capture platforms based on electrical properties. In this chapter, we discuss both types of techniques and how they are applied to isolate and characterize CTCs.

3.2 CTC Capture Based on Mechanical Properties

Circulating tumor cells, as inherent from primary tumor cells, are physically distinct from normal blood components in the aspect of size, density, and elasticity. Different technologies have been developed utilizing these properties and are discussed in the following sessions.

3.2.1 *Density-Based CTC Enrichment*

Density-based CTC enrichment is a standardized method for blood component separation. By layering blood onto a density gradient reagent and subsequent centrifugation, blood can be separated into plasma layer, buffy coat layer containing primarily mononuclear cells, and bottom layer containing granulocytes and erythrocytes (Fig. 3.1a). This can serve as a pre-enrichment step for CTC isolation since CTC will be retained in the buffy coat layer and isolated from granulocytes and erythrocytes. Further examination of CTC can be achieved through immunofluorescence labeling. However, density gradient centrifugation, although easy to handle and cost-friendly, suffers from poor retrieval rate as well as poor purity of CTC. Thus, several modifications have been made to enhance this assay for CTC isolation. The SepMate™ tubes developed by Stemcell Technologies employ a funnel shaped disk to enhance retrieval of the buffy coat and prevent mixing of the buffy coat layer with the bottom layer. Recently, the OncoQuick™ assay was developed employing a porous filter in the middle of the tube thus combining size-based isolation with density based isolation to achieve a higher retrieval rate of CTC [7].

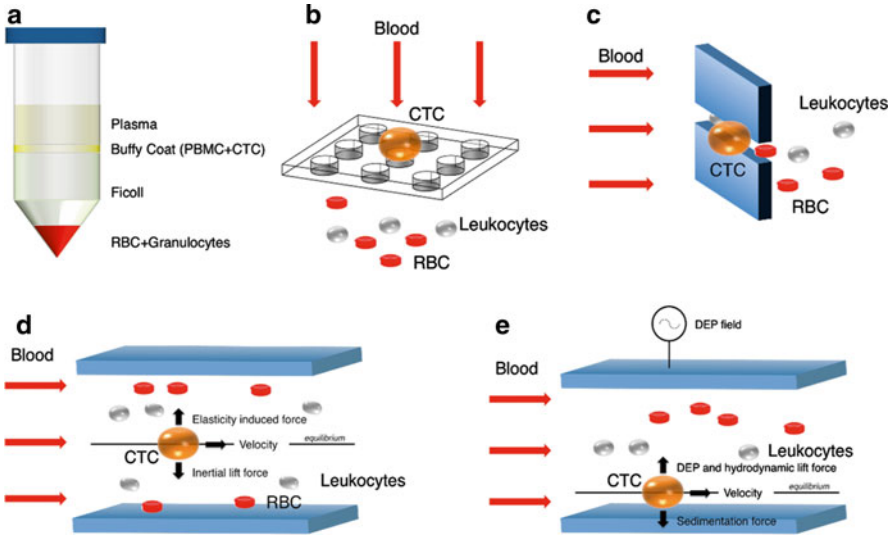

Fig. 3.1 Principles used for non-affinity based CTC enrichment. (**a**) Density based CTC enrichment. (**b**) Size-based microfiltration for CTC enrichment. (**c**) Size-based microfluidics for CTC enrichment. (**d**) Inertial focusing based microfluidics for CTC enrichment. (**e**) DEP based microfluidics for CTC enrichment

3.2.2 Microfiltration-Based CTC Enrichment

CTCs are inherently larger than other blood components. Thus, several technologies have been developed using size-based isolation as strategy. Microfiltration was one of the first concepts developed for size-based CTC isolation (Fig. 3.1b). The first microfiltration technology was developed employing a polycarbonate filter. Briefly, ^{35}U fission fragments were used to bombard a polycarbonate membrane, followed by warm sodium hydroxide etching to generate pores with uniform size of 8 μm. This size cutoff can efficiently capture large CTCs whereas letting small erythrocytes and leukocytes pass through [8, 9]. Post-capture, CTCs were analyzed by Immunohistochemistry staining (IHC) to distinguish CTC from leukocytes retained on filter by the criteria of Cytokeratin (CK) positive, CD45 negative and morphological features such as cytoplasm to nuclear ratio. This Isolation by Size of Epithelial Tumor Cells (ISET) technology was validated in several disease settings, including breast cancer, lung cancer, pancreatic cancer, cutaneous melanoma, and uveal melanoma [10–14]. Furthermore, downstream molecular characterization of CTCs can be performed, which provides additional information for disease management. Vona et al. demonstrated that, by Laser Capture Microdissection (LCM), Hep 3B tumor cells captured on filter can be dissected and analyzed for p53 gene. Also, the feasibility of DNA fluorescence in situ hybridization (FISH) is also demonstrated by hybridizing the cells captured the filter with probe for centromeric chromosome 1 [9]. Later, ALK-FISH was performed by Pailler et al. on CTCs captured from Non-Small-Cell Lung Cancer (NSCLC) patients to interrogate the ALK-rearrangement of these patients pre- and post-chemotherapy [15]. Another interesting phenomenon reported by Hou et al. using ISET technology was the capture of circulating tumor microemboli-contiguous groups of tumor cells on filter [16]. Following studies in small cell lung cancer and uveal melanoma further confirmed that presence of CTM could be correlated with worse prognosis [14, 17].

ISET technology, although it is effective for CTC isolation, can be limited due to the randomized generation of pores on filter. This could result in reduced filtration area and pore fusions, which could cause increased risk of clogging and loss of cells during filtration. To alleviate this concern, our group has developed a parylene-based microfilter, which is fabricated in a fashion that pores are evenly distributed in a well-controlled pattern. To achieve this, parylene C was deposited to 10 μm thickness and masked with photoresist material. Then the membrane was etched with reactive ion etching (RIE) followed by the last step, which was the stripping of photoresist using acetone. Using this novel microfilter design, Zheng et al. demonstrated on filter tumor cell capture and electrical lysis for PCR analysis [18]. Following validation reported by Lin et al. demonstrated >90 % recovery using this filter design and superior sensitivity over CellSearch™ affinity based CTC capture system's performance in the same cohort of cancer patients [19]. Another study reported by Birkhahn et al. demonstrated this novel microfilter could also be employed to enhance urine cytology performance [20].

RIE etching of parylene C technique not only provides us the advantage of controllable pore deposition but also enables us to alter the pattern and the shape of the

pores of the filter. By altering these factors, our group has developed next-gen filters that can be applied for viable CTC capture. For the first generation of filter described above, the sample needs to be fixed at end concentration of 1 % formalin for 10 min to achieve optimal retrieval rate of tumor cells, this is to prevent cell loss under the shear pressure of filtration. To reduce the shear pressure and achieve viable cell capture, Zheng et al. reported the fabrication of a 3D bilayer membrane filter. This filter is comprised of two layers, on the top layer, pores with 8 μm size were deposited and on the bottom layer pores with 9 μm size were deposited slightly off-setting the top layer pores. The gap distance between two layers is precisely engineered to be 6.5 μm. By this design, when cells are trapped on the top layer, the bottom layer can provide direct support in the opposite direction of the flow to reduce the pressure on trapped cells. This design is validated using model system and captured tumor cells remain viable and metabolically active at least 2 weeks post capture [21].

Another design of microfilter for viable CTC capture is the slot filter reported by Xu and Lu et al. By altering the geometry of the pores from round to slot, the fill factor is greatly enhanced thus reducing the shear pressure during filtration. Validation experiments confirmed tumor cells spiked into blood could be viably captured and cultured post capture. And telomerase activity can also be measured on the captured cells [22].

Another variation of the filter design for viable CTC capture is reported by Harouaka et al.—the Flexible Micro Spring Array (FMSA) design. This design utilizes a micro spring array to maximize the filtration area thus reduces the shear pressure during filtration. By using this design, spiked in tumor cells can be retrieved from blood viably and expand on filter [23]. FMSA device can also be used for on chip drug sensitivity test, and FMSA-derived cell culture can be reinject into animal models for in vivo drug sensitivity test as demonstrated by Gallant et al. in model system experiments [24].

Other microfilter platforms were also developed for CTC isolation. VyCap microsieves are fabricated using silicon nitride with evenly distributed pores of 5 μm size as reported by Coumans et al. [25]. Lim et al. also reported fabrication of microsieves using silicon-on-insulator (SOI) wafer with 10 μm sized pores [26]. Yusa et al. reported that, by fabricating a 3 dimensional phallodium filter with a pocket shape (with 30 μm sized pores on the top layer surrounding 8 μm sized pores on the bottom layer), blood sample could be processed without fixation and external pressure. The sample was processed through this 3D phallodium filter by gravity and the target cancer cells remained viable post capture [27]. Additionally, as reported by Tang et al., by replacing the cylinder shaped pores with conical shaped pores, a small differential pressure will be gained between the smaller pores facing the top and the larger pores facing the bottom of the filter, which will greatly reduce the leukocyte retention on filter and gain higher purity of CTC [28]. Also, Hosokawa, et al. reported a microcavity array (MCA) device fabricated from nickel by electroforming to generate arrays of cavities with the average diameter of 8.4–9.1 μm [29]. And later on, a MCA system was reported using a rectangular cavity design and validated in a pilot cohort of small cell lung cancer patients[30] (Table 3.1).

Table 3.1 Microfiltration-based CTC enrichment technology

Technology	Modeled cells	Clinical validation	Efficiency (*E*) and sensitivity (S)	Purity	Viability	References
ISET	Hep3B, Hep G2, HeLa, LNCaP, MCF-7	Breast, lung, pancreatic cancer, melanoma	S: Up to 1 cell/mL	N/S	N/S	[9–17]
Parylene C microfilter	LNCaP, MCF-7, SK-Br-3, J82, T24, RT4	Prostate, bladder, breast, colorectal cancer	S: Up to 1 cell/mL *E*: 89.0 ± 9.5 %	N/S	N/S	[18, 19]
3D bilayer parylene C filter	MCF-7, LNCaP	N/S	*E*: 86.5 ± 5.3 %	N/S	Y	[21]
Slot parylene C filter	PC3, DU145	Prostate cancer	*E*: ~70 %	1500-fold enrichment	90 %	[22]
Flexible micro spring array	MCF-7, MDA-MB 231, C8161, WM35	Breast, colorectal, lung cancer	*E*: 92.6 %	14,000-fold enrichment	>80 %	[23, 24]
VyCaP microsieves	SK-Br-3, MDA-MB-231, MDA-MB-468, MCF-7, PC3-9, COLO-320, SW-480, HL-60, K-562	N/S	*E*: 58 %	1000-fold enrichment	N/S	[25]
Silicon microsieve filter	HepG2, MCF-7, BT474	Breast, colorectal, prostate, cervical cancer	*E*: ~80 %	N/S	N/S	[26]
3D palladium filter	N-87, COLM-5	Breast cancer	*E*: >70 %	N/S	N/S	[27]
Filter with conical shaped holes	HT-29, U87	Lung, nasopharynx, mediastinal, cardiac, cervical, and breast cancer	*E*: ~95 %	96 % WBC clearance	95 %	[28]
Microcavity array	MCF-7, NCI-H358, SW620, AGS, SNU-1, Hs578T	N/S	*E*: >80 %	N/S	98 %	[29]
Rectangular microcavity array	NCI-H69, NCI-H82, NCI-H358	Small cell lung cancer	*E*: >80 %	7000-fold enrichment	Y	[30]

3.2.3 *Microfluidics-Based CTC Enrichment*

Microfluidic devices are being increasingly employed in analytical sciences and diagnostics because they enable miniaturization, integration, and automation [31–33]. One representative output of technology is the development of lab-on-a-chip (LOC) devices, also known as micro-total analysis systems, which combine lab-scale tasks on a single mini-scale chip [34, 35] to yield significantly interesting biological applications. Using intrinsic physical properties of cells, like size, density, shape, and deformability, or extrinsic properties of the cellular response to magnetic or electrical fields, or optical excitation, microfluidic platforms to allow manipulation of the cells can be created. Microfluidic platforms to manipulate cells can exploit affinity of cells to specific ligands such as antigens expressed on their surface. On the other hand, non-affinity based microfluidic platforms are interesting because no further steps are required to recover the cells following isolation. Hydrodynamic manipulations, such as inertial method [36], or the acoustophoresis method [37] are examples of non-affinity based manipulations. Apart from membrane filters, microfluidic systems are also popular in isolating CTC based on their biomechanical properties (Fig. 3.1c). An early example of such a microfluidic platform was reported by Mohamed et al. [38]. This massively parallel sieving device with 10 μm-wide and 20 μm-deep microchannels could trap neuroblastoma cells spiked into whole blood. A microfluidic platform has been developed by Tan et al. [39], which isolates the cancer cells of breast and colonic origin, using their larger size and stiffness characteristics. Their microdevice consists of multiple arrays of crescent-shaped isolation wells. A gap of 5 μm in each of the traps prevents any clogging issue. Pre-purification is happening utilizing a filter with 20 μm gap size to remove larger clusters before blood stream flows into the isolation section. Their experiment of screening 5 mL sample size, utilizing three microdevices at the same time, and under 5 kPa constant pressure, takes 2.5 h. The efficiency of at least 80 % capture for MCF-7, MDA-MB-231, and HT-29 has been reported.

Recent developments employ fluid flow dynamics to manipulate cells for isolation and separation. Fluid flow in microfluidics is considered a dominantly laminar regime due to the definition of non-dimensional Reynolds Number, which refers to the ratio of inertial force to viscosity [38]. Hydrodynamic particle manipulation methods are passive techniques and have high throughput. Inertial technique eliminates the physical barriers to trap the cells, instead, using hydrodynamic properties of the fluid flow to trap cells [38, 41]. Important design parameters in this technique are geometry of the microchannel, particle size, and flow rate. Different microchannel geometries have been explored, such as straight [42, 43], expansion–contraction [44, 45], spiral [46, 47], and serpentine [48].

Hou et al. [49] introduced an inertia-based microfluidic platform to isolate CTCs in a spiral microchannel configuration which is also known as Dean Flow Fractionation (DFF) method. Secondary flows, called the Dean vortexes, move the cells back and forth along the microchannel. Here, the cells are subjected to two forces, drag force and lift force, which exert differentially on cells with different

sizes and shapes. The ratio of inertial lift forces and Dean drag forces (F_L/F_D) will define final equilibrium position for the cells, which grows exponentially with cell size [50]. Their isolation device showed 100 % CTC detection efficiency using 3 mL of whole blood [49].

Hur et al. [51] developed a passive, continuous microfluidic platform utilizing "expansion–contraction" trapping reservoirs along microchannels. Stable vortices in the reservoirs trap larger cells (like CTCs), while the smaller cells flush along the microchannel to the outlet. The same group of researchers [52] developed a passive and label-free sorting technique exploiting both size and deformability differences in cells. Elasticity and viscosity impose differential lateral dynamic equilibrium positions on cells, thus separating different cell populations from each other. They observed that larger and more deformable tumor cells focus at the center of microchannel. They believe this method can also be a microfluidic platform to measure the deformability of cells. Bhagat et al. [53] proposed a two-stage chip design, which starts with contraction–expansion region where cells are focused by reaching an equilibrium between the counteracting wall-induced lift forces and viscous drag, and a region towards the end with pinched-flow configuration where large tumor cells are pinched by aligning to the central axis. They successfully reported the separation of spiked tumor cells from peripheral blood with 80 % efficiency at the throughput of 400 μL per minute. Augustsson et al. [54] developed a continuous separation method utilizing ultrasound wave radiation force. Acoustophoresis method is gentle, label-free and based on the intrinsic properties of cells such as density, size, and compressibility. The chip fabrication method has been demonstrated in detail by Moradi et al. [55]. This noncontact method does not affect cell viability or proliferation. A recovery of 87 % and 83 % of fixed cells (DU145 and PC3, respectively) with 4.2 mL/h throughput using a single microchannel has been reported [54].

Hyun et al. [56] utilized multi-orifice flow fractionation (MOFF) configuration in their device design to isolate the CTCs based on their size. The device consists of an initial filter and several parallel MOFF microchannels. There are a series of alternating contraction channels and expansion chambers, which initiate inertial forces, separating larger CTC from smaller blood cells. This platform was validated for CTC capture in a pilot study of 24 breast cancer patient samples. Microfluidic platforms are thus increasingly shown to be suitable for clinical and biological applications, with advantages such as rapid, label-free, high throughput, and cost-efficient analysis. Process parallelization can be designed in microfluidic platforms to achieve simultaneous analysis for several samples, thus expediting the process of diagnostics, therapeutics and fundamental studies in cancer biology.

3.2.4 CTC Capture Using Nanoroughened Surfaces

Mechanical property based label free isolation of CTC can not only be achieved based on cell size/density/inertial force but also by other properties that are not as well understood. Chen et al. reported that, CTC, when compared with normal blood

cells, has a different adhesion preference to nanorough surfaces. Thus, when applied to nanoroughened surfaces, tumor cells can be selectively captured by the surface. Preliminary results demonstrated that, when MCF-7 and MDA-MB-231 breast cancer cells were spiked into blood, the capture efficiency of the nanoroughened surfaces reached 93.3 ± 1.5 % and 95.4 ± 2.2 % respectively [57] (Table 3.2).

3.3 Capture Based on Electrical Properties

As addressed above, Circulating Tumor Cells are inherently distinct from the normal blood components. This distinct feature of CTC is not only reflected in its biomechanical properties, such as size, inertial force, and density as described above, but also reflected in its electrical properties. One popular example of using electrical property to isolate CTC is the use of Dielectrophoresis (DEP). DEP is first studied by Herbert Pohl in 1950 [58]. He described a phenomenon—DEP, that particles can be moved using polarization forces in an inhomogeneous electric field. This phenomenon is strongly dependent on volume and shape of the particle, the electrical property of the particle as well as the gradient of the field and the medium. Thus, DEP can be applied to isolate cells based on their size and electrical properties (Fig. 3.1e). Later on, in 1995, Becker et al. reported the distinct dielectric property of tumor cell, erythrocytes and lymphocytes and how DEP can be used to isolate breast cancer cells from blood [59]. Cheng et al. also reported isolation of HeLa cells spiked into blood using a DEP chip [60]. Additionally, An et al. reported that malignant breast cancer cells (MCF-7 breast cancer cell line) could be separated from healthy breast cells (MCF-10A cell) using DEP since they have distinct dielectric property [61]. In 2005, Park et al. reported that, by fabrication of a chip with 3D asymmetric electrodes, mouse embryonic carcinoma cell P19 can be separated from erythrocytes using DEP. This is a stepping-stone towards isolation of cancer cell from whole blood using a DEP microfluidic chip [62]. Additionally, Jen et al. reported a handheld microfluidic chip that is able to concentrate HeLa cells controlled by DEP generated from circular microelectrodes [63].

One of the first microfluidic devices that can continuously separate tumor cells from whole blood is reported by Alazzam et al. in 2011. This article described a method for continuous flow separation of CTC from blood. Briefly, interdigitated activated comb-like electrodes were positioned divergent and convergent with respect to the flow and CTCs were isolated due to their distinct response to the alternating current frequencies as compared with normal blood cells [64].

Other DEP microfluidic chips use combination of DEP of other microfluidic principles. Wang et al. reported that, by combining DEP with field-flow-fractionation (FFF), cell separations could be achieved efficiently. In this design interdigitated microelectrodes were mounted into rectangular chambers. Cells with distinct electric properties were levitated to distinct heights where the DEP forces were equilibrated with sedimentation forces. And by field flow, cells at different heights were carried at different velocities and thus separated. The authors demonstrated efficient

Table 3.2 Microfluidics-based CTC enrichment technology

Technology	Modeled cells	Carrier medium	Clinical validation	Efficiency (*E*) and sensitivity (S)	Purity	Viability	References
Crescent shaped structure	MCF-7, MDA-MB-231, HT-29	Diluted blood	N/S	*E*: >80 %	>80 %	Y	[39]
DFF spiral microchannel	MCF-7	Whole blood	N/S	*E*: >85 %	10^4–10^6-fold enrichment	>98 %	[49]
Microscale vortices	HeLa, MCF-7	Diluted blood	N/S	*E*: 10–23 %	~85 %	Y	[51]
Deformability based inertial microfluidics	HeLa, MCF-7, SAOS-2	Diluted blood	N/S	*E*: ~97 %	3.2–5.4-fold enrichment	~92 %	[52]
Pinched flow coupled inertial microfluidics	MCF-7, MDA-MB-231	Diluted blood	N/S	*E*: ~81 %	3.25×105-fold over RBC $\sim1.2\times10^4$ over leukocytes (2nd process)	>90 %	[53]
p-MOFF device	MCF-7, MDA-MB-231	Lysed blood	Breast cancer	*E*: 91.6–93.75 %	Elimination of 90.8 % leukocytes	N/S	[56]
Nanoroughened surface	MCF-7, MDA-MB-231	Lysed blood	N/S	*E*: ~94 %	N/S	Y	[57]

separation of several cell types including separation of MDA-435 breast cancer cells (later revealed to be melanoma cells) from normal t-lymphocytes [65].

DEP can also be combined with multi-orifice flow fractionation (MOFF) to achieve continuous separation of cancer cells from blood at higher flow rate as reported by Moon et al. In this microfluidic device, cell mixture will first enter a separation region, where MCF-7 cells will be focused in the center of the channel together with few contaminating blood cells. This enriched population will then enter a focusing region where all cells will be aligned at the sides of the channel by DEP force. At the end, the aligned population will enter a second separation region and tumor cells will be selectively isolated via DEP. By this design, 75.81 % recovery rate was achieved with the removal of 99.24 % RBCs and 94.23 % of WBCs at a flow rate of 126 mL/min as demonstrated by a model system using MCF-7 cell spiked blood [66].

Recently, a commercial DEP based microfluidic platform ApoStream™ was launched. In this device design, similar to the concept of DEP-FFF, CTCs were drawn close to the channel walls by the DEP force and thus collected in a collection chamber and other cells were flushed into a waste collection chamber due to inefficient DEP dragging forces. To test the system, SKOV-3 and MDA-MB-231 cancer cells were mixed with 12×10^6 peripheral blood mononuclear cells (PBMC) as model system to test the platform, and respectively 75.4 ± 3.1 % and 71.2 ± 1.6 % recovery rate was achieved. Since the cell isolation is fixation-free, the captured tumor cell can be then maintained in culture [67] (Table 3.3).

3.4 Conclusion

CTC is consisted of a very heterogeneous population, thus affinity based CTC isolation has its limitations on using specific antigen expression to capture CTC. As Zhang et al. reported, a subpopulation of CTC that has the potential to cause brain metastasis in breast cancer is EpCAM negative [68], which would have been missed if using EpCAM based CTC capture, a common antigen utilized in majority of the affinity based CTC capture platforms, including CellSearch. In contrast, non-affinity based CTC isolation has the potential to lead to a more comprehensive understanding of CTC, as supported by Barradas et al., who demonstrated that CTC can be captured from CellSearch waste using size based CTC capture [5].

To address this concern, some CTC capture platforms are employing a combinational strategy to combine affinity based capture with mechanical based capture to enhance capture efficiency, such as geometrically enhanced differential immunocapture (GEDI) device reported by Gelghorn et al. which employs a device geometry so that desired cells with larger size will get into contact with antibody coated walls more often due to streamline distortion [69] and CTC-iChip device reported by Ozkumur et al. which combines inertial focusing with antibody based magnetic negative depletion of leukocytes [70].

However, although mechanical and electrical property based isolation of CTC has gained its popularity due to the label free process to reduce bias, the understanding

Table 3.3 DEP-based CTC enrichment technology

Technology	Modeled cells	Carrier medium	Clinical validation	Efficiency (*E*) and sensitivity (S)	Purity	Viability	References
DEP based separation	MDA-MB-231	Diluted blood	N/S	N/S	>95 %	>98 %	[59]
Bioelectronic chip	HeLa	Whole blood	N/S	N/S	N/S	N/S	[60]
3D-asymmetric microelectrodes	P19	1×10^7/mL RBC	N/S	N/S	81.5 ± 7.6 %	Y	[62]
MOFF-DEP	MCF-7	Mixture of RBC and WBC	N/S	*E*: 75.81 %	162.4-fold enrichment	N/S	[66]
ApoStream™	SKOV-3, MDA-MB-231	12×10^6 PBMC	N/S	*E*: ~70–75 %	99.33 ± 0.56 % PBMC reduction	97.6 %	[67]

of the mechanical and electrical property of CTC is still largely based on cultured cell lines and more studies need to be conducted on clinical samples to help us to understand these features better. For example, a study conducted in castration-resistant prostate cancer by Coumans et al. demonstrated that, CTC isolated by CellSearch, whether their Cytokeratin area is larger than $4 \times 4\ \mu m^2$ or not, is predictive of survival [71]. This contradicts the standard size cutoff adopted in size based CTC isolation. Hence, more studies need to be conducted in order to define clinically relevant CTC better and to develop better technologies to isolate CTCs in a more efficient and more comprehensive manner with less contamination with undesired cell population.

As mentioned above, Herbert Pohl first described the unique DEP signature of tumor cell in 1950, and after years of improvement, the first commercial system based on this principle was finally developed in 2012. Similarly, other technologies are also being applied on CTCs to acquire a unique signature of CTC as compared with other cells. Some of these studies aim to develop a higher throughput/more efficient isolation technology, some aim to interrogate CTC to provide more information for disease management.

One example of this is interrogation of CTC by atomic force microscopy (AFM). Chen et al. used AFM to measure isolated CTC from prostate cancer patients, and demonstrated that CTCs exhibited mechanical phenotype resembling highly metastatic cancer cells in culture [72]. Additionally, Electrical impedance spectroscopy (EIS) was also applied to analyze cancer cells. Han et al. reported that, by using EIS, the membrane capacitances and resistance of cells can be measured, and the readout is distinct in MCF-10A, MCF-7 and MDA-MB-231 cells [73]. These technologies, due to their low throughput, are still limited at the level of single cell characterization. Future development is required for these findings to be translated into high-throughput, streamlined CTC isolation/characterization platforms.

Another promising area in CTC research is the functional characterization of CTC ex vivo. As demonstrated by Yu et al., ex vivo culture of CTC established from breast cancer patients can be used for drug sensitivity test and could potentially benefit the concept of personalized therapy [74]. This type of assays required CTCs to be captured viably with minimal manipulation, and label free, non-affinity based technologies can potentially benefit this field since several technologies including next-gen microfilters, microfluidic based isolations, and DEP based isolations are compatible with viable CTC capture and the elimination of labeling step can potentially benefit CTC culture due to the less manipulative process and shorter time to perform the capture process.

To conclude, CTC can be isolated from blood due to their distinct mechanical and electrical properties as compared with normal blood components. This will enable label free CTC capture without introducing bias by relying on the expression of certain antigen(s) on CTC. However, future studies will be needed to understand the physical properties of the clinically relevant CTC better. Additionally, future developments will not only focus on high-throughput, high purity CTC isolation/characterization technologies but also focus on viable CTC capture to enable functional characterization of CTC.

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Chapter 4
Molecular Assays for the Detection and Molecular Characterization of CTCs

Evi S. Lianidou, Athina Markou, and Areti Strati

Abstract Detection of Circulating Tumor Cells (CTC) in peripheral blood can serve as a "liquid biopsy" approach and has thus emerged lately as one of the hottest fields in cancer research. A variety of molecular assays are continuously been developed for CTC detection and molecular characterization. Molecular assays are based on the nucleic acid analysis in CTCs like RT-qPCR, multiplex RT-qPCR, methylation specific PCR, ARMS-PCR, and next-generation sequencing technologies. The main strategies are based on total RNA isolation and subsequent mRNA quantification of specific genes, and isolation of genomic DNA for DNA methylation studies and mutation analysis. Molecular characterization of CTC holds considerable promise for the identification of therapeutic targets and resistance mechanisms in CTCs as well as for the stratification of patients and real-time monitoring of systemic therapies. Quality control and standardization of these methodologies is very important for the incorporation of CTCs into prospective clinical trials testing their clinical utility. This review is mainly focused on the basic principles and clinical applications of molecular assays that are currently used for the detection and molecular characterization of CTCs.

Keywords Circulating tumor cells • CTC • Liquid biopsy • Molecular assays • Multiplex RT-PCR • Molecular characterization • Quantitative PCR (qPCR) • Reverse transcription (RT)-qPCR

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4.1 Overview Molecular Assays for the Detection and Molecular Characterization of CTCs

Circulating Tumor Cells (CTCs) are without doubt a very hot topic in cancer research [1] nowadays. Their presence in peripheral blood has been linked with worse prognosis and early relapse in numerous clinical studies and in various types of solid cancers [2]. Almost 10 years ago, FDA has cleared the CellSearch™ system (Veridex) for metastatic breast, colorectal, and prostate cancer [3]. Especially after that time, the critical role that CTCs play in the metastatic spread of carcinomas has been widely recognized, since in several clinical studies CTC detection has shown a correlation with decreased progression-free survival and overall survival in both operable breast cancer and metastatic breast cancer [4]. A meta-analysis of published literature on the prognostic relevance of CTC has clearly indicated that the detection of CTC is a reliable prognostic factor in patients with early-stage and metastatic breast cancer [5].

It is getting clear nowadays that the molecular characterization of CTC, in addition to CTC enumeration, that is based on the well-established FDA cleared CellSearch assay and far beyond their simple detection, may be considered as a "liquid biopsy" approach that may provide real-time information on patient's disease status. In the near future, identification of specific therapy-related molecular targets on CTC such as gene expression, chromosomal translocations, or gene mutations expressed on CTC could offer important information, early on to choose for the correct treatment and moreover explain resistance to established therapies [4, 6, 7]. Moreover, molecular characterization of CTCs has the potential to expand our knowledge of basic molecular pathways of invasion, migration, and immune surveillance and might contribute even to the identification of metastatic cancer stem cells with important implications for the development of improved therapies in the near future [8, 9]. Molecular characterization of CTCs is a very promising and potentially successful approach to identify therapeutically relevant targets expressed on CTC and thus stratify cancer patients for individual therapies [10]. Large scale translational trials, many currently in progress, will provide critical data to progress CTC analysis towards wider clinical use in personalized treatment [11].

Many excellent research groups worldwide are putting a lot of effort towards the development and perfection of novel systems for CTC isolation, detection, and molecular characterization [12, 13] since CTC analysis represents a very promising diagnostic field for cancer patients. We have to keep in mind though, that this task is very challenging and demanding, since CTCs are not only rare but highly heterogeneous as well, even within the same patient [14]. Moreover, an additional analytical and technical challenge is the fact that the amount of available sample is very limited, while the number of interesting targets to be evaluated on CTCs is constantly increasing.

CTC detection and characterization is currently based on highly sensitive CTC detection platforms that include quantitative PCR (qPCR) and reverse transcription (RT)-qPCR and multiplex RT-qPCR based methods, image-based approaches, and

microfilter and microchip devices [13, 15, 16]. There is, however, substantial variability in the rates of positive samples using existing detection techniques mainly due to CTC heterogeneity, and different systems used for CTC isolation, that are actually isolating different CTC populations. Implementation of CTC measurement in clinical routine practice is thus hampered in a severe way. Direct comparison of different methodologies for detecting CTCs in blood samples from patients with breast cancer has revealed a substantial variation in the detection rates [17, 18]. Moreover, many questions still remain unanswered regarding the optimal method to enumerate and characterize CTCs and the path to regulatory and general clinical acceptance of technology platforms currently under development [19].

This review is mainly focused on the main principles and clinical applications of molecular assays that are currently used for the detection and molecular characterization of CTCs. Quality control issues regarding CTC molecular analysis are also discussed.

4.2 Molecular Assays for the Detection and Molecular Characterization of CTCs

Molecular assays for CTC detection and molecular characterization take advantage of the extreme sensitivity and specificity of PCR. They are based on the isolation of total RNA from viable CTCs, and subsequent RT-PCR amplification of tumor-specific or epithelial-specific targets, or isolation of genomic DNA from CTCs and subsequent detection of mutations or DNA methylation in CTCs [15, 20, 21].

The main advantages of molecular assays for CTCs detection and molecular characterization (Table 4.1) are the extremely high sensitivity offered by PCR amplification, and the fact that by careful handling the isolated CTC sample, they

Table 4.1 Major advantages and disadvantages of molecular assays for the detection and molecular characterization of CTCs

Advantages	Disadvantages
• Amenable to automation • Amenable to quality control • Can give information both at the RNA and DNA level • High throughput • Highly sensitive • In silico assay design • Low cost • Multiple targets • Objective measurements • Quantitative • Small sample volume required • RNA can be isolated only from viable CTCs	• Absolute quantification of cell numbers is not feasible • Pre-analytical issues concerning CTC stability during sample shipment and storage • Require immediate handling of samples for CTC isolation and downstream analysis • Require specially designed lab areas to avoid PCR contamination • Lab personnel should be especially trained

can give information both at the RNA and DNA level. Moreover, they can be a priori designed in silico, and take advantage of databases and specific software programs to avoid cross reactions with non-target genes. Molecular assays can be quantitative, high throughput, and easy to perform, while they usually require a very small volume for analysis. Especially when multiplex PCR is used, many targets can be evaluated in the same sample, thus enabling a multiparametric approach in the precious and usually limited CTC sample. In contrast to imaging approaches used for CTC enumeration and molecular characterization, like CellSearch and immunofluorescence [22], measurements obtained by molecular assays are objective, and quantifiable, and are not subjected to personal estimations. It is also important to note that molecular assays are low cost, can be subjected to a quantifiable quality control system. Another major advantage is the fact that these methodologies could be easily automated if based on the existing know-how of fully automated systems for RNA and DNA isolation, and downstream PCR analysis, already used in the routine molecular in vitro diagnostics field, provided that the first crucial step of CTC isolation is also standardized.

The main disadvantages of molecular assays for CTCs detection and molecular characterization (Table 4.1) concern pre-analytical issues concerning CTC stability during sample shipment and storage, a problem that has already being solved in the case of CellSearch. Molecular assays currently require immediate handling of blood

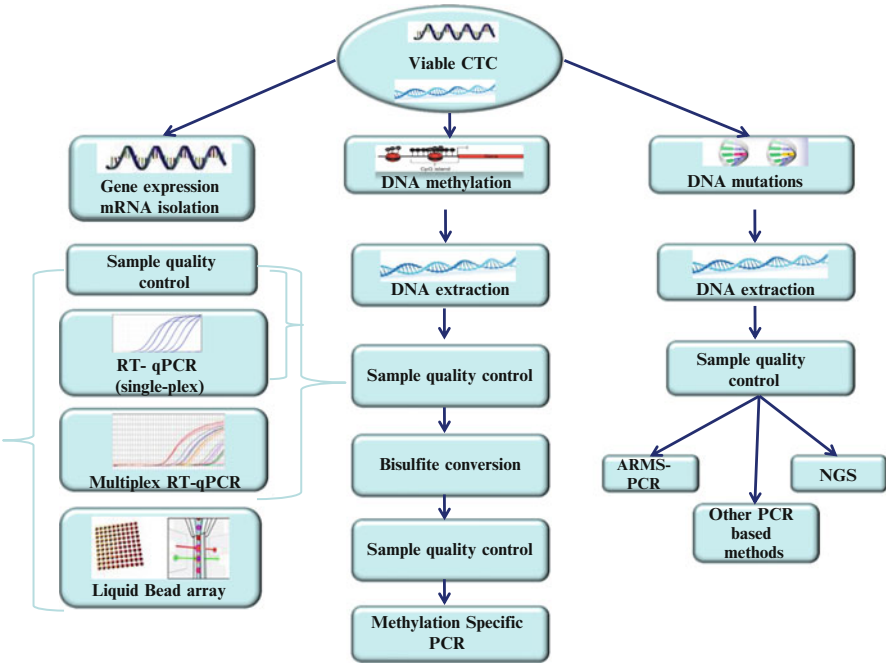

Fig. 4.1 Overview of molecular methods developed and used for the detection and molecular characterization of CTCs

samples for CTC isolation and downstream analysis, a fact that hinders at the moment long-distance shipment of samples to certified centers of analysis. Another major issue is that this type of analysis requires specially designed lab areas to avoid PCR contamination, and different areas for (a) RNA/DNA isolation, (b) setting up the PCR reactions, and (c) amplification as well as different storage areas for pre-PCR and post-PCR reagents. A major issue is also the fact that through molecular assays, we can only get information on the total number of target transcripts in our sample, not knowing whether these targets are co-expressed in the same cell, or derive from different cell populations. By using PCR to perform high dimensional single CTC profiling, Powell et al. have found a high heterogeneity of CTC even among the same individuals when they directly measured high dimensional gene expression in individual CTC without the common practice of pooling such cells [14]. Since CTC are highly heterogeneous, even in the same patient, and different CTC may express the same target at a different level, absolute quantification of CTC numbers is not feasible with molecular assays, unless analysis is performed at the single cell level.

An overview of the molecular assays that are currently being used for CTC detection and molecular characterization is presented in Fig. 4.1.

4.2.1 RT-qPCR

Development of real-time PCR technology had an enormous impact on cancer diagnostics, since it can provide significant and quantitative information on gene expression in an automated, rapid, versatile and cost-effective way [23]. Especially quantitative reverse transcription PCR (RT-qPCR) has been applied for risk assessment of cancer recurrence, and has been widely used for the detection of CTCs in a variety of cancers up to now.

RT-qPCR is a sensitive and specific homogeneous assay that can detect and measure minute amounts of nucleic acids in a wide range of samples. The main advantage of RT-qPCR is that quantitation is based on the exponential phase of the PCR instead of using the endpoint accumulation of PCR product at the end of the stationary phase of the PCR. Moreover, both amplification and analysis steps are automated, and there is no need for slab gels, and complicated sample manipulation after PCR. Moreover, the analytical performance of these assays can be validated in detail through a series of standard experiments. Although RT-qPCR is potentially sensitive and specific enough to detect one cancer cell in the presence of more than 10^6 leukocytes, this requires the use of appropriate mRNA markers for CTC.

Cytokeratin 19 (CK-19) is stably and abundantly expressed in epithelial tumors, but not in mesenchymal hematopoietic cells and has been successfully used as a marker for the detection of tumor cells in the bone marrow, lymph nodes, and peripheral blood by RT-PCR. Almost 10 years ago we have reported the development and analytical validation of a real-time quantitative RT-PCR methodology for CK19-mRNA using the LightCycler™ (Roche) system [24]. By carefully selecting primer sequences, so as to avoid amplification of known pseudogenes for CK-19, as

well as by discarding the first milliliters of blood after venipuncture, to avoid contamination by skin epithelial cells, false positive results in CK-19 mRNA determination can be avoided [25].

4.2.2 Multiplex RT-PCR

Many research groups have described the usefulness of using multiple markers to characterize CTCs using RT-qPCR in the blood of cancer patients [26–28]. However, molecular characterization of CTCs has been hindered by the very limited amount of available sample. Towards this direction, multiplex RT-qPCR assays for CTC detection and characterization offer a unique advantage since they can detect many gene targets at a time, using a very small amount of precious nuclei acid sample isolated from CTC [7, 26, 29]. Several mRNA markers may be useful for RT-PCR-based detection of CTCs. Quantification of these mRNAs is essential to distinguish normal expression in blood from that due to the presence of CTCs. Few markers provide adequate sensitivity individually, but combinations of markers may produce better sensitivity for CTC detection. The AdnaTest BreastCancer detect is a commercially available kit, where isolated mRNA from CTC is transcribed into cDNA and can be amplified in a following multiplex-PCR for the transcripts of EpCAM, MUC-1, and HER-2. Sieuwerts et al. established a robust method to perform mRNA expression analysis of multiple genes by a real-time reverse transcriptase (RT)-PCR on small numbers of CTCs enriched from whole blood by the CellSearch system. This method allows molecular characterization specific for as little as one CTC, and can be used to expand the understanding of the biology of metastasis and, potentially, to improve patient management [30].

4.2.3 Liquid Bead Array

A multiplexed PCR-coupled liquid bead array was designed to detect the expression of multiple genes in CTCs. This assay is based on isolation of mRNA from immunomagnetically enriched CTCs and multiplex PCR for CK-19, HER2, mammaglobin A, MAGEA3, TWIST-1, and PBGD. Biotinylated amplicons were hybridized against fluorescent microspheres carrying gene-specific capture probes and incubated with streptavidin-phycoerythrin and were then quantified by Luminex flow cytometry. Using this assay, the expression of six genes in CTCs can be measured simultaneously and reliably, thereby saving precious sample and reducing the costs and time of analysis. This novel assay forms an efficient basis for a multiplex approach to study the expression of up to 100 genes in CTCs [31].

4.2.4 Methylation Specific PCR (MSP)

Methylation specific PCR (MSP) has been used for the molecular characterization of CTC has been recently explored at the DNA methylation level. Epigenetic silencing of key tumor suppressors and metastasis suppressors known to affect hallmark properties of tumor cells, including growth and proliferation, invasiveness, epithelial phenotype, and stemness like *CST6*, *BRMS1*, and *SOX17* was detected in CTC by MSP [32]. Interestingly, highly methylated gene promoter sequences were found in the CTCs fraction of EpCAM positive/*CK-19* negative patients. This suggests that multi-parametric evaluation of CTCs is of critical importance. Breast Cancer Metastasis Suppressor-1 (*BRMS1*) was highly methylated and down regulated in CTC [33]. *SOX17* promoter was also found to be highly methylated in primary breast tumors, in CTCs isolated both from early and metastasis verified breast cancer patients, and in corresponding cell free DNA (fDNA) samples [34]. A key note of these findings is that it has been shown for the first time that *SOX17* promoter methylation in CTCs and in matched cfDNA is highly correlated. This finding shows towards a direct connection between the presence of CTCs and cfDNA in operable breast cancer patients, after surgical removal of the primary tumor.

4.2.5 Mutation Analysis on CTC

Detection of mutations in CTC is very difficult and challenging, since there are only a few circulating cancer cells isolated from patients and in most cases the cells carrying the mutations consist a minority in the cancer cell population. So far, the number of studies addressing this topic is very limited and moreover, even in these limited number of studies a relatively low percentage for DNA mutations is reported in a very small number of clinical samples. Mutations in known driver genes, e.g., BRAF, KRAS, PIK3CA, or EGFR, found in the primary tumor and metastasis were also detected in corresponding CTCs. Molecular assays are extremely powerful in this area, since they are far more sensitive than the conventional Sanger sequencing approach.

4.3 CTC Analysis at the Single-Cell Level

Next-generation sequencing (NGS) technologies are extremely powerful and in combination with reliable single CTC isolation offer a new dimension in the area of CTC molecular characterization. Using the DEPArray system, that allows isolation of single CTC, Peeters and colleagues have shown that they could obtain reliable gene expression profiles from single cells and groups of up to ten cells [35]. Very recently, Heitzer et al. performed the first comprehensive genomic profiling of CTC

in patients with stage IV colorectal carcinoma using array-CGH and NGS. Mutations in known driver genes found in the primary tumor and metastasis were also detected in corresponding CTC. Mutations that were initially exclusively present in CTC were found by additional deep sequencing to be present at sub-clonal level in the primary tumors and metastases from the same patient [36].

4.4 Clinical Significance of CTC Detection Using Molecular Assays

4.4.1 Breast Cancer

According to a recent meta-analysis of published literature that was performed to assess whether the detection of CTCs in patients diagnosed with primary breast cancer can be used as a prognostic factor, detection of CTCs in the PB indicates poor prognosis in patients with primary breast cancer [37]. The numerous ongoing trials that evaluate CTCs as markers for early prediction of treatment efficacy have been recently reviewed [11] (Table 4.2).

4.4.2 Early Breast Cancer

By using an RT-qPCR method for CK-19, our group has shown already many years ago that the detection of CK-19 mRNA positive cells in the peripheral blood of patients with operable breast cancer before, during, and after adjuvant treatment is an independent prognostic factor associated with an increased risk of disease relapse and shorter survival [27, 38–42]. The same research group has also detected CK-19 mRNA-positive CTCs in patients with early breast cancer. Expression analysis of CTC can also be an efficacy indicator of treatment. Using a real-time RT-PCR assay it was found that taxane-based chemotherapy resulted in a higher incidence of CK-19 mRNA-positive CTCs elimination, lower disease-free survival (DFS), and low incidence of deaths than taxane-free regimens [39]. Ignatiadis et al. reported a different prognostic value of cytokeratin-19 mRNA positive circulating tumor cells according to estrogen receptor and HER2 status in early-stage breast cancer [43]. By using a multimarker reverse transcription-PCR assay for cytokeratin 19, mammaglobin A, and HER2, the prognostic significance of CTC in early breast cancer was evaluated [44].

Benoy and colleagues compared the prognostic value of DTC and CTC in early breast cancer and came into the conclusion that only the presence of DTC was highly predictive for OS [45]. Persistent detection of CTC during the first five years of follow-up was associated with an increased risk of late disease relapse and death in patients with operable breast cancer and indicates the presence of chemotherapy- and hormonotherapy-resistant residual disease [46].

Table 4.2 CTC detection:clinical significance of molecular assays in various types of cancer

Cancer type	Molecular markers	Molecular assay	Clinical significance	References
Early breast cancer	CK-19	RT-qPCR	OS	[42]
	CK-19	RT-qPCR	DFS, OS	[27, 38–40, 43, 46]
	CK19, hMAM, and CEA	RT-PCR	DFS	[47]
Metastatic breast cancer	CK-19	RT-qPCR	DFS, OS	[41]
	CK19 and MGB1	RT-PCR	OS	[50]
	EpCAM, CK19, and hMAM	RT-PCR	DFS, OS	[51]
Dukes' stage B and C CRC	CEA/CK/CD133	RT-qPCR	DFS, OS	[54]
Early colorectal cancer	CEA/CK/CD133	RT-qPCR	DFS, OS	[55]
Colorectal cancer with liver metastasis	CD133, survivin	RT-qPCR	OS	[56]
Early prostate cancer	PSA, PSMA	RT-qPCR	DFS	[57]
Operable NSCLC	EpCAM/MUC1	RT-PCR	OS, DFS	[58]
	CK19, TTF-1	Nested real-time RT-PCR	DFS	[60]
	LUNX	RT-qPCR	OS, DFS	[61]
Advanced lung adenocarcinoma	Surviving, hTERT, CK-7, TTF-1	RT-qPCR	DFS	[59]
Early pancreatic cancer	CK19, MUC1, EPCAM, CEACAM5, and BIRC5	RT-qPCR	DFS	[63]
Stage III melanoma	MART-1, MAGE-A3, and GalNAc-T	Multimarker RT-qPCR	DFS	[65]
Stage IV melanoma	MART-1, MAGE-A3, and PAX3	RT-qPCR	DFS	[66]
	MART-1, GalNAc-T, PAX-3, MAGE-A3, and Mitf	RT-qPCR	OS	[68]
Metastatic uveal melanoma	Tyr and MelanA/MART1	RT-qPCR	OS, DFS	[67]
Ovarian cancer	EpCAM, MUC-1, and HER-2, CA 125	AdnaTest BreastCancer (RT-PCR)	OS	[69]
	PPIC, EpCAM	RT-qPCR	OS, DFS	[70]

Chen et al. investigated the diagnostic, predictive, and prognostic value of the detection of CTCs using a three-marker (CK19, hMAM, and CEA) RT-PCR assay in patients with early breast cancer. The detection rate of three-marker-positive CTCs in the blood of patients with early breast cancer was 54.0 %, significantly higher than in patients with benign breast disease and healthy blood donors. After 3 years of follow-up, detection of three-marker-positive CTCs was significantly associated with locoregional recurrence and/or distant metastasis. Detection of three-marker-positive CTCs in peripheral blood was an independent risk factor for reduced median relapse-free interval. The three-marker RT-PCR assay enhanced the sensitivity and specificity of CTC detection compared to each single marker assay [47].

In a prospective study, Chong et al., by using a multimarker real-time quantitative PCR platform, detected CTC in peripheral blood in 56 % (53 out of 94) of patients with operable breast cancer. The specificity was 95 %. Seventy-two patients who received systemic adjuvant chemotherapy were followed up. According to the results of this study, systemic adjuvant chemotherapy had a significant impact on CTC status, and this effect could be observed after three cycles of chemotherapy. Circulating tumor cells detection had the potential to be used to evaluate the efficacy of systemic adjuvant chemotherapy immediately after the chemotherapy was finished in operable breast cancer patients [48].

Obermayer et al. aimed to identify new gene markers for the PCR-based detection of CTC in female cancer patients. To achieve this goal they performed RT-qPCR for 380 gene targets using the AB TaqMan® Low Density Arrays. Then, 93 gene targets were analyzed using the same RT-qPCR platform in tumor tissues of 126 patients with primary breast, ovarian, or endometrial cancer and in blood samples from 26 healthy women and from 125 patients (primary breast, ovarian, cervical, or endometrial cancer, and advanced breast cancer). According to their results six genes were overexpressed in blood samples from 81 % of patients with advanced and 29 % of patients with primary breast cancer. EpCAM gene expression was detected in 19 % and 5 % of patients, respectively, whereas hMAM gene expression was observed in the advanced group (39 %) only [49].

4.4.3 Metastatic Breast Cancer (MBC)

Androulakis et al. detected CK-19mRNA-positive CTCs before the initiation of front-line treatment in 298 patients with metastatic breast cancer (MBC) using real-time PCR. The median PFS and the OS were significantly shorter in patients with detectable CK-19mRNA-positive CTCs compared with patients without detectable CTCs [41].

Reinholz et al. investigated the associations between baseline and posttreatment CTC gene expression and outcome of patients enrolled in four North Central Cancer Treatment Group metastatic breast cancer (MBC) trials in which specimens were shipped (at 4 °C) from community-based sites to a reference laboratory. According to the results of this study, CTC gene expression analysis conducted by a reference

laboratory is feasible when blood is collected from treating sites and processed 24–30 h postcollection. The presence of baseline CK19+mRNA CTCs was associated with poor prognosis, while a decrease in mammaglobin positive mRNA CTCs may help predict response to therapy of MBC patients [50].

The prognostic value of CTCs in 98 patients with MBC and 60 controls were evaluated by RT-PCR, by detecting the presence of EpCAM, CK19, and mammaglobin (hMAM). Triple-marker-positive CTCs were detected in 86 of 98 (87.8 %) patients with a significantly higher rate than the control group. Compared to single-marker detection, the triple combined marker detection exhibited significantly higher rate. Furthermore, the specificity of triple combined markers of serial test was 100 %. The expression of three genes was significantly correlated with lymph node metastasis, high histological grade, and high levels of serum CA153 and CEA. After 2 years of follow-up, the presence of CTCs with triple-marker positive in peripheral blood was an independent risk factor for reduced progression-free survival (PFS) and overall survival (OS), and the presence of CTCs before any chemotherapy predicts poor OS and PFS in patients with MBC [51].

Enumeration and molecular characterization of circulating tumor cells (CTC) may additionally help towards increasing the success rate during the clinical development of cancer drugs. According to recent results presented by Bao et al., CTC enumeration of patients in an all-comer study is feasible and may allow for patient stratification for PFS and OS to evaluate the clinical response of investigational agents. Gene expression profiling of isolated CTC by RT-qPCR may provide a means for molecular characterization of selected tumor targets [52].

4.4.4 Colorectal Cancer

The prognostic and predictive values of CTC analysis was evaluated in 60 colorectal cancer patients before systemic therapy—from which 33 patients were also evaluable for CTC analysis during the first 3 months of treatment. In this study, CTC were isolated through immunomagnetic enrichment, using the antibodies BM7 and VU1D9 (targeting mucin 1 and EpCAM, respectively), followed by real-time RT-PCR analysis of the tumor-associated genes CK19, MUC1, EPCAM, CEACAM5, and BIRC5. Results from this study suggest that in addition to the current prognostic factors, CTC analysis represent a potential complementary tool for prediction of colorectal cancer patients' outcome [53].

Shimada et al. investigated the prognostic value of CTC/CSC that express CEA CK19, CK20, and/or CD133 (CEA/CK/CD133) mRNA in the tumor drainage blood of CRC patients with Dukes' stage B and C by using RT-qPCR. Their results suggest that detecting CEA/CK/CD133 mRNA in tumor drainage blood by the real-time RT-PCR method would have a prognostic value in CRC patients with Dukes' stage B and C [54].

A novel marker PLS3 for CTC has recently been discovered which is expressed in metastatic CRC cells but not in normal circulation. PLS3-positive CTC are

independently associated with prognosis and it is particularly strong in patients with Dukes B and Dukes C [28]. Similar results have been obtained from Iinuma H et al., who studied the mRNA expression of antigen (CEA), cytokeratin (CK) 19, CK20, and/or CD133 (CEA/CK/CD133) in CTC in 735 patients with CRC. OS and DFS of patients with Dukes' stage B or C cancer who were positive for CEA/CK/CD133 were significantly worse than those of patients who were negative for these markers [55].

Pilati et al. tested whether the detection of CTC might identify CRC patients at high risk of dying of disease recurrence after apparently radical liver surgery. By studying the expression of a panel of cancer-related genes, as assessed by RT-qPCR they report that CD133-positive CTC may represent a suitable prognostic marker to stratify the risk of patients who undergo liver resection for CRC metastasis [56].

4.4.5 Prostate Cancer

CTC analysis is a promising biomarker in prostate cancer as well. Quantitative detection of CTCs in patients' pre- and post-radical prostatectomy (RP) using quantitative TaqMan® fluorogenic RT-PCR for prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA) mRNA was shown to improve the accuracy of the Kattan nomogram to predict the probability of recurrence-free survival (RFS) post-RP [57].

4.4.6 Lung Cancer

Zhu et al. evaluated the presence of EpCAM/MUC1 mRNA-positive CTCs in 74 NSCLC patients and showed that DFS and OS were significantly reduced in patients with EpCAM/MUC1 mRNA-positive CTC preoperation and postoperation [58]. In patients with advanced lung adenocarcinoma the expression of survivin, human telomerase reverse transcriptase (hTERT), cytokeratin-7 (CK-7), and thyroid transcription factor 1 (TTF-1) mRNA expression levels was evaluated. The sensitivity of these four markers combined was 82.3 %, which was significantly higher compared with single marker detection. High expression levels of survivin, hTERT, CK7, and TTF-1 mRNA were positively correlated with distant metastasis and with disease progression [59]. Another recent study suggests that thyroid transcription factor-1 (TTF-1) mRNA-expressing CTCs might be a useful surrogate predictor of disease progression before clinical manifestations are apparent, and that monitoring of TTF-1((+)) CTCs status after surgery may be useful for identifying high-risk patients among surgically resected NSCLC cases [60].

LUNX mRNA expression is strictly limited to normal lung tissue and non-small-cell lung cancer (NSCLC) tissue. A very recent study investigated whether the detection of LUNX mRNA-positive CTCs in peripheral blood at different time points is useful for predicting disease recurrence, DFS, and OS in NSCLC patients

undergoing surgery. Results have shown that detection of LUNX mRNA-positive CTC after surgery and the completion of adjuvant chemotherapy in patients with stage I-IIIA NSCLC are highly predictive for DFS and OS [61]. A multi-marker quantitative real-time PCR of a panel of marker genes (CK7, CK19, human epithelial glycoprotein (EGP), and fibronectin 1 (FN1)) was used to explore CTC detection in advanced NSCLC. ROC curve analysis showed capability of discrimination between advanced NSCLC patients and healthy controls [62].

4.4.7 Pancreatic Cancer

A multimarker RT-PCR assay was used to evaluate CK-19, MUC1, EpCAM, CEACAM5, and BIRC5 expression in CTC of pancreatic cancer patients. CTCs were detected in 47.1 % and patients who had at least one detectable tumor-associated transcript showed shorter median progression-free survival compared with patients who were CTC negative [63]. Sergeant et al. prospectively studied the value of a real-time RT-PCR assay for EpCAM detection in the peripheral blood and peritoneal cavity of patients undergoing pancreatectomy for pancreatic ductal adenocarcinoma (PDAC). Despite a significant increase in EpCAM counts in postoperative blood and peritoneal lavage fluid this was not associated with worse prognosis after pancreatectomy for PDAC [64].

4.4.8 Melanoma

Hoshimoto et al. have recently reported on the utility of multimarker RT-qPCR detection of CTCs in patients with melanoma diagnosed with sentinel lymph node (SLN) metastases in a phase III, international, multicenter clinical trial. Blood was assessed using a verified multimarker RT-qPCR assay (MART-1, MAGE-A3, and GalNAc-T) of melanoma-associated proteins. They came to the conclusion that CTC biomarker status is a prognostic factor for recurrence-free survival, distant metastasis disease-free survival, and MSS after CLND in patients with SLN metastasis. This multimarker RT-qPCR analysis may therefore be useful in discriminating patients who may benefit from aggressive adjuvant therapy or stratifying patients for adjuvant clinical trials [65]. To verify circulating tumor cell (CTC) prognostic utility in stage IV resected melanoma patients in a prospective international phase III clinical trial, the same group has used RT-qPCR to study the expression of MART-1, MAGE-A3, and PAX3 mRNA biomarkers. They report that CTC biomarker(s) ($\geq$1) were detected in 54 % of patients and were significantly associated with disease-free survival and overall survival in resected stage IV melanoma patients [66].

The presence of CTC in patients with metastatic uveal melanoma was evaluated as a marker for systemic disease and to determine their prognostic relevance, by RT-qPCR for tyrosinase and MelanA/MART1. It was found that CTC as evidence for systemic disease can be found in the majority of patients with metastatic uveal

melanoma, including patients with visible disease confined to the liver and that the detection of CTC-specific mRNA transcripts for tyrosinase and MelanA/MART1 by PCR is a poor prognostic factor for progression-free and overall survival [67]. Serial monitoring of CTC with the use of multimarker RT-qPCR assays for five melanoma-associated CTC biomarkers (MART-1, GalNAc-T, PAX-3, MAGE-A3, and Mitf) was shown to be useful for predicting therapeutic efficacy and disease outcome in patients with stage IV melanoma [68].

4.4.9 Ovarian Cancer

By using the AdnaTest BreastCancer based on immunomagnetic enrichment, targeting common antigens on epithelial gynecological cancers, followed by multiplex RT-PCR for EpCAM, MUC-1, and HER-2 transcripts and CA 125 that was assessed in an additional single-plex RT-PCR, Aktas et al. detected CTCs in the blood of 122 ovarian cancer patients at primary diagnosis and/or after platinum-based chemotherapy. According to their results, CTC positivity significantly correlated with shorter OS before surgery and after chemotherapy. This methodological approach might help to identify molecular targets for specific biological therapies [69].

In patients with epithelial ovarian cancer a recent study identified novel markers for CTCs and at evaluated their impact on outcome. Gene expression of cyclophilin C gene (PPIC), and EpCAM was analyzed using RT-qPCR in blood samples taken from healthy females and from 216 epithelial ovarian cancer patients before primary treatment and 6 months after adjuvant chemotherapy. PPIC positive CTCs during follow-up were significantly more often detected in the platinum resistant than in the platinum sensitive patient group, and indicated poor outcome independent from classical prognostic parameters [70].

4.5 Molecular Characterization of CTC Using Molecular Assays

4.5.1 Breast Cancer

4.5.1.1 EMT

Epithelial–mesenchymal transition (EMT) is an essential process in the metastatic cascade [71]. However there are currently very few data demonstrating directly the existence of the EMT process in CTCs [72]. A recent study, using the commercially available ADNAtest assay that is based on multiplex RT-PCR evaluated the expression of EMT markers and ALDH1 in CTC from primary breast cancer patients. This study has shown that a subset of primary breast cancer patients shows EMT and stem cell characteristics and that the currently used detection methods for CTC are not efficient to identify a subtype of CTC which underwent EMT [73].

4.5.1.2 HER2

Anti-HER-2 therapies are prescribed according to HER-2 status of the primary tumor. However, there is a growing body of evidence that the HER-2 status can change over time and especially during disease recurrence or progression in breast cancer patients [20, 22, 29, 74–76]. In this context, reevaluation of HER-2 status by molecular characterization of CTC is a strategy with potential clinical application. An optimal individualized treatment could then be selected by characterizing HER-2 status in CTC and comparing it to the primary tumor. In a recent randomized study the effect of trastuzumab on women with HER-2 negative early breast cancer and detectable CK-19 mRNA positive CTC before and after adjuvant chemotherapy that were randomized to receive either trastuzumab or observation was evaluated. According to this study, the administration of trastuzumab can eliminate chemotherapy-resistant CK-19 positive CTC, reduce the risk of disease recurrence and prolong DFS [10].

4.5.1.3 Estrogen (ER) and Progesterone Receptor (PR) Expression

The expression of predictive markers including the estrogen (ER) and progesterone receptor (PR) expression can change during the course of the disease. Therefore, reassessment of these markers at the time of disease progression might help to optimize treatment decisions. Metastatic tissue may be difficult to obtain for repeated analysis. In this context, characterization of circulating tumor cells (CTCs) could be of relevance. Fehm et al. were the first to demonstrate in more than 400 primary breast cancer patients that the expression profile between CTCs and the primary tumor with regard to ER/PR/HER2 positivity differs. The concordance rate between ER, PR, and HER2 status of CTCs and the primary tumor was 29 %, 25 %, and 53 %, respectively [29]. Based on these results Aktas et al. reevaluated the ER/PR expression by CTCs and compared the hormone receptor status expression profile of CTCs with the primary tumor. In this study all samples underwent immunomagnetic enrichment using the AdnaTest BreastCancerSelect (AdnaGen AG, Germany) within 4 h after blood withdrawal followed by RNA isolation and subsequent gene expression analysis by reverse transcription and Multiplex-PCR in separated tumor cells using the AdnaTest BreastCancerDetect. CTCs were analyzed for the three breast cancer-associated markers EpCAM, Muc-1, Her-2, and actin as an internal PCR control. According to their results, most of the CTCs were ER/PR-negative despite the presence of an ER/PR-positive primary tumor [77].

4.5.1.4 Mutations

Mutation analysis of PIK3CA on CTCs has a potential clinical relevance with respect to drug resistance against HER2-targeted therapy. Recently, by using SNaPshot-methodology comprising PCR amplification and single nucleotide primer extension, 15.9 % of patients with metastatic breast cancer were found to host 12 PIK3CA hotspot mutations in either exon 9/E545K (6/12, 50 %) or exon 20/H1047R (6/12, 50 %) [78].

4.5.2 Colorectal Cancer

4.5.2.1 EMT

Yokobori et al. recently discovered Plastin-3 (PLS3) as a novel CTC marker for metastatic CRC cells that possesses significant prognostic value. They found that PLS-3 was expressed in metastatic CRC cells but not in normal circulation. They report that PLS3 was expressed in EMT-induced CTC in peripheral blood from patients with CRC with distant metastasis. Multivariate analysis showed that PLS3-positive CTC were independently associated with prognosis both in a training set and in a validation set of CRC patients. Moreover, the association between PLS3-positive CTC and prognosis was particularly strong in patients with Dukes B and Dukes C [28]. In clinical CRC cases, high expression of PLS3 in CTCs of tumor drainage venous blood (TDB) as well as peripheral blood was established as an independent prognostic factor of OS, and the copy number gain of Xq23, which is the locus of the PLS3 gene, was significantly related to PLS3 overexpression. PLS3 induced the EMT via transforming growth factor (TGF)-β signaling and resulted in the acquisition of invasive ability in CRC cells [79].

4.5.2.2 Mutations

It is now known that colorectal cancer patients with KRAS and BRAF mutations do not respond to anti-EGFR therapy. In metastatic colorectal cancer after isolating single CTC with the DEPArray, KRAS mutations were detected and there was a mutational concordance between CTCs and primary tumor in 50 % of matched cases [80]. When Heitzer et al. checked for APC, KRAS, and PIK3CA mutations in CTCs isolated from six patients they found that most mutations initially found only in CTCs were also present at subclonal levels in the primary tumors and metastases from the same patient [36]. Mostert et al. detected KRAS and BRAF mutations in CTCs using lower denaturation temperature-PCR (Transgenomic™), real-time PCR (EntroGen™), and nested Allele-Specific Blocker (ASB-) PCR [16].

4.5.3 Prostate Cancer

Molecular determinants can be identified and characterized in CTCs of prostate cancer patients as potential predictive biomarkers of tumor sensitivity to a therapeutic modality [81].

4.5.3.1 TMPRSS2-ERG Status

Abiraterone acetate (AA) is an androgen biosynthesis inhibitor shown to prolong life in patients with castration-resistant prostate cancer (CRPC) already treated with chemotherapy. AA treatment results in dramatic declines in prostate-specific antigen

(PSA) in some patients and no declines in others, suggesting the presence of molecular determinants of sensitivity in tumors. Danila et al. studied the role of transmembrane protease, serine 2 (TMPRSS2)-v-ets erythroblastosis virus E26 oncogene homolog (ERG) fusion, an androgen-dependent growth factor, in circulating tumor cells (CTCs) as a biomarker of sensitivity to AA by a sensitive, analytically valid reverse transcription polymerase chain reaction assay in CTCs enriched from ethylenediaminetetraacetic acid anticoagulated blood obtained prior to AA treatment. According to their results, TMPRSS2-ERG status did not predict a decline in PSA or other clinical outcomes. This finding demonstrates the role of CTCs as surrogate tissue that can be obtained in a routine practice setting [82]. Hormone-driven expression of the ERG oncogene after fusion with TMPRSS2 occurs in 30–70 % of therapy-naive prostate cancers. Its relevance in castration-resistant prostate cancer (CRPC) remains controversial as ERG is not expressed in some TMPRSS2-ERG androgen-independent xenograft models. Attard et al. have also used quantitative reverse transcription-PCR to show that ERG expression was maintained in CRPC and came to the conclusion that there is a significant association between ERG rearrangements in therapy-naive tumors, CRPCs, and CTCs and magnitude of prostate-specific antigen decline in CRPC patients treated with abiraterone acetate [83].

4.5.3.2 Mutations

Coding mutations in the androgen receptor (AR) represent a possible mechanism underlying the development of castration-resistant prostate cancer (CRPC). Jiang et al. detected AR mutations in CRPC patients by using PCR amplification for AR gene, and Transgenomic's WAVE denaturing HPLC technology followed by direct sequencing. The relative abundance of the mutants in the amplified products ranged from 5 to 50 % [84].

4.5.4 Lung Cancer

4.5.4.1 Mutations

Firstly, the group of Haber showed that lung cancer patients whose CTCs carried EGFR mutation known to cause drug resistance had faster disease progression than CTCs who lacked the mutation [85]. In late stage lung cancer patients EGFR mutations have been detected in single tumor cells enriched from blood using laser cell microdissection to isolate individual CTCs followed by whole-genome amplification of DNA and finally PCR sequencing [86]. In patients with advanced non-small-cell lung cancer (NSCLC) where mutational analysis with a six-gene mutation panel (EGFR, KRAS, BRAF, NRAS, AKT1, and PIK3CA) was performed, only one EGFR mutation (exon 19 deletion) was detected in CTC-derived DNA from 38 patient samples [87].

4.5.5 Melanoma

4.5.5.1 Mutations

In melanoma, a peptide nucleic acid-clamping PCR assay was used for BRAF mutation analysis after immunomagnetic enrichment by the group of Dr. Hoon. Mutated BRAF was detected in 81 % (17/21) of stage IV melanoma patients [88]. When single CTC isolated from patients with melanoma were subjected to BRAF and KIT mutational analysis, the BRAF sequences and KIT sequences identified in the CTC were inconsistent with those identified in autologous melanoma tumors, showing clonal heterogeneity [89].

A selection of information on the molecular characterization of CTCs using molecular assays is presented in Fig. 4.2.

4.6 Molecular Assays for CTC Analysis Quality Control Issues

Comparison studies between different analytical methodologies for CTC enumeration and characterization by using the same samples and quality control are very important issues since standardization of assays is essential before their

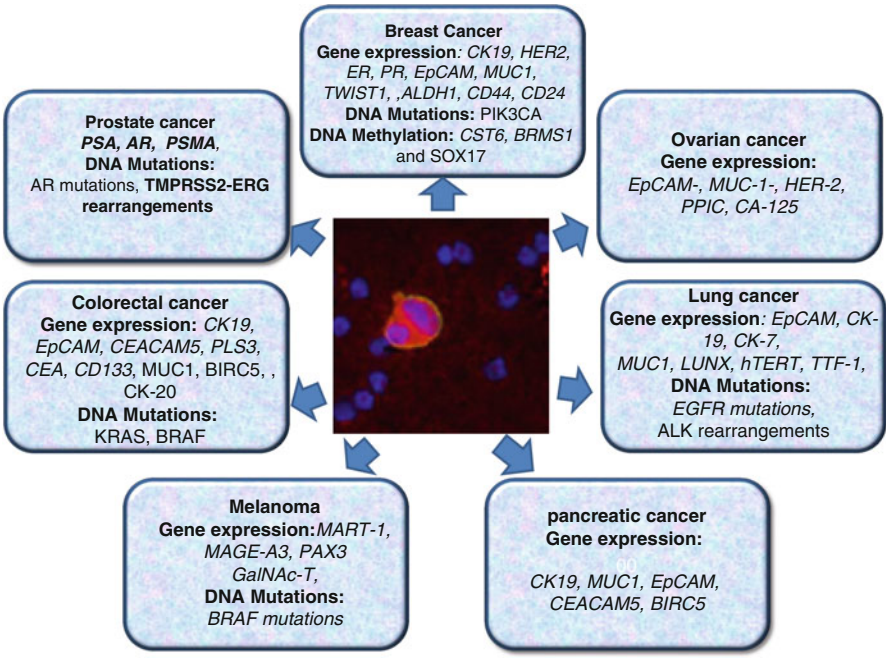

Fig. 4.2 A selection of information on the molecular characterization of CTCs using molecular assays

use in clinical practice. Following the path to regulatory and general clinical acceptance for technologies currently under development, and standardization of CTC detection and characterization methodologies are important for the incorporation of CTC into prospective clinical trials testing their clinical utility [19].

Critical issues concerning the standardized detection of CTC include (a) the standardization of the pre-analytical phase such as sampling itself (e.g., sample volume, avoidance of epidermal epithelial cells co-sampling in case that epithelial markers such as CK-19 will be later used for CTC detection), sample shipping (stability of CTC under different conditions) and storage conditions (use of preservatives, or anticoagulants), (b) standardization of CTC isolation through use of spiking controls in peripheral blood, (c) standardization of detection systems, and (d) inter-laboratory and intra-laboratory comparison studies for the same samples. The development of international standards for CTC enumeration and characterization is also very important especially in imaging detection systems that are observer-dependent [15, 19].

Concerning molecular assays, analytical validation is a crucial step prior to their application in precious CTC samples and is of vital importance. During the last few years, qPCR and RT-qPCR have become accessible to the majority of research labs, however, the in-house experimental design and validation processes applied to the related projects has resulted in wide variability in the quality, reproducibility, and interpretability of published data as a direct result of how each lab has designed their RT-qPCR experiments [90]. The "minimum information for the publication of quantitative real-time PCR experiments" (MIQE) was published to provide the scientific community with a consistent workflow and key considerations to perform qPCR experiments [91]. Taylor et al. have recently highlighted the serious negative ramifications for data quality when the MIQE guidelines are not applied and include a summary of good and poor practices for RT-qPCR [90]. Two surveys of over 1700 publications whose authors use quantitative real-time PCR (qPCR) reveal a lack of transparent and comprehensive reporting of essential technical information. Reporting standards are significantly improved in publications that cite the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines, although such publications are still vastly outnumbered by those that do not [92].

The following aspects are general and can be applied to any type of molecular assay, more specifically

4.6.1 Selection of Detection System in Real-Time PCR

A major key issue in designing novel real-time PCR assays for studying gene expression, in CTC, is to try avoiding SYBR green as a fluorescent dye, unless these are commercially available and very well tested. This dye is very cheap, and for this reason widely used in real-time PCR. However, SYBR green is not DNA sequence

specific, but is giving positive fluorescent signals generally, just in the presence of every double stranded DNA. Because of this, when using this reporter dye, nonspecific PCR products, or primer dimers are also detected and cause false positive signals. The only way to check this is to include a melting step at the end of PCR, to verify that the melting temperature of the PCR product that can be a priori, in silico estimated is the one expected. Hydrolysis (Taqman) and hybridization probes are sequence specific and should be preferred, even if they are far more expensive than SYBR green [91].

4.6.2 Analytical Specificity

The analytical specificity of the primers that are in silico designed and used (especially for multiplex PCR), as well as of the in silico designed probes (hydrolysis or hybridization) should be evaluated both in the presence and absence of each gene target. First, the analytical specificity should be assessed when only one individual gene target is used as a template. Secondly, analytical specificity should be also assessed in the absence of each individual gene-target. The assay should specifically discriminate the expression for each gene target in both cases.

4.6.3 Limit of Detection

A low detection limit is extremely important for CTC analysis. For this reason, before proceeding to patients' samples, the limit of detection of the developed molecular assays should be estimated by using spiking experiments of known number of cells in a known volume of peripheral blood. In case that the target gene is also expressed in healthy donors, a cutoff threshold value is usually set at three standard deviations from the mean expression level of the healthy controls.

4.6.4 Precision

Intra-assay variance (within-run precision) should be evaluated by analyzing the same sample in the same run, in three parallel determinations, following the entire analytical procedure. Inter-assay variance (between-run precision) should be evaluated by analyzing the same cDNA sample, kept frozen in aliquots at −20 °C, over a period of 1 month on five separate assays performed on 5 different days.

4.6.5 *Quality Control of Sample Integrity*

In every molecular assay, the quality of the sample material should be checked so there will be no false negative results. For this reason, the quality of total RNA isolated from a CTC sample should be checked, so that only non-degraded and not fragmented RNA should be used. Following MIQE guidelines, these pre-PCR evaluations have to be clearly documented in scientific publication to increase experimental transparency. Using the appropriate housekeeping genes and a suitable normalization method could partly reduce the impairing effect of total RNA integrity [93].

4.6.6 *Inter-Laboratory and Intra-Laboratory Comparative Studies for the Same Samples*

Up to now, in CTC analysis, there are a very limited number of inter-laboratory and intra-laboratory comparison studies for the same samples. In a recent study Strati et al. compared three molecular assays for the detection and molecular characterization of CTC after excluding all errors in the pre-analytic variables such as sample isolation, sample volume, logistics, and storage conditions, as well as important analytic variables such as CTCs isolation methodology, RNA isolation, and cDNA preparation steps. In this way, the effect of using different molecular transcripts on CTC detection was evaluated. When the same target was detected in the same cDNAs with the same set of primers and probes there was a very good concordance between singleplex RT-qPCR and multiplex RT-qPCR. When the same target was detected in the same cDNAs with a different set of primers, targeting different regions in the same gene sequence (HER-2), and by different detection systems, results were not statistically correlated. When the presence of CTC positivity was assessed based on completely different transcripts, there were discrepancies when the number of CTC was low, as in early breast cancer. On the contrary, in cases where the number of CTC was higher, as in verified metastasis, these assays gave comparable results even while targeting different transcripts. These data indicate the importance of CTC heterogeneity for their detection by different molecular assays [18].

A comparison study between the CellSearch system (Veridex, LLC) and a transcription-reverse transcription concerted reaction (TRC) method (a PCR-based technique), performed by using a colon cancer cell line and 42 whole-blood samples from patients with advanced or metastatic CRC has shown that the sensitivity and OS conclusions of the TRC method was similar to that of the CellSearch system [94].

The ability of three methods to detect CTCs in the blood of colorectal cancer patients was recently compared, by analyzing different aliquots of the same blood

sample for the presence of CTCs by a multimarker RT-PCR assay, the standardized CellSearch assay and dHPLC-based gene mutation analysis. In the population tested, none of the blood samples analyzed appeared to be positive by all three methods. The samples which were positive for CTCs by the CellSearch assay did not overlap with those that were positive by dHPLC. Interestingly, however, all of these samples were positive when assessed by RT-PCR. Conversely, of the samples that resulted negative by RT-PCR analysis, none appeared to be positive by either of the other methods. These data, therefore, indicate that of the three methods tested, the multimarker RT-PCR assay provides maximal probability of CTC detection [95].

A comparison study between the CellSearch system and a commercially available molecular assay (AdnaTest BreastCancer Select/Detect), evaluated the extent that these assays differ in their ability to detect CTCs in the peripheral blood of MBC patients. According to the reported results, the AdnaTest has equivalent sensitivity to that of the CellSearch system in detecting two or more CTCs. While there is concordance between these two methods, the AdnaTest complements the CellSearch system by improving the overall CTC detection rate and permitting the assessment of genomic markers in CTCs [96]. Another study was designed to directly compare three techniques for detecting CTCs in blood samples taken from 76 patients with metastatic breast cancer and from 20 healthy controls the CellSearch CTC System, the AdnaTest Breast Cancer Select/Detect and a previously developed real-time qRT-PCR assay for the detection of CK-19 and mammaglobin transcripts. According to the results reported in this study, 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 RT-qPCR approach for CK-19 and mammaglobin [17].

A very limited number of studies compared the clinical relevance of results obtained for the detection of CTC with different methods. The results of the DETECT trial, which was designed to directly compare the prognostic value of two commercially available CTC assays in MBC, have shown that the prognostic relevance of CTC detection depends on the test method [97].

4.7 Conclusion

CTC analysis is very challenging and demanding, since CTCs are not only rare but highly heterogeneous as well, even within the same patient. An additional analytical and technical challenge is the fact that the amount of available CTC sample for analysis is very limited, while the number of interesting targets to be evaluated on CTCs is constantly increasing. Molecular assays and especially multiplex RT-PCR offer a unique advantage for the detection and molecular characterization of CTC. The main advantages of molecular assays for CTC detection and molecular characterization are their extremely high sensitivity and the fact that they can be a priori designed in silico, and be quantitative, high throughput, and easy to perform, while they usually require a very small sample quantity for analysis. Analytical

validation of molecular assays before their application in precious CTC samples is of vital importance. Molecular assays are low cost, and can be subjected to a quantifiable quality control system. So far, their clinical applications in many types of cancer have shown that they can give clinically relevant information. In addition they have the potential to identify specific therapy-related molecular targets on CTC such as gene expression, chromosomal translocations, or gene mutations expressed on CTC. This information could be very important, especially in early steps of the disease to choose for the correct treatment and moreover explain resistance to established therapies.

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Chapter 5
Cancer Stem Cells and Circulating Tumor Cells: Molecular Markers, Isolation Techniques, and Clinical Implications

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Abstract There is now a considerable body of evidence that many cancers are hierarchically organized and driven by a cellular component termed "cancer stem cells" (CSCs). These cells have the ability to self-renew and to generate heterogeneous populations that constitute the tumor bulk. Preclinical studies have demonstrated that CSCs mediate tumor metastasis and resistance to chemotherapy and radiation therapy. CSC biomarkers have been identified and both in vitro and mouse models have been developed to facilitate the isolation of these cells as well as the elucidation of CSC regulatory pathways. Agents targeting CSCs have now entered early phase clinical trials. The development of these clinical trials highlights the important need to develop technologies to monitor CSCs in patients. Unlike hematologic malignancies, where tumor specimens are readily obtainable, in solid tumors obtaining serial biopsies to assess CSCs is difficult. Studies suggest that circulating tumor cells (CTCs) contain a highly enriched proportion of CSCs and thus monitoring these cells in blood may provide a *liquid biopsy* for CSC assessment in solid tumors. In parallel with developments of efficient CTC isolation technologies, assays to molecularly characterize these cells at single cell resolution are also being developed. In this chapter we will review the current status of CSC therapeutic technologies as well as microfluidic techniques for isolation and molecular characterization of CTCs in cancer patients. If CSCs are responsible for tumor metastasis, resistance, and recurrence, development of effective CSC therapies has the potential to significantly improve the efficacy of cancer treatments.

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5.1 Overview Cancer Stem Cells

It has been more than a century since the concept of stem cell origin of cancers was first proposed. It was suggested that a population of cells with self-renewal capacity would generate cancers from "embryonic rests" [1]. More recently, considerable evidence has supported the "cancer stem cell hypothesis." This hypothesis posits that cancers arise in self-renewing cell populations. These "cancer stem cells" also retain the ability to differentiate into non-self renewing populations that constitute the tumor bulk. The ability of these cells to self-renew as well as to differentiate parallels the properties of normal tissue stem cells. Preclinical and clinical studies have demonstrated that CSCs mediate tumor invasion and metastasis and by virtue of their resistance to chemotherapy and radiation therapy they may also contribute to treatment resistance. In addition to having important clinical implications, the CSC hypothesis has fundamental implications for understanding carcinogenesis and tumor biology. Although a number of controversies exist, the preponderance of evidence suggests that the majority of human cancers are hierarchically organized and thus follow a cancer stem cell model.

Different markers and assays have been developed to isolate and characterize cancer stem cells. Interestingly, many of these markers including CD44, CD24, CD133, and aldehyde dehydrogenase (ALDH) are also expressed in stem cells in normal adult tissues [2, 3]. CD44 is a specific receptor for hyaluronic acid that belongs to class I transmembrane glycoproteins with multifunctional properties. It is a key molecule that interacts with the extracellular matrix and regulates cell–cell adhesion, proliferation, survival, migration, and differentiation [4]. On the other hand, CD24 is a small protein on the cell surface of cancer cells that functions in cell adhesion and metastasis [5]. CD24 is not expressed in all cancers but has important roles in tumors where it is expressed [6]. Our group demonstrated that in human breast cancer, $CD44^{+}/CD24^{-/low}$ cells isolated from primary human breast cancers display stem cell properties [7]. In contrast, in human breast cancer cells with a $CD44^{+}/CD24^{+}$ phenotype are more differentiated and lack stem cell properties [8]. CD133 is another cell surface protein that has been studied in different cancers as a CSC marker. Expression of this membrane protein defines a subset of cancer cells that exhibit a drug resistant phenotype and enhanced tumor initiating ability in xenotransplantation assays [9]. It has also been reported that in some malignancies $CD44^{+}/CD133^{+}$ best identifies cells with tumor-initiating characteristics [10]. We have also found that in human breast cancer, cells with high ALDH activity displayed tumor initiating capacity and in mice, generated tumors that recapitulate the heterogeneity of the parental tumor [11]. Other investigators have reported that ALDH expressing cells are relatively resistant to chemotherapy and that presence of

residual ALDH positive cells following chemotherapy is associated with high probability of relapse and poor outcome [12].

Although the identification of markers of CSCs and in vitro assays for CSCs have been of great value [13], the definitive assay for CSCs depends on the ability of these cells to initiate tumors in immune suppressed mice [14]. In several tumor types, including human melanoma, the ability to initiate tumors in mice has been shown to be related to the level of immunosuppression in mice in which these cells were introduced [15, 16]. However CSCs also have been demonstrated to be present in tumors generated in transgenic mice when cells from these tumors are transplanted into immune competent syngenic mice. Together these studies point to an important role of the immune system in regulating CSCs [17]. Although these immune competent mice may represent a more physiologically relevant microenvironment, these studies are still open to the criticism that serial transplantation of tumor cells disrupts the microenvironment in which these tumors naturally develop. However, three recent landmark studies addressed these arguments by utilizing lineage tracing to demonstrate in three different tumor types that tumors originate in self-renewing stem cell populations generating tumors containing CSCs [18–20]. The relevance of these finding to human cancers was further demonstrated using NextGen sequencing of CSC populations in human leukemia [21]. Altogether, these studies indicate that the cancer stem cell and clonal evolution models of carcinogenesis are not mutually exclusive and in fact both probably apply to most human tumors. According to this "combined" model, cancers arise in self-renewing cell populations which then can develop further genetic or "epigenetic" changes allowing them to evolve. A fully developed tumor may thus contain multiple clones of CSCs and their progeny. In fact, cancer stem cells may continue to mutate and evolve even after full transformation. Therefore, individual cancers may contain multiple CSC clones.

Local microenvironment or "niches" regulate normal adult stem cells. In a similar manner, CSCs are also regulated by the tumor microenvironment [22, 23]. In fact, analogous to events involved in tissue repair after injury, the tumor microenvironment stimulates the self-renewal of CSCs. In addition, hypoxia and cytokines generated by stromal cells act as microenvironmental factors to regulate both normal and cancer stem cells. Interestingly, it has been shown that cytotoxic chemotherapy and radiation therapy similarly stimulate cancer stem cells by activating cytokine loops [24]. Microenvironmental factors such as BMP signaling in the lung may induce CSC differentiation thus serving to inhibit metastasis. It has been reported that the Coco gene regulates breast cancer stem cells to induce metastatic relapse to the lung via blocking this BMP inhibitory effect [25]. In addition, tumors may overcome metastasis inhibition through the production of TGFβ which in turn increases expression of ANGPTL4, a mediator of breast cancer lung metastasis [26].

The finding that CSCs display resistance to both chemotherapy and radiation therapy has important clinical implications [27, 28]. The observation that these therapies are able to cause tumor regression but that CSCs are increased following treatments such as neoadjuvant breast cancer therapy support this concept [29, 30]. This might also provide an explanation for why in most cancers tumor regression

does not correlate well with patient survival. Furthermore, since tumor regression is a measure of effects of therapy on bulk tumor cells rather than CSCs, other methods to evaluate the effect of therapies on CSCs are urgently needed.

Findings of several studies have suggested similarities between the CSC phenotype and acquisition of an epithelial–mesenchymal transition (EMT) state [31, 32]. It has been demonstrated that conditions which induce EMT in human breast cancers such as hypoxia or TGFβ also increase the proportion of cells expressing the CSC phenotype $CD44^{+}/CD24^{-/low}$ [33]. However, we have recently demonstrated that in human breast cancer, CSCs exist in alternate states which are characterized by different markers and properties. EMT like CSCs which have been characterized as $CD44^{+}/CD24^{-/low}$ are highly invasive but relatively quiescent. In contrast, the more epithelial or "MET" like CSCs, which are characterized by ALDH expression, show more proliferative phenotype with "self-renewal" characteristic. Furthermore CSCs display plasticity, being able to transition between EMT like and MET like states in a process regulated by the tumor microenvironment [34]. As determined by immunohistochemistry, the EMT like $CD44^{+}/CD24^{-/low}$ CSCs are primarily found at the tumor invasive front while MET like $ALDH^{+}$ CSCs are primarily located more centrally. This suggests a model in which EMT CSCs at the tumor invasive front enter the circulation where they metastasize to distant sites. These micrometastases are non-proliferative and remain dormant until they are induced to convert to an MET "self-renewing" state where they generate additional CSCs as well as the more differentiated cells which form the tumor bulk. This model is supported by studies which have demonstrated that both circulating tumor cells (CTCs) as well as disseminated micrometastatic (DTCs) cells in the bone marrow of breast cancer patients are enriched in $CD44^{+}/CD24^{-/low}$ cells which are Ki67 negative (i.e., non-proliferative). In contrast both primary tumors and macrometastases contain both $CD44^{+}/CD24^{-/low}$ and $ALDH^{+}$ CSCs. One of the characteristics of EMT CSCs is their low or absent expression of the epithelial marker EpCAM. Since antibodies to this protein are used to capture CTCs in methods such as the CellSearch™ assay, this technique may miss important populations of CTCs which display an EMT phenotype. This limitation as well as alternative CTC capture technologies is discussed below.

5.2 Circulating Tumor Cells (CTCs)

5.2.1 Importance and Implications

Metastasis, a major cause of cancer related deaths, starts with the dissemination of cancer cells from the primary site to the blood stream and ends with tumor formation in distant organs. Cancer cells that enter blood stream are called circulating tumor cells (CTCs) and are reported to be enriched in CSCs [35, 36]. The majority of the cells that enter the bloodstream from a primary tumor in cancer patients are dead as a consequence of shearing force or anoikis, or are eliminated by the immune

system. Therefore, only small fraction of CTCs survives and extravasates at distant sites. These successfully disseminated tumor cells may grow to form a metastasis, or remain dormant for many years [37]. Although most of the studies in the past focused on identifying CTCs of an epithelial phenotype [38–40], it has been increasingly recognized that CTCs consist of several subpopulations with different characteristics and circulating CSCs and/or cells with the EMT phenotype might be the drivers of metastasis. Overall, the prospect of a noninvasive liquid biopsy that could elucidate metastatic mechanisms makes CTCs an active area of cancer research.

It has been shown in xenograft models that metastasis initiating cells (MICs) within the CTC populations are positive for the breast cancer stem cell marker CD44. These $CD44^+$ MICs also have been reported to express other markers including MET and CD47 [7, 41]. In a cohort study, FACS-profiled samples showed that the frequency of CTCs with a $CD44^+/MET^+/CD47^+$ expression profile increased in parallel with the clinical progression without significant alterations in the bulk CTC numbers [42].

There is accumulating evidence that CTCs may display phenotypes distinct from those in primary tumors. This discordance may reflect tumor evolution as well as differential expression of markers on CSCs and bulk tumor cell populations. A prominent example of the later is HER2 expression in breast cancer. We have previously demonstrated that HER2 is a potent regulator of breast CSCs [43]. More recently we have reported that in luminal breast cancers HER2 may be selectively expressed in CSCs in the absence of HER2 gene amplification [44]. This might account for the surprising finding that clinical benefit of HER2 blockade in the adjuvant setting might extend to woman whose breast tumors do not display HER2 gene amplification. In addition this might account the reports that women with HER2-negative breast cancer may have CTC that express HER2 [45, 46].

The role for EMT in the blood-borne dissemination and disease progression was explored in a serial CTCs monitoring study in human breast cancer. This study showed simultaneous expression of epithelial and mesenchymal markers is rare in primary tumor cells, but only mesenchymal cells were found to be highly enriched in patients' CTCs. They also reported that mesenchymal CTCs were found both as single cells and as multicellular clusters. We also observed single cell and cluster of several cells in isolated CTCs from breast cancer patients in our studies [Unpublished data]. Isolated CTCs showed expression of TGFβ pathway components and the FOXC1 transcription factor [47]. Interestingly, the same study monitoring CTCs revealed a correlation in changes in the CTCs content and type in serial blood samples of one patient with ER^+/PR^+ lobular carcinoma with primary response to experimental regimen followed by resistance, response to a new experimental therapy, and finally disease progression.

An artificial neural network (ANN) analysis has been conducted as a prognostic tool to determine the relationship between CTCs as a continuous variable and overall survival in molecularly defined primary breast tumors. Based on the cutoff value of 5 CTCs per 7.5 ml of blood in metastatic breast cancer (MBC) patients, ANN revealed a linear increase of risk of death in MBC patients with increasing CTC

counts in a panel of molecularly different tumor subtypes. However, in $HER2^+$ patients treated with targeted therapy, this CTCs prognostic effect was less evident. This study may support the concept that the number of CTCs along with the biologic characteristics, should be taken into consideration for data analysis. The highest hazard of death was reported in $ER^+/HER2^-$ and triple negative (TN) MBC patients between the 12th and 18th month. The hazard of death decreased over the time after 18 months. Conversely, the hazard in $ER^+/HER2^+$ and $ER^-/HER2^+$ MBC patients treated with anti-HER2 therapy progressively increased with time. The hazard ratio (HR) for $HER2^+$ patients after anti-HER2 treatment was less than that for $ER^+/HER2^-$ and TN patients treated with chemotherapy [48].

5.2.2 CTC Isolation Techniques

There are several approaches that are widely used for the isolation of circulating tumor cells. The majority of techniques can be classified into two categories: (1) immunoaffinity based isolation and (2) biophysical properties based separation. Both of these approaches have certain advantages and disadvantages that are discussed below briefly along with their relevance and applicability for CTCs/CSCs isolation.

The most widely used approach for CTC isolation is the immunoaffinity capture of CTCs using distinct antibodies that are expressed exclusively on tumor cells and not present on the blood cells. The most commonly used antibody for the isolation of CTCs is against the epithelial cellular adhesion molecule (EpCAM). In these techniques, the antibody may be chemically tethered to a capture surface or magnetic beads, ultimately allowing CTC isolation. The immunoaffinity based approaches result in highly specific CTCs isolation and have demonstrated the prognostic, monitoring, and molecular diagnostic potential of CTCs. Systems making use of immunocapture have evolved from the macroscale operation of the first FDA approved CTC separation technology to a host of microfluidic devices being developed today.

An example of macroscale immunocapture is the CTC detection and enumeration system CellSearch™ (Veridex LLC), which has been approved by the FDA for use in metastatic breast [49], colon [50], and prostate cancer patients [51]. It consists of three components the CellPrep system, the CellSearch Epithelial Cell Kit, and the CellSpotter Analyzer. Sample processing begins with dilution and centrifugation before it is added to the CellPrep system for incubation with an antibody-coated ferrofluid from the CellSearch Epithelial Cell Kit. The antibody in this instance is against the EpCAM, allowing for the separation of epithelial cells from the blood. The interaction of anti-EpCAM on the magnetic beads with the EpCAM expressed by CTCs allows them to be isolated in the presence of magnets. The captured cells are then labeled using the remaining reagents from the CellSearch Epithelial Cell Kit including DAPI, a nuclear stain; fluorescently labeled antibodies against cytokeratins 8, 18, and 19, which are intermediate filaments expressed by epithelial cells; and fluorescently labeled antibodies against CD45, a surface protein

expressed by white blood cells. The cells solution is then transferred to the CellSpotter Analyzer fluorescence microscope using the MagNest Cell Presentation Device, where samples are then scanned and a selection of images are exhibited to an operator for ultimate approval. CTCs are determined to be those cells which have a rounded morphology, are nucleated (DAPI+), have stained positive for the cytokeratins, and have stained negative for CD45. A study of 964 patients with metastatic disease, 199 patients with nonmalignant disease, and 145 healthy volunteers showed ability of the system to detect at least 2 CTCs/7.5 ml blood in 36 % of samples taken from metastatic patients and only 0.3 % of healthy and nonmalignant samples, and confirmed the consistency amongst multiple operators [52].

Although it is sufficiently robust for FDA approval, the CellSearch™ system is limited in its purity, recovery, and sensitivity, leaving room for improvement by other cell capture systems. Given the advantages of minimized footprints, costs, and reagent expenditures coupled with a library of well-documented fabrication methods [53], it is unsurprising that the field of CTC research turned to microfluidics. Microfluidic devices have been applied in biological analysis in the form of "labs-on-a-chip" for the polymerase chain reaction (PCR), molecular separation, and immunosensing [54, 55] through the construction of systems with small scale mixers, pumps, reservoirs, and valves [56].

Microfluidics made its first appearance in CTC isolation in 2007 with the advent of the CTC-chip [40] (Fig. 5.1a). Features of this device include viable cell isolation, allowing for further potential downstream analysis, and the ability to process whole blood. The CTC-chip consists of an array of 78,000 microposts etched into a

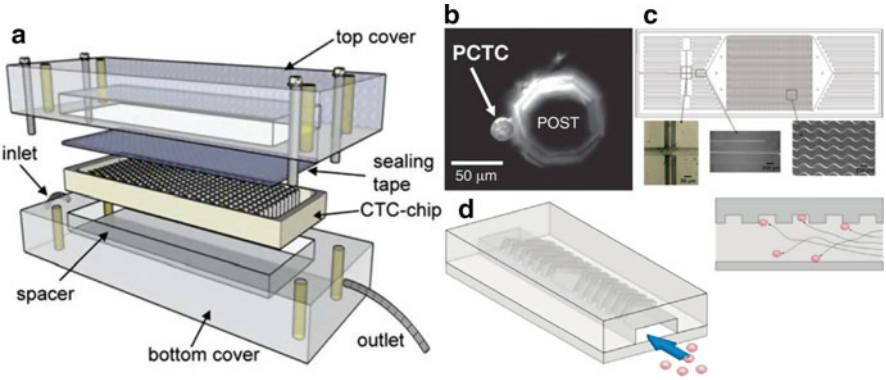

Fig. 5.1 Microfluidic circulating tumor cell (CTC) isolation systems. (**a**) CTC-chip device schematic [40], Copyright 2007 Nature Publishing Group. (**b**) A prostate cancer circulating tumor cell (PCTC) captured on an octagonal post in the geometrically enhanced differential immunocapture (GEDI) chip [57], Copyright 2010 The Royal Society of Chemistry. (**c**) Schematic and optical micrographs of the components of the high throughput microsampling unit (HTMSU) [58], Copyright 2008 American Chemical Society. (**d**) The polydimethylsiloxane (PDMS) ceiling molded in a series of chevron patterns serves as a chaotic micromixer in the Herringbone-chip [59], Copyright 2010 National Academy of Sciences of the United States of America

silicon substrate. Antibodies against EpCAM are then conjugated to the microposts to capture cells expressing that epithelial marker. The clinical utility of this device was demonstrated by processing 116 samples from 68 patients with breast ($n=10$), colon ($n=10$), non-small-cell lung ($n=55$), pancreatic ($n=15$), and prostate ($n=26$) cancers, with CTCs detected in 99 % (115 of 116) of patient samples. The CTC-chip represented a divergence from the previous macroscale separation paradigm, and in the process sparked the development of a multitude of microfluidic devices for use in CTC research.

Microfluidic CTC separation devices that make use of immunocapture have been further engineered with respect to capture antibodies, geometries, and materials. The circular posts of the CTC-chip have been adapted both in cross section and layout in the geometrically enhanced differential immunocapture (GEDI) chip [57] (Fig. 5.1b). The use of the transparent and inexpensive PDMS bonded to a transparent glass slide facilitates imaging, allowing for on-chip fluorescence in situ hybridization (FISH). Cheap and easy to pattern and mold, polymers can also be used as a capture substrate in microfluidic devices. For example, poly (methyl methacrylate) (PMMA) was used in a high throughput microsampling unit (HTMSU) for CTC capture by anti-EpCAM and enumeration by integrated conductivity sensors [58] (Fig. 5.1c). The microposts in this device are then functionalized with tissue-specific antibodies against the prostate specific membrane antigen (PSMA) to capture prostate cancer circulating tumor cells (PCTCs). The "Herringbone-chip" is designed to increase the number of collisions between the antibody-functionalized capture surface and cells expressing the antigen of interest through the inclusion of a chevron-patterned poly dimethylsiloxane (PDMS) ceiling [59] (Fig. 5.1d). Materials optimization can also be used to improve capture and purity. The novel nanomaterial graphene oxide (GO) has been patterned onto a silicon capture surface and conjugated with anti-EpCAM, yielding both high yields and unprecedented purity [60] (Fig. 5.2).

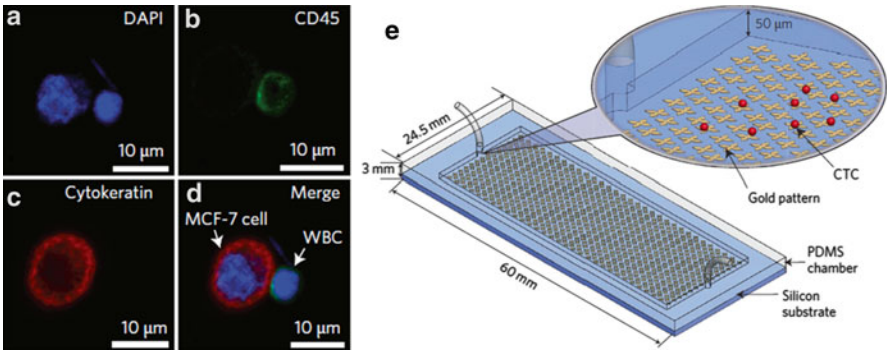

Fig. 5.2 Immunostaining of a captured breast cancer cell and white blood cell on the graphene oxide chip. Captured cells were stained with (**a**) DAPI, a nuclear stain, (**b**) with primary antibody against CD45, a WBC marker, and a *green* fluorescent secondary antibody, and (**c**) cytokeratin, an epithelial intermediate filament, and a *red* fluorescent secondary antibody. (**d**) The merged image of the three fluorescent channels. (**e**) A schematic representative of the graphene oxide chip [60], Copyright 2013 Macmillan Publishers Limited

Immunoaffinity techniques have the advantage of high specificity. However, they presume the phenotype of the cells and can only isolate the cells with the specified antigens. Therefore, these immunoaffinity based techniques may not be appropriate when it comes to the isolation of CSCs from blood, as the knowledge of the specific and unique antigens that can distinguish CSCs from hematopoietic stem cells is limited and still evolving. Hence sorting the cells based on currently available surface markers that are not shared by blood cells may result in only a subpopulation of CSCs that may not be sufficiently comprehensive to give the full insights into full potential of CSCs. Realizing these limitations, researchers have started developing the techniques based on the biophysical properties of the cells, also termed as "label-free" isolation techniques.

With any immunocoupling-based methods, capture is dependent on the expression of the specifically chosen biomarker. This is often at odds with heterogeneous and metaphoric nature of CTCs, particularly given changes associated with expression during the epithelial to mesenchymal transition (EMT). Given these limitations, CTC capture systems have been designed to take advantage of the distinct biophysical properties of CTCs including membrane potential, dielectric properties, the increased size of cancer cells relative to blood cells, and their difference in adhesion preferences.

In an attempt to move away from label based isolation approaches, researchers turned towards size based technologies. Tumor cells derived from solid tumors are of larger size when compared to majority of the blood cells. These differences are smaller yet consistent enough to make for an attractive strategy for separating CTCs from blood cells. Size based filtration techniques are emerging [61, 62] in which the cells are passed through pores etched in membranes, physically retaining larger cells on the top of the membrane. Zheng et al. presented an efficient membrane microfilter device made of parylene-C for the isolation of prostate cancer cells from whole blood [63]. The membrane filter contains 16,000 evenly distributed pores of 10 μm diameter and 20 μm space in between. The membrane was integrated with electrodes for direct electrolysis of the retained cancer cells and then polymerase chain reaction (PCR) was carried out on the cell lysate. In successive approaches, researchers used two-layer membranes to filter viable prostate and breast cancer cells [64]. The captured cells were cultured on device for 2 weeks. Two issues arose with increasing volumes of blood processed the membrane was easily clogged and whole blood needed to be diluted before filtering.

Kuo et al. demonstrated a microfluidic filtration system which can separate breast cancer cells spiked into whole blood with 50–90 % recovery rate [65]. The device consisted of a serpentine channel interconnected with two outer filtrate channels with rectangular apertures. The force experienced by cells during the filtration process was carefully assessed and the dimensions of the apertures were adjusted accordingly to minimize cell damage.

The concerns about these approaches include clogging of pores, high pressure drops in these devices as the cells start collecting on the pores, and CTCs squeezing through the pores due to their viscoelastic nature. Hence, many of these studies resort to pre fixation of the cells, limiting potential downstream analysis. Additional major limitations are low throughput and excessive nonspecific cell retention. Although the processing speed is good compared to immunoaffinity capture based methods, the amount of sample that can be processed without sacrificing the efficiency and purity is still limited.

To address the throughput and the sensitivity limitation of size based filtration techniques, researchers have been developing alternative techniques, again based on the cell size, using inertial microfluidics. The underlying fundamental principle is that cells of different sizes experience distinct magnitude of forces, causing them to focus at different lateral positions in microfluidic channels. Using this differential hydrodynamic focusing, the cells can be isolated.

The small scale nature of microfluidic channels lends itself to use in rare cell separation given that the cells of interest are of a different size from the other contaminating cells. Initially dispersed particles in microchannels have been observed to focus at equilibrium positions as a result of balancing forces. In straight rectangular microchannels, focusing is a two-step process beginning with the equilibration in terms of height due to the balance of shear and wall-induced lift forces followed by equilibration along the channel walls due to the rotation-induced lift force [66] (Fig. 5.3a). To separate particles based on size, curved microchannels can be used to include an additional balancing force the Dean drag force [67] (Fig. 5.3b). This is an inertial force which creates vortices that cause large particles to migrate toward the inner

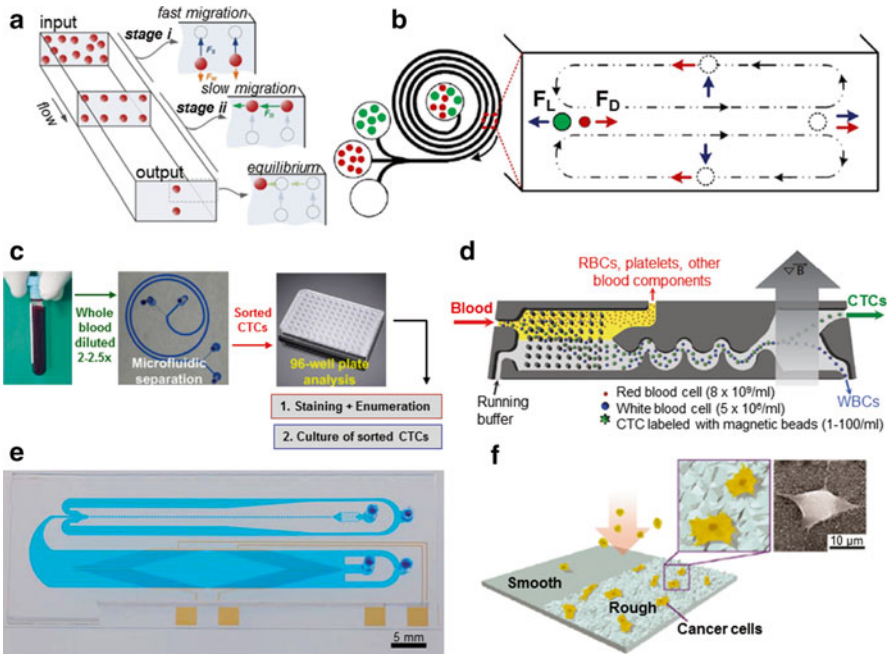

Fig. 5.3 Principles behind and examples of label free cell capture. (**a**) Force balance within a straight microchannel [66], Copyright 2013 The Royal Society of Chemistry. (**b**) Force balance and separation in a spiral microchannel [67], Copyright 2013 AIP Publishing LLC 2013. (**c**) An example of a spiral device used to separate CTCs from diluted whole blood [68]. (**d**) The three functional units of the CTC-iChip [69], Copyright 2013 American Association for the Advancement of Science. (**e**) Integrated multi-orifice flow fractionation (MOFF) and dielectrophoresis (DEP) device [70], Copyright 2011 The Royal Society of Chemistry. (**f**) CTC capture on a nanoroughened surface [72], Copyright 2012 American Chemical Society

wall while the smaller particles localize toward the outer side of the channel. Through use of a branched outlet, these separate particle streams are then collected.

The variation of channel geometric parameters allows for the separation of particles such as cells, and has therefore been used for CTC isolation. A spiral device has been optimized such that a complete Dean cycle allows the sorting of CTCs from diluted blood into bifurcated outlets with the outer outlet containing separated blood cells and the inner outlet containing viable CTCs [68]. These cells could then undergo downstream analysis including enumeration and culture (Fig. 5.3c). Inertial sorting has been coupled with both positive and negative cell sorting in the CTC-iChip [69]. Following the labeling of the sample with magnetic beads conjugated with antibodies against either epithelial cell antigens or blood cell antigens, the sample with a running buffer as it flows through a deterministic lateral displacement chamber where small blood cells are removed through hydrodynamic sorting. The remaining cells then pass through an inertial focusing channel to align the cells for enhanced magnetic sorting in the final chamber. In this last step a magnetic force either isolates the cells of interest (positive sorting) or removes the remaining contaminating cells (negative sorting), based on the antibody conjugated onto the magnetic beads (Fig. 5.3d). In the case of negative sorting, the CTCs are never labeled, allowing the separation of cells from multiple cancers and phenotypes.

These inertial microfluidics based techniques offer high throughput and sensitivity, yet suffer from the lack of sufficient specificity of isolated cells. When these techniques are used as truly label free techniques, they yield a large number nonspecific and non-tumor cells, mostly blood cells. This provides a formidable challenge for downstream assays used to characterize these cells and for meaningful functional assays. Despite these limitations, this class of techniques offers the ideal platform for the label free isolation of CSCs.

Cancer cells can be removed from a mixed cell population using their inherent dielectrophoretic properties by aligning cells of interest using electrodes. Moon et al. combined both hydrodynamic focusing and dielectrophoresis to isolate high purity cancer cells from blood at high flow rates [70] (Fig. 5.3e). Diluted blood was passed through a multi-orifice microchannel to separate blood cells from cancer cells by exploiting the different equilibrium positions of different cell types. Cancer cells, now pooled with fewer blood cells, were then flowed into a nonuniform electric field for further separation. This integrated system recovered 75.81 % of MCF-7 cells that entered the system while removing 99.24 % of red blood cells and 94.23 % of white blood cells. In an another study, single cells from a solution of cancer cells suspended in a buffer of the appropriate conductivity have been viably isolated using a DEP array wherein pre-fluorescently labeled cells are removed from a 320×320 array through the manipulation of DEP forces [71].

Overall, although the DEP based techniques offer specificity, the low throughput and the restrictions on the cell suspension media to maintain conductivity limit their wider applicability. These techniques can be adapted to isolate stem like cells from the normal tumor cells as it is believed that the stem cells have distinct membrane potentials. However, the cells of interest needed to be pre-sorted using another strategy prior to targeted CSC isolation.

There is a class of emerging label free techniques based on the tendency of cancer cells to adhere and spread on rough and textured surfaces. This property is the basis behind a recently published capture technology consisting of a nanoroughened glass slide patterned using reactive ion etching (RIE) [72] (Fig. 5.3f). While this device initially featured conjugated anti-EpCAM, the capture antibody did not significantly increase capture and was therefore no longer used as it is associated with limitations on the types of cancer cells that may be captured. These surfaces yield capture efficiencies up to 95.4 % for cell lines, although issues with purity leave room for optimization.

Yet another strategy that is slowly gaining popularity is negative selection. Here any of the above discussed (mostly immunoaffinity based) techniques is used to separate the unwanted blood cells of the remaining cells contain all CTC subpopulations including CSCs. Chen et al. presented a microfluidic disk to negatively deplete non-tumor cells via immunomagnetic principles to achieve isolation of rare cancer cells [73]. Non-target cells were labeled with magnetic beads and as samples passed through a multistage magnetic field, trapping the labeled cells. Compared to positive immunoaffinity selection, negative depletion accommodates the need to capture CTCs that do not express the typical surface markers, such as cells undergoing EMT [74].

An array of precisely spaced posts traps cells that are not flexible enough to travel through the gaps, yielding a population of cells that were shown to have the tumor initiating capacity expression pattern $CD44^{+}/CD24^{-}/claudin^{low}$ in gene expression array and analysis [75]. Because of the flexibility of blood cells, this technology could be utilized as a secondary enrichment step following CTC separation from the blood using a preexisting method. Similar to the DEP array, the deformability array requires solutions that either pre-labeled or presorted solutions, making it ideal for integration with other techniques described above in order to obtain the rarest desired cells from an already sparse population.

Nanomaterials present a number of advantages such as interaction with cells on a mutual length scale, leading to adhesion and spreading that may facilitate capture and culture. Multiple conjugation chemistries also permit the linking of capture moieties and other entities to the surface, presented at a higher number because of the increased surface area offered by the nanomaterial of choice. In addition to the graphene oxide (GO) chip, this strategy is used by devices constructed with vertically aligned carbon nanotubes (VACNTs) and structures such as nanopillars, nanowires, and nanofibers. VACNT "forests" have been shown to provide the advantages of high surface area antibody presentation combined with manipulated streamlines to increase collisions with the modified surfaces [76]. This approach has been demonstrated for $CD4^{+}$ T cell, *Escherichia coli*, and *Streptococcus pneumonia* isolation [77], and could conceivably be applied to CTC isolation. Similarly, deposited or etched silicon nanopillars and nanowires have been used to display anti-EpCAM for increased rare cell capture. Such nanopillar arrays have shown increased sensitivity relative to the CellSearch™ system CTCs were detected in 20 of 26 patients compared with 8 of 26 using CellSearch™ [78]. Nanowires have been engineered with the added benefit of controlled release using the temperature sensitive polymer poly *N*-isopropylacrylamide (PIPAAm) [79]. This device captured over 70

% of spiked MCF7 cells and released 90 % of them with 90 % viability. Electrospun titanium oxide nanofibers can coat a substrate in a horizontal orientation to exhibit anti-EpCAM while simultaneously providing nanoscale features with which the cells may interact [80]. The efficacy of this substrate was shown by processing colorectal and gastric cancer patient samples. While these techniques all feature the disadvantages inherent in specific antibody capture, the advantages afforded by nanomaterials may be harnessed in the future in the form of the direct interaction between these materials and CTCs.

While a great deal of work has already been done in the field of CTC capture, each type of capture does have drawbacks. Although the relatively gentle capture technique of the CTC-chip improves upon the inability of CellSearch™ to capture viable cells, both methods feature the disadvantages of immunocapture. The antibody–antigen binding interaction is strong and specific, but therefore necessarily limiting. The focus of immunocapture thus far has been epithelial CTCs, captured based on the expression of the epithelial cellular adhesion molecule (EpCAM). However, expression patterns of CTCs are variable both due to tumor heterogeneity and survival tactics such as the EMT. This process may down-regulate epithelial markers [81] such as EpCAM, causing these cells to be missed by traditional immunocapture devices. Solutions to this problem include the use of tissue-specific markers such as the prostate specific membrane antigen (PSMA) as in the GEDI chip; this still restricts the type of cell that may be captured even within a population that may not uniformly express a given marker.

Label free isolation presents an answer to the challenges faced by immunocapture but is not without its own difficulties. Inertial capture is frequently preceded by preprocessing steps such as centrifugation and dilution. In addition to being heterogeneous in surface marker expression, CTCs can vary in size, and the size cutoffs necessitated by outlet design allow the loss of smaller cancer cells, particularly those which overlap in size with white blood cells. Nanoroughened capture surfaces are dependent on neither surface expression nor size, but this capture generality lends itself to increased nonspecific binding. This is problematic for downstream analysis, particularly in the contamination of extracted genetic material.

The future directions of CTC capture will aim to solve these problems, ideally increasing the specificity, yield, and throughput. The approaches will in all likelihood take the direction of integrated modules that allow the advantages of multiple techniques and the use of nanomaterials. Integrated devices such as the CTC-iChip use both inertial and immunomagnetic sorting, making feasible the selection of cells of multiple tissue types, stages, and transitional phenotypes. These devices can integrate preprocessing into the device and increase throughput while simultaneously increasing the populations of cells targeted.

Although two of the inertial separation devices discussed above have been used to segregate cells that have been shown to express CSC markers, the field is still evolving. Cells isolated using the spiral device were shown to contain a subpopulation that stained positively for CD133, a marker associated with high tumorigenicity and stem like characteristics in cells [68]. While these $CD133^{+}$ cells also stained positive for cytokeratin, an epithelial marker, it still stands to reason that since the

isolation itself was not based on the presence of an epithelial marker, this technology could be used to recover and study CSCs with variable membrane expression patterns. The CTC-iChip run for negative selection also presents an opportunity to isolate cells that do not present traditional epithelial markers [69]. In addition to being able to separate cells from non-epithelial cancers such as melanoma, 15 captured cells from a castration resistant prostate cancer patient underwent multiplex qRT-PCR to reveal expression profiles for a panel of prostate specific, epithelial, mesenchymal, stem, proliferation, and white blood cell markers. The CTCs expressing stem markers did overlap with those expressing epithelial markers, and these CTCs were initially identified through staining against EpCAM. Once again, however, because the initial separation did not use epithelial markers, these results present a proof of principle that CSCs may be isolated using this method.

The ultimate goal of CTC separation is to have the maximum clinical utility. As such, it is important that isolation technologies obtain consistent results optimized for yield and purity. The cells captured should represent the heterogeneity of the primary tumor as well as the changes undergone to allow the traveling cells to survive circulation and form metastases at secondary locations. These isolated cells must also be pure enough to allow useful downstream analysis. Achievement of these goals will allow both the use of enumeration data to reliably predict the course of disease and treatment and the study of rare cells such as cancer stem cells which may provide the clues needed to generate further treatments and cures.

5.3 CSC Targeted Therapy

CTC capture and analysis of CSC populations within the isolated CTCs offers a unique opportunity to examine those cells that have already entered the blood stream and are thus a step farther in the metastatic cascade than primary tumor cells. As well as presenting the chance for a noninvasive *liquid biopsy*, CTCs samples may be enriched for the CSCs that may hold the key to therapeutic cancer relief. While current technologies can be limited in their specificity for epithelial capture, the trend of the field is the development of platforms that will enable the isolation of different phenotypes of CTCs, allowing the field of CSCs research to reach its full potential in the fight against cancer.

Several studies attempted to demonstrate correlation between patient clinical outcome and the frequency of cancer stem cells [82, 83]. In addition, both CSC and embryonic stem cell profiles expressed in tumors can be used as predictive markers for patient outcome. If self-renewing cell populations can develop tumors, then targeted therapy against these populations may be considered for chemoprevention. Studies have shown that aspirin as an anti-inflammatory agent and metformin as an antidiabetic drug can decrease risk of cancer development. Interestingly in mouse models, metformin has been able to target breast CSCs [84]. Furthermore, curcumin found in turmeric and sulforaphane found in broccoli have been shown to be able to

inhibit pathways in stem cell self-renewal and suggest usefulness of these dietary compounds for cancer prevention [85, 86].

The ultimate test to prove the cancer stem cell hypothesis will be the demonstration that patients' clinical outcome significantly improves following effective targeted therapy against cancer stem cells. Furthermore, effective targeted therapies in the adjuvant settings will support the cancer stem cell hypothesis [87, 88]. Selective targeted therapies against CSCs that have no harmful effects on normal stem cells will have great potentials in treatment of many human malignancies [89]. These targeted strategies may include (a) antibodies against CSC-surface markers, (b) inhibitors of signaling pathways that are essential for CSC self-renewal, (c) reversal agents that overcome innate resistance to chemotherapy and radiotherapy in CSCs, and (d) inducers of cellular differentiation to terminally differentiate CSCs [90, 91].

Trastuzumab, an anti-HER2 antibody, was the first successful targeted therapy became available for treatment of HER2-positive breast cancers. Unfortunately, resistance to this drug in some HER2 positive breast cancers resulted in incurable metastatic disease [92, 93]. This failure in targeted therapy led to the development of a dual inhibitor for EGFR/HER2 tyrosine kinase. This dual inhibitor, Lapatinib, was able to inhibit breast CSC activity in HER2 positive tumors in a neoadjuvant setting. In addition, combination of Lapatinib with chemotherapy showed a decrease in time to progression of trastuzumab-resistant patients [94, 95].

It has been shown that both normal mammary and breast cancer stem cells are capable of surviving in non-adherent culture conditions via forming floating colonies known as mammospheres [2, 96]. This in vitro culture system has been extensively used and proven to be a valuable method to assess CSC activity in breast cancer cell lines. Using this method, the role of IL8 in regulating the breast CSCs has been reported to be partly due to a novel SRC and EGFR/HER2-dependent pathway. It has also shown that in HER2-positive breast cancers, blocking the effect of IL8 on mammosphere formation by CXCR1/2 inhibition increases the efficacy of Lapatinib. Furthermore, Lapatinib prevented the promoting effect of IL-8 on mammosphere formation in both HER2-positive and negative breast cancers. These data together suggest that adding CXCR1/2 inhibitors to the current HER2-targeted therapies may be considered as an effective therapeutic strategy to decrease CSC activity in breast cancer and improve the survival of HER2-positive patients [97].

Our group demonstrated that IL-8 acts via CXCR1 and CXCR2 receptors and their inhibition reduces the CSC population, self-renewal and increases the efficacy of Docetaxel in reducing tumor size in xenografts [98]. SCH563705, a small molecule CXCR1/2 inhibitor, inhibited mammosphere formation by recombinant IL-8. Since this inhibition was not observed when the compound alone was used, it was postulated that IL-8 probably acts via a paracrine by stromal cells rather than an autocrine route by CSCs [99, 100]. Our group also demonstrated that CXCR1 blockade by a specific blocking antibody or Repertaxin, a small-molecule inhibitor, selectively targets CSCs in two human breast cancer cell lines in vitro. We also reported that CXCR1 blockade induces cell death in $CXCR1^-$ cells via a bystander effect [98]. Furthermore, our data, suggest that CXCR1 blockade (Fig. 5.4) may

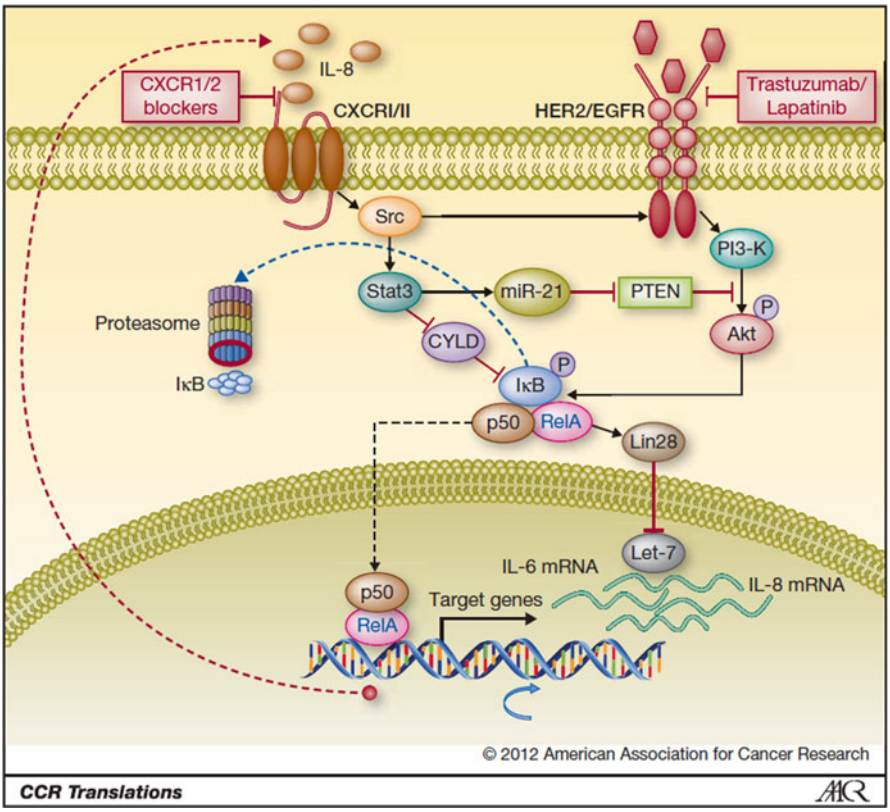

Fig. 5.4 IL8/CXCR1/2 and HER2/EFGR pathways in CSCs [101]. Copyright 2012 American Association for Cancer Research

provide a novel means of targeting and eliminating breast CSCs [101]. In Phase I clinical trials Repertaxin was well tolerated and trials are ongoing to determine its efficacy in breast cancer patients [101, 102].

Results of studying CSC self-renewal regulatory and survival pathways have introduced several novel targets for therapeutic development. Indeed, developing CSCs targeting agents is one of the main focuses of several biotechnology and pharmaceutical companies. In this concept, agents are under development to target pathways such as Notch, Hedgehog, Wnt, Akt/mTOR, and NFκB. Small-molecules that inhibit the Wnt and Notch pathways in colon cancer have been identified [103]. But due to equal importance of these pathways in regulating normal stem cells, potential cytotoxic side-effects on normal tissues raised concerns for using these inhibitors.

Inhibiting the Notch signaling pathway is potentially promising in targeted CSCs therapy (Fig. 5.5). Small molecules with γ-secretase inhibitory activities (GSIs) or monoclonal antibodies (mAbs) against Notch ligands and or receptors are currently

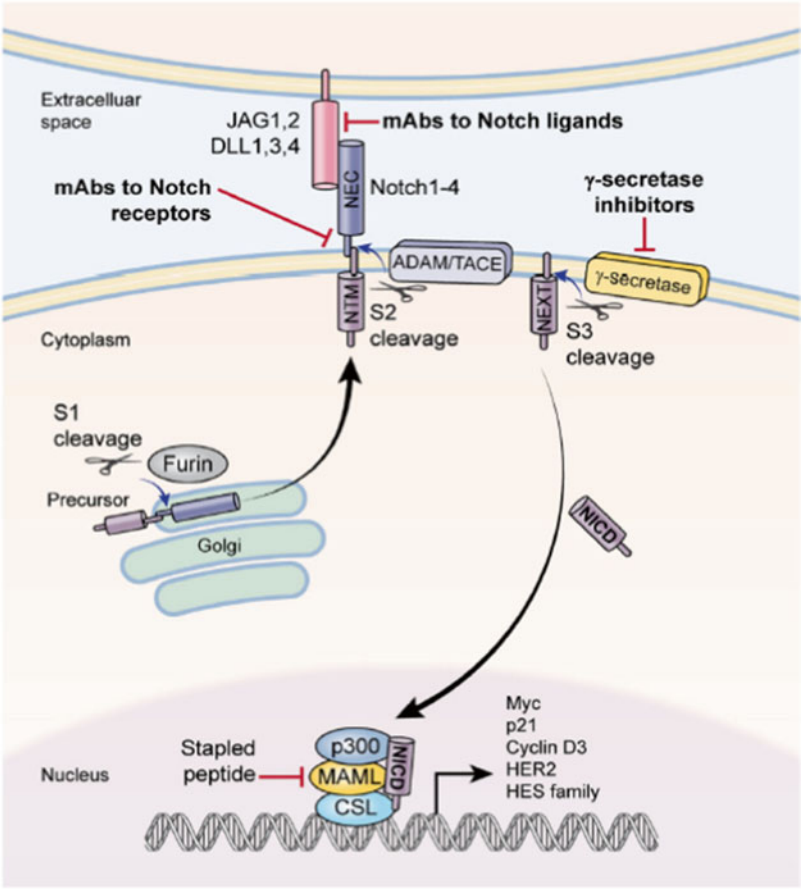

Fig. 5.5 Targeting Notch signaling pathway in CSCs [104]. Copyright 2013, Pharmacology & Therapeutics (2013), http//dx.doi.org/10.1016

in clinical development [104]. Gamma secretase inhibitors (GSIs) have been reported to be effective in targeting CSCs of solid tumors. To overcome toxicities associated with these inhibitors on normal tissues especially in gastrointestinal tract, dose scheduling was changed to an intermittent schedule along with co-administration of corticosteroids that appeared to have almost the same clinical efficacy with much less toxicity [105, 106]. A significant antitumor activity has been reported in about 50 % of the breast cancer xenograft models in a combination therapy using inhibitors of CSC self-renewal with antiangiogenesis compounds and chemotherapeutics that induces apoptosis and inhibit proliferation [107].

Another possible approach in CSC specific targeting is inhibition of signals that induces resistance to chemotherapy and/or radiation therapy. Efflux activity of ABC-transporters such as BCRP in CSCs has been used in side-population experiments to identify CSC populations. In melanoma, antibodies were used to inhibit

the activity of these membrane transporters [108]. In addition, chemosensitizing effect of antibody against IL-4 on apoptosis induction of chemotherapy has been demonstrated in CD133$^+$ colon CSCs [109].

Finally, CSC specific therapy using differentiation inducing compounds might be an effective strategy in converting CSCs to terminally differentiated tumor cells lacking self-renewal capability. In this context, Salinomycin as the first compound has been described with capability to induce terminal epithelial differentiation in breast CSCs [110]. It is well-established that certain types of CSCs exist in a quiescent state and thus are resistant to DNA-damaging agents. Induction of these quiescent stem cells to enter the cell cycle to become target of conventional chemotherapy has been successfully demonstrated in a mouse model for AML by treatment with colony stimulating factors (e.g., G-CSF) [111].

Due to commonalities between the pathways regulating CSCs and normal tissue stem cells, patients on these studies require careful monitoring for potential toxicities related to these agents. Fortunately, limited toxicities have been so far reported in the preliminary results from phase I trials of these agents. Of course, more will be known about efficacies versus harmful effects of these targeting agents during Phase II and III studies.

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Part II
Fundamental Studies of CTC

Chapter 6
Circulating Tumor Cells and Tumor Dormancy

Alison L. Allan and Ann F. Chambers

Abstract Metastatic cancer can recur months or even years after apparently successful treatment of the primary tumor. While the exact mechanisms leading to cancer recurrence remain poorly understood, failure to completely eliminate dormant micrometastases and solitary metastatic cells is believed to be a major contributor. Thus, while not of initial clinical concern, metastatic dormancy is still a significant clinical problem. The emerging use of circulating tumor cells (CTCs) as prognostic and predictive biomarkers for monitoring and understanding metastatic disease may provide an opportunity to address this challenge. In this chapter we discuss the current knowledge relating to CTCs and tumor dormancy, and the relationship between the two with regard to metastasis biology and treatment. We also consider the clinical impact of monitoring for CTCs in the absence of symptomatic tumor recurrence and what is needed for such an approach to providing "actionable" information that will improve patient outcome.

Keywords Metastasis • Circulating tumor cells • Molecular characterization • Tumor dormancy • Oligometastasis

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6.1 Overview: Circulating Tumor Cells and Tumor Dormancy

Most cancer deaths are due to metastasis, which occurs when tumor cells spread from the primary tumor to establish themselves as secondary tumors in distant and vital organs, where they can cause physiological damage. Cancer that is diagnosed when it is localized to the primary site is easier to treat successfully, however most therapies eventually fail in the metastatic setting. Adjuvant therapy is given when there is a suspicion that the cancer has already seeded undiagnosed micrometastases, in order to prevent their subsequent growth, and has been shown to improve survival in many settings. Metastatic cancer can recur months or even years after apparently successful treatment of the primary tumor, and failure to completely eliminate dormant micrometastases and solitary metastatic cells is believed to be a major contributor to this recurrence. Thus, metastatic dormancy is a significant clinical problem.

Metastasis itself is a complex process, since the successful metastatic cell must traverse multiple steps in order to ultimately develop into a clinical relevant metastatic lesion. These steps include escape from the primary tumor, intravasation (invasion) into the lymphatic or hematogenous vasculature, survival in the circulation, arrest and extravastion into the secondary organ site, initiation of metastatic growth at that site, and maintenance of growth into macrometastases [1, 2]. Given the multistep nature of this process, there should be several opportunities for early identification of disseminating cells before they become a clinical problem. Indeed, in cancer patients with either metastatic or apparently localized disease, there is growing evidence that the presence of circulating tumor cells (CTCs) in the blood is an important indicator of metastasis and poor outcome (reviewed in Refs. [3, 4]). The emerging use of CTCs as prognostic and predictive biomarkers for monitoring and understanding metastatic disease may provide an opportunity to address the challenge of metastatic dormancy. In this chapter we discuss the current knowledge relating to CTCs and tumor dormancy, and the relationship between the two with regard to metastasis biology and treatment.

6.2 Circulating Tumor Cells

Although CTCs have been recognized for well over a century [5], only recently has technological advancement allowed for detailed investigation of these rare cells and their consideration for use in the clinic. Even in patients with known metastatic disease, these cells are present at a very low frequency in the circulation (~1 CTC per 10^5–10^7 leukocytes) [6–8], necessitating the development of sensitive and specific approaches for their isolation, enumeration and molecular characterization. The enormous promise of CTCs for monitoring disease recurrence and treatment response in clinical oncology has resulted in an explosion of interest in developing biomarker

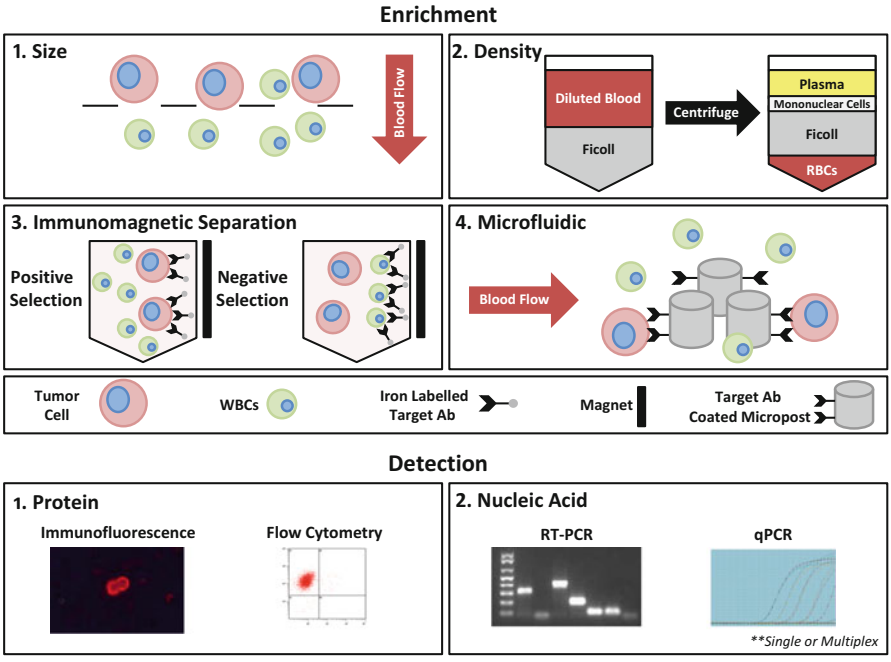

Fig. 6.1 An overview of the most commonly utilized techniques for the process of CTC enrichment and detection. In general, four approaches currently exist for CTC enrichment (*1*) size-based; (*2*) density-based; (*3*) immunomagnetic separation; and (*4*) microfluidic-based. Using size-based enrichment techniques, diluted whole blood is passed through a filtration device with specific sized pores (typically 8 μm). CTCs are captured based on differences in cell size between CTCs (typically >8 μm) and white blood cells (WBCs; typically <8 μm). Density-based enrichment utilizes Ficoll (or similar density gradient medium) to enrich for mononuclear cells (including CTCs) from other blood components. Immunomagnetic separation involves the use of iron-conjugated antibodies targeted toward CTCs (e.g., EpCAM; positive selection) or contaminating blood cells (e.g., CD45; negative selection) and incubation in a magnetic field. For microfluidic-based techniques, whole blood is slowly passed across a chip-based surface and isolated using either CTC targeted antibody-coated microposts (CTC Chip and iChip), or dielectrophoresis (DEPArray). Current CTC detection techniques use either a protein-based approach (i.e., immunofluorescence or flow cytometry) expressed by whole cells or secreted proteins (EPISPOT assay), or nucleic acid-based approaches such as RT-PCR or RT-qPCR, applied at the level of single genes or using a multiplex approach. *Re-printed from Lowes LE, Allan AL. Recent advances in the molecular characterization of circulating tumor cells. Cancers (Basel). 2014 Mar 13;6(1):595-624. doi: 10.3390/cancers6010595 (Open Access)*

approaches for analyzing CTCs in the clinical setting as well as understanding the underlying biology of these cells and their functional relationship to metastasis and tumor dormancy. As a result, more than 40 different CTC technologies are currently under development [9], many of which are described in greater detail elsewhere in this book. However, common themes are emerging that help to categorize these technologies into some key approaches that are required for successful enrichment, isolation, detection, and/or molecular characterization of CTCs (Fig. 6.1).

For enrichment, approaches include size-based, density-based, or immunomagnetic enrichment (i.e., positive selection of CTCs using epithelial-specific or tumor-specific markers, or negative selection using markers expressed by contaminating cells such as leukocytes). For detection and characterization, approaches include cytometric techniques such as immunofluorescence or flow cytometry using antibody-mediated detection; or nucleic acid-based techniques such as reverse transcription polymerase chain reaction (RT-PCR), quantitative-PCR (qPCR), microarray, or sequencing. The advantages and disadvantages of each of these approaches have been extensively reviewed elsewhere [9–12] and therefore not discussed in detail here.

6.2.1 Clinical Utility of CTCs

The intense interest in CTCs is evidenced by the fact that more than 400 clinical trials have or are utilizing CTCs as correlative biomarkers, and PubMed lists more than 14,000 publications involving CTCs [13]. However, despite the number and scope of these studies, the CellSearch® system (Janssen Diagnostics) remains the only CTC platform presently cleared by the FDA for detection and enumeration of CTCs in the clinical setting. This platform enriches for CTCs using positive immunomagnetic selection based on EpCAM (epithelial cell adhesion molecule) expression, followed by immunofluorescent staining for cytokeratins (CK 8/18/19), CD45 (to identify contaminating leukocytes), and the DNA dye DAPI (4′,6-diamidino-2-phenylindole). Using semi-automated fluorescence microscopy, positive CTCs are then identified as cells >4 μm in diameter with an intact cell membrane and a CK+/DAPI+/CD45− phenotype [14]. The CellSearch® system is currently FDA-cleared for prognostic use in metastatic breast, prostate, and colorectal cancers, where the presence of ≥5 (breast [14] and prostate [15]) or ≥3 (colorectal [16]) CTCs in 7.5 mL of blood has been correlated with poorer prognosis compared to patients with fewer CTCs in the same blood volume. Using this platform, CTC enumeration has been utilized not only to assess CTC number at baseline but also for serial assessment of response to treatment. It has been demonstrated that CTCs are correlated with patient outcome and that a change in CTC number during treatment is predictive of therapy response, often sooner than currently utilized techniques such as imaging [15, 17–19].

Thus far the greatest clinical utility for CTCs has been observed in the metastatic setting for breast and prostate cancer, with growing evidence in colorectal and lung cancer [14–16, 20–23]. In addition, the amount of CTC data available in the literature has facilitated several meta-analyses that have highlighted the prognostic value of CTCs in various cancers, including pancreatic [24], lung [23], colorectal [25], breast [26], and prostate cancer [27]. In particular, Zhang et al. (2012) analyzed data from thousands of breast cancer patients and demonstrated that CTCs are a stable prognosticator in both metastatic and early-stage breast cancer [26]. Importantly, CTCs are now taken into account in the American Joint Committee on

Cancer (AJCC) TNM (tumor-node-metastasis) cancer staging manual for breast cancer; as classification cM_0(i+) ("No clinical or radiographic evidence of distant metastases, but deposits of molecularly or microscopically detected tumor cells in circulating blood, bone marrow, or other nonregional nodal tissue that are ≤0.2 mm in a patient without symptoms or signs of metastases") [28]. Taken together, this body of clinical data provides convincing evidence to support the use of CTCs for tracking, understanding and treating metastatic disease in cancer patients.

6.2.2 Challenges and Potential of CTC Analysis

Several challenges still exist in the analysis of CTCs, including technological and statistical challenges (sensitivity, specificity, reproducibility, capacity for single-cell molecular characterization); biophysical parameters (clustering of CTCs with each other and/or with leukocytes and platelets, reduced capture of CTCs due to size restriction in small capillary beds); and biological factors (immune surveillance, loss of epithelial phenotype through epithelial–mesenchymal transition (EMT), molecular and cellular heterogeneity between individual CTCs in the same patient, and the relationship with cancer stem cells) (reviewed in Refs. [13, 29, 30]). These challenges are highlighted by the fact that although CTCs are useful in many epithelial cancer types discussed above, they have been found to be only minimally informative in other cancer types, either because of the biology of how the disease progresses/metastasizes (i.e., localized versus distant dissemination in cancers such as ovarian, liver, and brain cancer) and/or the lack of expression of epithelial markers which may impact the ability to detect CTCs from these cancers by most current clinical CTC approaches (i.e., renal cancer). It is also important to note that current CTC technologies are not always sensitive enough to reproducibly detect the lower numbers of CTCs that may be present in patients with early-stage disease or those in the adjuvant setting, where the risk of recurrence or metastasis is unknown [6, 29].

Although the CellSearch® system is considered the current "gold standard" for clinical CTC enumeration, its sensitivity and capacity for downstream molecular analysis is limited [6, 9]. Emerging technologies such as Epic Sciences' HD-CTC fluid biopsy (developed by Peter Kuhn and colleagues) [31–34], and the CTC Chip/iChip (developed by Mehmet Toner, Daniel Haber and colleagues) [20, 35–42] have shown great promise with regards to reproducibility in a clinical lab setting and have the added advantage of increased sensitivity for assessing earlier stage disease as well as the capacity for molecular characterization of CTCs. The HD-CTC fluid biopsy assay identifies CTCs without using surface protein-based enrichment, instead using sophisticated imaging and software algorithms to identify and present CTCs as high definition (HD) diagnostic pathology quality images [31–34]. The most developed version of the CTC Chip (the iChip) also does not rely on the presence of EpCAM or other known tumor antigens on the cell surface of CTCs, taking the approach of combining microfluidics with sequential negative and positive

enrichment methods on a herringbone microchip [37]. Both assays (HD-CTC and iChip) have been demonstrated to have improved sensitivity over the CellSearch® system and can provide CTCs in an ideal format for downstream characterization using various approaches including fluorescence in situ hybridization (FISH), immunofluorescence and mutational analysis.

6.3 Molecular Characterization of CTCs

Given our increasing awareness of tumor heterogeneity and the ability of tumors to evolve at the molecular level during disease progression, it is becoming apparent that simple enumeration of CTCs fails to capitalize on their full potential as biomarkers of metastatic disease. Perhaps the greatest promise that CTCs hold for oncology lies at the level of molecular characterization. Given the fact that metastasis determines the ultimate outcome for a patient, treatment decisions may be more effective if they are based on the genetic characteristics of metastatic lesions rather than on those of the primary tumor alone. However, obtaining biopsies from metastatic tumor tissue is an expensive, invasive and often painful procedure, limiting its widespread use in clinical practice [43]. Since CTCs are the intermediaries between primary and metastatic disease and are believed to be surrogates of a patient's metastatic tumor [44–46], molecular characterization of CTCs may provide an opportunity for noninvasive "real-time" biopsies during disease progression in order to track these molecular changes and potentially incorporate them into clinical decision making.

Several studies suggest that molecular characterization of CTCs may have clinical utility from the perspective of identifying loss or acquisition of molecular features in individual patients' tumors that may open up new avenues for targeted therapy that were not options based on the characteristics of the primary tumor alone. For example, Meng et al. demonstrated that almost 40 % of metastatic breast cancer patients who were initially HER2-negative (based on their primary tumor) acquired amplification of HER2 in their CTCs. When treated with Herceptin based on CTC HER2 amplification, some of these patients demonstrated a partial or complete response [47]. Several other subsequent studies have demonstrated discordance between HER2 status in patients' CTCs versus their primary tumor [48–53] and demonstrated that HER2-positive CTCs are a poor prognostic factor in patients with both early-stage and metastatic breast cancer [53–55]. Similar studies in prostate cancer have demonstrates an evolution in important disease-related markers such as AR, PTEN, and TMPRESS2ERG between primary tumors and CTCs that may help identify those patients most likely to respond (or not) to targeted therapies [35, 56–59].

In addition to examining individual molecular markers on CTCs that may have prognostic or predictive value, the CTC field has also benefited from technological advances in more sophisticated downstream analysis approaches such as genomic

sequencing. Recent studies have reported isolation and analysis of genomic DNA from CTCs and single cell analysis of copy number variation patterns, array-CGH, and next-generation sequencing [60–62]. Ni et al. (2013) analyzed single CTCs from lung cancer patients and observed that every CTC from an individual patient exhibited reproducible copy number variation (CNV) patterns, similar to those of the metastases (but not primary tumor) of the same patient [61], supporting the idea that CTCs can serve as a reflection of the molecular features of metastatic disease. Another study by Heitzer et al. (2013) observed that in CTCs from colorectal cancer patients, mutations in known driver genes such as APC, PIC3CA, and KRAS could be found in matched primary tumors, metastases, and CTCs; however mutations exclusive to CTCs were also observed. Interestingly, additional deep sequencing of tumor tissue demonstrated that most mutations initially found only in CTCs were actually present at a subclonal level in the primary tumors and metastases from the same patient [60], suggesting that CTCs are representative of the complex and heterogeneous tumor genome.

Although large-scale clinical data is still lacking with regards to the value of molecular characterization of CTCs as a clinical decision making tool, the studies described above suggest that this type of analysis holds tremendous promise with regards to developing personalized approaches to therapy and providing valuable insight into the underlying biology of metastasis and tumor dormancy.

6.4 Implications of CTCs for Understanding Metastasis Biology

In contrast to most areas of cancer research, the study of CTCs began in the clinic rather than at the laboratory bench. As a result, the majority of CTC studies have focused on technology development and clinical utility, with minimal investigation into the biology of CTCs until fairly recently. Initial CTC work in experimental systems utilized immortalized human cancer cell lines and xenograft mouse models, demonstrating that CTCs can be serially tracked over time in preclinical models and that increasing numbers of CTCs are correlated with increased metastatic burden [10, 46, 63, 64]. Additional studies in both preclinical models and patient samples have started to define some of the mechanisms underlying CTC progression to metastases, including hypoxia [65, 66], epithelial–mesenchymal transition (EMT) [41, 67–73], and stem cell-related signaling [42, 44, 69, 71, 74]. Importantly, it has now been demonstrated that viable CTCs can be isolated from patients and grown in culture [75–77] as well as injected into immunocompromised mice to initiate metastases [44, 77]. These studies highlight the critical role that CTCs play in disease progression and metastases development, and open up exciting possibilities for future experimental and clinical studies aimed at interrogating the role of CTCs in tumor dormancy.

6.5 Tumor Dormancy

6.5.1 Clinical Tumor Dormancy

One of the most problematic aspects of cancer is its ability to recur after apparently successful primary treatment. In some cases, these recurrences can be years or even decades after initial diagnosis and treatment. While some patients with melanoma, kidney cancer and breast cancer are often believed to be at particular risk for late recurrences, these can occur at low frequency for many cancer types; see Table 1 in Ref. [78]. Uncertainty about which patients will have late recurrences makes ongoing care of these patients difficult. Several clinical trials, including the MA.17 trial of long-term hormonal treatment in women with hormone responsive breast cancer, have indicated that tumor dormancy and late recurrences are a clinical reality, and that long-term therapy does offer some benefit in preventing micrometastatic disease from progressing [79–81]. However, these recurrences occur in a relatively small proportion of these patients, and this benefit to the group needs to be weighed against the toxicities associated with long term therapies for many patients [78, 82, 83].

6.5.2 "Cure" vs. "Clinical Dormancy"?

A dilemma about tumor dormancy and late recurrence is illustrated in Fig. 6.2. Figure 6.2a depicts the clinical situation following apparently successful treatment of a primary cancer. It is not known whether this patient has truly been cured, or if there is undiagnosed, micrometastatic but dormant disease present that will (or may?) recur. It is only after a cancer does recur that the patient can be categorized as having had dormant cancer that subsequently began to grow (Fig. 6.2b). Thus, a clinical identification/diagnosis of "dormancy" currently can only be made after the fact of recurrence. However, evolving technologies for monitoring for evidence of dormancy and micrometastatic disease, including CTCs and other blood biomarkers, as well as improving imaging approaches for detection of minimal residual disease may lead to an improved ability to detect small volumes of residual cancer. This information will then need to be appropriately integrated into cancer management strategies, discussed below.

6.6 Biology of Clinical Tumor Dormancy

Much more needs to be learned about the biology of tumor dormancy, which patients are at risk for dormancy and recurrences, and also whether micrometastatic disease is destined to recur or if there are lifestyle or therapeutic/preventive interventions that can

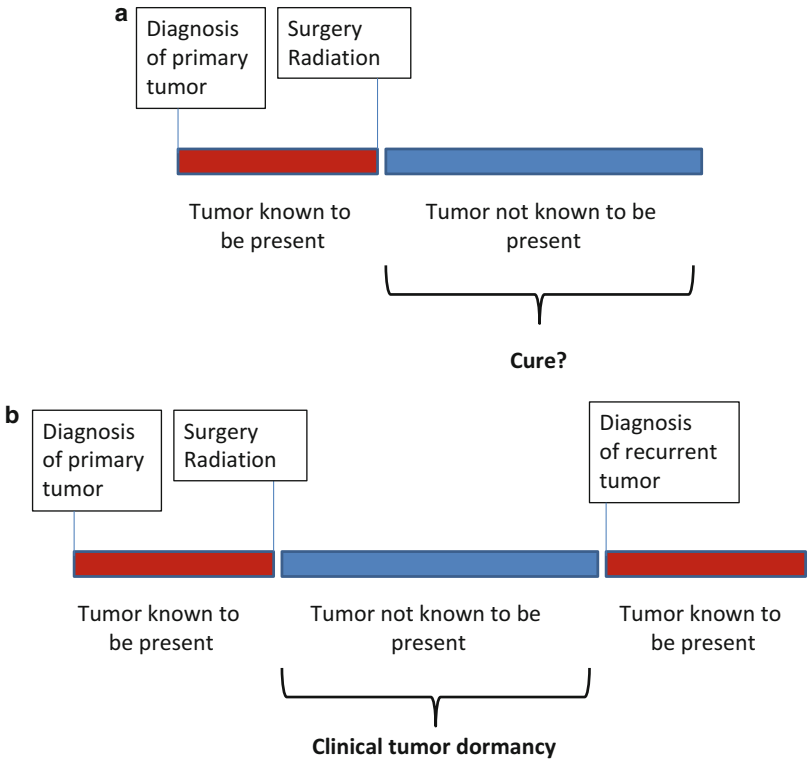

Fig. 6.2 Clinical tumor dormancy can be defined only after the fact of tumor recurrence. (**a**) Depicts the situation of a patient who was diagnosed with cancer and received local therapy (surgery, radiation) and perhaps adjuvant systemic therapy designed to eliminate any disseminated cancer. The *red bar* depicts the clinical situation of cancer known to be present. The *blue bar* depicts the situation of cancer not known to be present. The dilemma is that it is not known if this patient is cancer-free and cured, or if undiagnosed cancer still remains. If the *blue bar* represents a "long" period of time (e.g., 5 years, or more), the patient might be considered to be "cured." (**b**) Depicts the situation of a patient whose cancer recurs. It is only after cancer recurrence that it can be known that undetected cancer was present, and if the *blue bar* represents a "long" period of time, that the cancer was indeed persistent but in a state of "clinical dormancy." This diagram has no implications of mechanisms of maintenance of dormancy, location or state of cancer cells in this interval, or causes of cancer recurrence, which remain important research questions

minimize recurrence. Progress toward better understanding of the biology of tumor dormancy has come from both experimental and clinical studies (many focused on breast cancer), which in turn have led to hypotheses about clinical tumor dormancy.

Early case observations and thoughts on clinical tumor dormancy have been reviewed by Meltzer in 1990 [84]. Discussions about the possible kinetics of tumor growth and dormancy have been presented by Demichelli and colleagues, who concluded that clinical tumor dormancy likely is a consequence of arrested and restarted growth, rather than very slow, continuous growth [85]. A recent review by Uhr and Pantel concludes that "Clinical data suggest that a majority of breast cancer survivors

have cancer cells for decades but can remain clinically cancer-free for their lifetime" [86]. Clearly, the identification of molecular mechanisms responsible for this natural, long-term cancer control in patients, inherent to the state of clinical tumor dormancy, will be important to understand and may lead to interventions to maintain or prolong dormancy therapeutically

6.7 Experimental Models of Tumor Dormancy

A variety of in vivo and in vitro models have been used to try to understand the biology underlying tumor dormancy. Tumor dormancy has been observed in experimental metastasis models from many cancer types, reviewed in Refs. [87–89]. Experimental dormancy has been described as both cancer cell quiescence, evidenced by solitary cancer cells that persist in vivo without cell division [90, 91], and as pre-angiogenic micrometastases with balanced cell division and apoptosis, such that there is no net growth [92, 93]. When mice are treated with cytotoxic chemotherapy that targets dividing cells, cancer cells that are in quiescent state at the time of treatment have been shown to be insensitive to this therapy, leading to subsequent late recurrences [94–96]. These studies are consistent with clinical late recurrences, which can occur in some patients following adjuvant chemotherapy [1].

Many studies have attempted to decipher the molecular mechanisms that can regulate tumor dormancy. Some common themes have emerged, including the ability of the microenvironment surrounding a cancer cell in secondary sites to influence entry into, or maintenance of, a dormant state [97–100], as well as properties of the cancer cells themselves, such as expression of metastasis suppressor genes, reviews [101, 102]. A few examples of studies supporting these mechanisms of regulation of dormancy are presented below, and the articles and reviews cited above provide more examples.

Early studies by Ossowski and Aguirre-Ghiso, using a chicken embryo in vivo model, showed that reduction of urokinase plasminogen activator, limited β1 integrin activity and consequent reduction in interactions with the extracellular matrix, were associated with dormant cancer cell behavior [103–106]. Barkan et al. [107] adapted assays developed by Bissell and colleagues [108], in which cells are grown in 3D Matrigel matrices. Using these assays, Barken et al. found that multiple cancer cell lines that showed prolonged dormant behavior versus active metastatic growth in experimental mice also showed parallel "dormant" versus "proliferative" behavior in vitro. This in vitro assay thus enabled identification of molecular properties that reflected these growth patterns. Dormant cells showed cell cycle arrest and nuclear expression of the cell cycle regulators p16 and p27. In contrast, cells that made the transition from quiescence to proliferation increased production of fibronectin and β1 integrin signaling, cytoskeletal filamentous actin stress fiber formation and phosphorylation of myosin light chain (MLC) via MLC kinase. This study also showed that inhibition of inhibition of β1 integrin or MLCK prevented the transition from dormancy to proliferation, suggesting that regulation of interactions between cancer

cells and the extracellular matrix may provide a therapeutic target to regulate tumor dormancy [100, 107, 109]. Other studies have also implicated regulation of cancer cell–microenvironment interactions in maintenance of tumor dormancy [110]. For example, recent work by Bissell and colleagues suggests that, in mouse models, that mature blood vessels can suppress metastatic outgrowth, whereas sprouting microvasculature may induce a proliferative phenotype in cancer cells [111].

While great progress thus has been made in clarifying how dormancy and proliferation may be regulated experimentally, much remains to be learned about molecular mechanisms that can regulate dormancy and, importantly, how then to translate these experimental findings to achieve regulation of tumor dormancy clinically. The ways in which CTCs may provide information about dormancy are discussed below.

6.8 Relationship Between CTCs and Clinical Dormancy

Meng et al. [112] observed that CTCs could be detected in breast cancer patients free of overt metastases up to 22 years after their initial diagnosis, suggesting that many apparently "cured" cancer patients may harbor detectable dormant tumor cells [112]. However, the clinical implications of these findings remain unclear. Although very few studies have definitively shown that CTCs can provide reliable evidence of occult metastases, minimal residual disease, and/or clinical dormancy, exciting advances in CTC technology provide promise for understanding the relationship between CTCs and clinical dormancy. The benefit of early detection of metastases depends on whether it is possible to distinguish those cancers that are likely to behave aggressively from those that are indolent, and/or will never leave their state of dormancy. To achieve this, one might envision that molecular characterization of CTCs using a "dormancy versus proliferation" expression signature (i.e., Ki67, MLCK, β1 integrin, fibronectin, p16, p27) and correlation with time to metastatic recurrence in patients may provide critical insight into how CTCs are related to dormancy. In addition, the recent demonstration that viable CTCs can be isolated from patients and grown in culture [75–77] as well as injected into immunocompromised mice to initiate metastases [44, 77], may facilitate the study of functional behavior of dormant versus proliferative CTCs derived from patients as well as the underlying mechanisms of this behavior in greater detail than has been previously possible. Finally, molecular and functional comparison of CTC characteristics in epithelial tumor types with relatively short latency periods (i.e., lung or colorectal cancer) to those cancers with long periods of latency (i.e., breast cancer) may provide a greater understanding of the relationship between CTCs and clinical dormancy [88].

Since monitoring for CTCs is relatively noninvasive, requiring only a blood sample, this approach would have advantages over more invasive approaches such as molecular imaging. However, at this stage it is not known whether detection of CTCs as an early signal of cancer recurrence will lead to patient benefit, and it is unclear whether cancers detected by presence of increases in CTCs are more readily

treated than cancer recurrence based on current clinical practice. The presence of CTCs in cancer survivors who have no other evidence of persistent disease indicates that these individuals have dormant cancer somewhere in their bodies, which are shedding and replenishing the CTC population. Biologically, this evidence of persistent but asymptomatic disease offers many important opportunities to learn about cancer metastasis and dormancy, including how their progressive growth is being controlled. This information could provide new approaches for cancer control, through maintenance of the dormant state and/or through improved knowledge about how to eliminate dormant cancer.

6.9 Conclusions and Future Perspectives

Several cancer types can recur after long periods of an apparent cancer-free state. Analysis of CTCs may have potential value for monitoring patients at risk for cancer recurrence, especially delayed recurrence. Clinically, however, knowledge of CTCs in otherwise healthy individuals may lead to treatment dilemmas and harm, or lack of clinical benefit, to these people. Cancer that has not spread to distant sites is often treated with curative intent, whereas distant metastatic disease is generally regarded as not curable, at least with currently available therapies. The question of whether early detection of metastatic, recurrent disease offers clinical benefit to patients is an important but unanswered question. Thus, earlier detection of metastatic disease may lead to earlier treatment of asymptomatic individuals with a limited number of anti-metastatic therapies, with the inherent toxicities of these agents and without demonstrated knowledge that "early" metastatic disease is more treatable than later, symptomatic metastatic disease. Recently, Jochelson et al. discussed this issue in the context of breast cancer, and concluded that there is no evidence that, for women with recurrent breast cancer, earlier detection has clinical benefit to the patient [113].

One possible exception to this concern may lie in the case of oligometastatic disease, sometimes defined as few (e.g., 1–3) metastases confined to a single secondary site. While the subject of ongoing debate, in some cases, treatment of oligometastases with local treatment, such as surgery or stereotactic radiation, has been shown to lead to long-term benefit for some patients [114]. In other cases, however, local oligometastatic therapy may initially be successful, only to have additional metastases become apparent; presumably these were present as additional, undetectable micrometastases at the time of diagnosis and treatment of the oligometastases. Whether metastases detected "early" are less-progressed, and more treatable, than those detected later remains an unanswered question.

In conclusion, when evidence of persistent cancer is detected using CTCs or other circulating biomarkers, then clinical utility of this knowledge will depend on the reliability of these biomarkers to detect cancer, as well as the availability of clinically beneficial therapies for patients in that setting. Biologically, monitoring patients for recurrent, metastatic disease will be important in our understanding of

metastatic disease and dormancy, and this knowledge may in future lead to improved clinical management. Metastatic disease, for breast and most other cancers, is generally considered to be ultimately incurable, so clinical implementation of CTCs or other biomarkers to monitor for tumor recurrence will vary with the specifics of the particular cancer. However, the wealth of biological information that may be gleaned from serially analyzing the presence and fluctuation of CTCs over time, as well as (and perhaps more importantly) their molecular and functional characteristics may offer the opportunity to lead to clinical benefit for patients with persistent, dormant cancer.

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Chapter 7
Prevention of Conversion of Tumor Dormancy into Proliferative Metastases

Dalit Barkan and Ann F. Chambers

Abstract Late recurrences of cancer are believed to be due to dormant disease that can persist for long periods following apparently successful treatment of a primary tumor. Clinical tumor dormancy thus creates uncertainty for cancer patients and their physicians, who cannot be certain that their cancer will not recur. We have a poor understanding about which individual patients are at risk for cancer recurrence following a period of tumor dormancy. Thus, in spite of the clinical importance of tumor dormancy, much remains to be learned about the mechanisms responsible for induction of, and release from, dormancy. Here we consider the clinical problem of tumor dormancy and discuss evolving ideas of how tumor dormancy and reinitiation of growth may be regulated, both naturally in the body and therapeutically. A better understanding of mechanisms by which dormancy can be regulated may suggest new therapeutic approaches to either eliminate dormant cancer cells or promote the maintenance of dormancy.

Keywords Metastasis • Tumor dormancy • Disseminated tumor cells • Molecular characterization • Tumor microenvironment • Cellular dormancy • Angiogenesis • Immune regulation

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7.1 Overview of Cellular Dormancy and Micrometastases

Tumor dormancy is a clinically important problem and can be an obstacle to successful cancer treatment. A cancer may be treated with apparent success, only to return years or even decades later [1]. While cancers such as breast, renal, and melanoma have been reported to recur many years after primary treatment, data suggest that dormancy is a phenomenon not restricted to these tumor types, with evidence suggesting that tumor dormancy can occur in many cancer types [1]. However, our ability to predict which patients will have disseminated cancer cells that subsequently will recur is limited, making therapy decisions difficult.

Additionally, our knowledge about how dormancy can be regulated, and what may trigger dormant cancer cells to reinitiate growth, is also limited. Recently, tumor dormancy has become increasingly recognized as a growing clinical problem, stimulating research into this phenomenon. Here we discuss some important clinical issues surrounding dormancy and consider some evolving concepts of mechanisms through which dormancy and cancer regrowth may be regulated.

Metastases are responsible for most cancer deaths. Much is known about the steps involved in metastasis, from seeding of cancer cells from the primary tumor into the blood or lymphatic circulation, transport of cells to distant sites in the body, arrest in new organs, and growth in these new sites (reviewed in Ref. [2]). Fortunately, metastasis is an inefficient process, with few cancer cells that escape into the circulation actually leading to the formation of metastatic tumors [3, 4]. Many more cancer cells delivered to the circulation either die or go into a dormant state. It is also recognized that cancer cells shed from a primary tumor early during the growth and progression of a primary tumor [5–7]. Thus, many cancer patients may have disseminated and occult metastatic disease at the time of diagnosis of the primary tumor. Prediction of patients with disseminated but undiagnosed metastatic disease is based on population characteristics of patients with similar stage/grade of disease, rather than specific knowledge about an individual patient. Thus, some patients are overtreated with adjuvant therapy to benefit only a subset of them, while other patients with apparently "favorable" tumors may in fact be undertreated (e.g., [8, 9]).

The fact that cancer can remain in a dormant state for years or even decades is a testament to the body's ability to inhibit growth of cancer cells, at least some of the time, or perhaps to a cancer cell's ability to suppress its own growth, at least in some microenvironments. The challenge, of course, is in understanding how dormancy and subsequent reinitiation of growth is regulated in the body. This information could then be applied to the development of new therapeutic approaches, to induce and maintain disseminated cancer cells in a state of dormancy, or alternatively to kill these cancer cells.

Here we discuss a growing list of potential mechanisms by which circulating tumor cells (CTC) exiting the circulation and entering the secondary sites to become disseminated tumor cells (DTC) may be induced to enter a dormant state (either cellular dormancy, or pre-angiogenic, micrometastatic dormancy [1, 10, 11]) via microenvironmental cues they encounter in secondary organs. These cues may trig-

ger the cells to resume active growth after a period of dormancy. An improved understanding of ways by which cancer cells can enter and leave a functional state of dormancy may lead to new opportunities to target therapy directed against dormant cancer cells, to either destroy them or to maintain them in a non-growing state.

7.2 Mechanisms Underlying Quiescence and Survival of Dormant Tumor Cells

Metastasis, the spread of tumor cells, is an inefficient process where few of the disseminated tumor cells will successfully survive their journey. DTCs that survive the hemodynamic forces and the immune surveillance may seed secondary sites, encountering a new microenvironment that will determine their fate [1, 12, 13]. The DTCs may survive, become dormant, or progressively grow to form metastases [10]. The majority of the DTCs do not survive the initial colonization, whereas those that adapt and survive may persist to reside in a quiescent state (cellular dormancy) for many years (reviewed in Refs. [1, 12, 14]). This long term survival and quiescence of the DTCs may account for the latent recurrence years and decades after primary tumor resection and adjuvant therapy [15].

Three scenarios have been proposed to induce quiescence and survival of DTCs [16]. These include (1) the tumor microenvironment at the secondary site, (2) the tumor microenvironment at the primary site, and (3) early dissemination of tumor cells. We consider evidence in support of each of these scenarios.

7.2.1 Tumor Microenvironment at the Secondary Site

The idea that the tissue microenvironment at a secondary site may play a role in determining the fate of cancer cells that have spread throughout the body is a concept that was put forward over a century ago by Stephen Paget. Paget proposed that metastasis will occur only when the tumor cell (the "seed") and the microenvironment of a given organ (the "soil") are compatible [17]. Willis and Hadfield further developed this concept [18]. They coined the term "tumor dormancy" and specified tumor dormancy as a process involving growth restraints exerted by the ectopic tissue leading to reversible mitotic arrest (reviewed in Ref. [13]). Hadfield noted that "*When the interval* (between surgical excision and appearance of secondary tumors) *is prolonged to six years or more it seems impossible to escape the conclusion that the cells of the dormant growth are in a state of temporary mitotic arrest, no matter how long the period may be*" [18]. Consistent with this concept, it has been demonstrated in experimental models that cancer cells may be seeded throughout the body, where they may remain dormant, only growing in specific "favorable" organs (e.g., [19, 20]). Hence, a foreign, ectopic microenvironment may promote quiescence (cellular dormancy) of some DTCs.

Several mechanisms underlying DTC quiescence and long-term survival have recently been proposed. We previously demonstrated potential mechanisms by which the microenvironment may regulate tumor dormancy [21–23]. Solitary tumor dormancy and the transition to proliferation were recapitulated in vitro by utilizing a 3D in vitro culture system constituted from growth factor-reduced basement membrane extract (BME), to mimic components of the extracellular matrix (ECM). Our results revealed that in the 3D culture system, cells with dormant behavior in vivo remained cell cycle arrested with elevated nuclear expression of p16 and p27. Our findings that the ECM can impose growth inhibitory signals on tumor cells were in concordance with previous reports [24, 25] (Fig. 7.1). Interestingly, the dormant tumor cells displayed distinct cytoskeletal organization with evidence of only transient adhesion to the ECM [21]. However, we demonstrated that the switch from quiescence to proliferative metastatic growth was strongly influenced by interactions with the ECM as a result of cytoskeletal reorganization and formation of actin stress fibers. During this transition the tumor cells formed actin stress fibers via β1 integrin signaling and downstream phosphorylation of myosin light chain by myosin light chain kinase [21, 26]. These findings are consistent with previous work implicating β1 integrins in microenvironmental regulation of cell behavior [27] and were subsequently confirmed by others [28], emphasizing the important role of the full engagement of the dormant tumor cell with the ECM as a mechanism to escape tumor dormancy [21, 23].

These observations are also consistent with previous studies in which downregulation of the urokinase receptor was shown to mediate signaling through α5β1 inte-

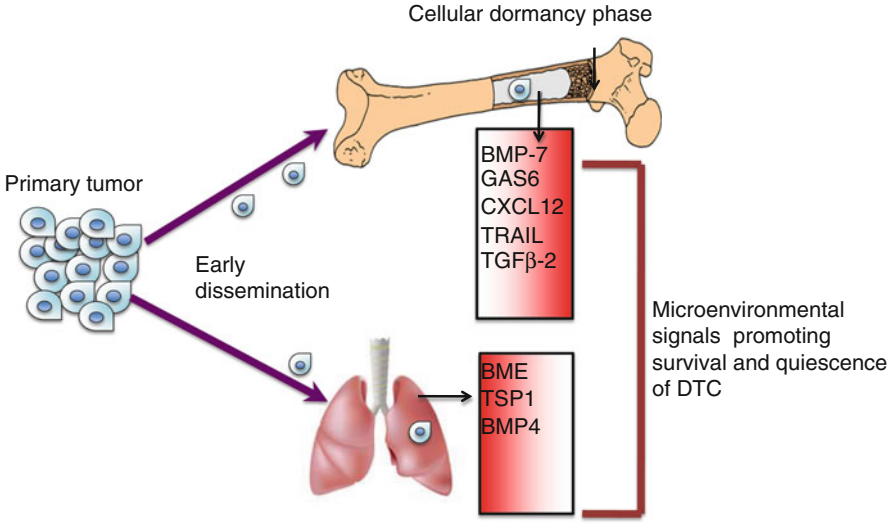

Fig. 7.1 Microenvironmental factors regulating survival and quiescence of DTCs in the lungs and bone marrow. The long-term survival and quiescence of DTCs in the bone marrow and or lungs is dependent on the microenvironmental cues within each site. BMP-7, BME, and TGFβ2 impose quiescence, whereas CXCL12 and TRAIL promote survival of dormant DTCs

grin, forcing the cells into dormancy [29, 30]. Furthermore, in transgenic mouse models for mammary or pancreatic beta cell cancer, knockdown of β1 integrin resulted in inhibition of proliferation of the mammary tumor cells and senescence of the pancreatic beta tumor cells [31, 32]. Thus, multiple lines of evidence indicate that lack of adhesion of the tumor cell to the ECM via integrins can lead a tumor cell to enter a dormant phase. A solitary dormant tumor cell that fails to properly adhere to the ECM can initiate, under these stress conditions, mechanisms that lead to its long-term survival. Pioneering work by the Aguirre-Ghiso laboratory demonstrated that endoplasmic reticulum (ER) stress signaling pathways contribute to growth arrest and survival programs during tumor cell dormancy. They showed that failure of squamous carcinoma cells (HEp3) to engage with the ECM led to inhibition of ERK1/2 signaling and activation of p38α/β signaling pathways. The reduction in ERK/p38 signaling ratio induced the stress adaptive response known as the unfolded protein response (UPR) [33–35]; and reviewed in Ref. [16]. These signals lead to an epigenetic reprogramming and induction of quiescence, by activation of RNA-dependent protein kinase–like ER kinase (PERK) [33, 34], survival and adaptation of dormant HEp3 (D-HEp3) cells in vivo by activation of ATF6alpha-Rheb-mTOR signaling independent on Akt signaling [36]. Interestingly, several metastasis suppressor genes which selectively inhibit the growth at secondary sites, such as MKK4 and MKK6, are activated by stress signals and are upstream activators of p38 [37]. The transcription factors BHLHB3/41/Sharp1 and NR2F1 are regulated by p38α/β and are required for dormancy of tumor cells in vivo [37]. Therefore, the growing family of metastasis suppressor genes, including KISS1, MKK6, BHLHLB3/Sharp-1, and Nm23-H1 among others, may inhibit the growth of DTC at secondary sites (reviewed in Ref. [38]), further supporting the notion that the microenvironmental cues can regulate DTC quiescence.

Indeed, quiescent DTCs are found in the bone marrow (BM) of patients [39]. Several recent studies have demonstrated how the BM could produce factors that will impose dormancy of their residing DTC (Fig. 7.1). Bone morphogenic protein 7 (BMP7) in the BM was shown to trigger dormancy of prostate DTCs by activating p38 signaling, upregulating the metastasis suppressor gene NRDG1, and thus inducing reversible growth arrest [40]. Secretion of growth arrest-specific 6 (GAS6) by osteoblasts and tumor cells was shown to induce dormancy of prostate cancer tumor cells [41]. Recently, Bragado et al. have demonstrated that transforming growth factor-beta2 (TGF-β2) highly expressed in the bone marrow induced ERK/p38 low signaling ratio resulting in induction of quiescence of highly malignant DTCs [42]. Intriguingly, in addition to growth factors regulating tumor dormancy in the BM, a recent report demonstrated that the transfer of miRNAs from BM stroma to breast cancer cells induced quiescence of the breast cancer cells [43]. Hence, microenvironmental factors in the BM may define metastasis-restrictive microenvironment activating stress signals in DTC leading to their quiescence (Fig. 7.1).

Collectively, DTCs residing at secondary sites can be exposed directly to stress signals upon their failure to properly adhere to the ECM, and or their exposure to factors defining restrictive microenvironment. These stress conditions may initiate mechanisms that will promote their quiescence and survival. However, can these mechanisms initiate programs that will ensure quiescent DTC long-term survival?

Autophagy is a highly regulated self-digestion process that produces nutrients and energy for the cell through the breakdown of cytosolic components, and can lead to long-term cell survival under stress conditions [44]. Evidence in the literature suggests that abrogated adhesion of epithelial cells to the ECM may induce autophagy through growth factor and nutrient sensing pathways, energy-sensing pathways, and integrated stress response [45]. Thus, restrictive microenvironments and induction of stress signals may trigger autophagy, thereby promoting long-term survival of the quiescent DTC [46].

In addition to the stress signals generated by microenvironment that may regulate DTC quiescence and long-term survival, there are additional microenvironmental factors that can promote the survival of DTCs. CXCL12 and TRAIL were shown to induce the survival of disseminated breast tumor cells in bone by upregulating Akt signaling via c-Src [47]. Similarly we have shown previously that activation of Src and ERK signaling is required for the switch of dormant breast cancer cells to metastatic growth [22], and combined inhibition of Src and MEK signaling was shown recently to reduce the survival of the dormant tumor cells in the lungs [48].

Overall the microenvironment at the secondary sites can promote stress regulated signals in the DTCs, directly or indirectly, thus determining their fate.

7.2.2 Tumor Microenvironment at the Primary Site

The microenvironment at the primary tumor site may prime the disseminated tumor cells to enter a quiescent state that will be maintained once the cells will colonize the distant site with matching microenvironmental cues. Gene signatures present in the primary tumors have been shown to predict long-term metastatic relapse [45, 49, 50]. Furthermore, gene expression signatures from surrounding histologically normal tissue proximal to the tumor were also shown to predict breast cancer patient survival [51]. It is possible that these gene signatures may be generated by stress signals present at the primary site such as hypoxia. These stress signals were shown to promote autophagy of the tumor cells, thus promoting the induction of quiescence and survival signals [44, 52, 53] that may protect tumor cells from programmed cell death induced upon cell detachment from extracellular matrix (anoikis) [45]. Hence, one can envision that a subset of cells in a primary tumor that disseminate from a hypoxic microenvironment may already be in a dormant state. These cells may be already primed with survival mechanisms such as autophagy and or gene expression patterns that may be enable their successful seeding of distant sites and their continued survival in a quiescent state.

7.2.3 Early Dissemination of Tumor Cells

Early-disseminated tumor cells may not possess the genetic input required to initiate growth at secondary sites [6, 54, 55]. Therefore, tumor cells that disseminate early from the primary site may be an additional instigator of DTC dormancy. There

are several reports demonstrating early dissemination of tumor cells in experimental mouse models. In MMTV-ErbB2 mice with pre-malignant lesions, DTC were already present in their BM [55]. In a uveal melanoma mouse model, it was shown that dissemination occurred at a very early stage and dormant DTCs were detected in several distant organs [56]. In a model of mammary hyperplasia GATA-3 loss facilitated early dissemination and eventually metastasis [57]. Importantly, early dissemination of tumor cells is further supported in clinical settings as well. Several reports have demonstrated that in breast cancer, DTCs are found in BM in ~10–30 % of breast cancer patients with noninvasive lesions (e.g., atypical ductal hyperplasia (ADH) or ductal carcinoma in situ (DCIS)) (reviewed in Ref. [16]). Furthermore, late recurrence of uveal melanoma in the liver (>10 years) was shown to be due to DTCs that were disseminated at least half a decade before diagnosis [58]. Intriguingly, Klein et al. [59] demonstrated that tumor cells in patients with different metastatic diseases had a homogeneous profile and exhibited several aberrations at a genomic level. In contrast, DTCs from patients with nonmetastatic disease were genetically heterogeneous, and their chromosomal abnormalities were very different from their matched primary tumors [60]. Hence, accumulating evidence in the literature suggests that early disseminated DTCs seeded to restrictive microenvironments will remain dormant and may require additional genetic or epigenetic alterations that will allow them to escape their dormant state.

7.3 Molecular Mechanisms Mediating the Transition from Tumor Dormancy to Metastatic Growth

Here we focus on three mechanisms that have been proposed to regulate the dormancy of cancer cells disseminated to secondary organs (1) cellular dormancy, (2) dormancy regulated by a pre-angiogenic state, and (3) dormancy maintained by aspects of the immune system. Enhanced knowledge about all of these mechanisms will be necessary in order to exploit these mechanisms for new therapeutic strategies.

7.3.1 Cellular Dormancy

Mechanisms underlying the reactivation of quiescent DTCs (cellular dormancy) are still largely unknown. Yet several reports in the literature demonstrate that reciprocal interactions between DTCs and their surrounding microenvironment can lead to intracellular signaling in the tumor cells that will reactive their proliferation. We and others have demonstrated that integrin beta 1 (Intβ1) activation is a key regulator in the switch from cellular dormancy to metastatic growth in vitro and in vivo [21, 22, 28, 29]. In vitro studies used a 3D culture system, constituted from growth factor-reduced basement membrane (BME), to model dormancy, and found that dormant vs. proliferative behavior in this model mimicked the dormant vs. metastatic

behavior of multiple cell lines in vivo [21]. Using this 3D system, it was demonstrated that supplementation of the BME with either fibronectin or type I collagen induces Intβ1 downstream signaling [21, 22], leading to activation of focal adhesion kinase (FAK) by Src. This activation results in downstream activation of the extracellular signal regulated kinase (ERK), a key regulator in cell cycle and cytoskeletal reorganization. ERK in turn induces phosphorylation of myosin light chain (MLC) by myosin light chain kinase (MLCK), culminating in f-actin stress fiber organization, followed by translocation of cyclin-dependent kinase inhibitor p27 to the cytoplasm [21, 22]. The following induced cascade culminates in the transition from dormancy (quiescence) to proliferation. Additionally, previous studies in head and neck and breast cancer cells demonstrated that high uPAR expression induces α5β1 integrin and in turn this complex recruited EGFR and FAK, which in a fibronectin-dependent manner induces sustained ERK activation [30]. Hence, Intβ1 plays an important role in the cross talk between disseminated tumor cells and their microenvironment. Furthermore, the activation of Intβ1 was dependent on the remodeled ECM enriched with fibronectin and or Type I collagen (Col-I) reminiscent of a fibrotic/desmoplastic microenvironment (Figs. 7.2 and 7.3). Thus, the establishment of a permissive microenvironment is required to promote the outbreak of dormant DTC along their ability to engage with it.

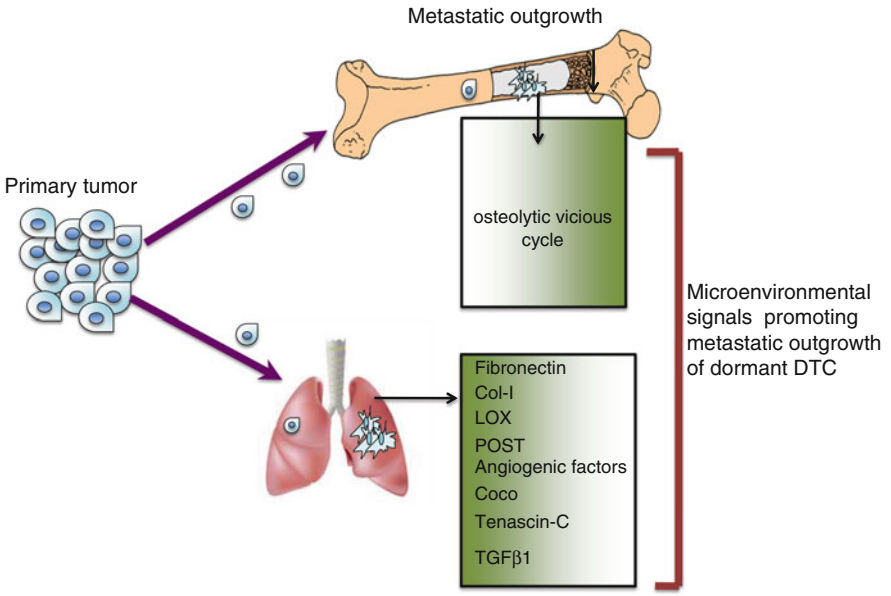

Fig. 7.2 Microenvironmental factors regulating the metastatic outgrowth of quiescent and pre-angiogenic DTCs. ECM remodeling and release of its bioactive factors are key microenvironmental signals promoting the emergence of DTCs from tumor dormancy to metastatic growth. ECM remodeling occurs during the vicious osteolytic cycle in the BM and upon establishment of a desmoplastic/fibrotic like microenvironment in the lung characterized by increased Col-I expression and its cross linking by LOX, formation fibronectin fibrils and release of ECM factors such as TGFβ1, POST, and pro-angiogenic factors

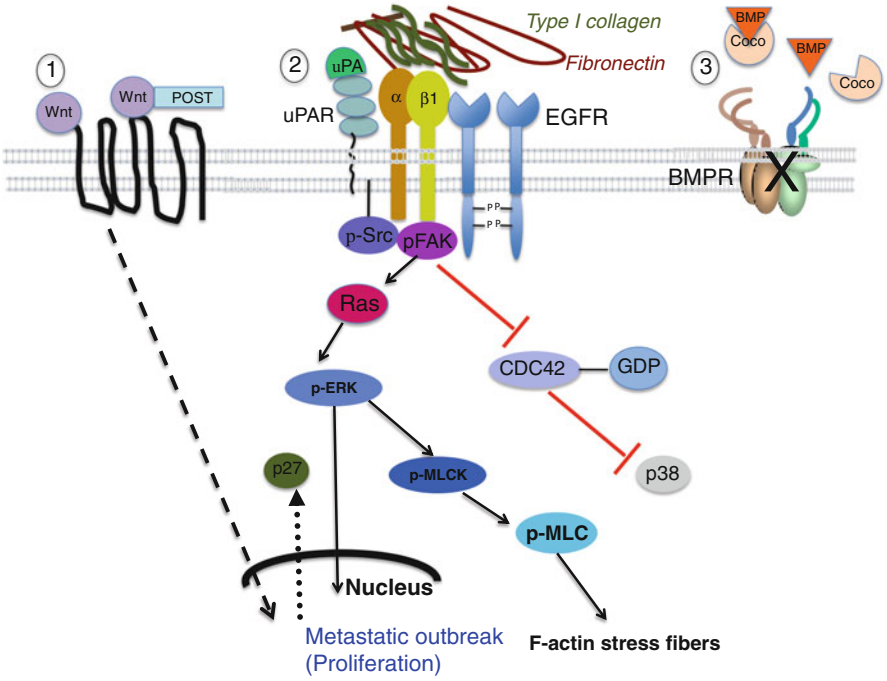

Fig. 7.3 Mechanisms leading to metastatic outgrowth of dormant DTC in the lungs. (*1*) Stroma-derived POST interacts and thus recruits WNT ligands activating the WNT signaling pathway. (*2*) β1-integrin activation through fibronectin/type I collagen and/or uPAR initiates downstream signaling via Src and FAK, inducing high ERK/p38 ratio which in turn activates MLCK leading to cytoskeletal reorganization and metastatic growth. (*3*) COCO derived from DTCs can antagonize the inhibitory effect of BMP-4

Indeed, we recently demonstrated that induction of fibrosis at the metastatic site such as the lung, by either the residing DTCs or by exogenous intervention, switches dormant tumor cells to metastatic growth in the lungs ([22]; reviewed in Ref. [23]; Figs. 7.2 and 7.3). Furthermore Cox et al. demonstrated that Lysyl oxidase cross-linking of Col-I in a fibrotic lung enhanced the outgrowth of DTCs [61]. Accordingly, matrix stiffening is induced by increased Col-I deposition and cross-linking and has been shown previously to promote malignant transformation and progression [62, 63] and was shown recently to regulate tumor dormancy [64]. Therefore, changes in the mechanical compliance of the matrix along with the biochemical composition that can occur as a consequence of therapy and or aging of tissue (reviewed in Ref. [23]) can promote permissive microenvironments that can support transition of dormant DTCs to metastatic growth. Importantly, dormant DTCs can emerge from their dormant state even in a restrictive microenvironment such as the lungs (Fig. 7.2). A report by Gao et al. demonstrated that dormant 4T07 breast cancer cells overexpressing Coco, an antagonist of transforming growth factor beta (TGF-β) ligands, transitioned from dormancy to metastatic growth in the lungs [65]. Coco

blocked the binding of microenvironmental BMP4 ligands to the BMP receptor on the cancer cells, thus overriding the restrictive cues produced by the lung microenvironment. In contrast, blocking endogenous expression of Coco in counterpart metastatic 4T1 breast cancer cells, induced tumor dormancy [65]. Along these lines, suppressive cues imposing dormancy of DTCs residing in stable microvasculature such as in the lung and BM are lost in sprouting neovasculature. Ghajar, Bissell, and colleagues demonstrated that in the sprouting neovasculature, the expression of tumor suppressive factors such as TSP1 is diminished, and conversely enriched with expression of tumor promoting factors such as TGFβ1 and the extracellular protein periostin (POST), thus instigating the outbreak of otherwise dormant breast tumor cells [66]. Establishment of a supportive niche in the BM for metastatic outgrowth of indolent breast tumors is fostered by increased local osteoclast activity. Lu et al. demonstrated that elevated expression of VCAM-1 on dormant breast tumor cells allowed dormant tumor cells to interact with osteoclasts, yielding paracrine signals and enhancing osteolytic metastatic growth [67]. Hence, establishment of a permissive microenvironment is required to support reactivation of the dormant tumor cells Fig. 7.2.

The microenvironment at the metastatic niche may also promote the residing dormant tumor cells to acquire a tumorigenic capacity by converting them to cancer stem cell like cells (CSC) (reviewed in Ref. [68]). Increasing evidence indicates that the tumor cells that initiate metastatic outgrowth possess several attributes of cancer stem cells (reviewed in Ref. [68]). Tenascin C, which is often found in stem cell niches, supports the outgrowth of breast cancer cell colonizing the lungs by elevating both Notch and Wnt signaling [69]. Furthermore, Malanchi and colleagues recently demonstrated that only tumor cell with CSC like properties will colonize the lungs and expand to form metastatic lesions [70]. These CSC induced the stromal cells in the lungs to express POST, a stromal factor of normal stem cell niches. POST expression in the resulting niche environment supported the growth of metastases by promoting Wnt signaling (Fig. 7.3). Hence, components of the metastatic niche may induce or maintain properties associated with stemness of DTCs. Importantly, DTCs can obtain a stemness phenotype at the metastatic site upon their loss of an epithelial phenotype and acquisition of a mesenchymal phenotype (epithelial–mesenchymal transition; EMT) [71]. Indeed, EMT has been shown recently, in addition to endow DTCs with CSC properties, to directly promote the outbreak of otherwise dormant tumor cells by increasing Intβ1 expression necessary for metastatic outgrowth [72] (Fig. 7.3).

7.3.2 Pre-angiogenic Dormancy

Cancer growth requires an expanding blood circulation to support continued growth, both for the primary tumor as well as metastases (reviews in Refs. [73, 74]). Angiogenesis, the growth of new blood vessels, depends on a balance between pro-angiogenic and antiangiogenic molecular stimuli. Angiogenesis has thus been seen

as a target for anticancer therapy, and the complexities of this approach are well recognized [75]. Angiogenesis has been shown to play a role in regulating cancer growth and dormancy. Folkman and colleagues documented that antiangiogenic factors secreted by a primary tumor could restrict distant micrometastatic growth, holding the metastases in an "active" state of functional dormancy in which cell division and apoptosis were balanced, with no net increase in metastatic tumor size [11, 76]. Tumors in a state of pre-angiogenic dormancy thus are distinct from quiescent, dormant tumor cells, and consequently may present a distinct therapeutic target [10]. Antiangiogenic therapies thus have the potential to inhibit tumor growth (at the primary or metastatic sites), and also to maintain pre-angiogenic micrometastases in a functionally dormant, non-expanding state.

Recent work from Naumov and colleagues have shown, in mouse models of primary tumor growth, that the angiogenic phenotype may be plastic and regulatable, raising hopes for development of agents that could revert vascularized metastases to a pre-angiogenic, non-growing state [77]. Along these lines, Almog and colleagues recently identified a set of 19 small noncoding RNA molecules (miRNAs) that control the phenotypic switch of human dormant breast carcinoma, glioblastoma, osteosarcoma, and liposarcoma tumors to exponential growth [78]. Downregulation of 16 of the highly expressed miRNAs correlated with the switch of dormant tumor to the fast-growing angiogenic tumor. Moreover, reconstitution of miR-580, 588, or 190 promoted prolonged tumor dormancy of otherwise actively proliferation angiogenic tumors. Hence, metastasis may potentially be maintained long-term in a pre-angiogenic dormant state by antiangiogenic therapy as was demonstrated previously [79] and as was predicted recently by the mathematical modeling by Benzekry et al. [80].

7.3.3 Dormancy Regulated by the Innate and Adaptive Immune System

Micrometastatic dormancy is characterized by active equilibrium between proliferation and apoptosis. This equilibrium was suggested to be regulated by immune surveillance in addition to the angiogenic switch [81]. In a mouse model of melanoma, the outgrowth of early DTCs at distant sites was controlled partially by CD8+ T cells. CD8+ T cells inhibited the growth of disseminated tumor cells, surprisingly, not by cytotoxic effects, but through cytostatic effects and their depletion led in turn to the emergence of DTCs from their dormant state [56]. Accordingly, recent reports demonstrated the role of T-lymphocytes as regulator of tumor dormancy [82] and active suppression of T cells by IFN-γ or IL-12 blocking induces escape from dormancy (reviewed in Ref. [83]).

In the DA1-3b mouse model of acute myeloid leukemia, dormant tumor cells were resistant to cytotoxic lymphocytes (CTL) by overexpressing B7-H1 and B7.1. B7-H1 interacts with programmed death-1 (PD-1) expressed on T cells, and inhibits T-cell activation and CTL-mediated lysis [84]. Hence, dormant tumor cells may become more resistant to specific CTL mediated killing. Indeed, recent reports have

demonstrated that PDL-1 (the ligand of PD-1) was upregulated in irradiated tissue. In contrast, administration of anti-PD-L1 enhanced the efficacy of ionizing irradiation (IR) through a CTL-dependent mechanisms leading to antitumor immunity in mice [85, 86]. Along these lines, methylation of suppressor of cytokine signaling (SOCS1) and its downregulation in dormant tumor cells was reported to deregulate JAK/STAT pathways within the dormant tumor cells, thus promoting resistance to CTL-mediated killing [87]. Hence, inhibition of T-lymphocytes and preventing the resistance of the dormant tumor cells to CTL mediated killing may be the mechanisms accounting for the escape of the dormant tumor cells from the immune response.

Overall, the studies described above in several animal models of tumor dormancy support the potential role of the immune system in keeping the micrometastases indolent for prolonged periods of time. However, controversies exist regarding the role of the immune system in regulating tumor dormancy in the clinical settings (reviewed in Ref. [88]). Furthermore, a recent report by Magnus et al. adds another complexity to the role of the immune system in regulating tumor dormancy [89]. They demonstrated that expression of Tissue Factor in indolent human glioma cells led to a stepwise transition of dormant tumor cells to metastatic outgrowth, a process that was preceded by recruitment of vascular (CD105+) and myeloid (CD11b+ and F4/80+) cells, thus demonstrating that the immune system might actually augment an escape of tumor cells from dormancy.

7.4 Conclusions of Metastatic Tumor Dormancy as a Clinical Target

Metastasis continues to be responsible for the majority of cancer deaths, in spite of our enhanced understanding of tumor biology. When cancer is detected early, before it has spread, it is more likely to be successfully treated, while metastatic disease is considerably more difficult to treat. Compounding this difficulty is the ability of an apparently successfully treated cancer to recur, sometimes years or decades later, following a protracted period of tumor dormancy. Here we consider some of the clinical and biological issues about tumor dormancy, and our relatively limited understanding of how dormancy may be regulated.

An increase in recent years in studies on mechanisms contributing to regulation of tumor dormancy is providing a growing wealth of information about dormancy. Clearly, tumor dormancy is a complex and multifaceted problem, and we have much to learn about how dormancy arises and persists, as well as how cancer cells can be released from dormancy and reinitiate growth. The fact that cancer can be naturally maintained in a state of dormancy gives hope that these processes can be studied and utilized in future therapies. However, the complexity of factors that contribute to dormancy and release from dormancy will make this approach challenging. Here we outline some of the factors that have been identified as contributers to tumor

dormancy, and thus suggesting ways to either maintain cancer in a dormant state or kill dormant cancer cells. It is clear that many aspects of the tissue microenvironment surrounding dormant metastatic disease contribute to the dormant phenotype. Potential therapeutic approaches to prevent dormant cancer cells from reinitiating growth include blocking microenvironmental signals that promote tumor growth, inhibiting angiogenic stimulation of micrometastatic growth, and enhancing immune regulation of dormancy. We have much to learn about dormancy and its regulation, but models are becoming increasingly available for experimental study. Additionally, there is a growing recognition that we need to learn much more about dormancy in patients. Which patients harbor dormant cells, and which patients can be considered cured of their disease? In patients who do have persistent cancer cells, what factors—either inherent to the tumor cell or modifiable factors in the patient—contribute to maintenance of dormancy vs. reinitiation of tumor growth? In order to address the clinical problem of tumor dormancy, we need continued and enhanced experimental efforts to understand the biology of tumor dormancy, coupled with increased understanding of the clinical status of disseminated disease in patients. This enhanced knowledge is crucial to improve the survival of cancer patients.

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Chapter 8
Genesis of Circulating Tumor Cells Through Epithelial–Mesenchymal Transition as a Mechanism for Distant Dissemination

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Abstract Epithelial–mesenchymal transition (EMT), a developmental process through which epithelial cells lose their characteristic apicobasal polarity and acquire the morphology of solitary migratory cells, has been implicated in the progression of carcinoma. EMT may contribute to the formation of cancer stem cells, evasion of immune surveillance, and induction of resistance to chemotherapeutics and targeted therapeutics. Metastasis is governed by a complex set of processes that are far from being fully understood and difficult to recapitulate through the current suite of

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in vitro experimentations. Circulating tumor cells (CTCs) in the peripheral blood have received much attention recently, as they may represent the first critical stage of cancer dissemination and their prevalence in metastatic patients is associated with worse prognosis. CTCs exhibit significant phenotypic heterogeneity across the EMT spectrum and preliminary studies have prompted the need to unravel the mechanisms by which CTCs are generated and how this diversity is attained in primary tumors. As such, improved methodologies are required to exhaustively characterize the full spectrum of CTC phenotypes and to identify the clonogenic cells. An understanding of the EMT phenotypes in CTCs should help in the design of more appropriate targeted therapeutics to abrogate the malignant potential of CTCs.

Keywords Epithelial–mesenchymal transition • Circulating tumor cells • Phenotype

Abbreviations

ALDH1	Aldehyde dehydrogenase-1
BMP	Bone morphogenetic protein
CEpC	Circulating epithelial cells
CGH	Comparative genomic hybridization
CK	Pan-cytokeratin
CTC	Circulating tumor cells
CTLs	Cytotoxic T lymphocytes
DEP	Dielectrophoresis
DTC	Disseminating tumor cells
E-cad	E-cadherin
ECM	Extracellular matrix
EGF	Epithelial growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial–mesenchymal transition
EMTed	Epithelial–mesenchymal transitioned
EpCAM	Epithelial cell adhesion molecule
FACS	Fluorescence-activated cell sorting
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FISH	Fluorescence in situ hybridization
Gsk	Glycogen synthase kinase
H & E	Hematoxylin and eosin
HDAC	Histone deacetylases
HER2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor

HMLE	Human mammary epithelial cell line
HMLR	Ras-transformed human mammary cells
ILC	Invasive lobular carcinoma
IVVM	Intravital videomicroscopy
JNK	Janus kinase
KLF4	Kruppel-like family member 4
LOXL2	Lysyl oxidase-like 2
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby Canine Kidney
MET	Mesenchymal–epithelial transition
MMPs	Matrix metalloproteases
MSCs	Mesenchymal stem cells
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PI3K	Phosphoinositide 3-kinase
PRC	Polycomb repressor complex
PTEN	Phosphatase and tensin homolog
RBCs	Red blood cells
RTK	Receptor tyrosine kinase
RT-PCR	Reverse-transcriptase polymerase chain reaction
SH2	Src-homology 2
TGFβ	Transforming growth factor
TMEM	Tumor microenvironment of metastasis
TNF	Tumor necrosis factor
TRAF	TNF receptor associated factor
Vim	Vimentin
WBCs	White blood cells
WISP2	Wnt1-inducible signaling pathway protein 2
ZO-1	Zonula occludens-1

8.1 Overview of Epithelial–Mesenchymal Transition

8.1.1 Epithelial–Mesenchymal Transition in Development

Epithelial–mesenchymal transition (EMT) is a unique developmental process of fundamental importance in morphogenesis and organogenesis in metazoans [1, 2]. EMT is one of the most highly conserved mechanisms throughout evolution generating mesenchymal cells from an epithelial layer. EMT operates in gastrulation of numerous invertebrate and vertebrate embryos leading to the formation of the three primary germ layers ectoderm, mesoderm, and endoderm. Although the morphogenetic movements operating during gastrulation differ among species, the general principles to induce the delamination of cells from an epithelial layer still apply.

These mechanisms are initiated at precise embryonic locations, including the ventral furrow in *Drosophila*, the vegetal pole in sea urchin, and the primitive streak in chicken, mouse, and human embryos. The sequence of events leading to the EMT of a defined subset of epithelial cells has been partially uncovered. In *Drosophila*, signaling is activated through Spatzle, the ligand of the Toll receptor, which induces Twist expression, a basic loop helix transcription factor and a much-studied master gene in EMT. Snail, another transcription factor controlling EMT, is also induced in the ventral furrow and, in cooperation with Twist, contributes to the execution of the EMT program, including the apical constriction of invaginating epithelial cells from the blastoderm and their subsequent delamination. In mammals, EMT in gastrulation is induced through the localized production of growth factors, including members of the fibroblast growth factor (FGF), transforming growth factor (TGFβ) and Wnt families. These distinct signaling systems will contribute to the downregulation of E-cadherin in part through the repression of its transcription by Snail1. The neural crest, a unique, transient neural-ectoderm structure of vertebrates, undergoes EMT before engaging into migration to populate distinct territories in within the embryo and before further differentiating into melanocytes, neurons, and glia of the peripheral nervous system. Multiple signaling pathways have been described to participate in the induction of neural crest progenitors and EMT, initiating with opposed gradients of FGF and retinoic acid. Subsequent steps in the transduction pathways involve Snail expression, which is itself controlled by Sox9, bone morphogenetic protein (BMP) 4-Noggin opposed gradients, and Wnt1. Additionally, Wnt1 controls the G1/S transition in the cell cycle through β-catenin and cyclin D1. Proteolysis of neuronal (N)-cadherin, a protein involved in calcium-dependent cell–cell adhesions, by gamma secretase will contribute to the activation of β-catenin cotranscriptional activity. Most interestingly, Sox9 and Snail2 overexpression can promote EMT ectopically throughout the neural tube. Finally, the epigenetic mechanisms mediated by Jumonji2A, a histone-demethylase, also operate to control the expression of Snail and Sox10, a SoxE family member that maintains the undifferentiated state of neural crest progenitors.

Heart morphogenesis is also remarkably controlled by four successive waves of EMT and Mesenchymal–Epithelial Transition (MET). One of the EMT steps involves the delamination of endothelial cells to form the cardiac cushion at the origin of the atrioventricular valves and thus is designated “end EMT.” This process is of the upmost importance in cardiac development and involves TGF-β members and the Notch–Jagged interaction. Furthermore, dysregulation of these pathways leads to cardiac valve malformations.

Multiple pathways are involved at different stages of embryonic development. In all cases, transcriptional regulators are involved in the control of intercellular adhesion and cytoskeletal remodeling. Multiple inducers and epigenetic controls refine the spatiotemporal regulation of EMT that targets initially apicobasal cell polarity. The creation of polarized mesenchymal cells in the subsequent steps includes the downregulation of junctional complexes, extensive remodeling of the cytoskeleton, and activation of cell–substrate adhesion.

8.2 Epithelial–Mesenchymal Transition in Tumor Progression

Carcinomas forming the vast majority of all tumors derive exclusively from the epithelial lining of tissues and organs. Following a long latency period, epithelial cells exposed to mutagens progressively lose their apicobasal polarity and growth control, leading to the formation of adenoma. The transition to in situ carcinoma, however, occurs with subsequent oncogenic events. In situ carcinomas are clinically manageable through surgery, since the malignant cells remain confined within a basement membrane. The most critical event is the transition from in situ carcinoma to the micro-invasive phase, during which some carcinoma cells are exposed to a stromal niche [3]. In situ carcinoma surrounding the stroma differs from those of normal tissues and organs with the presence of reactive myofibroblasts, inflammatory cells, neoangiogenic blood vessels, and new extracellular matrix (ECM) components. The formation of the primary, cancer-associated niche is a long process initiated at a very early stage during the acquisition of the first mutations in the epithelial lining. Once reaching the reactive stroma, invasive carcinoma cells become engage in a reciprocal interaction with resident macrophages, inflammatory cells and other stromal cells so as to increase their invasive behavior and to intravasate into blood vessels. Until recently, it was very difficult to capture the intravasation of carcinoma cells in tumor specimens using classical histopathological stains; now, it is feasible to identify breast carcinoma cells in close proximity to endothelial cells and macrophages, designated the "tumor microenvironment of metastasis" (TMEM) [4]. However, this has limited prognostic value, as the identification of carcinoma cells interacting with endothelial cells and macrophage has only been established for ER-positive tumors [5].

Once in the blood or lymph, circulating tumor cells (CTCs) are passively transported to distant sites. CTCs can be found as single cells or as aggregates, designated microemboli, with or without adherent platelets [6]. In 1975, the number of carcinoma cells released per gram of tumor was estimated as one million per day [7]; however, this number seems quite overestimated, based on the enumeration of CTCs and their expected half-life in the blood [8, 9]. Furthermore, the time of residence in the blood is still a matter of debate, estimated to be several hours to several days [8]. Experimental models indicate that more than 80 % of injected cells can reach a distant organ, such as the liver, and studies show that the majority of carcinoma cells can then reside for an extended period in the parenchyma; however, only a very small proportion will give rise to clinically detectable metastases while others will remain dormant for years [8, 10]. Breast cancer autopsies led to the designation of organ site-specific metastasis and the theory of the "seed and soil," a theory of metastasis posited in 1889 that suggests that a tumor cell—the seed—either remains dormant or thrives within the specific microenvironment now designated pre-metastatic niche of a certain organ—the soil [11, 12].

The final localization of these CTCs will depend on multiple factors, including the presence of fenestrated vessels, such as those found in the bone marrow, but also active processes that are driven by chemokines and other signaling systems operating in the pre-metastatic niche [3, 13].

Over 10 years ago, the hypothesis was put forward that carcinoma cells can "hijack" these EMT and MET developmental processes during the metastatic cascade [14] (Fig. 8.1). However, not all carcinoma cells will acquire a full-blown transitioned (EMTed) phenotype but rather will exhibit a phenotype reminiscent of one of various intermediate EMT stages [15, 16], which confers different characteristics as compared to full epithelial or mesenchymal stages. Some intermediate stages may be metastable [17]; this has been observed in numerous carcinoma cells that exhibit considerable heterogeneity in their morphology, motility, and clonogenicity, as recently described for the adenocarcinoma cell line, A549 [16, 17].

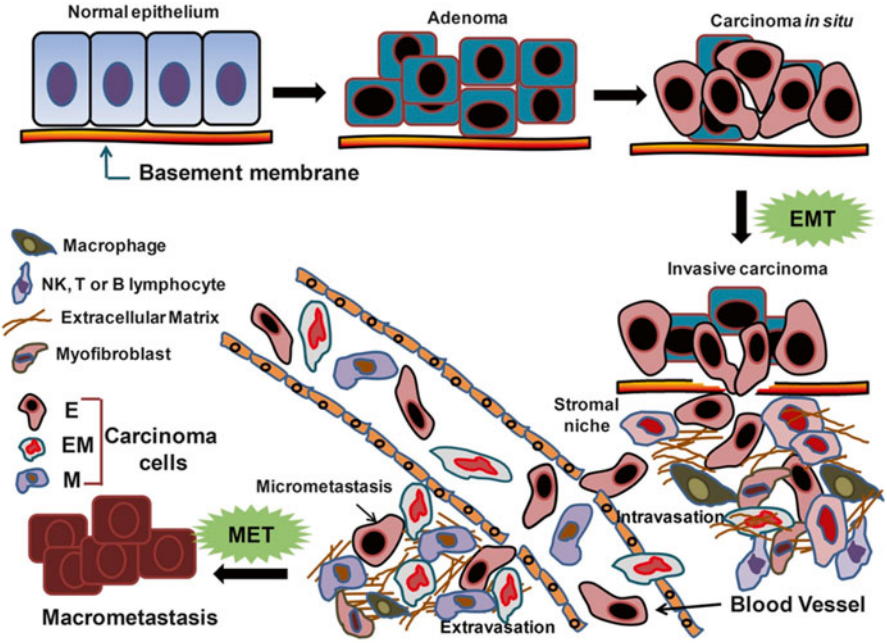

Fig. 8.1 The epithelial–mesenchymal/mesenchymal–epithelial (EMT/MET) hypothesis for carcinoma cell dissemination. Adenoma cells lose their apicobasal polarity and growth control and undergo a progressive remodeling of intercellular junctional complexes in in situ carcinoma. Some carcinoma cells undergo an EMT process leading to their progressive delamination from the surrounding carcinoma cells. Dissociated carcinoma cells invade the stroma through continuous reciprocal interactions. Some of the carcinoma cells encounter and intravasate into small vessels, often derived from an angiogenic or lymphangiogenic process. Circulating carcinoma cells, designated Circulating Tumor Cells (CTCs), can arrest in distant organs and eventually extravasate to form micrometastases. Localized micrometastatic cells can resume growth following a MET process

8.2.1 Inducers of EMT

The seminal discovery that polarized, epithelial Madin-Darby Canine Kidney (MDCK) cells transformed into migratory, mesenchymal-like cells in the presence of a conditioned medium from cultured fibroblasts led the way to uncovering one of the first mechanisms of EMT induction [18]. The subsequent identification of scatter factor (SF) as hepatocyte growth factor (HGF) in the fibroblast-conditioned medium triggered numerous investigations into c-Met receptor, its cognate tyrosine kinase surface receptor [19]. Around a similar time, HGF and other growth factors, such as FGF-1, epithelial growth factor (EGF), and TGF-α were uncovered to induce EMT in a bladder carcinoma line [20–22]. These signaling pathways were found to synergize with the activity of integrin alpha 2-beta to promote EMT and invasion [23]. It is clear that epithelial-like carcinoma lines of different origins can respond to distinct inducers [2]. Some carcinoma cell lines are found to respond more acutely to TGFβR signaling for EMT engagement whereas others may require Wnt signaling, inflammatory cytokines, and/or hypoxic conditions [24].

8.2.2 Signaling During EMT Execution

A plethora of pathways triggered by extracellular signals are now shown to be involved in EMT induction and maintenance of the mesenchymal state, which requires cooperation among several pathways. These pathways include canonical Ras and PI3K pathways resulting from receptor tyrosine kinase (RTK) activation. TGFβR activation can generate canonical, regulatory Smad phosphorylation and noncanonical pathways that trigger the loss of epithelial cell polarity through the ubiquitination of RhoA by Smurf1, which is itself activated by TGFβR-induced Par6 phosphorylation. Other TGFβR noncanonical signaling pathways lead to actin cytoskeleton remodeling through the activation of the small GTPases Rho, Rac, and Cdc42. In addition, the binding of tumor necrosis factor (TNF) receptor associated factor (TRAF)-6 to the TGFβR will lead to activation of TGFβ-activated kinase 1 (TAK1), which, in turn, activates P38 mitogen-activated protein kinase (MAPK) and Janus kinase (JNK) pathways. TGFβR can also activate the phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathway. These signal transduction pathways are also activated by RTKs and, to some extent, by integrins, emphasizing the entwined complexity of the networks that could ultimately induce EMT [24–28].

Activation of RTKs, TGFβR and Wnt receptors, among other receptors, will converge to induce the master transcriptional regulators of EMT. The proteins most commonly shown to be able to activate EMT on their own in epithelial cells include the Zinc finger proteins, Snail1 and Snail2, the zinc-finger E-box binding homeobox proteins, Zeb1 and Zeb2, the basic loop helix transcription factor, E12/47, the zinc-finger Kruppel-like family member 4 (KLF4), and the T-box protein, Brachyury. All

of these factors repress the E-cadherin gene through their binding to E-boxes in its proximal promoter. Other transcription factors are also documented to induce EMT, the most notorious one being the basic loop helix factor, Twist1. In addition to transcriptional repression of epithelial genes such as E-cadherin and tight junction proteins, epigenetic controls contribute to and stabilize the repressed state of these genes. This repression is ensured by histone deacetylases (HDAC)-1, -2, -3 and SIRT1 by methyltransferases (EZH2, SUZ12, and SUV39H1) of the polycomb repressor complex (PRC) 2; these proteins can cooperate with BMI1, a member of PRC1. For instance, Snail1 can recruit EZH2 and SUZ12 to trimethylate Histone3K27 in the vicinity of Snai1 when bound to its E-box DNA cognate site; this is followed by an additional interaction with PRC1 chromodomain proteins together with BMI1. HDAC1 and HDAC2 can associate with NuRD or Sin3a complex [29, 30], which bind in the vicinity of Snail1 to repress chromatin through histone H3K9 and K14 deacetylation. G9a, another methyltransferase, and LSD1, a Histone demethylase, are also involved in establishing histone marks. G9a dimethylates H3K9, which is subsequently trimethylated by Suv39h1, leading to heterochromatization that is reinforced by additional CpG island methylation by DNA methyltransferases [31, 32].

Other control mechanisms for EMT implicate the involvement of microRNA, particularly the Mir-200 family members and Mir-205, which form a negative regulatory loop with Zeb masters genes. Mir-200 family members can also antagonize SIRT1 and SUZ12. Recent studies provide additional evidence for complex regulatory networks in the control of EMT. Direct transcriptional control by various master genes is clearly not sufficient to establish graded levels of EMT and to confer plasticity of the epithelial phenotype. As shown above for E-cadherin, epigenetics plays a central role in setting the EMT spectrum but noncoding RNA can create additional regulatory loops showing exquisite sensitivity [33].

Finally, the half-life of these master genes can be regulated by posttranslational modifications. Snail1 can be phosphorylated by glycogen synthase kinase (Gsk)-3β and subsequently ubiquitinated and degraded by the proteasome, whereas Pak1 phosphorylation and lysine oxidation by lysyl oxidase-like 2 (LOXL2) can stabilize the nuclear localization of Snail1 [34].

8.2.3 Molecular Alterations of Epithelial Cells in Transition toward a Mesenchymal Phenotype

Different markers of EMT have been used to provide evidence for the existence of morphological transitions. These markers were initially uncovered in vitro through the establishment of EMT in normal epithelial cells or during early embryonic development. One must be cautious in considering these markers when analyzing EMT in carcinoma, since most carcinoma may have already engaged into some of the EMT programs, such as the loss of cell polarity. In normal epithelial cells, the

first event in EMT is the loss of cell polarity, implicating the downregulation of the Crumbs3, Pals1, and Patj polarity complex. This initial event directly impacts the stability of tight junctions, which directly interact with the polarity complex. Tight junction components, such as claudins, occludins, and zonula occludens-1 (ZO-1; one of the cytoplasmic partners) are also rapidly downregulated in the early phase of this morphological transition. Of note, studies have shown that growth control is also much affected, since the Hippo-Yap pathway function is intimately linked to the epithelial cell polarity status [35–37].

Another most profound change concerns the adhesive properties of epithelial cells. The expression of E-cadherin, the prototypic epithelial cell adhesion molecule, is progressively lost and is substituted by N-cadherin or even by much weaker intercellular type-2 cadherins such as cadherin-7 or -11 [38]. Desmosomes are disassembled during EMT induction, implicating the loss of the desmosomal cadherins—desmogleins and desmocollins. The cytokeratin network anchored to desmosomal desmoplakins in epithelial cells collapses around the nucleus and is replaced by vimentin intermediate filaments. Actin microfilaments are profoundly remodeled and shift their localization from the cortex to form cytoplasmic and basal networks [28, 39]. The transition to a mesenchymal state is also accompanied by the production of ECM components such as collagens, fibronectin, and matrix metalloproteases (MMPs). Thus, the EMT program sets the stage for cells to migrate and invade extracellular matrices.

8.2.4 Protection from Anoikis, from Senescence and Acquisition of Stem Cell-Like Properties

Anoikis is defined as programmed cell death that is engaged during epithelial cell detachment from the ECM. Integrins, the main receptors for ECM components, are therefore considered to be essential for anoikis resistance. Epithelial cells interacting with the ECM can resist anoikis through FAK binding to the cytoplasmic domain of integrin subunits. FAK is autophosphorylated on Y397, which allows for the recruitment of Src kinase; in turn, Src kinase phosphorylates FAK on Y925, creating a Src-homology 2 (SH2) binding site for activation of the MAPK pathway. This initial cooperation between FAK and Src leads to the activation of the Akt and MAPK pathways, both inactivating pro-apoptotic proteins by phosphorylation [40]. Anoikis can be circumvented by changing the integrin expression profile as to adapt to different environments during invasion at metastatic sites, since some integrin heterodimers are more suited for enhancing the anti-apoptotic program. Such is the case for skin squamous carcinoma, where αvβ5 integrin is substituted by αvβ6 integrin [41]. Some integrins such as αvβ3 have more ambiguous functions either in promoting or abrogating anoikis [40]. Aside from integrin activation or in cooperation with integrin signaling, multiple other pathways involved in EMT can protect carcinoma from anoikis. Numerous surface receptors stimulated by ligands produced by tumor stromal cells can induce EMT and anoikis resistance. For instance,

the neurotrophic tyrosine kinase receptor, TrkB, once activated mediates EMT through MAPK activation with the expression of downstream transcriptional repressors Twist Snail1 and Zeb1, whereas anoikis is mediated by PI3K-AKT signaling [42].

Intercellular cadherin-mediated adhesion is also essential for epithelial survival. The abrogation of E-cadherin adhesion either through antibodies directed against E-cadherin or inhibition of translation by shRNA leads to apoptosis. However, Ras-transformed human mammary cells (HMLR) become refractory to anoikis following the downregulation in E-cadherin. These E-cadherin-negative cells express Zeb1 and Twist1, resulting in an EMT phenotype that promotes invasion and metastasis [43]. In addition, studies show that the targeted knockout of *E-cadherin* in a p53-null mouse mammary tumor model leads to the formation of invasive adenocarcinoma, invasive lobular carcinoma (ILC), and sarcomatoid carcinoma. In particular, metastases were found in animals bearing ILC, and anoikis resistance was found in the E-cadherin-depleted carcinoma cells derived from the primary tumors when cultured on non-adhesive substrates [44]. In another study, a large collection of ovarian carcinoma cell lines were allocated to one of four phenotypes using E-cadherin (E-cad), pan-cytokeratin (CK), and Vimentin (Vim) antibodies: epithelial ($E\text{-cad}^+/CK^+/Vim^-$), intermediate epithelial ($E\text{-cad}^+/CK^+/Vim^+$), intermediate mesenchymal ($E\text{-cad}^-/CK^+/Vim^+$), or mesenchymal ($E\text{-cad}^-/CK^-/Vim^+$) [16]. Each of these four phenotypes showed distinct patterns of expression of epithelial- and mesenchymal-specific genes, including the classical EMT drivers. Remarkably, the viability index of the different cell lines grown in suspension in ultralow adhesive substrates showed that intermediate mesenchymal phenotypes were more anoikis-resistant than their intermediate epithelial counterpart. A Src inhibitor could restore partial anoikis sensitivity through E-cadherin re-expression.

Another mechanism driving anoikis in normal epithelial cells is mediated by the activation of the Hippo pathway [45]. Cell detachment provokes a rapid depolymerization of actin microfilaments thus liberating the Hippo kinase Lats1/2, which inactivates YAP nuclear translocation by phosphorylation. However, the loss of E-cadherin expression, a hallmark of EMT, alters apicobasal polarity and thus disrupts the Hippo phosphatase and kinase signaling system, which is itself intimately regulated by the polarity Crumbs and Par3/Par6 complexes as well by tight and adherens junctions [35]. In addition, inactive phospho-YAP/TAZ sequestered by adherens junctions can be released in the cytoplasm to promote survival, and this possibly is mediated through MAPK activation [46]. The role of Yap in promoting anoikis resistance becomes more predominant in transformed cells, since the Hippo kinases are downregulated [45]. This pathway has been shown to interact with several other pathways, including the Wnt and TGFβR pathways. The loss of epithelial cell polarity, an early step during transformation, releases non-phosphorylated YAP/TAZ together with Smad3, which then can activate the transcription of genes affecting proliferation and morphological changes associated with EMT. The canonical Wnt pathway further promotes these phenotypes by allowing the translocation of β-catenin into the nucleus [47] and releasing sequestered Yap from the β-catenin destruction complex [48].

Clearly, one of dominant mechanisms linking EMT and anoikis include oncogenic kinases, such as Ras, and the loss of tumor suppressors, such as Phosphatase and tensin homolog (PTEN), leading a strong AKT activation [40]. However, oxidative stress is now to be considered to be central in tumor progression and EMT [49]. These mechanisms implicate multiple enzymatic systems generating oxygen free radicals and hydrogen peroxide. Src, one of the targets of reactive oxygen species (ROS), can, in cooperation with EGFR, promote anoikis resistance in carcinoma cells. Hypoxia, a very predominant mechanism operating during tumor growth, is able to induce EMT and anoikis resistance through the classical Snail, Zeb, and Twist as well as through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB, another driver of EMT, is a central player in promoting anoikis resistance through Akt activation, as it increases the expression of anti-apoptotic proteins and represses the expression of pro-apoptotic proteins. Hypoxia can also trigger autophagy and rescue cells from anoikis [40].

Recent studies have revealed a complex network of miR in EMT and anoikis. The discovery that miR-200 family members establish a double regulatory negative feedback loop with Zeb1 and Zeb2 offers new opportunities to understand how to regulate the EMT status of cells [33, 50, 51]. A number of experiments have shown that miR-200 family members are direct targets of p53, which can sensitize cells to anoikis [52, 53]. In the absence of p53, cells can be rendered anoikis-resistant by restoring Zeb expression and that of other targets of these miRNAs, such as the oncogenic tyrosine Trk [54]. In contrast, other miRNAs have been identified, such as miR-155 and miR-30a and miR-181, as EMT promoters. For instance, miR-181 inactivation elicits a dramatic effect on the ability of breast cancer cells to acquire and maintain an EMTed, metastatic and anti-anoikis phenotype. High miR-181 levels repress Bim expression, which is required for anoikis following the loss of cell substrate interaction [55].

It is now apparent that a complex network of EMT transcriptional inducers, miRNAs, and effectors of oxidative stress and hypoxia can impact autophagy and energetic metabolism. These intricate networks allow cancer cells to escape anoikis and acquire an EMTed phenotype [40]. Evasion of anoikis represents a critical barrier that carcinoma cells must overcome in order to metastasize.

8.2.5 Senescence

Senescence is a biological program occurring when normal cells have exhausted their proliferation potential. Cells, however, do not undergo apoptosis or anoikis but reach a resting dormancy-like state, albeit they remain metabolically active. A senescent state can be reached by premalignant cells that have kept a fail-safe program, which prevents further oncogenic events from occurring. Typically, cells expressing an oncogene may undergo senescence through Rb- and p53-driven pathways abrogating cell proliferation. Recent studies have exemplified how the senescence program can bypass the acquisition of an EMT phenotype by Twist or Zeb1 [56].

One mechanism by which the fail-safe program can be escaped is through a Twist-mediated transcription of the polycomb protein, BMI1, which directly targets the cell cycle inhibitor, INK4A [57].

8.2.6 Stemness

The hypothesis that cells can acquire stemness when acquiring an EMT phenotype was initially shown in the immortalized human mammary epithelial cell line, HMLE cells [58, 59]. Ras transformation or forced or inducible expression of TWIST1 or SNAIL1, which induces the typical morphological transformation observed in EMT, leads to the acquisition of spheroidogenic and tumorigenic properties and the expression of a particular phenotype. Snail or Twist EMT-induced cells can self-renew but also give rise to differentiated cells from the basal and luminal layers of the mammary gland. Such cells, sorted from normal mouse and human mammary gland, exhibit an EMTed phenotype.

It is worth noting that the $CD44^{high}/CD24^{low}$ stem cells isolated from carcinoma also exhibit an EMTed phenotype. $CD44^{high}/CD24^{low}$ stem cells can give rise to $CD44^{low}/CD24^{high}$ non-stem cell cancer cells in vitro. However, quite surprisingly, a non-stem cell can give rise to stem cells following a spontaneous detachment of so-called "floating" cells from the monolayer, which are enriched in stem cells as compared to the original population of HMLE cells [60]. A similar reversal was observed with HMLE cells rendered tumorigenic. In a subsequent study, the expression of SLUG and SOX9 was shown to be sufficient to induce stemness in the mammary epithelial cells, and these two transcription factors were also able to render MCF7 cells highly clonogenic [61].

However, a recent study points to two distinct cancer stem cells in breast carcinoma [62]. The classical $CD44^{high}/CD24^{low}$ stem cell exhibits a mesenchymal-like phenotype, whereas aldehyde dehydrogenase-1 (ALDH1)-positive cells exhibit an epithelial-like phenotype, even though the cells are primarily localized in the tumor core; likely, in a hypoxic zone. Two similar stem cell populations can be isolated from breast carcinoma lines. The enriched $CD44^{high}/CD24^{low}$ and ALDH1 fractions give rise to a heterogeneous population. This includes the two stem cell fractions in culture, thus showing the plasticity of the two stem cell compartments. Interestingly, normal mammary gland possesses both of these stem cells. Indeed, a MET ALDH1-positive fraction can reconstitute a ductal and alveolar bilayered structure; these MET ALDH1-positive cells are located in the lobulo-alveolar structures, whereas the EMT $CD44^{high}/CD24^{low}$ fraction is located in the basal layer and also exhibit self-renewal capacity.

8.3 EMT Can Alter Immune Response

EMT has been implicated in immune escape. One pioneer study showed that B16F10 melanoma cells constitutively expressing SNAIL1 undergo EMT in vitro [63]. Also, the co-culture of spleen cells with the B16F10 Snail-expressing cells

in vitro showed a remarkable decrease in the proportion of CD4- and CD8-positive cells, albeit FOXP3 expression was induced in the $CD4^+CD25^-$ regulatory cell population. The immunosuppressive function of FOXP3 is shown to be controlled by the production of thrombospondin by melanoma cells. In vivo, the Snail-expressing cells form primary tumors and metastases, which induce $CD4^+FOXP3^+$ T-regulatory (T_{reg}) cells and abrogate dendritic cell responses. This study demonstrated the critical role of EMT in immune escape through a complex mechanism involving the genesis of specific T_{reg} cells and preventing cytotoxic T lymphocytes (CTLs) from infiltrating and lysing the tumor cells.

CTL lysis is also found to be inhibited in a human breast cancer cell line model. MCF7 cells exhibiting a robust epithelial phenotype can undergo EMT following forced expression of wild-type, constitutively activated SNAIL1 or following long-term exposure to TNFα [64]. A CTL clone engendered to specifically lyse MCF7 has a much reduced killing activity when co-cultured with mesenchymal-like MCF7. The immune synapse appeared to be immature and, in addition, the mesenchymal-like MCF7 engaged in an autophagic program that would be likely to promote survival. The immature immune synapse may result from defective cortical actin dynamics to position Major Histocompatibility Class 1 antigens and associated adhesive and regulatory proteins. A similar finding was observed when WISP2 (Wnt1-inducible signaling pathway protein 2), a secreted protein, was downregulated in MCF7. shRNA against WISP2 leads cells to acquire a strong mesenchymal phenotype through the activation of the TGFβR pathway. In these cultures, the WISP2-downregulated cells showed much lower levels of miR-7-5b and a higher level of the stem cell transcription factor KLF4, one of its target genes. Clusters of carcinoma in basal-like breast cancer were also found to express KLF4, TGFβ, Smad2/3, and Twist [65]. CTL lysis is impaired in low WISP2-expressing cells, as they cannot establish a functional immune synapse in part due to the diminished antigen presentation but also likely resulting from defective cortical machinery in the postsynaptic cytoplasmic domain; this is similar to the mechanism described above for mesenchymal MCF7 cells.

8.4 CTCs in Cancer Progression

Disseminated, malignant cells from primary tumors are found in blood and in the bone marrow of the iliac crest bone, the only tissue which can be conveniently biopsied without posing a major threat to patients. Indeed, bone marrow aspirates were practiced in a number of European Institutions, and this led to a landmark meta-analysis paper published in 2005 showing a breast cancer-specific mortality risk of 2.44 for patients with disseminated tumor cells (DTCs) [66]. The presence of bone marrow DTCs correlated with increased metastatic burden, aggressive disease, and a decreased time to relapse. However, studies of CTCs in blood are now becoming prevalent, with more than 15,600 papers on the subject as on October 2014.

CTCs provide a prospective source of information for clinicians, and can be conveniently isolated by liquid biopsy. CellSearch-based (Veridex LLC, Raritan, NJ, USA) enumeration of CTCs in the blood samples from 177 breast cancer patients revealed that metastatic breast cancer patients with five or more CTCs per 7.5 ml of blood had a shorter median progression-free survival and shorter overall survival than those who did not [67]. This data has since been supported by a recent meta-analysis of CTCs in 1,944 metastatic breast cancer patients prior to treatment using the same search parameters and thresholds (≥5 CTCs/7.5 ml), and found an overall survival hazard ratio of 2.78 [68]. More importantly, CTCs can originate from different regions of a tumor, thereby potentially representing tumor heterogeneity. They also provide a sample source for understanding cancer cell survival in the harsh conditions of blood circulation. Despite recent advances, CTCs need to be much better characterized to support their clinical utility [69, 70]. As today, there are very few indicators with which to identify CTCs that harbor metastatic potential. Xenografts of CTCs in immunocompromised mice appear to be a promising strategy but the success rate is still very low [71]. Aside from murine models [10], we do not know what fraction of CTCs can localize in a target organ. In particular, large CTCs (diameter 20–30 μm) or microemboli theoretically should be cleared from the peripheral blood circulation by being trapped in small capillaries (~8 μm). However, they have been detected as intact CTC clusters by various CTC enrichment techniques [72–75], especially those that require minimal manipulation. It is unknown how these clusters manage to evade capture and persist in the circulation. One study showed that CTCs from localized breast cancer patients had a half-life of 1–2.4 h [76]. A potential mechanism by which CTCs can survive in the blood flow is to form mixed aggregates with platelets [6]; indeed, platelet-deficient mice are much less prone to forming metastases following IV injection of melanoma cells in an experimental model [77]. In addition, CTC–platelet mixed aggregates can be protected from natural killer (NK) cell lysis [78]. There is an urgent need to know more about their ability to escape the immune system by forming microemboli and/or through the acquisition of an EMTed phenotype that renders CTCs unfavorable for CTL lysis [79, 80]. Last, but not least, CTCs have also been detected in treated cancer patients without clinically detectable disease, revealing cancer dormancy [76], a state which needs to be better defined since it may reflect a steady state whereby an increase in cell number is accompanied by an equal number of cell deaths [81].

As mentioned, the recent discovery that CTCs exhibit a spectrum of EMT phenotypes suggests that the population of CTCs isolated with existing technologies may be highly underrepresented, and this prompts for the development of methods that can securely detect most CTCs to deepen our understanding of CTC biology.

8.4.1 Methods of CTC Detection and Isolation

The specificity and selectivity of current CTC enrichment methods are hindered by technical limitations, as well as by the insufficient understanding of CTC biology. Most of these strategies hinge on cell-surface antigen recognition or cell size. Current technologies are critically analyzed for their limitations to enrich heterogeneous CTCs exhibiting a spectrum of the EMT phenotype (Table 8.1).

Table 8.1 Methods of CTCs detection and isolation

Types	Term	Enrichment concept	Clinical trials	Mean blood vol. per test/ml	Estimated range CTCs/ml	Microemboli detected	CTC detection threshold/ml	Specificity (disease detection)	Purity (%)	References
Immunophenotyping	CTC-chip	Microfluidics, Ab-coated micropillars	Yes	2.7	5–1,281	No	ND	ND	9.2±0.1 %	[83]
	HB-chip		Yes	4	12–3,167	Yes	10	14/15 (93 %)	14.0±0.1 %	[74]
	Nanostructured silicon substrates with integrated chaotic micromixers		Yes	1	1–33	No	ND	20/26 (76.9 %)	ND	[37]
	Geometrically enhanced differential immunocapture (GEDI)		Yes	1	27±4	No	ND	18/20 (90 %)	62±2	[82]
	Microelectro mechanical system	Microfluidics, Ab-coated microchannels	No	NA	NA	NA	NA	NA	ND	[84]
	Nanotube-antibody biosensor array	Microfluidics, Ab-coated nanotubes	No	NA	NA	NA	NA	NA	ND	[85]
	MACS cell separation systems	Immunobeads	Yes	5–15	1–571	No	5	24/49 (49 %)	ND	[94]
	CellSearch	Microfluidics, Ab-coupled ferrofluid	Yes	7.5	1–1,491	No	0	66/92 (70 %)	ND	[90]
	MagSweeper	Ab-coupled ferrofluid	Yes	9	1±3	No	0	47/47 (100 %)	100 %	[199]

Table 8.1 (continued)

Types	Term	Enrichment concept	Clinical trials	Mean blood vol. per test/ml	Estimated range CTCs/ml	Microemboli detected	CTC detection threshold/ml	Specificity (disease detection)	Purity (%)	References
	CTC-chip ephesia	Microfluidics, Ab-coupled ferrofluid	No	NA	NA	NA	NA	NA	ND	[200]
	Fiber-optic array scanning technology (FAST)	Flow cytometry	No	NA	NA	NA	NA	NA	ND	[91]
	Multiphoton intravital flow cytometry		Yes	0.5	1–153	No	6	10/12 (83.3 %)	ND	[87]
	Multiparameter flow cytometry		No	NA	NA	NA	NA	NA	0.01 %	[88]
			No	NA	NA	NA	NA	NA	0.01–0.001 %	[89]
	Antibody-functionalized fluid-permeable surfaces	Microfluidics, filtration	No	NA	NA	NA	NA	NA	NA	[86]
	AdnaTest	Ab-coupled microbeads	Yes	5	NA	No	1 of 3 markers >0.15 ng/µl	29/55 (53 %)	NA	[201]
Size and deformability	3D microfilter	Filtration	No	NA	NA	NA	NA	NA	ND	[113]
	ISET		Yes	6	1–4	Yes	0	3/7 (42.9 %)	ND	[163]
	Microcavity array	Microfluidics, filtration	No	NA	NA	NA	NA	NA	ND	[202]
	CT-chip		No	5	NA	NA	NA	NA	80–90 %	[96]

	Spiral inertial biochip	Microfluidics (inertial focusing)	Yes	7.5	5–100	Yes	5	20/20 (100 %)	~10 %	[73]
	Slanted chip		Yes	7.5	3–125	No	4	10/10 (100 %)	0.6–25 %	[114]
	CTC-iChip	Microfluidics (inertial focusing), ferrofluid	Yes	10	0.5–610	Yes	0.5	37/41 (90 %)	7.80 %	[100]
	Vortex chip	Microfluidics (inertial focusing)	Yes	7.5	23–317	Yes	5	12/12 (100 %)	57–94	[104, 203]
Electric properties	DEP-FFF (dielectrophoretic field-flow fractionation)	Membrane capacitance	No	NA	NA	NA	NA	NA	ND	[106]
		Morphologic and electrical conductivity differences	No	NA	NA	NA	NA	NA	ND	[204]
Invasive properties	Vita-Assay™/ Collagen Adhesion Matrix (CAM) technology	Ingestion of fluorescent CAM fragments	Yes	1	18–256	No	0	38/64 (59.4 %)	0.5–35 %	[205]

8.4.2 Immunoselection of CTCs

CTCs are commonly enriched using immunomagnetic beads or other supports coated with antibodies directed against epithelial cell adhesion molecule (EpCAM), a cell-surface protein not exclusively expressed on epithelial cells and whose functions remain to be fully defined. Other membrane or cytoplasmic antigens, ranging from epithelial, mesenchymal, or intermediate types, can also be targeted for CTC detection. CTC isolation methods can be mediated by the interaction of target CTCs with antibody-coated features, including micropillars [37, 74, 82, 83], microchannels [84], nanotubes [85], or nanoporous surfaces [86] under precisely controlled laminar conditions with reliable efficiency. Recently, the rise of immunomagnetic techniques via the use of commercially available systems based on fluorescence and magnetic-activated cell sorting (MACS) has provided simplified enrichment methods for CTC isolation. Immunomagnetic methods range from the common flow cytometry [87] and MACS cell separation systems to the more sophisticated use of macro-iron beads, magnetic beads, ferrofluid (colloidal iron)-based systems [88–90], and fiber-optic array scanning technologies [83, 91]. In magnetic-based cell detection and isolation, whole blood or mononuclear cells are placed in contact with particle (magnetic beads or ferrofluid particles)-bound antibodies. Labeled cells are collected by applying a magnetic force while non-labeled cells remain in the supernatant and are discarded. Since a large number of leukocytes still remain trapped with the target cells [92], some methods include a "negative" selection of leucocytes (e.g., with anti-CD45) after the "positive" selection with antibodies specific to epithelial cells [93, 94]. In addition, further phenotyping via immunostaining, amplification of genetic loci, or single-cell analysis should be applied to ascertain the malignant properties of the CTCs. Furthermore, novel markers that are unaffected by the EMT process, such as plastin 3 (PLS3) [95], are being investigated to replace the use of these conventional biomarkers for more reliable CTC detection.

8.4.3 CTC Enrichment Based on Size, Deformability, and Other Physical Properties

Label-free sorting methods have been often proposed to overcome problems encountered by immunophenotyping methods (see Sect. 2.2.1). One method relies on the filtration of whole or lysed blood using track-etched membranes or microfabricated planar isoporous filters [96]. Filtration provides a sensitive means of CTC isolation under low hematocrit levels and low blood volumes [97]. Other physical label-free methods have also been utilized, including density gradient centrifugation [98] and isolation by optical properties [99, 100]. Density-based separation is performed using Ficoll or other reagents to separate the denser, nucleated cells [CTCs, epithelial cells and white blood cells (WBCs)] from the red blood cells (RBCs) and plasma. This separation technique can be complemented with other approaches to generate enhanced assays, such as the OncoQuick [98], which removes contaminating WBCs

with an additional filter step. Microfluidics is also favored for CTC detection because it can fractionate cells by modulating the flow [101]. Several systems based on different flow parameters and principles [73, 96] have been developed for CTC isolation from whole or lysed blood. Recently, high-throughput, label-free cell sorting based on centrifugal forces, coupled with inertial focusing microfluidics effect on particle migration (Dean Flow Fractionation) in curvilinear microchannels, has been reported [102]. The process is passive and the selection threshold can be adjusted by varying microchannel dimensions, fluidic forces (i.e., inertial lift and Dean drag forces) and particle size to achieve separation of high yield and purity [103, 104]. However, CTCs may exhibit different cell sizes and so other label-free assays, such as photo-acoustic flow cytometry [105] or technologies that involve dielectrophoresis (DEP) [106], have been suggested to improve the capture efficiency of CTCs.

8.4.4 Techniques Based on Proliferative Capability

CTCs are rare events, and the lack of significant numbers of CTC samples poses a real challenge for defining the phenotypic and genotypic characteristics of CTCs. Previous attempts to expand these cells under conventional culture conditions (normoxia, 2D substrate surface, 5 % CO_2, high humidity) have been unsuccessful; these techniques generally result in the formation of immortalized cell lines from CTCs pre-enriched with either fluorescence-activated cell sorting (FACS) [107], negative enrichment [108], or other affinity-based microfluidics assays [109]. To date, the efficacy of current techniques in obtaining a positive CTC culture is very low [FACS-enriched, 3/28 (7.9 %); CTC-iChip-enriched, 6/36 (16.7 %); Rosette Sep, 6/32 (18.8 %)], which renders the generation of a novel, high-throughput method pivotal for the reproducible generation of CTC cultures and for actual use in clinical therapeutics. These previous methods have also required large volume of samples for detection (FACS-enriched, 25–40 ml; CTC-iChip-enriched, 20 ml; Rosette Sep, 8 ml). Primary cell cultures may reflect the original disease rather than immortalized cell lines, as the latter may undergo selection and give rise to phenotypes that can no longer represent the primary disease [110, 111]. More recently, a microwell assay technique has been developed, yielding 50 % positivity in early-stage breast cancers and almost 80 % positivity in metastatic breast cancers [79].

8.5 CTC Biology and Their Effects on Existing CTC Enrichment Technique

8.5.1 Rarity of Occurrence

CTCs are estimated to be present as few as 1 in every 10^9 blood cells [112]. CTC counts per 1 ml of whole blood can be detected by various assays, and these numbers fluctuate with different cancer types. The CellSearch System is currently the only

Food and Drug Administration (FDA)-approved clinical utility for CTC detection and isolation, and has been reported to recover a range of CTC counts per 7.5 ml of whole blood for certain cancer types (i.e., per 7.5 ml whole blood breast cancer, 2; prostate cancer, 8; lung cancer, 0) [72]. The technique is based on the identification of EpCAM on CTCs, and results in counts that are extremely low as compared with the 5×10^9 RBCs and $5–10 \times 10^6$ nucleated WBCs present per ml of whole blood. Most filtration devices, such as ISET (Rarecells US Inc., Austin, TX), demonstrate a similar level of sensitivity (i.e., median CTC counts per 7.5 ml whole blood breast cancer, 2; prostate cancer, 17; lung cancer, 6). In addition, many of these devices are limited to low working blood volumes [113] due to blood clotting and processing speed. Thus, a novel technology is warranted. Recently, the development of spiral microfluidics has spun off a series of devices that could detect CTCs at high-throughput, with CTC counts ranging from tens to hundreds of cells per 7.5 ml of blood [73, 102, 114]. A CTC culture assay has also been developed to enrich and expand CTCs precisely based on proliferative potential from small volumes of blood [79]. Low CTC counts present a tremendous technical challenge to define the phenotypic and genotypic characteristics of CTCs, especially via techniques which require a minimum working concentration (such as PCR and karyotyping). Such methods to detect and enrich CTCs at low amounts is imperative for early cancer detection and treatment prognostics, since CTC counts and proliferative capability have been correlated with disease progression and treatment efficacy [67, 115, 116].

8.5.2 CTC Heterogeneity

8.5.2.1 Biomarker Expression

The rarity of CTCs hinders the discovery of unique CTC biomarkers for diagnosis and therapy. To date, a limited number of markers are utilized for the detection of CTCs, and a combination of unique antigens must be identified for the detection and enrichment of CTCs [72, 117]. Thus far, most CTC identification employs the use of epithelial markers (EpCAM and cytokeratins) or organ-specific markers (Table 8.2) which are not unique to CTCs. EpCAM, first reported in 1979 as a colorectal carcinogenic antigen by Koprowski and colleagues [118], has a varied range of functions, such as stem cell proliferation and regulation of cyclin expression, and its overexpression leads to cells with increased epithelial characteristics. EpCAM-knockout mice exhibit abnormal extraembryonic tissue development, leading to early embryonic lethality phenotypes [119]. Contrary to common understanding, there is little evidence for the adhesive properties of EpCAM, and its biology remains rudimentary. EpCAM was reported to confer fibroblastic cells with calcium-independent adhesive properties [120] and to regulate claudin expression [75]. Hence, EpCAM does not necessarily reflect an epithelial state, and may not be the most secure candidate for CTC detection and analysis. On the other hand, the so-called tumor markers are also expressed in benign cells; hence non-CTCs may

Table 8.2 Biomarker list for the positive selection of CTCs or negative selection of blood cells to eliminate contaminants

Selection	Marker type	Organ type	A/B[a]	Marker	References
Positive	Epithelial markers	All	A	EpCAM	[72]
			A	Cytokeratins 7, 8, 18, 19, and 20	[72, 73]
			A	EGP-2	[206]
	mRNAs		B	Telomerase	[207]
			B	Human telomerase reverse transcriptase (hTERT)	[208]
	Tumor-related markers		B	Alpha-fetoprotein (AFP)	[209]
			B	Carcinoembryonic antigen (CEA)	[210]
			B	Squamous-cell carcinoma antigen (SCCA)	[211]
	Organ-specific markers	Breast	B	Mammaglobin	[212]
			B	HER2-neu	[117]
			B	Mucin-1	[213]
		Prostate	B	Prostate-specific antigen (PSA)	[214]
			B	Prostate-specific membrane antigen (PSMA)	[74]
Negative	Leukocyte markers		CD45		Various literature

[a]A, Epithelial cells; B, Altered expression level associated with malignancy

thus be misrepresented as cancer cells during detection and identification processes because of the presence of circulating epithelial cells (CEpC) in blood [121] or as a result of nonspecific or false-positive labeling. Nonspecific binding is usually associated with Fc receptor-bearing WBCs or illegitimate expression of EpCAM in normal plasma cells or hematopoietic precursors [117]. The range of false positives detected varies with each method of CTC detection, and these values are estimated by processing healthy blood samples as controls (0–20 %; [121–123]). Most false positives reported are WBCs and these counts may be reduced by negative selection with WBC biomarkers (e.g., CD45). In fact, activated leukocytes are found at higher counts in the blood samples from cancer patients, and may even be coated or express epithelial markers (EpCAM or cytokeratins) [124, 125]. However, negative selection with CD45 antibodies to remove leukocytes cannot deplete CEpCs and other progenitor hematopoietic cells [126]. These CEpC are usually associated with benign epithelial proliferative diseases or inflammation [122]. The presence of contaminating WBCs, stromal bone marrow-derived cells, mesenchymal stem cells (MSCs), and CEpCs isolated along with CTCs will affect the downstream analysis of pooled cell populations. Such degrees of impurity in CTC samples generate inconsistent results, hindering data interpretation and analysis.

To complicate matters, epithelial antigens (EpCAM and cytokeratins) are often downregulated in the subpopulation of CTCs that are potentially most invasive, due to a partial or complete EMT. Thus, assays based on affinity-binding principles lose huge numbers of CTCs that do not express epithelial markers [121, 127, 128]. Breast cancer cells are highly heterogeneous, and can be currently classified loosely into six main molecular subtypes, namely normal-like, luminal A and B, human epidermal growth factor receptor 2 (HER2)-positive, basal, and claudin-low [129, 130]. Each subtype exhibits a varied expression pattern and, consequently, affinity-based assays will definitely lose breast cancer CTCs that do not express the antigen of interest. For example, CTCs from triple-negative cancers may evade detection due to a lack of antigen expression. This is also true for other breast cancer subtypes which are characteristic of basal, epithelial and adipose cells [131]. Furthermore, mesenchymal antigens may be expressed solely or in conjunction with epithelial markers (double-positive) in CTCs. The complexity of cancer and the lack of a specific cancer antigen thus reduce the specificity of antigen-based CTC detection methods.

To date, the lack of a specific CTC biomarker means that current CTC identification based on primers and antibodies alone is not specific and only states a "probability" that CTCs are present. Conventional CTC isolation processes also have lengthy protocols and conditions that might result in alterations of biomarker expressions in the span of time required for blood processing. A high-throughput assay for CTC detection and isolation is thus warranted.

8.5.2.2 Cell and Nuclear Morphology

A standardization of the immunocytochemical detection of breast carcinoma cells in the bone marrow was established in 1999 [132]. Breast carcinoma cells are defined as those with "a clearly enlarged nucleus," with a presence of clusters (≥2 cells) and a "high nucleus–cytoplasmic ratio." This method of detection has been previously standardized by investigating DTC numbers from bone marrow [132]. Further, cytopathological evaluation has been used to complement immunochemical methods of CTC identification (Fig. 8.2). An analysis of CTC morphology at different stages can also enable oncologists to monitor the evolution of the disease and track efficacy of treatment. To ease the limitations of CTC isolation by affinity-binding methods (see Sect. 2.221), the physical properties (density and size) of CTCs have instead been exploited for enrichment. The filtration concept adheres to the previous immunocytochemical definition [132] by selecting cells based on size. Filtration generally reduces the loss of CTCs through multiple manipulation steps, but only isolates large cells depending on pore size. Another limitation is the working blood volume, which is reduced as filter pores become clogged. Microfluidics is an emerging alternative to filtration, and is potentially able to process large volumes of blood under high hematocrit levels, thus minimizing this issue of volume loss. Biochip parameters can also be flexibly altered to extract cell populations of various sizes.

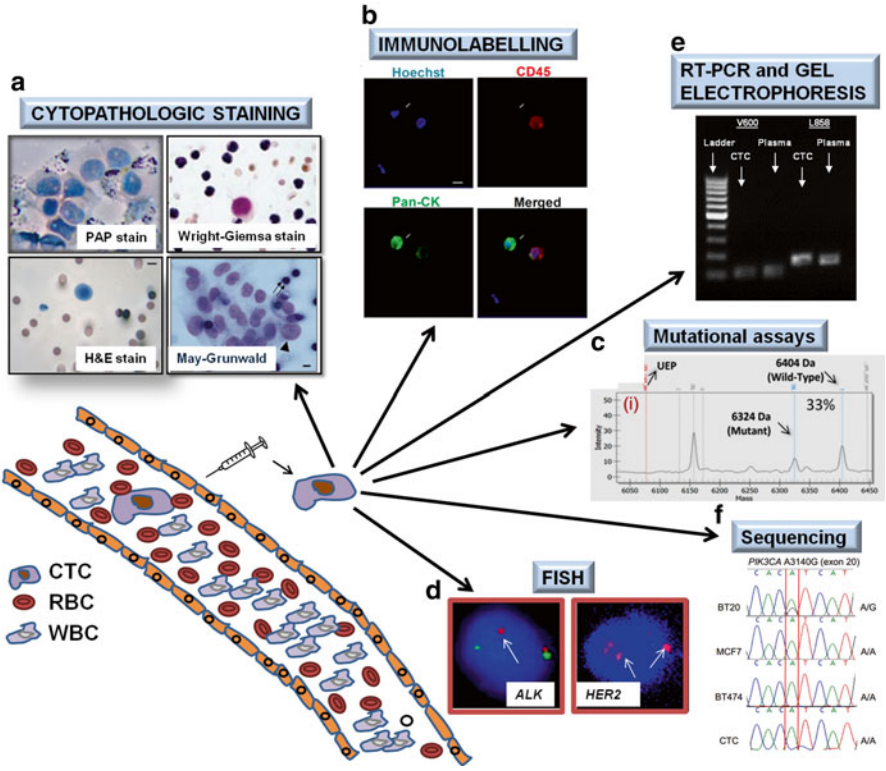

Fig. 8.2 Circulating tumor cell (CTC) detection methods. Cellular and nuclear morphology. (**a**) Cytopathological staining of enriched CTCs via Papanicolaou stain (PAP) [102], Wright-Giemsa stain [133], hematoxylin and eosin stain (H&E) [195], or May-Grunwald stain [196]. (**b**) Immunolabeling of antigens associated with cancer cells. The epithelial-like subpopulation of CTCs can be defined as cytokeratin-positive/CD45-negative and Hoechst-positive [102]. (**c**) Mutational assays (e.g., MassArray spectra) can be used to detect known cancer mutations, such as EGFR L747_P753>S, from enriched CTCs [102]. (**d**) Fluorescence in situ hybridization is commonly used to detect CTCs expressing copy number alterations in cancer genes, such as ALK (using a break-apart probe) [102] and HER2 [114]. (**e**) Real-time polymerase chain reaction (RT-PCR), coupled with gel electrophoresis assays, helps identify alterations in genetic expression of single or pooled cell samples. (**f**) Sequencing of single or pooled cell samples allows for the detection of pathogenic mutations, such as in PIK3CA, in breast cancer [94]. (**a**) Wright-Giemsa stain, Copyright © 2010 Dena Marrinucci et al.; H&E stain, Reprinted by permission from Macmillan Publishers Ltd Journal of Investigative Dermatology, Copyright © 2010; May-Grunwald stain, Reprinted from Hofman et al., with permission from John Wiley and Sons Copyright © 2010; (**d**) HER2 staining, Reproduced from Majid et al. Analyst (2014) with permission from The Royal Society of Chemistry; (**f**) Copyright © 2014 Deng et al.

In view of recent findings, however, the strict definition established in 1999 may not be able to fully describe the complete spectrum of CTCs. Numerous studies have reported intrapatient CTC variability in terms of morphology and antigen expression [72, 73, 79, 97, 102, 133]. Indeed, some CTCs resemble the size and

nuclear morphology of WBCs and epithelial cells, making them indistinguishable from the contaminants. Cells may also be malignant in the absence of a "clearly enlarged nucleus" or aneuploidy. Besides, bone marrow cancer cells are more likely to comprise a higher proportion of invasive cells to enable extravasation, and these may be morphologically distinct from a large portion of the transiting CTCs, which may not be as malignant or invasive. A lack of understanding of this CTC heterogeneity has led to a restrained definition of identifying enriched CTCs (1999 standardized criteria; see above) [72]. These selection criteria result in the loss of smaller, WBC-like and benign-like CTC subpopulations, which may be those cells that escape sequestration from capillaries and contribute directly to metastatic spread.

8.6 Association with Blood Cells, Platelets, and Other Cancer Cells

CTCs can aggregate into clusters, forming microemboli. The utilization of most CTC enrichment devices is not favorable for identifying CTC clusters, as the process generally induces breakage of cell clumps. Culturing and size-isolation methods have been useful in observing microemboli in patient blood samples. However, the roles of these clusters in systemic spread and their origin remain unknown. It has been speculated that CTC microemboli could arise from shredded tumor cell clusters and that they may enter the peripheral circulation through engulfment by invading neo-vessels [2]. They might also originate from single cells, which then undergo proliferation mediated by adhesion molecules (such as one or more of the various cadherins). However, a recent study has revealed that these microemboli are formed by oligoclonal carcinoma cells, which may cluster through a plakoglobin-mediated mechanism prior to intravasation [134].

Clusters are more sheltered from external shear and from detection by immune cells [135, 136], which allows them to persist in the peripheral circulation and attach at distant sites. The detection of CTC microemboli also correlates with worsened disease prognosis [137, 138], and clusters are believed to be able to generate metastases independently (within the vessels) without the need for extravasation [139]. In addition, platelets are observed to associate with CTCs via tissue factor or selectins, and are thought to promote cohesion and a partial EMTed phenotype potentially through TGFβ, which is released from these platelets [6] prior to or with restrained blood flow [2].

To date, the mechanism(s) through which microemboli contribute to metastasis is unclear. Microemboli can execute collective migration [140], a phenomenon correlated with survival and proliferation advantage; some studies have reported the attachment of CTC clusters to blood vessel walls in confined regions (e.g., arterioles or capillaries), which promotes proliferation and eventual rupture of the capillary walls to form micrometastases [134, 139].

8.6.1 Circulation and Viability of CTCs

The precise enumeration of CTCs is difficult to achieve, and current methods likely considerably underestimate their number. CTCs have a brief transition period in the circulation [141], with estimates that a time frame of 5 min is all that is required to sequester 85 % of CTCs initially shed into the bloodstream [142, 143]. The lifetime of a CTC and the stimuli required to trigger its release into the bloodstream are still debated. Studies have detected a large increase of cells with epithelial (EpCAM or cytokeratins) markers in blood after iatrogenic procedures [144], and results from animal studies indicated that these cells were suggested to be able to persist in the circulation for up to 1 month [145]. The circulation of these cells vary according to their origin and phenotype [76, 144], but it remains inconclusive whether this rapid turnover reported in the literature actually corresponds to CTCs or CEpCs because of the lack of tumor-specific biomarkers. Cells that are positive for epithelial (EpCAM or cytokeratins) markers have also been detected in trace amounts in blood samples from healthy donors, which are also likely to correspond to the circulating epithelial cells or CEpC subpopulations. Further specific analyses will be required to explain the trace amounts of "CTCs" circulating in samples from healthy donors, or redefine the identification criteria of these cells.

Currently, all existing CTC isolation assays are limited by the stringent selection process chosen for CTC enrichment, leading to specific subpopulations of CTCs detected, amongst the entire range of CTCs present in the blood. Most of these enriched CTCs are rendered nonviable due to long processing time or cellular stress incurred during manipulation. However, several efforts in the past 2 years have led to various reports of successful CTC cell lines. These cell lines were generated from spontaneous immortalization of pre-enriched CTCs for at least 6 months under in vitro expansion, and may be potentially useful in drug screens or for prognosis. Clonal growth from prostate CTCs also generated organoids, which preserved various morphological features as compared with the original, heterogeneous sample before culture [108]. New methods to expand CTCs in vitro without the need for affinity-based, pre-enrichment assays are currently developed [79].

8.7 Epithelial–Mesenchymal Transitioned Phenotypes in CTCs

The heterogeneity of CTC phenotypes has resulted in a great difficulty in identifying and characterizing CTCs. This variation in phenotypes has been attributed to a partial or complete EMT. CTCs may also exhibit one or more phenotypes in their entire transit time within the peripheral circulation particularly if aggregated with platelets.

The EMT process has been closely linked to the invasive potential of cells. Primers and antibodies identifying biomarkers such as E-cadherin, cytokeratins EpCAM, N-cadherin, Vimentin, Snail, Zeb, and Twist have been routinely used to identify EMTed cells. The EMT process leads to the generation of cancer cells with stem-like properties and increased invasiveness.

8.7.1 In Vivo Analysis

There is currently no data to track human CTC evolution in vivo. Existing in vivo studies are based on animal models that focus on CTC detection and isolation methods or the extravasation process via immunolabeling of tumor cells [87, 88]. Common techniques supporting the establishment of in vivo models include the use of intravital videomicroscopy (IVVM) and flow cytometry (Fig. 8.3). The local invasion of tumors and intravasation processes are usually not examined, as they are difficult to replicate in animal models [146].

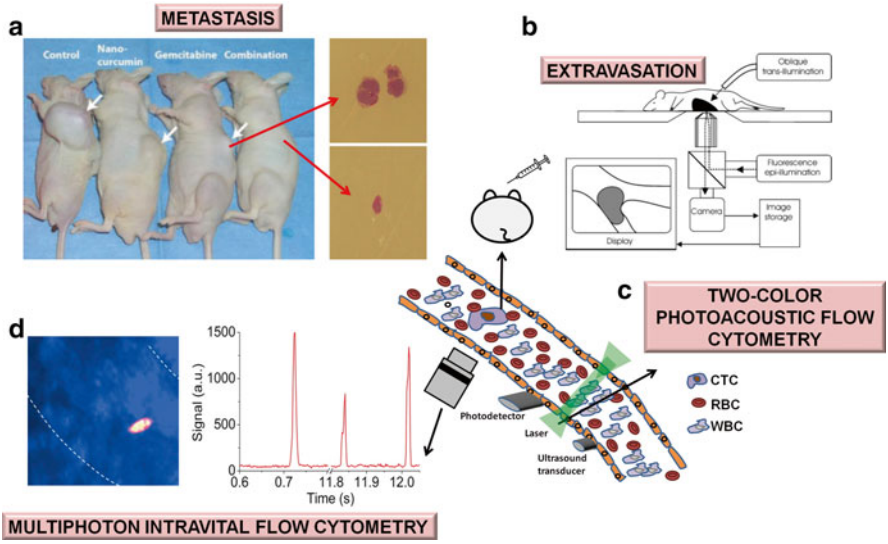

Fig. 8.3 Potential and current in vivo analysis or detection of circulating tumor cells (CTCs). (**a**, **b**) Existing cancer models that can be coupled to the CTC field for a better understanding of cancer biology. (**a**) *Left*, Potential applications of orthotopic or xenograft tumor models for analyzing CTCs [197]. (**b**) Extravasation model [198]. (**c**) In vivo detection of CTCs with two-color photoacoustic flow cytometry [105] and (**d**) multiphoton intravital flow cytometry [87]. Reprinted from Cancer Cell, Vol. 15(3), Ebos JML et al., Accelerated Metastasis after Short-Term treatment with a potent inhibitor of tumor angiogenesis p 232–239. Copyright (2009), with permission from Elsevier. (**b**) Reprinted from MacDonald et al., with permission from Wiley Periodicals, Inc., Copyright © 2002. (**c**, *left*), Reproduced from He et al., with permission from the National Academy of Sciences, USA

The establishment of metastases by CTCs is often investigated by murine mouse models injected with fluorescence-labeled cancer cell lines [147]. Previous studies utilizing IVVM illustrate the sequestration of CTCs by arterioles or capillaries according to size. When sequestered, CTCs may sense the microenvironment, which, in turn triggers EMT processes that promote their invasiveness and proliferation, eventually leading to rupture of the microvessels and the promotion of metastasis [139, 148, 149]. However, according to Groom and colleagues, CTCs do not demonstrate rolling but become arrested and alter their morphology by membrane distortion [150]. This is associated with the initiation of metastasis, which allows the cell to extravasate from the vessel.

It should be reemphasized that CTCs are highly heterogeneous and only a small subpopulation is postulated to carry metastatic or tumorigenic potential. Current studies established that 2.5 % of the seeded, post-enriched CTCs (1/40) establish micrometastases in animal models, and only 0.01 % are tumorigenic and could proliferate into macrometastasis [143]. However, these values do not truly reflect the proportion of metastatic or tumorigenic cells; instead, the inefficiency of CTCs to metastasize is likely attributed to cell dormancy, apoptosis, and a requirement of "companion cells" to generate tumor development and angiogenesis for tumor maintenance [143]. Indeed, single cancer cells or micrometastasis may be arrested in the midst of the cell cycle [151] under "unfavorable" micro-conditions and evade immune responses for years [152]. Detailed mechanisms may vary according to cancer type and are not fully understood.

Despite the lack of advancement for in vivo models to illustrate the role of CTCs in tumor development, such models will continue to serve as the most reliable means for investigating the complexity of the system until the microenvironment can be faithfully replicated via in vitro models.

8.7.2 In Vitro Analyses

CTCs obtained via isolation and enrichment assays or devices are often further processed for downstream analysis. Current research focuses on the identification of novel biomarkers or to deepen the understanding of the proposed role of CTCs in cancer progression via the EMT process. As previously mentioned, a large population of CTCs demonstrate a downregulation in epithelial markers (EpCAM, cytokeratins) (see Sect. 8.4.2.1—Biomarkers), which are also common "EMT tags." The presence of EpCAM has been associated with only 70 % of tumors of different subtypes [153] and cytokeratin-negative cell populations have also been observed to form the majority of CTCs in certain instances [154]. Furthermore, cultured cells derived from DTCs or CTCs demonstrate phenotypic changes, including the loss of epithelial markers [155] and cultured CTCs are reported to be highly heterogeneous in terms of size, morphology, and antigen expression. Epithelial biomarkers are part of the set of proteins responsible for cell adhesion. Downregulation or the absence of these biomarkers in cancer cells generates a phenotype that promotes detachment and dissemination [2].

The heterogeneity of CTCs is highly credited to cancer cells engaged in different stages of the EMT process. Other studies have demonstrated further variations in the expression of EMT biomarker combinations, generating subpopulations of putative stem cells (breast cancer, $CD44^{high}/CD24^{low}$; lung cancer, $CD166^{high}/CD133^{high}$) [102] in CTC populations that correspond to a cancer stem cell (CSC) phenotype. CSCs are another rare subpopulation of cancer cells with tumor origins [156] that have tumor-initiating properties [2] and certain levels of drug resistance [157]. These cells can be artificially induced, forcing normal epithelial cells to enter a CSC-like stage [58, 59]. Cancer cells under the EMT process are conferred increased drug resistance, which benefits their metastatic capability [158]. However, it is difficult to speculate the origin of these tumor-initiating subpopulations and whether EMT is important in inducing the first CSCs. Besides, various cancer subtypes (e.g., luminal type breast cancer) are associated with mature cell phenotypes and low CSC counts [130, 159]. Signals triggering the EMT process have also remained unclear. Hypoxia has been demonstrated to be involved in promoting the EMT process via LOX and Snail induction [160, 161]. It is always possible that other pathways are present and working hand-in-hand with the EMT process to generate the uniqueness of CTCs and CSCs.

8.8 Methods for Diagnostic Identification of CTCs

Ascertaining the presence of CTCs is usually supported by cytological analyses including the nuclear–cytoplasmic ratio and anisokaryosis. Cytopathological methods utilize simple blood smears to identify CTCs from small volumes of whole blood. With the advancement in various technologies, CTCs can now be enriched from clinically significant volumes of blood. Thus, cytopathological analyses can provide reliable and consistent data, assuming that the original cell morphology is intact after blood processing techniques. Such tests can also highlight cell micrometastasis and their associations with a worsened prognosis (see Sect. 2.23). Furthermore, comparisons can be made between CTCs and tumor-derived cells of the same patient to access similarity in morphology and differentiate non-tumor cells from tumor cells in these CTC populations.

But when will we ever be certain of the proportion of CTCs amongst epithelial cells in blood? Current diagnostic methods are not able to arrive independently at a clear diagnosis, and the former method (affinity-binding) is largely restricted by the nonspecific markers used in isolation. A brief comparison of multi-marker, real-time RT-PCR, filtration, and immunochemical techniques for CTC isolation demonstrates that the highest sensitivity is achieved with real time RT-PCR [162]. Although data from RT-PCR assays cannot be interpreted as "counts," they can label a sample as positive for CTCs, and the reliability of the data depends on the sensitivity of the protocol and the blood volume sampled. However, due to technical limitations, genome-based analyses are more commonly used for characterization [92, 154, 162, 163] rather than routine diagnosis. Depending on the methodology, RT-PCR

may demonstrate reduced sensitivity in CTC detection due to low cell sample counts or the types of reagents used in an attempt to increase the signal-to-background ratio [164]. The specificity of RT-PCR also hinges on the proportion of CTCs per ml of blood, which varies with each sample. Fluorescence in situ hybridization (FISH) probes are not able to hybridize targeted genes in all cells, and this limitation gives rise to false negatives, especially when CTCs are enriched under low purity. The definition of a "positive" sample via FISH also varies with individual studies and needs to be better assessed. Other technologies, such as comparative genomic hybridization (CGH) and mutational assays are costly, require single-cell analysis [163], and cannot evaluate samples under a "real-time" basis. For these assays, CTCs can be specifically picked up by hand-pipetting or microdissection. Evaluations of single-cell CTCs via FISH and other genome analyses have been demonstrated by several groups [165–167]. Yet there are few characteristic genetic alterations that correspond to all cancer cells of a solid tumor, thus reducing the sensitivity of this technique.

Another fundamental issue involving the accuracy of diagnostic methods revolves around the fluctuation of CTC counts in blood samples per patient at different periods of time [168]. Several genomic and immunochemical analyses have demonstrated that the sensitivity of the assay increases (number of cases tested positive) when repeated blood samples are taken from the same patient for testing. The origin and mechanism of CTC release into the circulation still remains unclear. How, when and at what rate are CTCs shed from the tumors? These unknown clearly prevent clinicians from obtaining an optimal number of blood samples at an optimal time of the day. A new technology (http://www.gilupi.de/) permits the collection of CTCs using an EpCAM-coated medical wire inserted into a blood vessel, which may serve to answer some of these questions once the device is optimized for reproducible analyses [169].

8.9 EMT as a Portal to Stem Cell Characteristics

The first evidence of stem cells in cancer came in the 1960s by Stevens and Bunker, whereby they reported the generation of teratomas from primordial germ cells [170]. This hypothesis was supported by various other works, which demonstrated the multipotency of CSCs [171, 172]. Further, in 1994, an acute myeloid leukemia (AML)-initiating cell population was identified from AML patients by transplantation into severe combined immune-deficient (SCID) mice [173]. By then, human CSCs had been successfully identified in various cancer types [174, 175]. Multiple markers have since been used to isolate and enrich CSCs from different tumors. $CD44^{+}CD24^{-/low}Lineage^{-}$ and $ALDH^{+}$ were characterized as markers for CSCs from breast [176, 177]; $CD133^{+}$ for colon, brain and lung; $CD34^{+}CD38^{-}$ for leukemia; $CD44^{+}$ for head and neck cancer; $CD90^{+}$ for liver; $Cd44^{+}/CD24^{+}/ESA^{+}$ for pancreas. CSCs have the ability to generate more stem cells that differentiate through asymmetrical cell division. As one progeny retains the stem cell identity, the other

undergoes rounds of cell division and subsequent post-mitotic differentiation. There are different theories to explain the origin of CSCs. The prevailing is that they arise from normal stem/progenitor cells and obtain the ability to generate tumors after encountering genetic mutation(s) and/or environmental alteration(s). The $CD44^{+}CD24^{-/low}Lineage^{-}$ cell population used to identify CSCs in breast cancer patients is reminiscent of the $CD44^{+}CD24^{-/low}$ cell population acknowledged to be mammary gland progenitor cells [178]. Another theory suggests that CSCs arise from somatic cells that acquire stemness characteristics and malignant behavior through genetic alterations. It is still not well understood whether breast CSCs originate by the oncogenic transformation of normal mammary stem cells or after the dedifferentiation and acquisition of stem cell characteristics by carcinoma cells, or if both mechanisms contribute to the generation of breast CSCs. EMT contributes to the acquisition of stem cell-like characteristics in cancer cells, and EMT results in mesenchymal traits and the expression of stem-cell markers in human mammary epithelial cells. Further, these cells have an increased ability to form mammospheres [58], and various studies have confirmed that mammospheres contain stem-like cells that can generate an entire mammary ductal tree when implanted into a cleared mouse mammary fat pad. CSCs might promote the invasion and metastasis of tumors by acquiring some properties of mesenchyme. When cancer cells interact with fibroblast, granulocytes, macrophages and other cells in certain environmental niches, these cells may release the signal to induce EMT, resulting in the increased invasion of cancer cells. Recent studies show that reprogramming MCF-10A non-tumorigenic mammary epithelial cells with OCT4, SOX2, Klf-4, and c-MYC can generate breast CSCs [179].

The induction of EMT enhances self-renewal and the acquisition of CSC characteristics. Emerging evidence suggests that breast cancer stem cells and EMT cooperate to produce CTCs. CTCs and CSCs have been identified in the bloodstream of patients with metastatic disease. The characterization of CTCs from patients as cells with an EMT signature has provided evidence that EMT also occurs during the dissemination of cells from a primary tumor [80]. In the circulation, epithelial cells tend to die from anoikis, and it has been posited that the prior activation of EMT during the initial invasion of metastasis may render the cells resistant to anoikis. Indeed, the EMT-induced loss of cell polarity in metastatic cancer cells downregulates the Hippo pathway and leads to such resistance [45].

8.10 Potential Clinical Application Components of EMT Pathways as Therapeutic Targets

EMT-linked pathways can provide targets for novel drug discovery. A recent bioinformatics analysis has exemplified how to leverage this EMT phenotype to guide therapeutic intervention [180]. EMT has a profound impact on neoplastic progression and patient survival as well as on the resistance of cancers to therapeutics.

New therapeutic combinations using genotoxic agents or EMT signaling inhibitors are therefore expected to circumvent the chemotherapeutic resistance displayed by these cancers. Thus, targeting critical orchestrators at the convergence of several EMT pathways, such as NF-kB, AKT/mTOR, MAPK, β-catenin, protein kinase C, SMAD factors, and microRNA family members will likely be of clinical significance.

In a study to identify inhibitors of EMT in carcinoma, an EMT inhibition screening assay was developed for the high-throughput/high-content screening of small molecule compounds. Druggable targets ALK5, MEK, SRC, and, to some extent, PI3K may play a significant role in EMT modulation and cancer progression [181]. A knockdown in the expression of genes promoting EMT was found to be an efficient way to revert the EMT phenotype, with the knockdown of Snail or Twist able to restore the sensitivity of the cells to cisplatin [182]. Targeting miRNAs is another interesting approach that would complement conventional or targeted therapies in cases where there is evidence of EMT-mediated resistance. Indeed, downregulation of miR-221 and miR-222 restored the sensitivity to tamoxifen [183], and there is evidence to show that curcumin, epigallocatechin-3-gallate, indol-3-carbinol, and flavonoids each can modulate miRNA expression and EMT [184].

Sorafenib, a small molecule that inhibits the kinase activities of Raf-1 and B-Raf, inhibits HGF-mediated EMT in hepatocellular carcinoma [185]. Celecoxib, a chemopreventive and therapeutic drug, inactivates EMT both in vivo and in vitro in colon cancer experimental models, thereby contributing to the inhibition of cancer cell growth by modulating β-catenin signaling [186]. Metformin, in association with chemotherapy, reverses multidrug resistance and the TGF-β-induced EMT phenotype, resulting in cancer cell death in human breast cancer cells. Metformin can also inhibit the growth of melanoma and human colon cancer cells [187]. Finally, an in vitro study found that colon cancer cells were sensitized to 5-fluorouracil paclitaxel and etoposide. Dasatinib, a small molecule tyrosine kinase inhibitor, targets a wide variety of tyrosine kinases and also inhibits tumor cell migration and invasion in a number of different tumor types. Also dasatinib was reported to inhibit the development of metastases in mouse models [188]. Collectively, these preclinical data underline the possibility of increasing cell sensitivity and reversing drug resistance by interfering with pathways that lead to EMT [189].

Various studies have revealed that ovarian cancer cells with epithelial features are more sensitive to chemotherapy. Epimorphin, also known as syntaxin-2, is a morphogenic protein previously studied for its role in MET. It is also a pro-epithelial factor in ovarian cancer cells and has been shown to promote the conversion of mesenchymal cancer cells towards a more epithelial-like phenotype, as characterized by the loss of mesenchymal phenotypic traits and the acquisition of epithelial ones [190]. Thus, epimorphin induces morphological changes reminiscent of MET in ovarian cancer cells, and this leads to their enhanced sensitivity to carboplatin. Elemene, an active component of the herbal medicine *Curcuma wenyujin*, has been clinically used to treat leukemia and carcinomas in brain, breast, liver, and other

tissues. It has been shown that elemene blocks and reverses the EMT process in the MCF-7 cells through the Smad3-mediated downregulation of nuclear transcription factors [107]. Another compound reported from cultured microorganisms from the bark of the yew tree, alternol, has been shown to have an inhibitory effect on cells derived from mouse lymphocyte leukemia and human gastric and prostate cancers. Alternol also inhibits the migration and invasion of human hepatocellular carcinoma cells by reversing the process of EMT [191]. And finally, Nutlin-3 has been identified as a potent and selective small-molecule MDM2 antagonist with an inhibitory effect on metastasis. Nutlin-3 has been shown to prevent TGF-β1-induced downregulation of E-cadherin and abrogate the increase in mesenchymal markers in p53-deficient cancer cells [192].

8.11 Summary and Perspectives

Extensive research on CTCs has fully demonstrated their independent prognostic relevance for the survival of patients with metastatic breast cancers [68], but current attempts to expand upon this prognosis have been unsuccessful, indicating a pressing need to develop novel strategies for CTC retrieval and characterization [193]. EMT is increasingly being explored in human carcinoma to more clearly define its involvement in cancer progression and metastasis as well as in the onset of drug resistance or treatment relapse [194]. Preliminary attempts for CTC cultures have demonstrated the potential to utilize expanded primary cell populations in the clinic, such as for monitoring treatment efficacy, patient overall survival, and early-stage cancer detection. There are also efforts to generate new therapeutic combinations, such as the use of small molecule compounds, against these rare cell populations with heightened drug resistance or tolerance that are undergoing EMT (e.g., CSCs). A complete understanding of EMT is key for the development and success of therapeutic interventions against these cells. Additionally, the identification of an EMT signature will open the doors for direct and targeted therapies for new diagnostics of particular advantage. A true transition from basic research to clinical research strongly requires investigating new therapeutic targets and the development of new drugs. Indeed, EMT inhibition or stem cell reversal to a more differentiated, epithelial phenotype through MET could enhance cell death or sensitize cancer stem cells to conventional therapies. The role of EMT with existing therapeutics as well as new drug advancements will be vital for the discovery of new strategies to prevent metastasis.

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Part III
CTC Analysis

Chapter 9
CTC Analysis: FISH, ISH, Array-CGH, and Other Molecular Assays

Verena Tiran, Marija Balic, and Nadia Dandachi

Abstract Recent findings have unveiled a remarkable heterogeneity and complexity behind cancer diseases. Specifically, intratumoral heterogeneity has been associated with therapeutic failure and drug resistance, posing considerable clinical challenges to finding effective treatment modalities. Therefore, assessing tumor heterogeneity is clinically important for developing novel and efficient targeted therapy concepts. Molecular analysis of circulating tumor cells (CTCs) may be an exciting and promising surrogate biomarker to assess tumor heterogeneity. With recent advances in molecular analysis, single-cell profiling is possible and allows assessment of the extent of heterogeneity between individual cells. Molecular profiling of CTCs may help to identify specific subsets of CTCs with tumorigenic potential and paves the way for developing therapies specifically targeting these cells. Here we focus on currently used molecular assays, including (fluorescence) in situ hybridization, array-CGH, next-generation sequencing, qualitative and quantitative RT-PCR, and microarrays. We show how information obtained by these technologies has contributed to a better understanding of tumor and CTC biology and how this knowledge may be translated into a meaningful clinical benefit.

Keywords Cancer • Metastasis • Circulating tumor cells • Molecular profiling • Tumor heterogeneity • Prognosis • Prediction • Molecular assays

9.1 Introduction

Large-scale sequencing studies of solid tumors have revealed extensive tumor heterogeneity within individual primary cancers providing evidence for a remarkable genetic complexity of malignant tumors [1, 2]. This intratumoral heterogeneity may

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be responsible for therapeutic failure and drug resistance in cancer, posing considerable clinical challenges to designing effective treatment modalities. Importantly, intratumoral heterogeneity is not limited to the primary tumor, but is also observed in distant metastases. This is especially important, given that distant metastases are responsible for the majority of cancer related mortality, and that therapeutic decisions are typically based on analysis of primary tumors [3]. Indeed, several studies have described the discordance in diagnostic markers between primary and metastatic tumors [4]. Therefore, assessing tumor heterogeneity is clinically important for a proper management of cancer patients and developing novel and efficient treatment concepts [3].

Molecular analysis of circulating tumor cells (CTCs) may be a valuable potential surrogate biomarker to assess tumor heterogeneity. Furthermore, molecular profiling of CTCs may prove a promising alternative and complementary biomarker for identification of predictive targets and monitoring of disease progression. With recent advances in molecular analysis, single-cell profiling is possible and allows assessment of the extent of heterogeneity between individual cells [5–7].

Recent findings suggest that treatment failure and tumor relapse may be attributed to a minor subpopulation of tumor cells, termed cancer stem cells (CSCs), which sustain tumor growth, seed metastases and resist conventional therapies [8, 9]. CTCs and CSCs share several properties, leading to the hypothesis that circulating CSCs initiate metastases. CSCs can be identified by cell surface markers depending on the type of cancer like CD44, ALDH, or by their expression profile of epithelial-mesenchymal transition (EMT) proteins or stem cell specific factors [10]. In addition, cancer cells can change their genetic phenotype during disease progression and drug treatment, making it more difficult to target them [11, 12]. Molecular characterization of CTCs may better represent heterogeneity of disease and may help identify novel therapeutic targets and understand therapy resistance mechanisms in these cells.

9.2 Enrichment and Molecular Analysis of CTCs

Very few CTCs are present in patients even with metastatic disease, with an estimated frequency of only one cell per milliliter of blood. Because CTCs are so rare compared to circulating blood cells, specific and sensitive enrichment methods for their detection and characterization are needed [13]. As a result, a variety of enrichment techniques have been used. In general, they are based on either physical or biological properties of CTCs that distinguish them from non-tumor cells. Here we provide a brief outline and refer the reader to several comprehensive reviews available on this topic [14–16].

Antibody-based methods are the most widely used approaches and depend on antigens that are differentially expressed by CTCs but not blood cells. The most commonly targeted antigen on CTCs is the epithelial cell adhesion molecule (EpCAM, also known as HEA or Ber-Ep4). The semiautomated CellSearch® platform is the most successful and recognized antibody-based enrichment technique using magnetic beads that are linked to an EpCAM antibody [13]. Although EpCAM

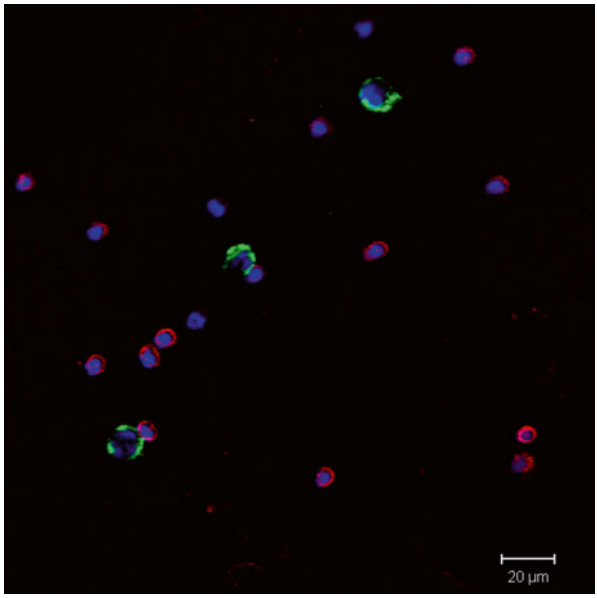

Fig. 9.1 Immunofluorescence staining of captured tumor cells expressing CK (*green*) and peripheral blood mononuclear cells expressing CD45 (*red*). DAPI (*blue*) was used for nuclear visualization

is expressed in most cancers of epithelial origin, CTCs with low or no expression may be missed using such an enrichment technique. For example, EpCAM is frequently not expressed in some epithelial tumors such as renal cell carcinoma, urothelial cancer, and squamous cell cancers [17]. Evidently, non-epithelial cancers such as melanoma cannot be analyzed using such methods. In breast cancer, heterogeneous expression of EpCAM has been observed with lobular subtypes showing no or weak expression [17–19]. There is also evidence that a subset of CTCs undergoes EMT, characterized by downregulation of epithelial markers such as EpCAM [20, 21]. Enrichment techniques depending on EpCAM might fail to capture an important subpopulation of CTCs.

Other enrichment approaches are based on physical properties such as differences in cell density. Examples of such approaches are Ficoll Hypaque separation [22] and OncoQuick [23]. Another property of tumor cells is cell size and has been successfully used for CTC enrichment [24–28]. Since this enrichment approach is independent of antigen expression on CTCs, it is applicable to a broader range of tumor types and has the potential to enrich different subpopulations of CTCs enabling assessment of tumor heterogeneity. At this point it becomes clear that different enrichment technologies do not always detect the same subpopulations of CTCs and may limit significance of subsequent conclusions.

Currently, none of the available enrichment platforms provides a pure population of tumor cells and therefore all separation techniques require subsequent analysis to distinguish CTCs from nonspecifically captured cells. The most common approaches involve either immunocytochemical/immunofluorescent detection of tumor-specific antigens such as cytokeratins (Fig. 9.1) or varying molecular approaches [15].

Molecular assays are based on the identification and characterization of nucleic acid sequences and have various advantages including high sensitivity and multiplexing capacity. New technical advances in DNA and RNA analysis of CTCs have resulted in an increase in scientific knowledge leading to a better understanding of tumor and CTC biology and tumor heterogeneity. Specifically, genomic analysis of single CTCs now enables assessment of tumor heterogeneity. Moreover, investigation of changes in molecular profiles of CTCs during treatment has become increasingly important, since this may serve as biomarkers of treatment response or resistance and lead to the design of novel molecular therapies specifically targeting CTCs with tumorigenic potential [29, 30]. Several studies have already confirmed the feasibility of molecular CTC analysis in order to associate genomic alterations with cancer progression and monitoring response to targeted therapy [29, 30]. However, the clinical value of molecular CTC analysis has yet to be confirmed by large prospective trials.

Here we focus on currently used molecular detection technologies for CTC analysis and how resulting discoveries have contributed to a better understanding of tumor and CTC biology and how this knowledge may be translated into a clinical benefit.

9.3 In Situ Hybridization (ISH) Techniques

In situ hybridization (ISH) assays are used to localize nucleic acid information or visualize gene expression products within a morphologic context. These methods rely on hybridization of a labeled nucleotide sequence to a complementary RNA or DNA target sequence. In contrast to immunohistochemistry (IHC) methods, where the protein content of the cell is detected, ISH is able to localize RNA or DNA at the cellular level in heterogeneous tissues [31]. Also, RNA ISH can be the method of choice if antibodies are not commercially available, because virtually all sequences can be custom labeled.

Typically, the sequence probes are subjected to fluorescence labeling to visualize chromosomal abnormalities in cancer cells with the possibility to stain several targets simultaneously [32]. The ability to visualize genomic alterations with cellular confirmation is one important advantage of ISH techniques. Other advantages of fluorescence in situ hybridization (FISH) include the availability of automated FISH enumeration systems and the ability to define threshold values based on quantifiable ratios of mutation to parent chromosome [29].

Alternatively, probes can be labeled with radioisotopes or can be visualized with chromogenic substrates similar to those used with IHC. Such chromogenic ISH methods allow the convenient use of bright field microscopy and the visualization of gene alterations within the histological context [33].

FISH has been successfully applied to assess HER2 amplification status in primary and metastatic tumors [34]. HER2 overexpression is associated with enhanced tumor aggressiveness, therapy resistance and poor prognosis for patients [35]. Treatment with HER2 targeting agents, such as trastuzumab, lapatinib, and

pertuzumab, significantly extend disease-free survival [36–38]. Recent studies revealed discordance in HER2 status between primary and metastatic site, suggesting that a subset of patients who might benefit from HER2 targeted therapies might be missed [39, 40]. Studies examining HER2 status in CTCs may provide a complementary and alternative surrogate assay. For example, Flores and colleagues used FISH to identify CTCs in breast cancer patients [41]. They revealed discordant HER2 amplification between CTCs and corresponding primary and metastatic breast cancer tumors. Similarly, Punnoose et al. determined HER2 status by FISH in CTCs from metastatic breast cancer patients receiving treatment with Herceptin [42]. Although the majority of patients showed concordance with HER2 status from patient tumor tissue, in a subset of patients (11 %), HER2 status in CTCs differed from HER2 status in the primary tumor. Meng et al. reported on a subset of patients who had acquired HER2 gene amplification in their CTCs during disease progression. In contrast, another study found a high concordance between HER2 status in CTC and primary tumors [43]. Taken together, these results suggest that molecular profiling of CTCs may serve as biomarkers of treatment response or resistance.

The feasibility of FISH to identify copy number alterations in CTCs was also demonstrated in other tumor entities. One study isolated CTCs of metastatic prostate patients and hybridized cells with androgen receptor (AR) and MYC probes. Patients with more than ten CTCs showed an increase in AR amplification and additionally a MYC copy gain, a molecular profile that has been reported for metastatic prostate tumors. These findings proved useful for late stage prostate cancer and the authors suggest this method as a noninvasive way for routine tumor profiling [44]. Attard et al. successfully applied FISH technology to characterize CTCs for heterozygous or homozygous deletion of PTEN in prostate cancer patients [45]. In their study, Swennenhuis et al. characterized CTCs of patients with metastatic castration-resistant prostate cancer using fluorescence labeled DNA probes to target specific chromosomes [46]. This allowed visualization of abnormal numbers of chromosomes and consequently identification of cancer cells. Again, results showed heterogeneity not only between patients but also among CTCs of the same patient. All these studies underscore the feasibility of FISH technique to molecularly characterize CTCs and provide important evidence that in the future genomic alterations in CTCs could be utilized for assessing disease progression.

More recently, Yu and colleagues developed fluorescent RNA-ISH to visualize WNT2 expression in CTCs of pancreas cancer patients. They found that overexpression of WNT2 in CTCs enhanced sphere forming capacity (an established in vitro surrogate marker for self-renewal activity of CSC) and metastatic potential. This study suggested a noncanonical WNT expression during the hematogenous spread of pancreatic cancer, because CTCs showed a higher expression of WNT2 in metastatic cells compared to the primary tumor [47]. The study by Payne and colleague is another example that illustrates the feasibility of multiplex fluorescent RNA ISH to detect and molecularly characterize CTCs [48]. They demonstrated that CTCs detected with RNA ISH predicted shorter progression-free survival in metastatic breast cancer patients, highlighting the functional importance of the CTCs detected with this technology.

9.4 Array-CGH

While multicolor FISH can simultaneously identify several chromosomal variations in a tumor cell, array comparative genomic hybridization (aCGH) is able to provide genome-wide information on copy number variations along each chromosome (Fig. 9.2). In a typical aCGH experiment, competitive hybridization between DNA from a patient and a healthy control is performed to identify copy number changes between the two samples. However, depending on the experiment, controls can vary, and information can be obtained, for example, regarding differences between CTCs and primary/metastatic lesions, or CTCs in treatment responders versus nonresponders [29]. With recent advances in the technique of aCGH it is now even possible to analyze the genome of single cells. Gene-profiling studies of CTCs using aCGH provide essential insight into tumor progression and the extent of tumor heterogeneity [49]. As with all molecular techniques, however, genomic alterations detected with aCGH might not necessarily reflect the CTC phenotype at the functional protein level [29].

Holcomb et al. examined the genome of a small number of CTCs isolated from prostate cancer patients using rare cell genomic amplification and aCGH. They established a protocol with consistently low levels of experimental noise, acceptable dynamic range for detecting chromosomal abnormalities, and reproducibility across biological replicates [50]. With this proof-of-principle study they demonstrated that CTCs from nonmetastatic prostate cancer patients had fewer genomic alterations compared to primary tumor cells and metastatic prostate cancer patients compared to normal.

In a later study, Paris et al. demonstrated that copy number profile of CTCs detected in castration resistant prostate cancer patients was similar to that of their paired solid tumor DNA, but distinct from corresponding DNA from the remaining depleted mononuclear blood cells after EpCAM enrichment of CTC [51]. Similarly, Magbanua et al. performed copy number profiling on CTCs isolated from castration

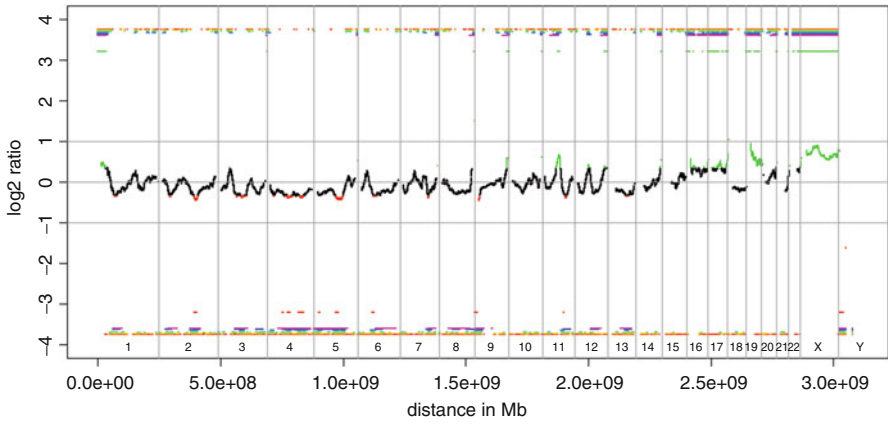

Fig. 9.2 Example of an aCGH profile, showing copy number variations in tumor cells enriched from pleural effusion of a breast cancer patient. Color represents copy number status *red*, lost regions; *green*, gained regions; *black*, balanced regions

resistant prostate cancer patients and found high-level copy number gains at the androgen receptor locus in a majority of CTCs, but not in the matched primary tumors [52]. In a more recent study, the same group successfully applied aCGH to profile CTCs from metastatic breast cancer patients [53]. In this study they also showed that serial testing of CTCs indicated genomic change over time, suggesting that this approach may be used to explore genomic alterations involved in cancer progression and to monitor efficacy of targeted therapies in clinical trials.

In another recent study, Heitzer and colleagues isolated single CTCs from patients with metastatic colorectal carcinoma and applied aCGH and NGS of a panel of 68 CRC-associated genes. In comparing the findings with primary tumors and metastases, they identified multiple copy number changes that were also present in the primary tumors. Some of the mutations demonstrated in CTCs were also present in the tumor but at subclonal levels. Other genetic variations were exclusive to the CTCs and might serve as targets for specific therapies [54].

9.5 Next-Generation Sequencing (NGS)

Identification of oncogenic gene alterations that cause uncontrolled cell growth and/or regulation is currently performed in the primary tumor and has successfully led to the development of cancer therapies. Examples are mutations such as EGFR [55] and ALK [56] in non-small-cell lung cancer (NSCLC) or BRAF/KRAS in colorectal cancer [57]. However, mutations may differ in metastases compared to the primary tumor, and hence targeting of all cell types not being possible [4].

NGS technologies have transformed molecular tumor genetics, since they provide a comprehensive analysis of genomic tumor landscapes, revealing important insights into tumor heterogeneity. While aCGH detects larger aberrations, next-generation sequencing methods can provide information about smaller genetic changes including point mutations, rearrangements, and small insertions/deletions. Sequencing technologies can be applied to both genomic DNA and transcribed RNA sequences and is now extensively used for various applications, including CTC analysis. Several NGS platforms are now available and an overview of the most commonly used platforms is provided in Table 9.1 [29, 58–61].

Table 9.1 Examples of NGS platforms

Sequencing platform	Mechanism	Read length per reaction	Time	Template preparation
Roche 454	Pyrosequencing	400 bp–400 Mb/run	10 h	Emulsion PCR
Illumina/Solexa	Reversible termination	2× 100 bp/run	11.5 days	Solid phase
ABI-SOLiD	Sequencing by ligation	2× 60 bp/day	8 days	Emulsion PCR
Ion torrent	H^+-ion sensitive transistor	320 Mb/run	8 h	Emulsion PCR

The first NGS platform was developed in the 2000s by the 454 Life Sciences Company, commercialized by Roche. This NGS method used PCR amplification and involved sequencing-by-synthesis based on pyrosequencing [62]. An alternative technology is the SOLiD™ system developed by the Applied Biosystems Company, which is a sequencing by oligonucleotide ligation technology. This sequencing platform yields high sequencing throughput but individual sequence readouts are relatively short [63]. More recently, a related sequencing technology was developed by the Illumina Company. Their Solexa system has even greater sequencing throughput but comparatively smaller individual sequence readouts. Currently, Illumina is one of the cheapest methods for the production of a high number of short reads along the genome. An advantage compared to microarray analysis is that the samples can be barcoded and pooled together in one reaction tube. In this way, up to 200 single cells can now be sequenced in one Illumina run [64].

One of the first studies to show feasibility of single cell RNA sequencing with improved coverage across transcripts allowed detailed interrogation of CTC transcriptomes [65]. Using this technique the authors were able to identify distinct gene expression patterns including candidate biomarkers for melanoma circulating tumor cells. A similar study applied RNA sequencing on EpCAM-positive CTCs from metastatic prostate cancer patients [66]. Another study by Hardt et al. demonstrated the technical feasibility of NGS by comparing NGS results with microarray results. They isolated CTCs from estrogen-positive breast cancer patients and performed gene expression profiling with both methods. The advantage of NGS compared to microarray is that it can also detect new transcripts and it is not limited to known genes [67]. However, transcriptional profiling is hampered by the labile nature of RNA, a problem that was particularly often observed in single cell RNA sequencing studies [65, 66]. Technical advances in the preservation and stabilization of RNA will help to overcome this limitation.

One of the first comprehensive genomic profilings of CTCs was performed by Heitzer et al. [54]. Ultra-deep sequencing of colorectal primary tumors and metastases revealed the presence of driver mutations at subclonal level that were initially missed but were found in CTCs. In addition, they also captured novel mutations that were unique to single CTCs, providing evidence for cell-to-cell heterogeneity. This study lays a firm foundation for the most important clinical benefit of genomic sequencing of CTCs, where this approach may be used to monitor genomic tumor evolution within a patient over time and subsequently may help to improve treatment decisions.

Most recently, Marchetti et al. assessed EGFR mutations in CTCs from NSCLC patients by ultra-deep NGS. They suggest a genetic heterogeneity for EGFR mutations in CTCs and confirmed earlier findings that EGFR mutations can differ between the primary tumor and metastatic sites. Also tumor heterogeneity can explain these results and may play an essential role in therapy resistance [68]. In another recent study, the investigators analyzed primary tumor and metastatic biopsies from renal cell carcinoma patients by using whole-genome, whole-exome, and transcriptome sequencing. At the molecular level, metastatic cells showed a high dissimilarity compared to the primary tumor, which in part explains poor prognosis for the patients [69].

As with other genomic technologies, it is yet unclear if genomic sequencing will provide functionally relevant information that can be used for predicting patient outcome and therapy response. Combination of this approach with other phenotyping platforms may help to overcome this limitation in order to draw meaningful information and conclusions for improved clinical treatment decisions [29]. Also, by improving the sequencing technology systems and developing third and fourth generation sequencing techniques, this methodology will become a more attractive and economical approach in the clinical setting [63].

It is important to mention that sequencing techniques have also been successfully applied to analyzing cell-free circulating tumor DNA (ctDNA). Currently, genotyping of ctDNA is a fast growing field with the greatest potential for clinical implementation due to its high specificity and technological simplicity. However, this topic was out of the scope of the present chapter. Despite these advantages, CTCs provide the unique ability to study viable tumor cells and obtain functionally relevant information on tumor biology and metastasis.

9.6 PCR Techniques and Other Molecular Assays

The most commonly used mRNA-based method for detecting CTCs is reverse-transcriptase polymerase chain reaction (RT-PCR) or quantitative real-time RT-PCR (qRT-PCR). The high sensitivity together with the multiplexing capacity make these approaches flexible, cost- and time-efficient. The main disadvantages of mRNA-based methods are that no information regarding the morphology can be obtained and the accurate number of CTCs cannot be estimated [15].

In breast cancer, CK19 has been most commonly used to detect CTCs [70, 71]. In addition to cytokeratins, several other potential markers have been studied, examples being mucin 1, CEA, mammoglobin, and HER2 [15, 29].

Multiplex qRT-PCR helps to address tumor heterogeneity and heterogeneous expression of markers [72–74]. Powell et al. confirmed heterogeneity of CTCs in their study, but more importantly, they found that individual CTCs did not cluster by patient or disease stage [75]. Their finding supports the concept that CTCs belong to a subset of cells with phenotypes fundamentally different from pooled tumor tissue and phenotyping the primary tumor alone might lead to suboptimal treatment decisions. A commercially available molecular assay, the AdnaTest™ (AdnaGen), uses multiplex RT-PCR to identify tumor-associated genes expressed in CTCs from breast cancer patients. This assay showed good concordance with other molecular assays and equal sensitivity to the CellSearch system [76].

Smirnov and colleagues were one of the first to perform global gene expression profiling of CTCs in colorectal, prostate, and breast cancer patients. They generated a list of CTC-specific genes and used qRT-PCR to differentiate the expression level of this gene set in patients compared to normal controls. Their study illustrated, for the first time, the feasibility of performing global gene expression profiling in CTCs [77].

Barbazan et al. applied whole transcriptome amplification and gene expression analyses on EpCAM-enriched CTCs isolated from metastatic colorectal cancer patients. They identified a 410-gene signature that characterized CTCs. Validation of a number of genes was performed by quantitative RT-PCR in an independent set of patients [78]. Sieuwerts et al. reported that profiling a low number of CTCs might result in discrepant estrogen receptor and HER2 status profile compared to primary tumor, a finding that could impact the use of current therapeutic strategies in breast cancer [79].

Gene expression profiling studies, such as those evaluating the expression profiling of EMT related and CSC signatures in CTCs [20, 21, 80–82], have enabled a more detailed evaluation of the biologic events associated with CTCs and cancer metastasis. These studies provide important evidence that genomic assessment of CTCs may serve as a research tool for exploring the biology of metastasis.

So far, only few studies have investigated epigenetic alterations in CTCs. DNA methylation is an important epigenetic mark controlling gene expression and altered DNA methylation patterns are hallmarks of human cancers [83]. Chimonidou et al. were the first to provide evidence that methylation of tumor suppressor and metastasis suppressor genes occurs in CTCs. In their studies, they tested EpCAM-positive CTCs from breast cancer patients and found by methylation-specific PCR that promoters of the genes cystatin M (CST6), breast cancer metastasis suppressor 1 (BRMS1) and SRY-box containing gene 17 (SOX17) were highly methylated [84–86]. Furthermore, they demonstrated heterogeneity among CTCs at the epigenetic level, as shown by different methylation profiles in individual patients. Sieuwerts et al. were the first to study miRNA expression in CTCs and demonstrated specific expression of ten miRNAs in CTCs isolated from metastatic breast cancer patients [79]. In contrast to single cell transcriptome analysis, investigation of DNA methylation patterns in single cells is primarily hampered because of more complex methodologies. Such studies will become more frequent as new technologies become available to study epigenetic alterations from single cells [87].

9.7 Conclusions and Future Directions

Advances in technologies have enhanced molecular characterization of CTCs, and recent results underscore the potential of CTCs as prognostic and predictive biomarkers, and ultimately as a research tool to identify novel therapeutic targets. Findings also unveil, however, the remarkable heterogeneity and complexity behind cancer diseases. Not surprisingly, this heterogeneity is also found in CTCs. For example, EpCAM-based techniques only enrich for a subpopulation of CTCs, suggesting that a subset of CTCs with EMT phenotype that are responsible for metastasis might be missed because they do not express epithelial markers. Genetic tumor heterogeneity has been associated with treatment failure and resistance and can pose essential clinical challenges. CTCs may offer an ideal opportunity to assess tumor heterogeneity and to identify cells with a metastatic and therapy

resistant phenotype in order to guide and improve therapeutic interventions for an individual patient. Rapid technological advances in molecular assays pave the way for exiting new insight into the biology of CTCs. Meanwhile, enrichment of CTCs remains the greatest technical challenge and limits the number of CTCs that can be analyzed in a patient. Despite the availability of single-cell analysis, accurate interpretation of CTC heterogeneity remains difficult. It is clear that development of novel technologies that enhance CTC capture are needed to resolve this limitation. The great challenge in the future will be to extract clinically meaningful information from this data and translating it into a clinical benefit for patients.

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Chapter 10
Genome-Wide Gene Copy Number Analysis of Circulating Tumor Cells

Mark Jesus M. Magbanua and John W. Park

Abstract Very little is known about the molecular biology of CTCs. The paucity of information can be largely attributed to the technical hurdles in isolating these extremely rare cells. Despite these challenges, there is a pressing need to elucidate the molecular characteristics of these tumor cells. In this chapter, we highlight recent studies on genome-wide gene copy number analysis of CTCs and comparisons with primary tumors. These initial studies serve as groundwork for future efforts in discovery and development of novel CTC-based genomic biomarkers. Further molecular profiling of CTCs may provide novel insights into mechanisms of disease progression and tumor evolution, and open new avenues for personalized treatment.

Keywords Circulating tumor cells • Molecular characterization • Copy number analysis • Array comparative genomic hybridizations • Genomic instability • Fluorescence-activated cell sorting • Genomic analysis • Copy number variation • Metastasis

10.1 Introduction

Metastatic spread involves the escape of tumor cells from primary tumors into the blood stream. These tumor cells, also known as circulating tumor cells (CTCs), can migrate to distant sites and initiate metastatic disease. Since the advent of

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technologies to sensitively detect and count ("enumerate") CTCs, it has been demonstrated that elevated numbers of CTCs in the blood of solid tumor patients can portend poor survival [1–5].

The underlying biology of the CTCs themselves remains poorly understood. In principle, molecular characterization of CTCs can provide new insights into the biology of cancer metastasis, as well as new biomarkers for personalized treatment. However, progress has been hindered by the formidable technical challenges in efficiently isolating these extremely rare cells (~ one CTC per billion of nucleated blood cells).

A hallmark of cancer is genomic instability [6, 7]. Genome-wide copy number aberrations can be assessed using microarrays (e.g., array comparative genomic hybridization, aCGH) [8] and more recently by next generation (massively parallel) sequencing [9]. Studies involving genome-wide copy number analysis of primary tumors have shown that certain chromosomal regions may be preferentially amplified or deleted in different types of cancers [7, 10–12].

Investigations of genomic alterations in CTCs, including comparative analysis with primary tumors, have recently been reported. In this chapter, we discuss results of genome-wide copy number analysis of CTCs in different solid tumors, including prostate, colorectal, and lung cancers.

10.2 Enrichment and Isolation of CTCs

10.2.1 EpCAM-Based Isolation

Most strategies to isolate CTCs have included EPCAM-based immunomagnetic enrichment methods (e.g., the widely used CellSearch system [13]), which involves the capture of CTCs using magnetic beads conjugated with EPCAM antibody. The enriched population, however, still retains a considerable amount of leukocytes requiring additional steps to further purify CTCs [14]. We have developed a protocol, referred to as "IE/FACS", to isolate highly pure CTC populations from blood [15–17]. IE/FACS consists of an initial immunomagnetic enrichment step similar to that of the CellSearch system. This is followed by the addition of fluorescently labeled monoclonal antibodies specific for leukocytes (CD45) and epithelial cells (EPCAM) to distinguish CTCs from leukocytes during sorting via fluorescence-activated cell sorting (FACS).

10.2.2 Non-EpCAM-Based Isolation

Alternative CTC collection strategies include those based on physical properties, e.g., size and density, which allow separation of CTCs from patients' peripheral normal blood cells [18]. Yet another enrichment approach is based on metastatic

cells' ability to adhere and invade collagen adhesion matrix (CAM) [19]. Using a specialized tube (Vita-Cap™), CTCs adhere to CAM-coated inner walls and non-adherent blood cells are washed away. Collagenase treatment releases the CTCs as well as leukocytes that are nonspecifically attached to the matrix [20, 21].

A comprehensive discussion on the methods involving CTC enrichment and isolation is presented in Chaps.2 and 3.

10.3 Genome-Wide Copy Number Analysis of CTCs

10.3.1 Early Breast Cancer

Since CTCs are much less frequent in early versus metastatic breast cancer, larger volumes of blood are necessary for routine screening to detect these rare tumor cells [22]. To address this limitation, a recent study demonstrated the feasibility of utilizing leukapheretic samples to capture CTCs in early breast cancer patients [23]. Examining whole circulating blood substantially increased CTC detection to about 90 % as compared to 5–24 % in ~10–30 mL of blood [22, 24]. Genomic profiling of captured single CTCs via metaphase CGH analysis detected genomic aberrations in a subset of cells. Aberrations were consistent with that of breast cancer, e.g., loss of 8p and gain of 8q. Additionally, higher levels of genomic alterations were correlated with increased risk of recurrence.

10.3.2 Metastatic Breast Cancer

Our group has combined IE/FACS and array comparative genomic hybridization (aCGH) to perform the genome-wide copy number analysis of CTCs from breast cancer patients [16]. This approach involves efficient and complete isolation of CTCs for molecular profiling; in contrast, methods to capture CTCs via cell adherence, microfluidics, or immunomagnetic separation alone typically entail substantial leukocyte contamination and can compromise the ability to perform genome-wide analyses. Our approach yields genomic profiles of CTCs without significant contamination from leukocytes or non-malignant epithelial cells.

Initial assay validation using breast cancer cells spiked into healthy blood confirmed that accurate aCGH profiles were obtained from small pools of cells, including single cells and showed no evidence of leukocyte contamination. We subsequently applied this approach to blood samples from 181 metastatic breast cancer patients, 102 of which were successfully profiled. Genomic profiling of CTCs revealed numerous copy number alterations, including many previously reported in primary breast tumors, confirming the malignant nature of the CTCs.

Frequent copy number aberrations identified in our series of 102 CTC samples included gains in 1q and 8q and losses in 1p, 2q, 4q, 8p, 11q, 13q, 15q, 16q, and 18q

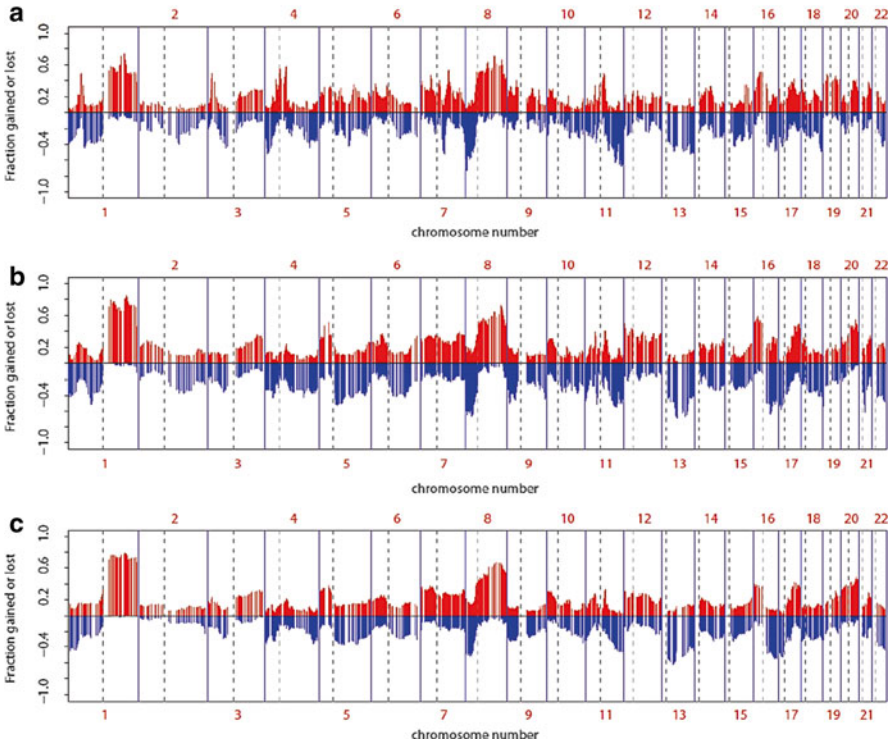

Fig. 10.1 Copy number analysis of breast CTCs and primary tumors. Recurrent gene copy number aberrations in (**a**) CTCs from 102 metastatic breast cancer patients [16] and (**b**) in 62 primary breast tumors [10] and (**c**) 137 primary breast tumors [25]. Gains and losses are shown in *red* and *blue*, respectively. (Reprinted from Magbanua et al. 2013, Cancer research 73(1):30–40)

(Fig. 10.1a). Focal amplifications included 8p11-12 (*FGFR1*), 8q24 (*MYC*), 11q13 (*CNND1*), 17q12 (*HER2*), and 20q13 (*ZNF217*). Comparative analysis between CTC profiles in this study versus primary breast tumor profiles from previously published aCGH datasets by Fridlyand et al. [10] ($N=62$) (Fig. 10.1b) and by Chin et al. [25] ($N=137$) (Fig. 10.1c) revealed high concordance of gains and losses. Recurrent focal amplifications in CTCs were also frequently observed in both Fridlyand and Chin datasets, e.g., on 8p (including *FGFR1*), 8q (*MYC*), 11q13 (including *CCND1*), 17q (*ERBB2*) and regions on 20q (including *ZNF217*).

Next, we compared our CTC CGH dataset and the Fridlyand et al. dataset [10] to explore genomic aberrations specifically prevalent in CTCs but not in primary tumors. Results of this exploratory comparative analysis suggested that specific aberrations including losses on 10q22 and 8p23 and gains on 5q13 (including *CCNB1*), 7q22 (including *MUC12* and *MUC17*), 9p13 and 9q31 were significantly more frequent in CTCs compared to primary tumors (Table 10.1). Because these two datasets were totally independent (i.e., CTCs and primary tumors were *not* from

Table 10.1 Genomic aberrations in CTCs versus primary tumors

Region	Cytoband location	Event	Genes	Freq. in CTCs (%) This study	Freq. in PT (%) Fridlyand 2006	Difference	*p*-value	*q*-bound	Representative genes
chr1: 201,992,107-202,349,947	1q32.1	Gain	18	41.2	74.2	−33.0	4.75E−05	4.06E−03	*CDK18, ELK4*
chr5: 68,169,016-71,536,626	5q13.1–q13.2	Gain	53	35.3	4.8	30.5	3.14E−06	5.66E−04	*CCNB1, CENPH, CDK7, RAD17, BIRC1, NAIP*
chr7: 100,186,769-100,368,731	7q22.1	Gain	6	40.2	8.1	32.1	4.79E−06	7.61E−04	*MUC12, MUC17, SERPINE1*
chr8: 5,847,273-5,941,302	8p23.2	Loss	0	65.7	35.5	30.2	2.00E-04	7.39E-03	
chr9: 33,837,365-34,550,533	9p13.3	Gain	30	45.1	8.1	37.0	2.51E−07	8.47E−05	*UBE2R2, KIF24, DNAI1*
chr9: 110,909,453-111,137,992	9q31.3	Gain	0	39.2	3.2	36.0	3.59E−08	1.62E−05	
chr10: 72,533,114-72,838,622	10q22.1	Loss	7	32.4	1.6	30.7	2.74E−07	6.42E−05	*CDH23*
chr13: 47,964,202-49,047,825	13q14.2	Loss	17	15.7	50.0	−34.3	5.22E−06	5.25E−04	*RCBTB2, RCBTB1*
chr17: 68,538,604-69,170,211	17q25.1	Gain	15	6.9	37.1	−30.2	2.10E−06	4.73E−04	*COG1, SDK2*

Gains and losses with ≥30 % difference in frequency between CTCs (Magbanua et al. 2013 Cancer Res) and primary tumor (Fridlyand et al. 2006 BMC Cancer) and with a *p*- and *q*-value (corrected for multiple testing) <0.05 were considered statistically significant. (Reprinted from Magbanua et al. 2013, Cancer Research 73(1):30–40)

the same patients), we also showed the feasibility of comparing aCGH profiles from CTCs versus matched primary tumors in a series of five cases.

Comparison of CTCs with matched archival primary tumors ($N=5$ pairs) confirmed shared lineage as well as some divergence. Our results indicated a clear clonal relationship between primary tumors and subsequent CTCs, and the appearance of new, as well as conserved, genomic alterations. Two examples are discussed below:

Case 1. Twenty CTCs were isolated at two different time points (Days 1 and 42) from a metastatic breast cancer patient #4013, a 50-year-old female with ER/PR positive and HER2 positive disease. Comparison of the CTC profiles revealed high concordance. Focal amplifications on 8q24, 12q15, 17q12 (*HER2*), and 20q13 were observed in both samples (Fig. 10.2a). Next, archival primary tumor from 6 years prior to CTC analysis was obtained and analyzed via aCGH. Copy number analysis showed multiple aberrations in common with the CTC samples, including the same focal amplifications. Interestingly, some aberrations, e.g., losses in 6q, 13q, 18q, and 20p, were observed only in CTCs and not in the primary tumor suggesting that these cells had acquired additional alterations.

Case 2. Twenty CTCs were isolated from a metastatic breast cancer patient #4015, a 54-year-old female with triple negative disease. Copy number analysis of CTCs showed losses in 3p, 5q, and 6q and focal amplification on 8q24, gains in 10p and 19q (Fig. 10.2b). The archival primary tumor and a lymph node metastasis from 2.5 years prior were then obtained and subjected to aCGH analysis. Copy number profiles of the primary tumor and nodal metastasis, were highly correlated with each other, but to a lesser degree with the CTC profile. CTCs exhibited additional genomic aberrations, e.g., gain in 20q and loss in 3p that were not observed in the primary tumor and nodal metastasis.

10.3.3 Prostate Cancer

Our group has also performed IE/FACS to isolate and analyze prostate CTCs from castration resistant prostate cancer patients [17]. Copy number analysis of CTCs from nine patients revealed a wide range of copy number aberrations, including those that have been previously reported in prostate tumors, e.g., loss in 8p and gain on 8q [11, 26] (Fig. 10.3). However, unlike primary tumors, high-level gains in a region containing the androgen receptor (*AR*) gene in the X chromosome was observed in seven (78 %) of the nine cases, while low level gains were observed in the remaining two (22 %) cases. Amplification of *AR* in CTCs is consistent with observations in castration resistant prostate solid tumors [26]. In addition, comparison of genomic profiles between CTCs from two patients with the corresponding pretreatment primary tumors revealed clonal-relatedness with some divergence including amplification of the locus containing the *AR* region in CTCs but not in the matched primary tissues. This study confirmed other findings by FISH analysis [27, 28] that the *AR* gene can be amplified in CTCs from castration resistant prostate cancer patients in association with hormone resistance.

A CAM-adherence approach was used by Paris and colleagues to enrich for CTCs from blood of 13 metastatic prostate cancer patients [21]. CAM-captured cells including co-purified background leukocytes were subjected to aCGH analysis. Analysis of recurrent aberrations in CTCs from nine patients successfully profiled revealed copy number alterations in cancer related genes (e.g., *POTE15* and *GSTT1*). In contrast to IE/FACS isolated CTCs (discussed above), amplification of the *AR* gene and aberrations (e.g., loss of 8p and gain of 8q) frequently seen in prostate cancers were not observed in CAM-enriched cells. Comparison of genomic profiles of CTCs with primary and metastatic tumors from two patients revealed high concordance.

10.3.4 Colorectal Cancer

In a study involving six metastatic colorectal cancer patients, CTCs were enriched using the CellSearch method followed by isolation of single cells via micromanipulation and aCGH analysis [29]. Analysis of genomic profiles revealed copy number aberrations in CTCs that were commonly seen in colorectal cancer, e.g., losses in 5q13–5q31 which contain the adenomatous polyposis coli (*APC*) gene. Despite some divergent genomic aberrations, genomic profiles of single CTCs were very similar to that of matched primary tumor and metastatic lesions. Additionally, copy number profiles revealed "private" aberrations that were unique to single cells.

10.3.5 Lung Cancer

A similar CellSearch-based approach was used to isolate single CTCs from 11 lung cancer patients [30]. Copy number data was inferred from next generation low pass whole genome sequencing (0.1× coverage). Comparisons of copy number profiles revealed high similarities of genomic profiles among single CTCs from the same patient and among patients. Clonal-relatedness was observed when single cell profiles were compared to primary tumors and available metastatic lesions from the same patient. Interestingly, distinct global copy number profiles were observed between small-cell lung cancer and lung adenocarcinoma.

10.3.6 Melanoma

A study by Chiu and colleagues [31] demonstrated the feasibility of genome-wide copy number profiling of CTCs from melanoma patients with regional metastasis. Antibodies against melanoma-associated cell surface gangliosides were utilized to capture circulating melanoma cells instead of epithelial markers. Array CGH

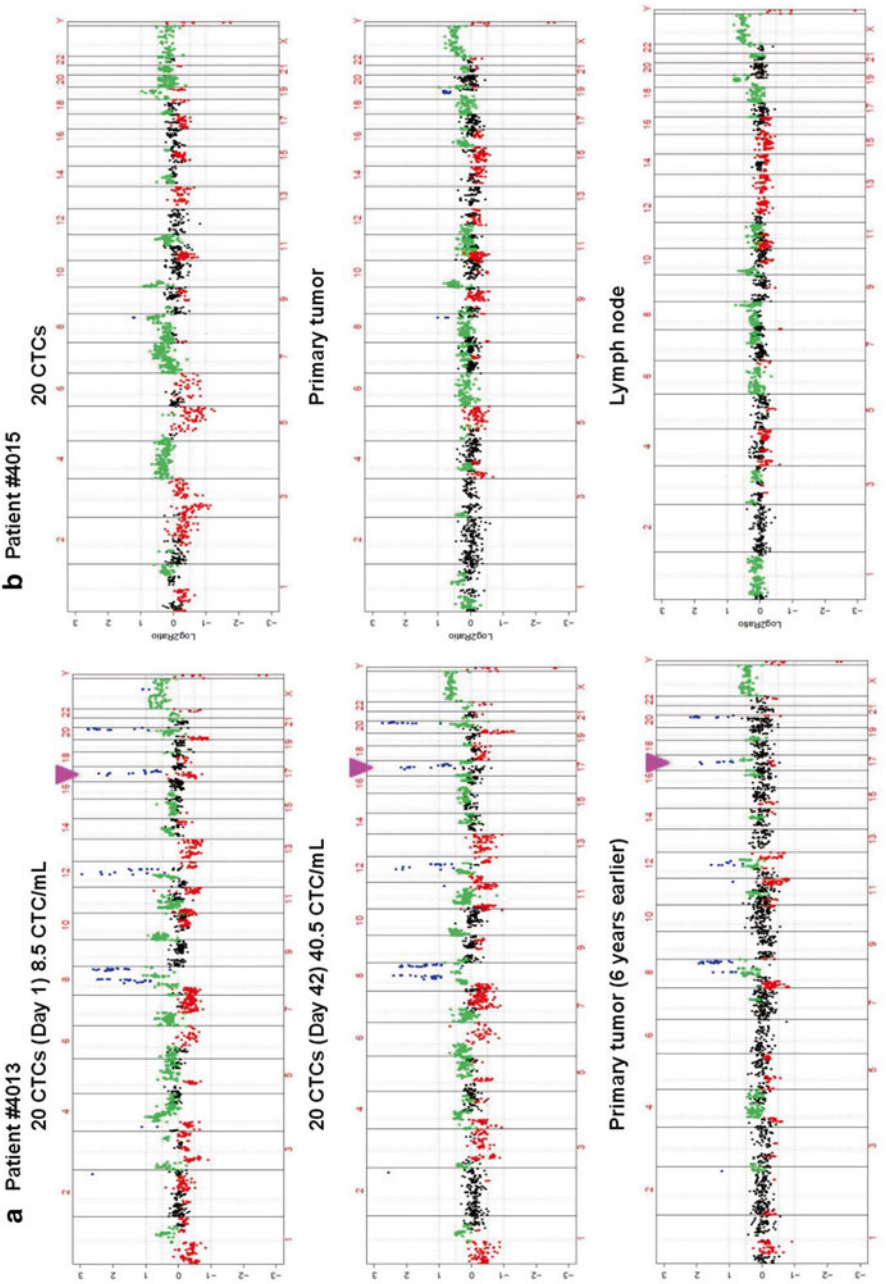
a
Patient #4013
20 CTCs (Day 1) 8.5 CTC/mL
20 CTCs (Day 42) 40.5 CTC/mL
Primary tumor (6 years earlier)
b
Patient #4015
20 CTCs
Primary tumor
Lymph node
Log2Ratio

Fig. 10.2 Case studies for genomic profiling of breast CTCs and matched primary tumor. (**a**) Genomic profiles of 20 CTCs isolated from two independent blood draws (days 1 and 42) and the primary tumor of patient #4013. The patient's primary tumor was surgically removed 6 years prior to the CTC assays. *Arrow* indicates *HER2* amplification on chromosome 17. (**b**) Genomic profiles of 20 CTCs, primary tumor and nodal metastasis from patient #4015. The patient's primary tumor and lymph node were surgically removed 2.5 years prior to the CTC assays. The log2 ratio value for each BAC clone is plotted on the *y*-axis. The *x*-axis represents the genomic position of each BAC clone on the array, with chromosome numbers indicated. *Vertical solid lines* indicate chromosome boundaries, and *vertical red dashed line* represents the centromeric region dividing each chromosome into the p- or short arm (to the left of the centromere) and the q- or long arm (to the right of the centromere). Color represents gene copy number status *red* = loss, *green* = gain, *blue* = amplification, and *black* = no change. (Adapted from Magbanua et al. 2013, Cancer research 73(1):30–40)

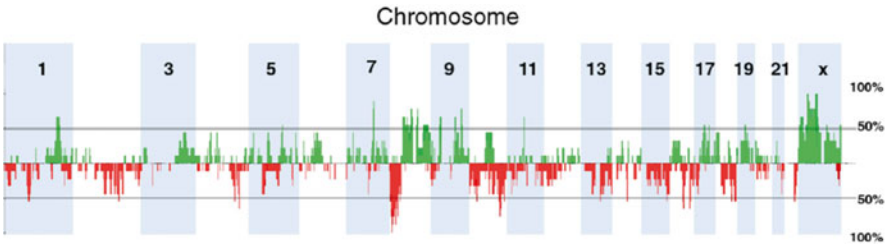

Fig. 10.3 Copy number analysis of CTCs from metastatic castration-resistant prostate cancer patients. Recurrent gene copy number aberrations in CTCs from nine patients; gains and losses are shown in *green* and *red*, respectively. Chromosome Y was not included in the analysis. (Reprinted with permission from Magbanua et al. 2012, BMC Cancer 12(1):78)

analysis of putative circulating melanoma cells revealed copy number gains (e.g., 2q35) and losses (e.g., 6q25.3 and 9q34.3) that were observed in all patient samples. Similar copy number aberrations were seen in circulating melanoma cells and in regional metastasis from the same patient. Interestingly, CTC-associated copy number aberrations were also identified in distant metastasis from advanced melanoma patients, suggesting that certain genomic aberrations are selected for in the course of disease progression. Finally, a biomarker panel composed of five CTC-associated genomic aberrations was able to identify stage IIIB/C melanoma patients with poor clinical outcome.

10.4 Discussion and Summary

Mechanisms involved in cancer progression, including distant metastasis and resistance to treatment, remain elusive. CTCs accessed from the blood may provide insights into how cancers spread and why patients fail to respond to therapies.

In-depth molecular analysis of CTC is fundamental to the elucidation of their role in metastasis. This includes the systematic survey of genomic aberrations throughout the genome, e.g., chromosome gains, losses and focal amplifications, which are hallmarks of malignancy [7]. Initial characterizations of CTCs using fluorescence in situ hybridization (FISH) showed the feasibility of assaying for particular amplification events in CTCs [32–34]. More recently, genome-wide copy number analyses have been reported. The results of these studies, as summarized in this chapter, provide clear evidence that cancer-associated genomic changes can be detected in CTCs [16, 17, 23, 29–31]

Approaches for isolation of CTCs are based on different biological and physical parameters, which may lead to a bias towards certain CTC populations [18, 35]. For example, CTCs from castration resistant prostate cancer patients captured via CAM-adherence [21] and EPCAM-based [17] methods revealed distinct copy num-

ber profiles. It is therefore important to consider the limitations of the capture methods utilized when analyzing CTC genotypes/phenotypes.

The genetic relationship between CTCs and solid tumor tissues obtained from the same patient may shed new light on the mechanisms involved in tumor evolution and progression. Comparative studies between CTCs and corresponding primary and metastatic lesions have confirmed clonal-relatedness [16, 17, 21, 29, 30]. Identification of specific molecular signatures and genetic changes that occur in CTCs could lead to new targets for anti-metastatic therapies and druggable biomarkers.

In the treatment of advanced cancer patients, reliance upon biomarkers obtained from primary tumors has always been a problematic and questionable practice. Recent studies have confirmed that CTCs have the potential to acquire new genomic aberrations, including those potentially associated with disease progression and treatment resistance [16, 29, 30, 34]. CTC-based biomarkers, therefore, may provide more relevant information about molecular target status as well as disease behavior. In the future, clinicians may be able to draw upon molecular profiles of CTCs to provide a more individualized and efficacious treatment.

In conclusion, molecular characterization of CTCs provides an opportunity to develop biomarkers for metastatic breast cancer treatment that are *more accessible* (via blood sampling as a "liquid biopsy") and *more relevant* (reflecting changes associated with metastasis and disease progression) than primary tumor tissue. CTC-based assays can in principle provide easily accessible biomarker information and may be more insightful than those based on primary tumors due to greater relevance to metastatic disease, serial sampling ability, and contemporaneous acquisition with cancer progression. Employing new and powerful approaches including genome-wide profiling of CTCs may provide new insights into mechanisms of disease progression and treatment response/resistance, and open new avenues for biomarker development and personalized treatment.

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Chapter 11
Perspectives on the Functional Characterization and In Vitro Maintenance of Circulating Tumor Cells

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Abstract Circulating tumor cells (CTCs) that detach and migrate from primary tumors are implicated in the metastatic spread of cancer. The identification of CTCs in peripheral blood samples has been associated with poor survival outcomes in various cancer types. As a readily accessible source of tumor tissue there is a vast potential to develop CTCs as a biomarker to advance cancer diagnosis, prognosis and the development of novel and targeted therapies. The fact that CTCs occur as extremely rare events in whole blood presents a technical challenge for characterization, requiring enrichment techniques that are both highly sensitive and sufficiently specific.

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The culture and expansion of CTCs is desirable as a means of yielding a population suitable for comprehensive functional characterization and drug testing. Reports of successful in vitro culture of CTCs are rare, but various approaches have been attempted and significant progress has been made. The development of protocols for reliable and efficient culture of viable CTCs will advance our biological understanding of cancer metastasis and facilitate the development of personalized therapies.

Keywords Circulating tumor cells (CTCs) • CTC Culture • CTC functional characterization • Personalized therapy • Chemoresponse

11.1 Introduction

Metastatic disease accounts for 90 % of cancer-related mortality, and is the most important determinant in the 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 can also occur through widespread dispersion of tumor cells via the lymphatic system and/or entry into the circulatory system. The metastatic spread 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. Additionally, it has been shown that the metastatic process is inefficient, with the overwhelming majority of tumor cells that break free from their primary sites failing to form secondary tumors. This inefficiency is presumably the result of a number of mechanisms including anoikis (activated contact-dependent apoptotic mechanisms following disengagement from the epithelial substratum), as well as by cell shearing induced by circulatory

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stress. Therefore, it is logical to assume that for the successful formation of secondary tumors, malignant cells must undergo a series of molecular changes at their primary site that enable their capacity to migrate and intravasate, survive as anchorage-independent cells, overcome the shear forces and oxidative environment of the circulatory system and ultimately target and colonize secondary organs.

Circulating tumor cells (CTCs) represent the population of cells that have acquired the means to gain access to the circulatory system, and therefore are the cell population that is ultimately responsible for the development distant metastases. As a result, CTCs have emerged in recent years as a biomarker with strong prognostic and diagnostic potential, and the enumeration of CTCs with respect to progression-free survival, overall survival, and therapeutic response has been widely reported on in a number of malignancies [1–6]. In contrast to other sites where tumor cells may have disseminated, such as lymph nodes, bone marrow, ascitic fluids 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. Beyond enumeration, recent advances in this field also suggest that the molecular characterization of CTCs could help improve therapeutic response prediction by directly evaluating the expression of drug targets expressed in malignant cells while in transit to secondary sites [7]. Although beneficial, the types of analyses done to date on CTCs have largely been in static cell populations; this is in part due to the fact that historically, many CTC enrichment approaches do not preserve cell viability. Without readily available methods to enrich viable CTCs, the functional characterization of CTCs is not possible. The ability to perform proteomic and transcriptomic analyses, as well as evaluating the tumorigenic capacity of CTCs in vitro and in vivo, could provide invaluable insight towards further elucidation of the metastatic process. Further, the ability to maintain viable CTCs in culture would not only be important in the research setting, but could enhance the clinical utility of CTCs for therapeutic monitoring through analysis of drug sensitivity. In this chapter, we plan to (1) provide a comprehensive review of recent advances in technologies that allow viable CTC enrichment, (2) discuss how these technologies are currently being applied towards functional CTC characterization, and (3) describe the advances in methods for rare cell culture, limitations and newly found progress in the challenging effort to expand viable CTCs in vitro (Fig. 11.1).

11.2 Technologies for Viable CTC Enrichment Functional Characterization and Cultures

11.2.1 Established from CTCs in Animal Models and Human Blood Samples

CTCs exist as rare cells in circulation, occurring at concentrations on the order of one in one million nucleated cells. It is highly desirable to enrich the CTC population to facilitate the feasibility and efficiency of downstream characterization and

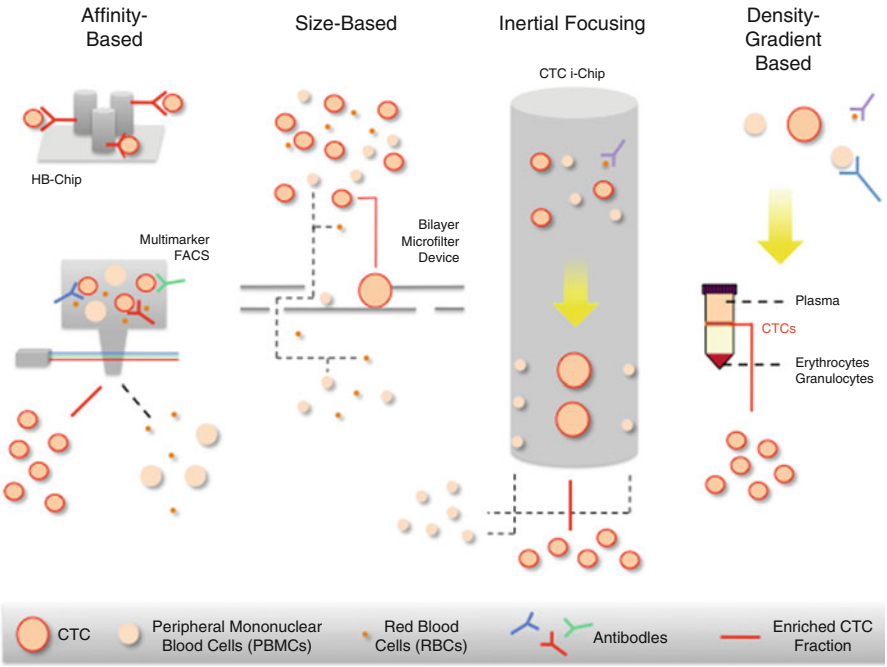

Fig. 11.1 Methods for viable CTC enrichment. By *density gradient centrifugation*, RBC-depleted fractions are layered onto a Ficoll separation medium and centrifuged. The CTC fraction is collected above the Ficoll layer in the buffy coat, while less dense plasma and denser, pelleted tetrameric antibody complexes bound to non-tumor blood cells are discarded. Although size-based methods of CTC enrichment typically require that samples be fixed prior to filtration, newer device architectures have been conceived such that blood can remain unfixed rendering cells of interest viable. Using *a bilayer filtration device*, shown both in top and side views, non-tumor blood cells are permitted to pass large and small pores on both filters to be discarded in the flow-through. In contrast, viable CTCs are trapped at the pore edges by a precisely defined gap distance between the two filter layers. *Immunoaffinity-based* techniques employ antibodies against surface antigens expressed on CTCs either by functionalization of solid supports (as in the HB-Chip) or by fluorescently labeled antibodies detecting specific wavelength emissions for efficient cell sorting. The CTC-iChip is an example of a non-affinity-based enrichment technique called *inertial focusing* where by RBCs are depleted, then differential inertial properties align larger CTCs at the center of a microfluidic tube for collection, while smaller, non-tumor blood cells are pushed to the perimeter and later flowed away from the device

culture assays. Various technologies have been developed to select for CTCs and deplete the blood cell background. Currently, isolation of CTCs by density gradient centrifugation [8, 9], RT-PCR based detection of CTCs [10–12] and affinity-based capture of CTCs using cell surface markers specifically expressed by malignant cells of interest [1–6, 13] and differential separation of CTCs from non-tumor blood cells by size and deformability [14, 15] are the strategies most commonly used to isolate and identify CTCs.

With density gradient centrifugation, whole blood samples from cancer patients are diluted 11 in 1× PBS, layered onto a separation medium (i.e., Ficoll-Hypaque)

and centrifuged. Following centrifugation, the buffy coat containing peripheral blood mononucleated cells (PBMCs) and CTCs, with similar buoyant density as PBMCs, are isolated, and placed onto glass slides for IHC and microscopic identification. More recently, this method has been improved by the development of adjunct technologies such as OncoQuick (Grenier Bio-One), which employs a 50 ml polypropylene tube with a porous barrier inserted above the separation medium that prevents unwanted mixing of blood fractions with differential density, allowing for better resolution of layers and higher CTC recovery, and RossetteSep (Stemcell Technologies) an approach that depletes the blood cell fraction prior to density-gradient separation using Tetrameric Antibody Complexes that recognize non-tumor blood cells and targets them for removal in the high buoyant density pellet fraction.

Affinity-based methods, where CTCs are separated away from non-tumor blood cell fractions by functionalizing support systems with tumor- and/or tissue-specific antibodies such as EpCAM, are typically the most popular and their use for CTC enrichment and characterization has been most widely reported of all available technologies. The first commercially available, and currently the only FDA-cleared technique for CTC characterization in the clinic, is the CellSearch platform (Veridex, LLC). While its efficacy has been demonstrated in a number of malignancies, it was solely designed for the enumeration of CTCs and has very limited flexibility to be used for more in depth molecular characterization. As a result, a number of groups have expanded the ability for CTC characterization by affinity-based capture through the development of newer assays that increase the availability of enriched cells for downstream applications. The Toner group has developed a series of chip-based technologies, where a pneumatic-pressure-regulated pump that moves blood samples across an array of silicon-etched microposts functionalized by EpCAM antibodies. As the blood flows across the "CTC-Chip," targeted cells bind to the microposts and can later be exposed for molecular characterization [13, 16]. Although originally believed to lack the sensitivity necessary to enrich rare cell populations, multimarker fluorescence-activated cell sorting (FACS) methods have been adapted and used successfully for CTC isolation and characterization (references). One such technology, ImageStream (X), combines the strengths of flow cytometry and fluorescent microscopy to isolate and simultaneously image CTCs as they flow past a detector [17, 18].

Alternative label-free approaches have been developed that separate CTCs from blood cells on the basis of physical properties. Microfiltration involves the use of membranes with pores that capture larger CTCs while allowing blood cells to pass through. Traditionally, filtration-based CTC enrichment systems require that blood samples are partially fixed prior to processing, which provides cells of interest the protection against shear stress during filtration, but renders them unavailable for functional assays [19]. To overcome this limitation, we have developed microfilters with precisely defined pore and device architecture modifications that allow for the capture of viable CTCs without any pre-fixation requirement. In one design, pores are fabricated as "slots" rather than circles, which allows for easier deformation and flow-through of non-tumor blood cells in the longitudinal direction. In addition, the slot pore design has a much larger fill factor than other microfilter designs containing round pores, which greatly reduces the flow resistance during filtration. Using this system, we have demonstrated the ability to assess telomerase activity in

metastatic prostate cancer blood samples [20]. In another design, a flexible micro spring array (FMSA) is functions to be the active filtration structure for viable CTC enrichment [21]. The dense, flexible structure helps reduce initial impact forces to cells, improves the porosity to 30–50 %, reduces the processing pressure and increases the sample volume that can be analyzed. Concurrently, we have developed a separable, bilayer CTC capture device (SB microfilter) by adding a secondary membrane layer to the structure [22]. 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. Contrastingly, larger tumor cells are unable to migrate laterally between the two membranes and remain on the device. Using our SB microfilter in blood samples from a mouse mammary tumor model system, our group has recently demonstrated the ability to sensitively and efficiently enrich viable CTCs from which sustainable cell cultures can be established directly on-chip [Williams A, et al., *manuscript in review*]. Additionally, the SB microfilter structure was designed such that the two membranes can be physically separated, allowing for mechanical release of captured cells onto other platforms (e.g., adherent culture flasks or Matrigel), for subsequent culture and molecular analysis.

In addition to these strategies, other techniques for CTC enrichment have been developed using microfluidic flow. Tan et al. used a series of crescent shaped traps in a microfluidic chamber to capture CTCs based on their size and deformability [23]. Inertial microfluidic approaches achieve higher throughput cell sorting through the application of hydrodynamic forces in designed microfluidic channels. This principle was applied in the development of a pinched flow coupled shear-modulated inertial microfluidic device for the isolation of CTCs [14]. 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. CTC are then collected from the axial center, while smaller non-tumor cells are flowed through side outlet channels and removed. Another design that traps tumor cells in generated microscale vortices has been reported [24, 25]. Inertial flow focusing is also exploited in spiral shaped microfluidic channels that isolate CTCs using drag forces [26–28]. Ozkumur and colleagues, in the third generation of the chip-based technology for CTC enrichment first reported by Toner et al., have incorporated microfluidic flow separation with affinity-based capture. Combining inertial focusing with a microfluidic debulking step for size-based separation of CTCs from RBCs and platelets, and deflection of magnetically labeled WBCs into a collection channel different from the CTC channel, the “CTC iChip” has been designed to overcome limitations associated with antigen-dependent enrichment systems [15]. Gleghorn et al. have also developed a novel platform for CTC enrichment that is similar to the original “CTC-Chip” micropost array first developed by the Toner group. 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 chip help to increase the collision frequency of larger CTCs while simultaneously decreasing the collision frequency of smaller non-tumor blood cells, thereby enhancing the opportunity to positively and negatively isolate cells of interest, respectively [29].

Beyond the use of these technologies for the enumeration and molecular characterization of CTCs, recent reports suggest their potential utility for the enrichment of viable CTCs, which would allow for their functional characterization. Using an adaptation of their previously described ^{HB}CTC-Chip for affinity-based CTC capture [13], Yu and colleagues successfully enriched CTCs from an endogenous mouse pancreatic cancer model, where viable CTCs were suitable for the performance of RNA sequencing. Such analyses identified Wnt2 as a gene upregulated in the mouse CTC fraction [30]. Yu et al. further found the expression of Wnt2 in pancreatic cells to be associated with suppression of anoikis, increased metastatic potential in vivo, and enhanced anchorage-independent sphere formation, an effect that was found to be similarly associated with WNT2 expression on human pancreatic CTCs in 5 of 11 cases tested [30]. The same group has extended their ability to perform molecular analysis on viable CTCs at the transcriptomic level in metastatic breast cancer, where RNA sequencing in addition to RNA in-situ hybridization (RNA-ISH) revealed that, an increase in the mesenchymal character of CTCs as compared to corresponding primary tumors correlated with disease progression, as well as reversible shifts between mesenchymal-dominant and epithelial-dominant cell fates throughout therapy in real-time [31]. Using gradient-based centrifugation for CTC enrichment in conjunction with the RosetteSep Human Circulating Tumor Cell Cocktail (Stem Cell Technologies), Hodgkinson and colleagues were able to successfully enrich viable CTCs from patients with small-cell lung cancer (SCLC) for direct injection and establishment of CTC-derived explants (CDXs) in immune-compromised mice [32]. Further CDX molecular analyses by Hodgkinson et al. reveled that CDXs mirror the donor patient's response to platinum-based and etoposide chemotherapy, and genomic analysis of CTCs demonstrated remarkable similarity to the corresponding CDX [32]. Similarly, Baccelli and colleagues used the RosetteSep kit in conjunction with MACS to enrich viable CTCs in metastatic breast cancer patient samples, where the existence of metastasis-initiating cell (MICs) subsets among bulk luminal breast CTC populations were identified and elegantly demonstrated to give rise to bone, lung, and liver metastases when injected into immune-compromised mice [33].

Adding to the suite of functional analyses possible following viable CTC enrichment, some groups have reported the ability to establish cell cultures from viable CTCs using animal model systems. By injecting GFP-labeled, immortalized PC3 prostate cancer cells into immune-compromised mice, Howard and colleagues were able to extend the functional analysis of CTCs through maintenance in vitro. Howard et al. drew blood samples from prostate-injected mice following 30 days of tumor growth, depleted the red blood cell (RBC) fraction using a hypotonic lysis buffer, collected cell pellets, and plated resuspended nucleated cells onto culture dishes in RPMI-1640. Using this methodology, Howard and colleagues established a novel CTC-derived cell line with a defined set of molecular characteristics that are

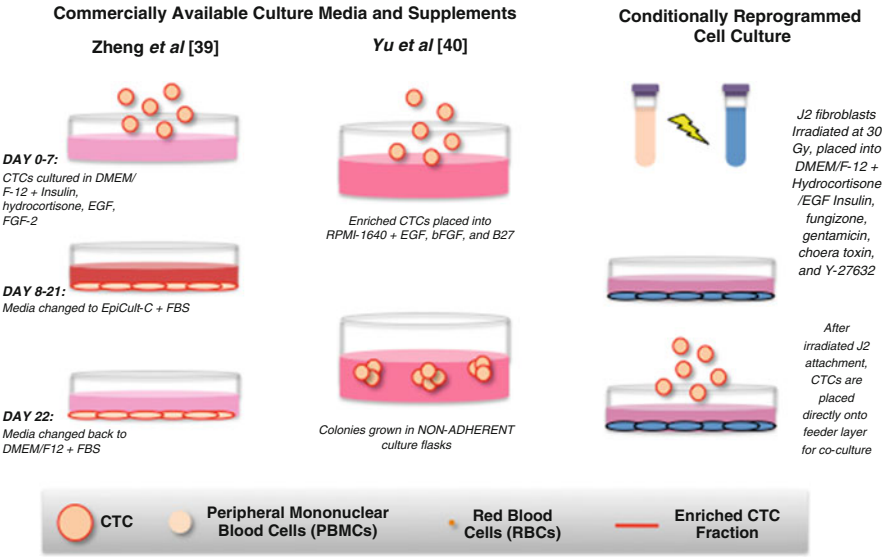

Fig. 11.2 Methods for in vitro maintenance of viable CTCs. Viable CTCs enriched by the methods described above are later available for functional characterization, as well as expansion in culture, either by modification of commercially available reagents or by novel reagent systems. Importantly, the system used by Yu and colleagues has to date been the only technique described that restricts viable CTC culture by use of non-adherent conditions

distinct from the corresponding primary tumor xenograft [34]. In comparison to the primary tumor established by parental PC3 cells, PC3 CTCs demonstrated decreased adhesiveness and downregulated expression of E-cadherin, β4-integrin, and γ-catenin, as well as upregulation of BCL-2 and suppression of GRP94 [34]. In a second mouse model, Carvahlo et al. enriched viable CTCs (verified by cytokeratin 8 and EpCAM immunostaining) from blood samples of transgenic adenocarcinoma of the prostate (TRAMP) mice by gradient density centrifugation, followed by RBC lysis and leukocyte depletion by anti-CD45 immunomagnetic bead separation [35]. Using this methodology, Carvahlo and colleagues later demonstrated the tumorigenicity and aggressiveness of enriched TRAMP CTCs in vivo, where two of nine immune-compromised mice developed massive liver metastases [35] (Fig. 11.2).

Although critical for the development of methodologies with applications in human blood samples, the use of animal model systems for viable CTC enrichment and culture we have described have inherent limitations. Achieving viable CTC capture and culture in human blood samples is a significantly more complicated task than in mouse models. In addition to the difficulties associated with viable CTC isolation, the optimal culture conditions for CTC expansion (discussed in greater detail later) must also be experimentally defined, possibly on a disease-specific basis. When using mouse model systems with xenograft implants, the tumor cells used to establish the primary tumor often come from previously immortalized, well-established cell lines for which the culture conditions have been already well-defined. Furthermore, the demonstration of in vivo tumorigenicity of CTCs derived

from xenograft implants typically require that enriched cells be introduced into immune-compromised mice. However, the presence of immune cells in human blood samples is a factor that significantly complicates the enrichment of rare CTCs and cannot be controlled or eliminated. Additionally, there is a substantially growing body of evidence suggesting that the immune system could play a central role in CTCs survival through immunosuppressive activity of myeloid-derived suppressor (MDSC) and or T-regulatory cell function to protect CTCs from immune surveillance [36]. Additionally, our group has noted the presence of CTC-immune cell clusters in patient sample blood, and we have hypothesized a direct mechanistic role for these clusters, where CTCs "hitchhike" through the circulatory system, exploiting immune cell abilities to extravasate from the circulation at secondary sites via their interaction with selectins and integrins to migrate across blood vessel barriers [36]. Thus, the use of immune-compromised mice removes a significant technical limitation for CTC enrichment, and eliminates any possibility of studying the potential contribution of immune function in the metastatic process. The study described by Howard et al. using fluorescently labeled PC3 cells exemplifies both of these limitations, as a well-established cell line was used to establish primary tumors, and CTCs derived from these primary tumors were enriched in the absence of any surrounding non-tumor blood cells. In the study performed by Carvahlo and colleagues, a transgenic mouse model was used, thus one would expect that CTC enriched from TRAMP mice would be compatible for use in inoculation of immune-competent mice. However, immune-compromised mice where used to demonstrate tumorigenicity. Further, although CTCs were derived from transgenic mice in the Carvahlo study, conditions for the culture of TRAMP tumors have been previously described [37]. In our own study, the use of syngeneic mouse models (4T1 and 4T07) were specifically selected such that the presence of non-tumor blood cells among CTCs could most accurately recapitulate human blood samples, but the mouse mammary tumor cells used in our study are immortal, and the conditions for in vitro culture of these tumor cells are well-established.

For the first time, a small number of groups have reported the ability to translate the success from others using these technologies for cultures from viably enriched CTCs in mouse model systems to human blood samples. Using gradient-based centrifugation for CTC enrichment in combination with a novel cell culture reagent (TrueCells, LLC), McGregor and colleagues successfully expanded viable CTCs in vitro from 80 % (12/15) patients with stage III ($n=4$) and stage IV ($n=11$) metastatic melanoma patents on a short-term basis [38]. Using the GEDI device, Kirby et al. performed on-chip treatment with taxol drugs on viably enriched CTCs, and reported microtubule organization alterations in CTCs [39]. Viable CTCs in metastatic breast cancer have been enriched using multimarker FACS technology by Zhang et al., where three (3) human CTC cultures were established and sustained in vitro [40]. In this study, a putative breast cancer brain metastasis tropism signature was identified, where a HER2+/EGFR+/HPSE+/Notch1+ subset of CTCs enriched from each patient was highly invasive and capable of generating brain metastases when inoculated in nude mice [40]. Viable CTCs were enriched by Yu and colleagues using the CTC iChip discussed above, where CTC-derived cultures were successfully established from six (6) different metastatic estrogen receptor

positive breast cancer patients, of which three were tumorigenic in mice [41]. Genomic sequencing of the CTC-derived cell lines demonstrated preexisting as well as newly acquired mutations, including variations in the ESR1, PIK3CA, and FGFR2 genes [41]. Interestingly, Yu and colleagues were only able to generate CTCs when patients were actively progressing in the face of therapy, and results from in vitro drug sensitivity of CTC-derived cell lines were concordant with the corresponding patients' past history of drug resistance and response [41].

11.3 Next-Gen Culture Methods for Sensitive Cell Expansion In Vitro

With the establishment in 1951 of the first human cancer cell line HeLa, the human tumor cell lines have had an important impact on cancer research, and have made the variety of cancer treatments possible. However, despite many decades of incremental improvements, it is still extremely difficult to routinely establish long-term permanent cell lines from human primary tumors reproducibly and with high efficiency. In conventional culture systems primary human tumor cells are typically growth-arrested after several weeks over 90 % of the time. In the remaining rare cases when tumor cell lines are eventually established in standard culture media, four sequential stages of growth are observed in general; a brief period of rapid growth for a few weeks, followed by a growth plateau, then wide spread cell death, and the occasional emergence of rare, rapidly growing tumor cell clones. The problems confronted by the establishment of long-term, primary tumor cell cultures is only exacerbated when the focus is turned towards the expansion of viably enriched CTCs, which to date has been not only been limited due to technological limitations for sensitive and efficient viable CTC enrichment, but also the result of a poor understanding of the correct combination of media supplements needed to drive CTC expansion in vitro. In this section, we review some of the most commonly used means to supplement and promote the growth of tumor cells in vitro, as well as the manner in which some of these methods are being adapted towards the culture of viable CTCs.

The addition of fetal bovine serum (FBS) has been the standard supplementary item in culture media since the inception of in vitro mammalian cell maintenance. Despite its wide popularity, there are many documented limitations to the use of FBS in cell culture, including variable lot-to-lot performance, and the potential to contaminate cultures with fungi, viruses, and bacteria from source animals. In response to these constraints, many shifted to the use of effective serum-free media supplements for the culture of mammalian cells. To do so, serum-free media cocktails such as Ham's F-12 have been developed, and further supplemented with purified extracts of growth-promoting molecules such as albumin, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), basal fibroblast growth factor (bFGF), and other animal-derived components to regain the proliferative characteristics lost by discontinued use of FBS.

Selective inhibition of rho-associated protein kinase (ROCK) activity has been a method traditionally employed to support the growth of human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs). Although ROCK inhibition can be achieved through shRNA targeting [42], the preferred method is to utilize synthetically derived small molecule inhibitors that compete for the ATP binding site in the rho kinase p160 catalytic domain. For chemical inhibition, Y-27632 is the most popular compound, and is widely available commercially. When used for cell cultures, inhibition of ROCK by Y-27632 functions to block anoikis, a form of programmed cell death that is induced contact dependent cells via detachment from the local tissue environment or culture substrate. Y27632 and similar compounds thereby can increase single cell survival in suspension, and has been found to enhance the adhesive properties of cells in primary cultures by mediating effects on the cellular cytoskeletal network. Further, some reports suggest that addition of Y-27632 to cell culture suspensions increases the survival of cryopreserved cells, as well as increases the post-thaw survival rate of cell cultures recovered from liquid N_2 [43]. Pipparelli and colleagues evaluated the effects of Y-27632 supplementation in human corneal endothelial cell cultures, finding that ROCK inhibition significantly enhanced cell adhesion and wound healing both in vitro and ex vivo with no induced toxicity, reduction of cell viability, or induction of corneal endothelial cell proliferation [44].

A long-standing approach to prolonging the proliferation of epithelial cells in culture reported by Rheinwald and Green is through the use of "feeder" cells, most commonly immortalized murine fibroblasts [45]. By this method, cells of interest are cocultured with a nearly confluent layer of mitogenically inactivated mouse or human fibroblasts. Mitogenic inactivation can occur through chemical treatment, such as via supplementation with Mitomycin C, but the most common method of mitogenic inactivation occurs through irradiation. Chemical treatment or high-energy gamma irradiation (typically around 3000 rad) functions to remove the capacity for cell division of the fibroblast layer, while simultaneously preserving their metabolic activity, thus allowing them to "feed" nutrients onto the non-irradiated cells of interest to stimulate proliferation. Coculturing cells of interest with feeder layers not only conditions media with an array of naturally produced factors other culture methods might provide artificially, the stromal cells used to establish the feeder layer are in direct physical contact with the cells of interest and serve to create a microenvironment that more closely resembles in vivo conditions in a manner that standard 2D culture systems do not. Irradiated feeder layers have also been used to aid in the establishment cultures from primary epithelial tumor tissues. As a major drawback in the selective culture of human mammary epithelial cells from primary tumors is the overgrowth of tumor-associated stroma, the use of irradiated feeder layers act to form a specific cellular substrate which constitutes a physical barrier to unwanted fibroblast attachment [46].

More recently, the supplementation of culture media with Y-27632 in combination with irradiated feeder cells has been shown to synergistically enhance the proliferative capacity of human keratinocytes versus either component alone. Chapman et al. first demonstrated that combining irradiated immortalized J2 mouse fibroblast feeder cells with supplementation of DMEM/F12 culture media with the Y-27632

ROCK inhibitor increased the long-term proliferation and unexpectedly enabled keratinocytes to efficiently bypass senescence and become immortal without detectable cell crisis [47]. Schlegel, Albanese and colleagues have further defined the use of irradiated feeder cells and Y-27632 to establish what are termed conditionally reprogrammed cells (CRCs) from vertebrate epithelial tissues and have demonstrated the ability to establish cell cultures from all epithelial types tested to date, both normal and malignant, across multiple species, including human, mouse, rat and dog with high efficiency [48]. Using this method to develop CRCs from primary epithelial cells, Suprynowicz et al. demonstrated the induction of CRCs to be rapid, reversible, and capable of reprogramming an entire cell population, rather than a selection of a minor subpopulation [49]. Importantly, Yuan and colleagues showed that positive response to drug therapy in a patient with a 20 year history of recurrent respiratory papillomatosis could be accurately predicted by in vitro chemosensitivity analysis of CRCs established from the primary tumor biopsy [50] and Pollock et al. have successfully used primary prostate CRCs to enhance the biological relevance of early stage preclinical drug development studies [51]. Chapman and colleagues later demonstrated that the observed effects of Y-27632 media supplementation on keratinocytes are rapid and conditional, supporting previous observations that the CRC conditions do not irreversibly impact the genotype or phenotype of cell cultures, and thus recapitulates the molecular activities observed with similar efficiency as other in vitro model systems established using conventional cell culture methods [52]. The ability to preserve the genetic characteristics of a heterogeneous cell population in vitro, establish cultures reliably from multiple tissue types from very few founder cells, and conserve the in vivo characteristics of cytotoxic drug treatment in vitro make the use of conditional cell reprogramming a particularly attractive method for the culture of CTCs. Although our groups, as well as others [41], have been unsuccessful to date, the appropriate optimization of conditional cell reprogramming for the culture of CTCs is currently underway.

Other groups have adapted commercially available cell culture methods to be used for the in vitro expansion and study of CTCs. As mentioned above, Zhang et al. successfully established three (3) human CTC cultures from three different patients with metastatic breast cancer using a relatively complex methodology [40]. CTCs were collected and cultured in DMEM/F12 media supplemented with insulin, hydrocortisone, epidermal growth factor (EGF), and fibroblast growth factor-2 (FGF-2) for the first 7 days, then switched to EpiCult-C medium (commercially available from Stemcell Technologies Inc.) supplemented with FBS from days 8 through 21. From day 22 on, the colonies that had been established were switched back to DMEM/F12 culture media supplemented with FBS. Using a comparatively simpler method, Yu and colleagues established CTC cultures from metastatic breast cancer patient blood samples with ultralow attachment plates in RPMI-1640 media supplemented with EGF, bFGF, and B27 [41]. Interestingly, Yu and colleagues reported a number of other unsuccessful culture conditions in similar blood samples, including those from the aforementioned Zhang group, Mammary Epithelial Cell Growth Medium (MEGM; Lonza), an epithelial cell culture medium reported by Sato and colleagues [53], and others. McGregor and colleagues have developed a novel, cell culture reagent system for in vitro CTC expansion currently being used

in clinical trials (NCT01528774) for which patients are currently being enrolled, but the contents of their media are proprietary and thus cannot be further described.

11.4 Considerations for Performing Functional Characterization and Cultures in Viable CTCs: The Known Unknowns

The integration of new enrichment and cell culture strategies for the functional analysis and culture of viable CTCs can be highly challenging. Two overarching physical and biological considerations must be addressed when designing systems and experiments that are aimed at viable CTC enrichment and subsequent functional characterization, that being the selection of an appropriate technology for sensitive and efficient CTC enrichment, and a method to reliably stimulate growth of viably enriched CTCs across multiple tumor types in vitro. For example, the use of an insensitive or inefficient technology for viable CTC enrichment will negatively impact even the best method for establishing cultures from rare cell populations, and conversely, poor methods and reagents to establish cultures will prevent success even in cases where the most sensitive and efficient CTC enrichment platforms are used. One of the more paradoxical relationships experienced to date is that methods that are best at capturing CTCs, historically, either have required cellular fixation or submit the cells to high shear stress, resulting in diminished viability. Conversely methods that best retain cell viability have been less effective and efficient at enriching CTCs. These two critical factors (cell viability and efficient CTC capture) have continued to represent a formidable barrier to appreciable progress made in this field. In this section, we highlight some of the limitations associated with the enrichment and cell culture strategies we have discussed previously.

The majority of studies that have shown success in the enrichment and functional characterization of viable CTCs have employed a density gradient based enrichment method, but due to its poor sensitivity, reported to be 10–65 % [8, 9], the use of Ficoll-Hypaque to enrich CTCs for any type of molecular study is severely limited; mainly through the possible loss of tumor cells that either migrate to the plasma layer, or due to the formation of aggregates to the bottom of the gradient. Additionally, whole blood can mix with the density gradient if the centrifugation step is not performed immediately. Although the introduction of RosetteSep and OncoQuick were designed to mitigate these technical problems, the enrichment of viable CTCs by density-based centrifugation still requires a high CTC burden in blood samples sufficient for functional characterization and the successful initiation of cell cultures. The studies conducted by Bacelli et al. and Hodgkinson et al. vividly demonstrate this limitation, where in the Bacelli study no less than 1109 enriched CTCs per 7.5 ml blood were required to establish tumors in mice [33], and in the Hodgkinson study at least 509 CTCs per 7.5 ml blood were required to establish tumors in mice [32]. The study conducted by Carvahlo and colleagues in the TRAMP mouse model, which used a gradient-based method in combination with negative depletion of non-

tumor blood cells, also reported unsuccessful attempts to apply the same methodology in human metastatic prostate cancer blood samples, further exemplifying this limitation [35]. High CTC burden, even for enrichment technologies with very high sensitivity and efficiency, is typically observed in patients with late stage disease at diagnosis, where treatment strategies are generally palliative rather than curable, thus the clinical benefit of applying such techniques would be limited.

While the efficacy of affinity-based enrichment strategies for viable CTC analysis has been demonstrated [40, 41], it requires that the cells of interest have robust expression of tumor-specific markers that can be exploited for their isolation. Thus affinity-based enrichment techniques are generally limited to very specific disease settings, and even within a specific disease defining an appropriate biomarker panel that accurately reflects the biologic heterogeneity of CTCs could be difficult. In the study by Zhang and colleagues, the subset of CTCs negative for EpCAM expression (the marker most commonly used for affinity-based CTC enrichment) contained the highest proportion of CTCs with the brain metastasis signature described as well as the highest level of tumor formation in vivo, further highlighting the importance of suitable biomarker selection for affinity-based CTC enrichment [40].

Filtration-based strategies, including those our group has designed, continue to demonstrate promise for their ability to enrich viable CTCs in an antigen-independent fashion, potentially overcoming the limitations of affinity-based enrichment systems discussed above. But to date, there have been no studies that have reported the successful use of a filter-based enrichment method for functional characterization and culture of human CTCs. Other non-affinity based platforms we have discussed, such as the CTC-iChip, also demonstrate promise and currently represent the most successful means to enrich, functionally characterize, and establish cultures from viable CTCs. Even in the midst of its success, the CTC-iChip is not without its own limitations. While the CTC-derived cell lines established by Toner's group have and will continue to yield invaluable data that provide insight into the mechanisms of metastasis, drug resistance and potential new drug targets for the research community, the initial culture doubling times for CTC cultures ranged from 1 to 3 weeks, even for samples containing as many as 3000 CTCs per 6 ml blood sample [41]. While acceptable in the research setting, the time required establishing cell cultures for use in in vitro drug sensitivity assays to predict therapeutic response by this methodology would be prohibitive in the clinical setting.

11.5 Conclusion

The ability to isolate, functionally characterize, and importantly, establish primary and long-term cell cultures from viable CTCs, could potentially transform cancer patient management. Such a facility would provide the opportunity for researchers to expand our knowledge of the metastatic process through establishment of in vitro and in vivo model systems for metastasis that more closely resemble disease while in transit to the secondary site. Of clinical importance, once fully optimized, one could envision the establishment of CTC cultures permitting the development of

drug sensitivity assays, such that an assessment can be made to predict whether a course of adjuvant systemic therapy is suitable for a patient *before the treatment begins*, and to monitor the efficacy of an ongoing treatment *in real-time* using viable CTCs enriched from serial blood draws during therapy. Such a facility would also translate from the bedside to the lab bench, as researchers would then be able to expose novel mechanisms for resistance to a given therapy in patients whose CTCs survive in vitro despite drug exposure. Although not yet widely available, substantial progress has been made in the ability to enrich viable CTCs, and optimized protocols amenable to clinical applications would constitute a major step forward towards evidence-based, individualized cancer patient management.

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Chapter 12
Prognostic Implications of CTC in Breast Cancer

Jeffrey B. Smerage

Abstract Circulating tumor cells (CTCs) represent an important conceptual link between a primary tumor and the development of metastatic disease, and in the setting of metastatic disease CTC have the potential to reveal important insights into the biology and behavior of the cells undergoing the metastatic process and contributing to the resistance and progression of disease over time. In breast cancer the enumeration of CTC has been demonstrated to be a strong prognostic factor for both progression and survival. The finding of elevated CTC after one cycle of cytotoxic chemotherapy are associated with a particularly poor prognosis, suggesting the need for innovative drugs and treatment strategies. Although prognosis can be important in treatment planning, enumeration of CTC has not yet led to predictive models for the selection of specific drugs or for when to stop or switch the current therapy. CTC can be effective in defining when and how often to perform radiographic extent-of-disease scans. Given the relatively modest impact the CTC enumeration has had on clinical care, the focus has been the development of new platforms to increase sensitivity to allow there detection in a larger fraction of patients and to allow biologic interrogation of these cells such that CTC might allow marker-driven treatment choices.

Keywords Circulating tumor cell • CTC • Breast cancer • Prognosis • Prediction • Liquid biopsy • Tumor marker

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12.1 Detecting Circulating Tumor Cells

The hematogenous spread of a primary tumor to distant sites has long been at the core of oncologic dogma [1, 2]. Conceptually circulating tumor cells are the link between the primary tumor and metastasis. The first published report [3] was in 1869 when T.R. Ashworth, an Australian physician performing an autopsy on a patient who had died of metastatic cancer, noted cells seen by light microscopy that were morphologically identical to cells taken from the tumor. He commented, "that if they came from an existing cancer structure, they must have passed through the greater part of the circulatory system to have arrived at the internal saphena vein of the sound leg." Clearly, to be clinically useful, these cells need to be detectable earlier in the course of the disease, and they must have at least prognostic significance and preferably a predictive role in the management of the treatment of the disease. Understanding the biology of CTCs should lead to a better understanding of the metastatic process, and in addition should lead to a better understanding of the behavior of metastatic disease after it has been established. Ultimately the goal is to improve clinical outcomes for patients with cancer.

The rarity of circulating tumor cells has been the central challenge in the development of CTC platforms. The technical limitations and the assumptions on how to define and quantify these cells affect the types of clinical questions that can be addressed and the outcomes the resulting clinical investigations. CTCs are estimated to occur at an average frequency of 1 in 10^6 nucleated blood cells. Early attempts at isolating tumor cells generally used physical properties of cells through either filtration or density gradient centrifugation to isolate CTCs, but these methods were labor intensive and had inadequate sensitivity and specificity. These earlier assays were associated with a significant loss in CTCs, with a recovery of only 10–65 % cultured tumor cells spiked into whole blood [4, 5]. There also remains a need to distinguish these cells from leukocytes. The majority of leukocytes are removed during the isolation process, but many still remain. The distinction is made even more challenging by the recognition that normal hematopoietic cells can transiently express epithelial markers such as EpCAM [5], cytokeratins [6], MUC-1 [7], and TAG-12 [8]. In the case of immunofluorescent cell identification this raises the importance of actively excluding cells by the use of leukocyte-specific markers such as CD45. For platforms that utilize rt-PCR to detect gene expression, the choice of target gene selection is important to reduce the chance of false positive results that could occur as a result of transient epithelial markers in the contaminating leukocytes.

The development of immuno-selection and automated image analysis were two major advances that allowed the field to move forward. Immunomagnetic techniques allow recovery rates of approximately 85 % [9, 10] from blood samples spiked with cultured epithelial tumor cells. Studies using patient samples have varied significantly in methodology, but in general 40–60 % of patients with metastatic breast cancer are defined by these assays as being positive for CTC (Table 12.1). Notably there are some patients who are CTC negative by immunomagnetic assays throughout the course of their disease, although the biologic significance of this observation is not well understood.

Table 12.1 Selected CTC clinical studies in metastatic breast cancer

Reference	Technology	CTC threshold	Clinical scenario	*N*	Patients with elevated CTC (%)	mPFS (months)	mOS (months)
Cristofanilli [11]	CellSearch	≥5 CTC	PS, Any line or type of therapy.	177	49	2.7 (≥5)	10.1 (≥5)
						7.0 (<5)	>18 (<5)
Budd [12]	CellSearch	≥5 CTC	PS, Any line or type of therapy.	138	43	NR	8.5 (≥5)
							22.6
Cristofanilli [13]	CellSearch	≥5 CTC	PS, first-line therapy.	83	52	4.9 (≥5)	14.2 (≥5)
			Any type of therapy.			9.5 (<5)	>18 (<5)
Cristofanilli [14]	CellSearch	≥5 CTC	RS, Any line or type of therapy.	151	44	NR	13.5 (≥5)
							29.3 (<5)
Bidard [15]	Density centrifugation, cytokeratin staining	≥1 CTC	RS, Any type or line of therapy.	37	41	NR	Approx.
							15 (≥1)
							22 (<1)
Liu [16]	CellSearch	≥5 CTC	PS, Any type or line of therapy.	74	35	3.2 (≥5)	NR
						5.1 (<5)	
Pierga [17]	CellSearch	≥1 CTC	PS, first line, chemotherapy.	267	65	Approx 8 (≥5)	Approx 22.5 (≥5)
		≥5 CTC			44	10 (1–4)	Not reached for 0 or 1–4 CTC
						20 (0)	
Muller [18]	CellSearch	≥5 CTC	PS, Any line or type of therapy.	254	50	9.3 (≥5)	18 (≥5)
						10.9 (<5)	27 (<5)
	Adna Test (Immunomagnetic isolation, rt-PCR detection)	>0.15 ng/μL			40	8.8 (+)	19.3 (+)
						10.7 (−)	23.8 (−)

(continued)

Table 12.1 (continued)

Reference	Technology	CTC threshold	Clinical scenario	*N*	Patients with elevated CTC (%)	mPFS (months)	mOS (months)
Wallwiener [19]	CellSearch	≥5 CTC	PS, Any line or type of therapy.	486	42	4.8 (≥5)	18.0 (≥5)
						7.6 (<5)	Not reached
Bidard [20]	CellSearch	≥5 CTC	RS (pooled analysis of 20 studies), Any line or type of therapy.		47	Approx.	Approx
						6.5 (≥5)	16.3 (≥5)
						11 (<5)	>34 (<5)
Smerage [21]	CellSearch	≥5 CTC on day 1 and day 22	PS (cooperative group), first line, chemotherapy.	595	54	4.9 (≥5, ≥5)	13 (≥5, ≥5)
						8.9 (≥5, <5)	23 (≥5, <5)
						11.1 (<5, <5)	35 (<5, <5)

Multiple studies of CTC have been performed in metastatic breast cancer, but they vary by technology used, CTC threshold used to define patients as positive, whether they were prospective studies (PS) or retrospective studies (RS), by allowed lines of therapy, and allowed types of therapy. Although most provided Kaplan–Meier curves, not all reported the actual mPFS or mOS. In cases without formal reporting the mPFS and mOS have been estimated visually and reported here as "approximate." NR indicated data not reported

These techniques led to the development of CellSearch® (Janssen Diagnostics, LLC), which is currently the only FDA-cleared platform for the enumeration of CTCs in patient care. This system is highly analytically validated [10]. The blood sample is prepared by adding an anti-EpCAM antibody that has been labeled with a magnetically active ferric particle. The red blood cells are lysed and the tube is then placed into a magnetic field that pulls the EpCAM positive cells to the edges of the tube, allowing removal of the lysed red cells and most of the unlabeled leukocytes. The sample is then suspended in a small volume of media and placed into a cartridge for viewing. Fluorescent stains for DNA (DAPI), cytokeratin, and CD45 are added to the sample, and the sample is then visualized via automated fluorescent microscopy. The computer identifies "events" that are DAPI positive, cytokeratin positive, and CD45 negative, and then presents them as images for a technician to review. This review is important to make confirm that these images have the morphology of a cell, and to assure that they are not a contaminating leukocyte. As a result of the analytic validity and the commercial availability, it became the largest source of clinical data. The reality that after 10–20 years of research there is only one FDA cleared CTC device speaks to the difficulty in creating a platform with both analytic and clinical validity.

This assay was initially studied in patients with metastatic cancer, and it utilizes a threshold of 5 or more CTCs in 7.5 ml of whole blood to define a patient as being positive for CTCs. This threshold was selected because it best separated patient populations based upon median progression free survival (mPFS). It was at a threshold of 5 CTC that Cox proportional-hazards ratio between the populations with slow versus rapid progression reached a plateau [11]. The use of a 5 CTC threshold also minimized the false positive rate in a normal control population without cancer. In this original publication, single epithelial cells are found in only a small number of individuals with an average of 0.1 epithelial cells per 7.5 ml blood, and none of the normal controls had and two or more epithelial cells. Three or more epithelial cells are only rarely found in people without cancer. The selected threshold affects both the questions that can be asked and the clinical outcomes observed. For example, in patients with early stage breast cancer, the frequency of CTCs is much lower, and in using a threshold of 5 CTC in 7.5 ml blood, only 3 % would be considered positive [22]. To achieve adequate sensitivity in the early stage breast cancer most investigators began using larger volumes of blood and a lower threshold to define positivity.

12.2 CTC and Prognosis in Metastatic Breast Cancer

Clinical decisions are a balance between risks and benefits, and in the care of patients with cancer prognosis is an important factor in this assessment. Cancer prognosis is important for many reasons. From the perspective of patients, prognosis is important so that one would know what to expect and so that appropriate planning can occur. From the perspective of clinical decision-making, it is important because prognosis is a significant component to the clinical assessment of risks and

benefits. In the adjuvant setting where the goal of therapy is cure, physicians and patients are more willing to accept increased short-term treatment toxicities if the disease risk is high. In the setting of incurable metastatic disease the goal of care is palliative, and if the prognosis is very poor with currently available drugs this might move physicians and patients toward either clinical trials of novel agents, or to less toxic therapy to maximize quality of life in the time that is remaining.

Multiple studies have demonstrated and confirmed the prognostic implications of CTC in patients with metastatic breast cancer. Patients with elevated CTC have a worse prognosis as measured by PFS and by overall survival (OS) (Table 12.1). Comparing the outcomes between different studies is difficult due to the use of different populations, different thresholds to define positivity, and difference in reporting of clinical outcome. The first published clinical study using CellSearch [11] evaluated 177 patients representing a wide cross-section of metastatic breast cancer, including patients starting hormonal therapy or chemotherapy and patients receiving first-line therapy or later-line therapy. Median PFS was significantly worse for patients with elevated CTC prior to initiation of a new therapy. Patients with elevated CTC at baseline had a mOS of 2.7 months compared to 7.0 months ($p<0.001$) for patients with low CTC at baseline. Similarly, survival was significantly worse with a mOS of 10.1 months vs. >18 months ($p<0.001$) for patients with elevated versus low CTC respectively. When looking at the subset of patients starting a new line of chemotherapy, the mPFS was 2.3 months vs. 6.8 months and mOS was 8.3 months vs. >18 months for patients with elevated versus low CTC. Original interpretation of the non-chemotherapy group was not as clear because it was a much smaller subset ($n=54$) and because the reported population received hormonal therapy, "immunotherapy," or both. The largest group represented by "immunotherapy" was trastuzumab for HER2 positive cancer. The difference in PFS and OS in this heterogeneous small subgroup was not as great and was not statistically significant ($p=0.44$). However in a subsequent analysis [13] it was found that patients starting first-line endocrine therapy ($n=23$) had a notable difference in mPFS of 11.3 months vs. >18 months, although due to the small sample size it remained nonsignificant ($p=0.15$). It was this data that led to the FDA clearance of CellSearch. Subsequently, similar CTC data have been published in colon cancer [23] and prostate cancer [24] has led to the expansion of the FDA clearance to those cancers.

The prognostic significance of CTC is also true for patients initiating first-line chemotherapy. A retrospective subset analysis of the original study [13] evaluated patients starting first-line cytotoxic chemotherapy and revealed a mPFS of 2.7 months vs. 7.0 months ($p<0.001$) and mOS of 10.1 months vs. >18 months ($p<0.001$) for patients with elevated versus low CTC respectively. A separate study by Pierga and colleagues [17] evaluated 267 patients initiating first-line chemotherapy and showed qualitatively similar results. In this analysis patients were grouped by CTC levels into cohorts of patients with ≥5, 1–4, and 0 CTC per 7.5 ml of whole blood. In this analysis the mPFS were 8 months, 10 months, 20 months respectively. Median OS had not been reached for the 0 and 1–4 CTC populations so comparison of mOS was not possible. These differences in prognosis for patients initiating first-line chemotherapy were confirmed by the prospective SWOG S0500

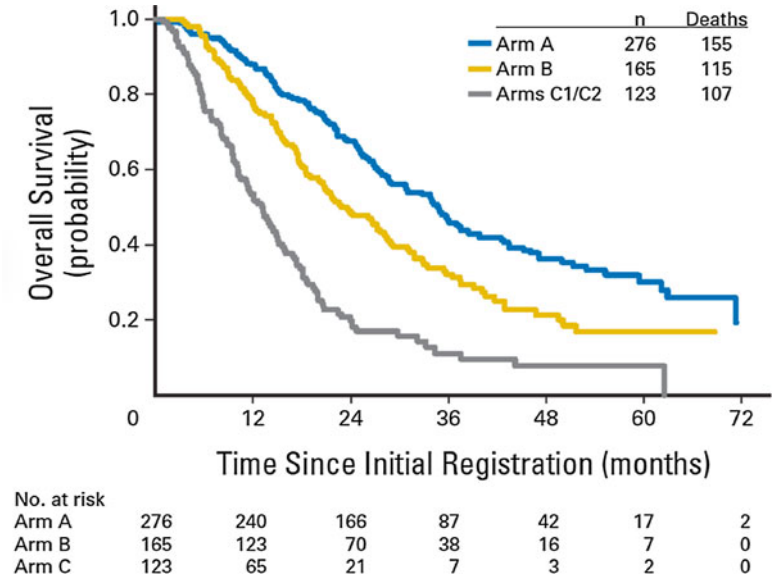

Fig. 12.1 SWOG S0500 Overall survival by CTC group—Patients had CTC levels drawn at baseline. Patients with low CTC (<5 CTC per 7.5 ml whole blood) at baseline were observed on Arm A. Patients with elevated CTC at baseline had CTC level repeated on day 22. Those that converted to low after one cycle of first-line chemotherapy were observed on Arm B. Patients with elevated CTC at both baseline and after one cycle of therapy were randomized to different treatment strategies on Arms C1 and C2. There were no differences in outcome between C1 and C2 (see Fig. 12.3), and the overall survival presented here is the combined C1 and C2 population. Adapted from Smerage, J. et al., JCO (2014) 32: 3483–3489 [21] with permission. Copyright® 2014 Journal of Clinical Oncology. All rights reserved

study. In this study of 595 patients, 123 had elevated cells at both baseline and after one cycle of chemotherapy, 165 had elevated cells (≥5 CTC/7.5 ml) at baseline but converted to low after the first cycle of chemotherapy, and 276 had low CTC at baseline. The mPFS were significantly different at 3 months, 9 months, and 11 months ($p<0.001$) and mOS were significantly different at 13 months, 23 months, and 35 months ($p<0.001$) respectively for the three groups (Fig. 12.1). Similar prognostic differences were seen in subgroups with ER positive, HER2 positive, or triple negative disease.

CTC may also be a better predictor of OS than radiographic staging studies. In a study 138 patients [25] with metastatic breast cancer, CTCs drawn about 4 weeks after starting a new therapy were compared to imaging done after a median of 10 weeks after the initiation of therapy. Inter-reader variability was greater for the radiologic evaluation compared to CTCs. For radiology there was a 15.2 % disagreement between interpreting radiologists when assessing radiographic status between indeterminate, stable disease or partial response, or progressive disease. There was only a 0.7 % disagreement in the assessment of CTC being <5 versus being ≥5. Patients with non-progression (stable disease or response) on scans but

low CTC had a better mOS of 26.9 months compared with the patients who had non-progression on scans but elevated CTC with a mOS 15.3 months. Notably patients with progressive disease on scans but low CTC also had longer mOS of 19.9 months when compared to patients with both radiographic progression and elevated CTC with a mOS of 6.4 months. As with the results from the SWOG S0500 study, this suggests that elevated CTC after initiating therapy is a clinically important finding that reflects a particularly unfavorable tumor biology and general resistance to current therapeutic options.

12.3 CTC in the Monitoring of Metastatic Disease for Progression

Several analyses have shown the monitoring of CTCs can predict patients whose cancers are beginning to progress or will progress in the near future. In a prospective study of 68 patients [16], CTC were collected at monthly intervals for 6 months and then every 3 months afterwards. Radiographic staging was performed every 3 months. Looking back from each staging scan, the investigators looked to see if the preceding CTC predicted the results of the imaging. Patients with elevated CTC 7–9 weeks prior to performing scans were 70 % likely to have progression on those scans. When performed 3–5 weeks prior to planned scans, CTC predicted a 60 % chance of progression on the scans. From the clinical perspective it is important to note that this means that 30–40 % did not have progression. Thus an elevated CTC value raises the concern for progression now or in the near future, but it does not define progression. A second similar study [26] followed 177 patients starting a new therapy for metastatic breast cancer and obtained CTC with each clinic visit (every 3–5 weeks). Of patients with elevated CTC at 3–5 weeks or 6–8 weeks after the first dose of therapy, 68 % and 53 % experienced progression within 3 months respectively. This population included both patients starting hormonal therapy and patients starting chemotherapy. It is possible that these two populations have different temporal patterns in their CTC levels related to the initiation of a new therapy and in their time to progression. Again, it is noted that a significant portion of these patients did not have progression on their subsequent scans. So the elevated CTC raised the possibility of progression but did not define progression. For patients with low CTC the risk of progression is much lower. If a patient continues to have low CTC, it might be reasonable to delay future scans. One clinical strategy would be to use CTC to determine how often to perform staging scans. Patients without significant symptoms or laboratory abnormalities might be able to delay scans as long as their CTC remain low, but as soon as the CTC rise to 5 or greater this would be an indication for further radiographic evaluation, even in the absence of symptoms. Such a strategy has the potential to reduce side effects from contrast agents, to reduce radiation exposure, to reduce patient inconvenience, and to save costs associated with radiographic scans.

12.4 CTC in Randomized Interventional Studies

The SWOG S0500 clinical trial, referred to above in the discussion about CTC and prognosis, was a randomized phase III study testing the hypothesis that patients who have elevated CTC after starting a new therapy are likely on ineffective therapy and that they would experience improved outcomes by switching to an alternative treatment. The basis for this hypothesis came from the observation from the original IMMC-01 trial [13] that patients who have elevated CTC after one cycle of first-line chemotherapy had a very short mPFS of 2.0 months and mOS of 9.2 months. It appeared that these CTC were identifying patients who had cancers that were very likely resistant to that first therapy. Thus it was hypothesized that patients would have improved outcomes if they switched immediately to an alternate chemotherapy in a different drug class. By switching after one cycle of therapy, they would potentially avoid cumulative toxicities from the initial (and presumed ineffective) chemotherapy, and would have a greater chance of having a response by switching to a drug with a different mechanism of action.

SWOG S0500 enrolled a total of 595 patients who were about to initiate first-line chemotherapy for metastatic breast cancer. The choice of therapy was determined by the treating physician. The schema is shown in Fig. 12.2. Patients had CTCs measured prior to starting chemotherapy using CellSearch. Patients who had low cells were believed to have lower risk disease and were observed for outcome without intervention. Patients who had elevated CTC at baseline (≥5 cells/7.5 ml

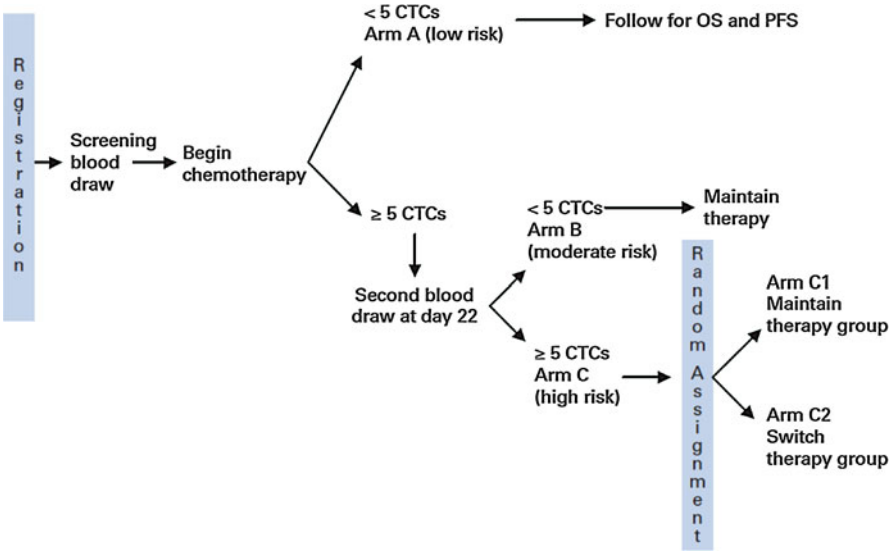

Fig. 12.2 SWOG S0500 Clinical trial schema—Adapted from Smerage, J. et al., JCO (2014) 32: 3483–3489 [21] with permission. Copyright® 2014 Journal of Clinical Oncology. All rights reserved

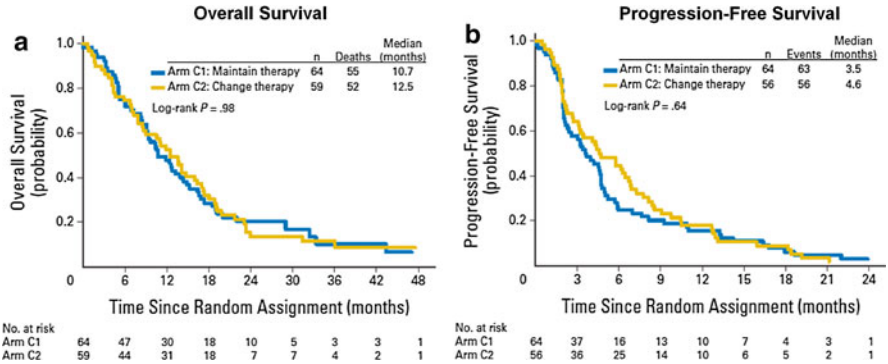

Fig. 12.3 SWOG S0500 Clinical outcomes for the randomized population—Patients with elevated CTCs (≥5 CTC per 7.5 ml whole blood) at baseline and after one cycle of first line chemotherapy were randomized to either continue current therapy (Arm C1) or switch immediately to a new class of chemotherapy drug (Arm C2). Kaplan–Meier curves are presented for (**a**) overall survival and (**b**) progression-free survival. Adapted from Smerage, J. et al., JCO (2014) 32: 3483–3489 [21] with permission. Copyright® 2014 Journal of Clinical Oncology. All rights reserved

whole blood) had a repeat CTC evaluation 3 weeks after the first dose of chemotherapy. Patients who had converted to low CTC (4 or less) were observed for outcome without intervention. Patients who continued to have elevated CTC were randomized to continue their current chemotherapy (standard of care) or to switch immediately to a new class of cytotoxic chemotherapy. Both arms were then followed until progression. Disappointingly there was no difference in outcome for PFS (HR = 0.92, $p = 0.64$) or OS (HR = 1.0, $p = 0.98$) (Fig. 12.3). Given the differences in prognosis based upon CTC, the investigators concluded that patients who have elevated CTC after the first cycle of chemotherapy are highly likely to have cancers that are generally resistant to cytotoxic mechanisms. These patients need better treatment options and might derive more benefit from early consideration of clinical trials of novel agents rather than simply following standard sequential lines of cytotoxic therapies.

There are additional randomized clinical trials for which results have not yet been reported [27]. The *STIC CTC METABREAST Study* is using CTC to assess prognosis in patients with newly diagnosed metastatic ER positive, HER2 negative breast cancer who have not received prior treatment for metastatic disease. Enrolled patients are randomized between standard therapies versus CTC-directed therapy. For the standard therapy arm, the choice of initial treatment is made by the treating physician based upon clinical judgement. For patients on the CTC-directed treatment arm, patients with low CTC are considered at lower risk and proceed to endocrine therapy, and the patients with elevated CTC are considered high risk and are started on cytotoxic chemotherapy. The *CirCe01 Study* also randomized patients between a standard therapy arm and a CTC-directed therapy arm. For the standard arm, patients receive standard clinical and radiographic evaluations during the course of their therapy, and changes in therapy are based upon clinical definitions of

progression. For patients on the CTC-directed therapy arm, CTC are obtained after cycle 1 of any new therapy. If CTC are elevated after one cycle they would switch immediately to an alternate therapy without waiting for clinical signs of progression. This same assessment would occur after starting each new line of therapy. Thus unlike SWOG S0500, which only used CTC to direct therapy for the first line of therapy, CirCe01 uses CTC to evaluate all lines of therapy. The *DETECT III Study* is evaluating patients with metastatic HER2 negative breast cancer starting first through third lines of therapy, and CTC are being tested for HER2 expression. Patients whose CTC are found to overexpress HER2 are randomized to receive or not receive the oral anti-HER2 tyrosine kinase inhibitor lapatinib. The goal is to determine whether HER2 on CTC predicts response to anti-HER2 therapy. The *Treat CTC Study* is evaluating patients with HER2 negative early stage (non-metastatic) breast cancer who have completed neoadjuvant chemotherapy and breast surgery. Patients with detectable CTC are randomized to receive 18 weeks of adjuvant trastuzumab versus observation. The CTC will be tested for HER2 expression and correlated with clinical outcomes. The goal is to determine whether HER2 over-expression on CTC is predictive for benefit from anti-HER2 therapy. Randomized studies will be key to knowing whether directing therapy based upon CTC numbers or based upon CTC marker expression provides clinical benefit. All four of these studies utilize CellSearch for CTC evaluation. The results of these studies are anxiously awaited.

12.5 Predicting Recurrence in Early Breast Cancer

The use of CTC in early breast cancer (stages 1–3) has been more limited due to the lower number of patients with detectable cells and due to the overall lower concentration of cells in those patients that do have detectable CTC. The CellSearch platform typically requires either a lower threshold to define positivity or increased sample volumes of 30 ml when used in the early breast cancer setting. Other technologies such as rt-PCR may be more sensitive and may play a role in the adjuvant setting [28].

The SUCCESS study evaluated CTC using CellSearch in 2026 women starting adjuvant chemotherapy for stage I–III breast cancer. All women had CTC samples drawn prior to starting therapy, and 1492 had samples drawn at the completion of therapy. To achieve adequate sensitivity, this study used 30 ml of whole blood. Prior to starting chemotherapy 21.5 % had ≥1 CTC and 3.1 % had ≥5 CTC. Across the population the number of CTC ranged from 0 to 827 per 30 ml blood. After completing chemotherapy, 22.1 % of patients had one or more CTC, but only 1.9 % had 5 or more cells. With a median follow-up of 35 months, the presence of CTC prior to chemotherapy identified a population of women at significantly higher risk of recurrence. In the primary analysis using 1 CTC as the threshold of positivity, the 3-year recurrence-free survival was significantly worse for those with ≥1 CTC at 88 % versus those with no cells at 94 %, $p = <0.0001$ (Fig. 12.4, panel a). In an exploratory analysis, they looked at the threshold of ≥5 CTC and found a 3-year recurrence-free

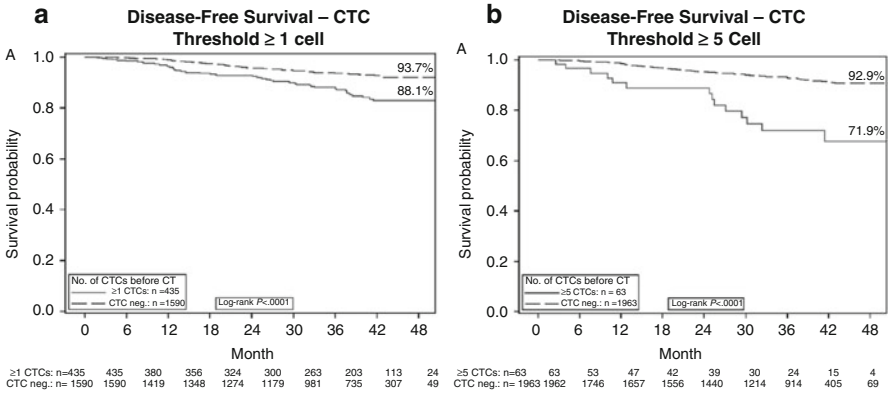

Fig. 12.4 SUCCESS study—Prognostic value of CTC in early stage breast cancer—Patients with stage I–III breast cancer had CTC samples drawn prior to starting adjuvant chemotherapy. Sample volume of 30 ml is larger than volumes used in metastatic setting. Presented are Kaplan–Meier curves based on different thresholds of CTC positivity. (**a**) When CTC threshold of ≥1 CTC per 7.5 ml whole blood is used 24 % of population is positive. (**b**) When CTC threshold of ≥5 CTC per 7.5 ml whole blood is used 3 % of population is positive. Adapted from Rack, B. et al., JNCI (2014) 106: dju066 [22] with permission. Copyright® 2014 Journal of the National Cancer Institute. All rights reserved

survival of 72 % versus 93 %, $p<0.0001$ (Fig. 12.4, panel b). Notably 14 % of the patients with ≥5 CTC had died within 3 years, which is a very high 3-year mortality for early stage breast cancer. The choice of CTC threshold also had a significant affect on the number of patient considered high risk. In this study population, 435 patients (21 % of total population) had ≥1 CTC, whereas only 63 patients (3 % of total population) had ≥5 CTC.

Another study reported outcomes for 302 patients treated at MD Anderson for stage I–III breast cancer. All patients had a biopsy-proven diagnosis of invasive breast cancer but had not yet undergone definitive surgery. None received neoadjuvant chemotherapy. CTCs were drawn prior to surgery. Analysis was based upon a 7.5 ml volume of blood using the CellSearch platform. Twenty four percent of this patient population had ≥1 CTC. Patients with increasing numbers of CTC had higher risks of recurrence. When using a cut-off of ≥1 CTC the hazard ratio for recurrence was 4.0 ($p=0.02$), for ≥2 CTC the hazard ratio for recurrence was 8.2 ($p<0.0001$), and for ≥3 CTC the hazard ratio for recurrence was 11.5 ($p<0.0001$). The Kaplan–Meier recurrence-free survival estimates also demonstrated significant separation. This study also showed significant decrease in the number of patients deemed to have elevated cells based on the threshold used. Patients with ≥1 CTC represented 24 % of the population, but patients with ≥2 or ≥5 CTC only represented 10 % and 5 % of the population respectively.

Other technologies may be more sensitive in early stage breast cancer, and one study using rt-PCR to detect cytokeratin-19 (CK-19) transcripts was able to separate patients into low and high risk of recurrence. A population of 167 patients with node negative breast cancer were evaluated for CK-19 mRNA with samples collected prior to initiation of adjuvant systemic therapy [29]. The population was mixed in

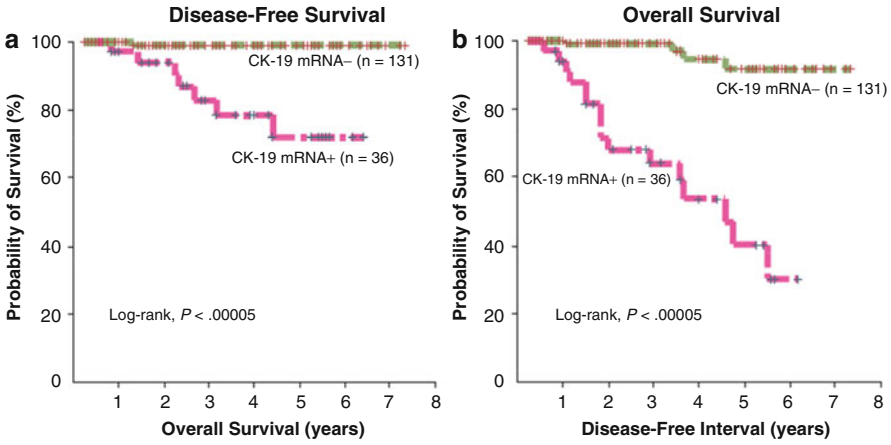

Fig. 12.5 Prognostic value of CTC detected by rt-PCR in patients with early stage breast cancer—(**a**) Progression-free survival and (**b**) Overall survival for 167 patients with lymph node negative early stage breast cancer based on the presence or absence of cytokeratins-19 messenger RNA (CK-19 mRNA) by quantitative RT-PCR (qRT-PCR) (Adapted from Xenidis, N. et al. JCO (2006) 24: 3756–3762 with permission. Copyright® 2006 American Society of Clinical Oncology. All rights reserved [29])

that some received chemotherapy, some endocrine therapy, and some both based upon tumor characteristics and standard of care risk assessment. Clinical outcomes were dramatically different depending upon the presence or absence of detectable CK-19 mRNA (Fig. 12.5). With a median follow-up of 32 months, 44 % of patients with detectable CK-19 mRNA experienced relapse compared to 3 % for those patient that were CK-19 mRNA negative. In addition, seven of the eight patients that died during follow-up were in the group with detectable CK-19 mRNA.

The threshold for defining positive CTCs is potentially more critical in this setting of early breast cancer because "circulating epithelial cells" can be identified in "normal" women and in women with benign breast diagnoses. Note that for the purpose of this discussion, these will be called epithelial cells rather than CTC because they are being isolated from women not known to have cancer. This finding complicates the interpretation of CTC in the early breast cancer setting because it introduces the risk of false positives when a low threshold is used to define patients as being positive for CTC. The numbers are generally small in patients without cancer, commonly being reported as an average of 0.1 CTC per 7.5 ml whole blood [10, 11, 30]. In one population of 145 "healthy" women CellSearch revealed that 5.5 % had one epithelial cells and none had two or more cells, and of 199 woman with benign breast diagnoses 7.5 % had one cell, 1 % had two cells, and 1 % had three cells [10]. In a second population of 84 individuals without cancer CellSearch revealed that 4.9 % had detectable cells, one with one cell, two with two cells, and one with three cells [22]. In a third population of 89 "healthy" women rt-PCR for CK19 revealed 2.2 % to be positive for the circulating epithelial marker [31]. There is no consensus on what level of CTC should define positivity in the early breast cancer, but caution should be used in using the low threshold of 1 CTC.

While CTC can identify early stage breast cancer patients at higher risk of recurrence, there are no studies demonstrating benefit from changing therapy. Notably, in both the SUCCESS study and the MD Anderson study all patients received adjuvant chemotherapy, and if appropriate, adjuvant endocrine therapy. Thus there are either few or no additional treatments within standard of care that could be added to augment the therapy that these patients received. The studies also differ in the timing of the CTC evaluation. The MD Anderson study evaluated the patients prior to surgery, and the SUCCESS study evaluated the patients after surgery. It is not known which time point is more clinically relevant. Thus this is a group of patients for which CTC are prognostic but do not yet drive therapy decisions. This is clearly a group that would benefit from new treatment options. Given their relatively high risk of recurrence, they would be good candidates for clinical trials of novel therapeutic approaches.

Despite the increased risk of recurrence for patients with detectable CTC, it is important to note that not all patients with detectable CTCs will have recurrence of disease. This is clear from the low recurrence rates seen in the SUCCESS and MD Anderson studies. This an area of CTC biology that is poorly understood. One study by Meng and colleagues [32] suggests that CTC can persists for long periods of time in the circulation without progression to clinically evident disease. They evaluated CTC in a group of long-term survivors of early breast cancer who were all without evidence of recurrent disease. Included were 36 women who had been treated with mastectomy for early stage breast cancer 7–22 years prior to enrollment in this study. None had clinical or radiographic evidence of recurrence. Of 36 patients, 13 (33 %) had 1–2 Circulating Epithelial Cells in 10–25 ml of blood. Fluorescence in situ hybridization (FISH) demonstrated abnormalities in these CTC including aneusomy in Chromosomes 1, 3, 8, 11, and/or 17. These abnormalities suggest that these are cancer cells. A control population without a history of cancer was also investigated, and only 1 of 26 control individuals had a single detectable epithelial cell, and that cell did not have any detected aneusomy. All of the breast cancer patients in this study were far enough out from their original diagnosis that it is reasonable to conclude that most of them were cured of their breast cancer. However, it is unknown if any of these patients might experience a recurrence in the future. Breast cancer is a disease known to have some very late recurrences [33]. These persistent CTC might be an explanation for these late recurrences. If these are tumor cells, they may have acquired the needed mutations for uncontrolled growth and migration, but they need a "second hit" to be able to form actual tumor masses at distant sites.

12.6 New Technology Platforms

While determining prognosis is clearly important in cancer care, the true vision for CTC is to use them to predict sensitivity to therapeutic agents and to help determine the best next therapies. Many investigators would describe this as using CTC as a

minimally invasive biopsy or as a liquid biopsy. CTC represent the potential of a biopsy that can be repeated over time, such that changes in tumor biology can be monitored as the cancer mutates [34, 35]. In that context CTC research is currently at a crossroads between the enumeration of CTC versus the biologic characterization of CTC.

The majority of current clinical outcomes data is based on platforms and techniques that primarily had the capability of quantifying cells. These platforms have the limitation that only about half of the patients have detectable cells. This is an advantage if the goal is to count cells because it separates patients into two populations that can be compared based upon the number of cells. However, if the goal is to understand the biology of the patient's cancer, then at sensitivity of only 50 % is a disadvantage. The ideal assay for phenotype determination would be able to isolate cells from all patients.

The majority of assays developed over the past 1–2 decades relied on epithelial cell surface markers such as EpCAM and cytokeratins to identify CTC. The rationale was that most tumors are of epithelial origin and that hematopoietic cells should not express epithelial markers. While this is generally true, there is growing evidence that some of the most important cancer cells may not express these markers, including tumor initiating "stem cells" and cells that have undergone epithelial–mesenchymal transition (EMT). Given the loss of epithelial markers, these cells would not be identified by assays that rely on epithelial markers for either isolation or detection/visualization [36, 37]. There is also data to suggest that CTC that are captured in clusters may represent these more stem-like cells and may be associated with a worse prognosis [38].

To circumvent this limitation there are several new strategies in development that aim to increase the sensitivity of CTC isolation such that a higher proportion of patients have detectable cells and to collect CTC in ways that are not reliant on epithelial marker alone for the isolation. Most new platforms utilize microfluidics or microfiltration to increase yield. Early generation microfluidic devices continued to use anti-EpCAM antibodies for capture, but had significantly higher capture rates. For one device using microposts [39], CTC were recovered from 115 of 116 patients tested, across an array of cancer types including breast, prostate, lung, colon, and pancreatic cancer. CTC counts ranged from 5 to 1281 cells/ml of blood, and these cells were at 50 % purity, which represents a significant improvement in purity over the immunomagnetic platforms. The average volume of blood used per patient was 2.7 ml. None of 20 healthy volunteers had detectable cells by this assay. A second device using graphene oxide nanosheets [40], detected CTC in all patients of a 20-patient cohort with metastatic breast cancer, early stage lung cancer, and metastatic pancreatic cancer. All patients had $\geq$2 CTC per ml, and the average was 5 CTC per ml. None of six healthy controls had cells by this assay. In a third platform using a herringbone surface pattern to create microvortexes [41], 14 of 15 patients with metastatic prostate cancer or lung cancer had isolated cells with a median of 63 CTC/ml and a mean of 386 CTCs/ml. This platform was also described as having rare "false positive" findings in healthy volunteers. They reported a median of 1 cell/ml and a mean of 3 cells/ml in these normal volunteers. As a result of these

findings, the investigators set an initial threshold for positivity at 10 cells/ml of whole blood. These false positives also raise the concern for false positive marker analysis if noncancer cells are captured and evaluated for RNA or protein expression. Other microfluidic platforms are using novel mechanisms to isolate CTC without the use of epithelial markers. This includes techniques such as deterministic lateral displacement, inertial focusing and magnetophoresis, immunomagnetic depletion of leukocytes, dielectrophoresis, as well as a large number of novel materials and nanostructures [42–45]. In addition, microfiltration devices are also in development allowing sized-based isolation of CTC without reliance on epithelial markers for the isolation process. One such device is was able to identify CTC in 51 out of 57 patients testing, compared to 26 of 57 matched samples evaluated by CellSearch [46]. Cells isolated with this platform are also highly viable and easily cultured after isolation from spiked whole blood samples [47]. All of these platforms are still very early in the analysis of their analytic and clinical validity, but if successful these technological advances have the potential to transform the CTC from a prognostic marker to a predictive marker that allows individualized selection of therapy based upon the biology of an individual patient's tumor.

12.7 Assessing Markers on CTC

Many biological markers have been detected on CTC. These include ER [48, 49], HER2 [32, 50–53], EGFR [54], MAGE [55], phosphorylated FAK [55], PI3K [55], androgen receptor [56], insulin-like growth factor [57], BCL2 and M30 [58, 59], and others. The comparisons of CTC immunohistochemistry and FISH analysis are quite remarkable. Meng and colleagues demonstrated the ability to perform both assays in breast cancer CTC (Fig. 12.6). With advances in technology even complex evaluations such as whole-genome sequencing and expression profiling are now becoming possible on single cells [61, 62], raising the possibility very sophisticated analyses of CTC, even when few in number.

The biologic interrogation of CTC raises the possibility of using CTC as a predictor of response to targeted therapies. As noted above, the *DETECT III Trial* and *Treat CTC Trial* are attempting to do this with HER2-targeted therapies. Another group is using this approach to develop a CTC-based assay to predict response to endocrine therapy in breast cancer [49]. In an ongoing prospective clinical trial, they are evaluating women initiating a new line of endocrine therapy for ER positive metastatic breast cancer. CTC are being collected and the cells stained for proteins associated with estrogen signaling, including ER, BCL-2, HER2, and Ki67. These markers are being combined to create an "endocrine therapy index." Expression of these proteins will be correlated with time to progression and OS. The clinical goal is to develop a test that will help clinicians know whether to continue with sequential lines of endocrine therapy because the index predicts that the tumor cells remain sensitive, or whether to switch to chemotherapy because the index predicts resistance to endocrine therapy. This is another example of an innovative clinical trial that is attempting to move beyond just enumeration of CTC.

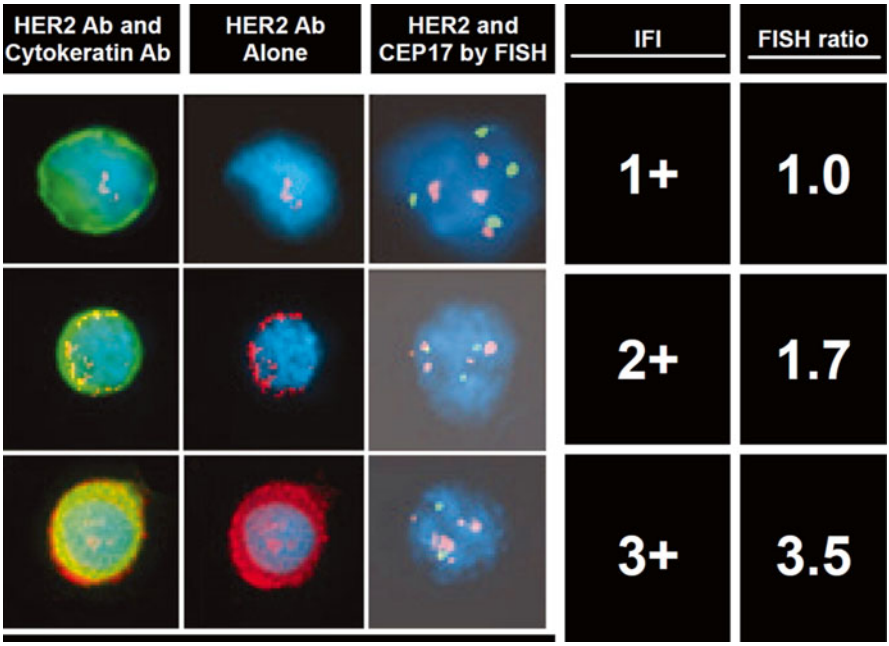

Fig. 12.6 Evaluation of protein expression and chromosome aneusomy using CTC. Displayed are three different CTC, each in a horizontal row. Cells were evaluated for HER2 protein expression by fluorescent immunohistochemistry, cytokeratin protein expression by fluorescent immunohistochemistry, and HER2 and Chromosome 17 centromere (CEP17) by FISH. All cells are counterstained with the nuclear stain DAPI. Adapted from Meng, S. et al., PNAS (2004) 101: 9393–9398 [60] with permission. Copyright® 2004 Proceeding of the National Academy of Sciences. All rights reserved

12.8 Summary

The technology in the detection of CTC has evolved quickly over the past decade. The majority of current clinical data is from platforms primarily designed to count cells based upon expression of epithelial markers, and it has been clearly demonstrated that elevated CTC are associated with a worse prognosis for women with breast cancer. This includes both early stage cancer and advanced cancer. While understanding prognosis is an important aspect of clinical decision-making, the counting of CTC has not been able to provide insight into what drugs to use and when to change therapy. Based upon the results of the SWOG S0500 study, it is clear that women starting first-line chemotherapy who have elevated cells after one cycle of chemotherapy have a very poor prognosis with currently available treatment options. This suggests that this population should be considered for clinical trials of novel agents early in their course of treatment. Ongoing platform development now focuses on the biologic characterization of CTC with the hope that such characterization will allow rational selection of targeted agents. The newer

platforms allow characterization to be done more easily, and they have a much higher sensitivity, allowing detection and characterization of cells in almost all patients with metastatic breast cancer. The true clinical value of these innovations awaits further analytic and clinical validation.

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Part IV
Potential Clinical Applications of CTC

Chapter 13
CTC in Advanced Breast Cancer Prognosis, Monitoring, and Clinical Utility

Massimo Cristofanilli

Abstract Circulating tumor cells (CTCs) are epithelial cells that can be found circulating in the blood of metastatic breast cancer patients and may represent a heterogeneous population including cancer stem cells and cells shed from the metastatic lesions. Interest and research continues in CTCs with the intent to detect and perform molecular analysis of those cells with the possibility to better understand the fundamental processes driving tumor metastasis. The technological advancement suggested the potential to measure the metastatic potential of a tumor at the single cell level rather than waiting until the cells established a metastatic lesion and proliferate until they are large enough to be visualized on imaging or cause symptoms. Therefore, earlier detection of these cells may be associated with more effective treatment of micrometastatic disease and lead to better outcomes. This chapter will provide an overview of the clinical utility of CTCs in breast cancer by reviewing the various techniques of CTC isolation; discussing their use as a prognostic indicator, as well as monitoring tool for response to therapy and detection of recurrence in the metastatic setting; reviewing their use in the neoadjuvant setting for prognosis and prediction of disease recurrence after surgery; evaluating CTC variation according to molecular subtype; discussing the detection of HER2+ CTCs in patients with HER2− tumors; and finally outlining ongoing trials and future directions of CTCs.

Keywords Circulating tumor cells • Breast cancer • Liquid biopsy

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13.1 Introduction

13.1.1 Background

Circulating tumor cells (CTCs) are epithelial cells that can be found circulating in the blood of metastatic breast cancer patients and may represent cancer stem cells or cells shed from the tumor. CTCs were first describe by Thomas Ashworth in 1869 who postulated that there was a connection between these cells and the primary tumor, and the tumor's ability to spread to distant body sites. Since their initial detection, there has been much research and continued interest in CTCs as they allow discovery and analysis of tumor cells that have escaped from their primary tumor and are circulating in the blood. These cells have gained the ability to migrate, a key step in the metastatic process, and may have the capacity to colonize at a different anatomic site as a metastatic lesion. The goal is to detect the metastatic potential of a tumor at the single cell level rather than waiting until the cells established a metastatic lesion and proliferate until they are large enough to be visualized on imaging or cause symptoms. Earlier detection may lead to treatment changes that provide better outcomes.

Our understanding of CTCs, along with our ability to isolate them has come a long way since their discovery but is still fraught with technical limitations. Several decades ago, there was a focus on disseminated tumor cells (DTC), which can be isolated from a bone marrow aspirate and were found to confer a negative prognosis [1]. The research done on DTC helped to establish morphologic criteria and the sample size needed to produce results that were clinically significant. The technologic advances gained in DTC research have been applied to the study of CTCs and has helped to guide methodology and future research. CTCs continue to be of clinical interest as they can offer important clues about the underlying biology of the tumor and the mechanisms it acquired that permit metastatic spread.

13.1.2 CTCs Isolation Techniques

Currently there are multiple methods for isolating CTCs. It is a technically difficult process given the relative rarity of the cells in blood, there is one CTC for every 1,000,000 cells. Most processes start with an enrichment step. Immunohistochemical assays isolate CTCs by positive or negative selection of their antigen expression. Positive selection is typically done with an antibody to epithelial cells markers such as epithelial adhesion molecule (EpCAM) [2]. Negative selection is done with a leukocyte antigen such as CD45 [3]. Positive selection is more commonly done because of its cost effectiveness; less antibody reagent is needed when selecting a few cells from many. However, positive selection may fail to capture some of the CTCs due to differential expression or regulation of the antigen expression, particularly in stem cell-like cells, which is an important limitation. As cells undergo

epithelial-mesenchymal transformation (EMT), they can acquire stem-like properties that are associated with tumor aggressiveness and capacity to metastasize [4]. Cells undergoing EMT may lose their epithelial markers and therefore would not be picked up by an antibody to the EpCAM antigen. Also, the normal-like breast cancer subtype does not have high expression of EpCAM therefore using this antigen may underestimate or miss CTCs in this subtype [5]. Therefore, negative selection appears to be a more favorable strategy for CTC isolation as it would capture the cells undergoing EMT as well as CTCs with epithelial markers. Inclusion of the EMT population is particularly important if the CTCs are studied for mutational analysis rather than simple enumeration.

Currently, the most commonly used and only FDA approved assay for CTC detection is the CellSearch™ assay. This technique uses enrichment with EpCAM antibody-coated ferrofluids mixed with 7.5 mL of the patient's blood. Those cells that react to the EpCAM antibody are magnetically separated from the other cells. These cells are then stained with an antibody to cytokeratin (an epithelial marker), then a CD45 antibody is used to identify any leukocytes, and finally a DNA stain, DAPI, is used to stain the nuclei of the CTCs and leukocytes. The cells are loaded onto the CellTracks Analyzer II which selects cells that stain for both cytokeratin and DAPI, and displays them for review by an analyzer. This is a rigorous selection process that isolates cells that express EpCAM and are CD45 negative, and are positive for cytokeratin and has a nucleus that binds DAPI. Unfortunately the cells are usually not viable after this process due to the permeability needed to stain for intracellular cytokeratin and DAPI. Therefore the cells cannot be used for genomic profiling.

Another positive selection technique is the "CTC-chip" [6]. This is a microfluidic platform using whole blood that captures CTCs as they pass by microposts coated with EpCAM antibody under laminar-flow conditions. The pilot study demonstrated a high rate of CTC detection (99 %) however the purity (ratio of cytokeratin+ to CD45+ cells) was low at 50 %. This technique has promise given the high detection rate and further studies are underway.

There has been some discussion regarding the viability of CTCs captured by various techniques and a question of whether some of the cells are apoptotic rather than viable CTCs. A functional assay has been developed called the EPISPOT (Epithelial ImmunoSPOT) [7]. This technique negatively selects cells with a CD45 antibody, and then cultures the cells on a membrane that captures the proteins produced or secreted by the cells over a 2-day period. These proteins can be analyzed to produce a CTC protein fingerprint.

An alternative to the immunohistochemical approach for CTC isolation is PCR-based assays which are mostly targeting RNA markers on CTCs via RT-PCR assays [8]. This method is challenging as tumor cells typically are genetically unstable therefore finding a standard genomic marker for identification is problematic. Another limitation is that RT-PCR cannot quantify CTC count which is significant since a CTC count of ≥ 5 versus <5 have a different prognostic value. One method developed by AdnaGen Technology, combines immunomagnetic separation techniques with quantitative RT-PCR [9]. The RT-PCR approach needs further validation studies before it has the credibility and reproducibility of the immunohistochemical approaches.

A completely separate approach to CTC capture is a size-based method. CTCs can be separated from hematopoietic cells by a microfilter due to their larger size relative to hematopoietic cells. There are several microfilter separation approaches including ISET (Isolation by Size of Epithelial Tumor Cells) [10] and MEMS (Micro Electro-mechanical system) [11]. Advantages of this approach are more frequent detection of CTC clusters [12] and that the cells intact so they can undergo genomic analysis after isolation. However, there is heterogeneity in the size of CTCs which can result in some of the CTCs passing through the filter [13]. Also the pores may become clotted by leukocytes which makes the enrichment process less effective and can contaminate the result.

Clearly there is a wide variety of methods for CTC isolation with varying levels of evidence to support them. A study comparing the ISET approach to CellSearch in various tumor types found that CellSearch was more sensitive for detecting CTCs in MBC but ISET was more sensitive for other tumor types such as metastatic lung cancer [12]. The DETECT trial compared CellSearch and AdnaTest and found that CellSearch had more prognostic value in MBC [14]. Given the number of methods and paucity of head-to-head comparisons, there is still debate on the best method for isolating them. Table 13.1 summarizes the methods of CTC enrichment and detection.

13.2 Clinical Utility of CTC Detection

13.2.1 CTCs in Metastatic Breast Cancer

Technology for the detection of CTCs has continued to develop over the years. As the technology has improved, the role of CTCs has become further defined. A pivotal study in 2004 demonstrated that enumeration of CTCs in metastatic breast cancer (MBC) was prognostic for PFS and OS [1]. Specifically, this study examined 177 women with MBC who had adjuvant or metastatic treatment, or were newly diagnosed, and were about to initiate a new line of therapy. They drew blood at baseline, at 3–4 weeks and then every 9–12 weeks. Data was collected from the first 102 patients and used to establish a cutoff threshold for CTC count which would distinguish patients at higher risk for progression. They found that detection of 5 or more CTCs from 7.5 mL whole blood was the threshold and validated this finding in the subsequent 75 patients. The patients that had ≥5 CTCs at baseline had median PFS of 2.7 months and median OS of 10.1 months compared to 7 months and >18 months respectively in patients with <5 CTC at baseline. CTCs were also prognostic when measured at the first follow-up visit, patients with ≥5 CTCs had a median PFS of 2.1 months and median OS of 8.2 months compared to 7 months and >18 months respectively for patients with <5 CTCs at first follow-up. Interestingly, those patients whose baseline CTC was ≥5 but at first follow-up was <5 had the same prognosis as patients whose baseline and first follow-up were both

Table 13.1 Methods of CTC enrichment and detection technologies

Detection methods	Advantages	Disadvantages
Immunocytochemical methods		
Immunocytochemistry	Analyzes blood and bone marrow samples	Operator dependent readout
	Assesses morphological criteria	Time-consuming
	Parallel analysis of multiple target antigens	Yields nonviable cells
	Quantification of CTCs	
	Single cell isolation	
CellSearch™	Semi-automated device	Blood samples only
	Validated technical standard	EpCAM-dependent
	(FDA approved device)	Yields nonviable cells
	Quantification of CTCs	Costs
CTC-chip	Quantification of CTCs	Blood samples only
	Small sample volume	EpCAM-dependent
	High detection rate	Technically demanding
EPISPOT assay	Analyzes blood and bone marrow samples	Depends on active release of target proteins
	High sensitivity and specificity	No availability of spotted cells for downstream analyses
	Functional test for viable cells	
	Quantification of CTCs	
	Independent of tumor antigen phenotype	
PCR-based methods		
RT-PCR	Operator independent readout	Difficult control of false positive results (low-level illegitimate expression; only improved in quantitative real-time RT-PCR)
	Analyzes blood and bone marrow samples	Difficult control of false negative results (deficient/low expression of target mRNAs, RNA instability)
	High sensitivity	No visualization and quantitation of CTCs
Adnagen test	Operator independent readout	EpCAM/MUC1-dependent
	Analyzes blood and bone marrow samples	No visualization and quantitation of CTCs
	High sensitivity	

<5. The same was true for the reverse—if the baseline CTC was <5 but first follow-up was ≥5, those patients had the same PFS and OS as patients whose CTC were ≥5 both at baseline and first follow-up.

Similarly, a retrospective study of 151 patients with metastatic breast cancer was analyzed for factors associated with poor prognosis and shortened survival [15]. Circulating tumor cells were enumerated with a cutoff of <5 termed negative and ≥5 was positive. There was a significant difference in median OS between the groups with negative versus positive CTCs, 29.3 months and 13.5 months respectively. They found that detection of ≥5 CTCs had prognostic value independent of factors such as disease subtype, tumor burden or line of therapy.

Multiple prospective and retrospective trials have supported the prognostic significance of ≥5 CTCs for PFS and OS; this was summarized in a recent pooled analysis of 1944 patients with MBC [16]. The authors created a clinicopathological prognostic model to determine the added impact of CTCs for PFS and OS. They found that adding CTC count (<5 or ≥5) to their predictive model significantly increased the prognostication for OS and PFS. This is in contrast to serum tumor markers, CEA and CA15-3, when checked at 3–5 or 6–8 weeks which did not add significant prognostic value in their model. The prognostic value of CTCs was consistent across all subtypes of disease. Finally, in the multivariate analysis, CTC count was the strongest prognosticator for PFS and OS. Table 13.2 lists the critical prospective studies using the CellSearch assay for CTC analysis.

13.2.2 Monitoring in the Metastatic Setting

In addition to their prognostic value, CTCs can be used for monitoring response to treatment and disease recurrence. Liu et al. conducted a prospective study to evaluate CTCs as a monitoring tool in conjunction with radiographic imaging [17]. CTCs were drawn at baseline and serially along with radiographic imaging to see if elevated CTC values correlated with or preceded progression of disease on imaging studies. They found that patients who had ≥5 CTCs had 6.3 times the odds of disease progression on imaging compared with patients who had <5 CTCs. Several subsequent studies have confirmed these findings [18, 19]. This is a significant finding for many reasons. CTCs were able to detect disease progression at an earlier stage than can be discerned on imaging. This allows physicians to change treatment earlier and prevent subjecting patients to toxic therapy that is not beneficial. As CTC isolation techniques improve and CTCs are seen as a reliable tool for monitoring disease, they may start to replace or decrease the use of radiographic imaging. CTC enumeration is a less invasive test that is more sensitive and cost effective, and has no associated toxicity.

Continued elevation of CTCs is known to be associated with a poor prognosis as well as a sign of chemo-resistance. A recent trial of MBC patients used persistently elevated CTCs as a marker of chemo-resistance and evaluated a strategy of early change in chemotherapy to see if it improved survival [20]. More specifically,

Table 13.2 Prospective studies of CTCs using CellSearch assay

Trial	Number of patients	Primary endpoint
Observational trials		
Cristofanilli et al.	177	PFS, OS
NEJM 2004		
Nole et al.	80	PFS, OS
Annals of Oncology 2008		
Liu et al.	68	PFS, correlate CTC enumeration and response on radiographic imaging
Journal of Clinical Oncology 2009		
Bidard et al.	67	Time to progression
Annals of Oncology 2010		
Nakamura et al.	107	OS
Breast Cancer 2010		
Consoli et al.	93	PFS, OS
Tumori 2011		
Hartkopf et al.	58	Correlate change in CTC count with changes on radiographic imaging and serum CA-15.3 levels
Anticancer Research 2011		
Pierga et al.	267	PFS, OS
Annals of Oncology 2012		
Muller et al.	221	Compare prognostic impact of CellSearch and AdnaTest assays
Breast Cancer Research 2012		
Martin et al.	99	PFS, OS and clinical benefit rate
Oncologist 2013		
Jiang et al.	294	PFS, OS
Annals of Oncology 2013		
Pierga et al.	44	Central nervous system objective response and OS
Annals of Oncology 2013		
Wallwiener et al.	393	PFS and OS
BMC Cancer 2014		
Interventional Trials		
Smerage et al.	595	OS
Journal of Clinical Oncology 2014		
Pierga et al.	996 (planned accrual)	PFS
STIC trial, currently accruing		
Pierga et al.	568 (planned accrual)	OS
CirCe01 trial, currently accruing		

patients initiating their first line of chemotherapy for MBC had CTCs drawn before cycle 1 and 2. If the CTCs were persistently elevated at the second draw, patients were randomized to either continuing that chemotherapy until radiographic progression or changing chemotherapy after the first cycle. Unfortunately, those patients with continually elevated CTCs after randomization continued to have a shortened OS, and there was no survival advantage to changing chemotherapy early versus continuing on initial therapy, essentially confirming their chemo-refractory disease. The study again confirmed the prognostic value of CTCs the group with no CTCs at baseline had the best median OS, and the group whose CTCs fell after 21 days of initiating chemotherapy fared better than the group with persistently elevated CTCs. A recent study by Wallwiener et al. concurred with these results [21].

A similar study, the CirCe01 trial, is still underway but rather than evaluating patients undergoing first line therapy, it is evaluating heavily pretreated patients who are starting their third (or later) line of chemotherapy [22]. CTCs are measured at baseline and after one cycle of new treatment, and if the patient does not have a sufficient decrease in CTCs, therapy will be changed to the next line of treatment and CTC evaluation repeated after one cycle. The patients will continue to change therapy every few weeks if there is not a sufficient decrease in CTCs. The primary outcome is overall survival but they are also evaluating PFS, quality of life and depression/anxiety.

Studies are underway evaluating CTCs as a means for molecular monitoring which could offer further disease characterization and possibly aid in treatment decisions. A recent study evaluated gene expression profiles of CTCs in MBC patients at baseline and follow-up [23]. The CTCs were isolated by CellSearch™, then RNA was extracted from the CTCs and gene expression was analyzed via quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). A panel of 22 breast cancer-specific genes was chosen for CTC analysis along with three control genes. In a previous study by the same group, expression of TFF1 in tumor tissue has been shown to be associated with metastasis to the bone [24]. In this study they found concordance with CTC expression of TFF1 and bone metastasis. They also analyzed ERβ expression in CTCs with response to endocrine therapy and found that ERβ expression was associated with sensitivity to endocrine treatment. This study, along with other studies like it, demonstrates the potential use of CTCs as a "liquid biopsy" for molecular characterization and monitoring.

13.2.3 CTCs According to Molecular Subtype

CTCs may play more of a role in predicting recurrence in certain subtypes of breast cancer. Breast cancer is a heterogeneous disease with varying degrees of aggressiveness and risk for recurrence, as well as susceptibility to hormone and cytotoxic therapy. Due to the heterogeneity of the disease, a study by Ignatiadis et al. investigated the difference in prognostic significance according to disease subtype of CTC detection using CK-19 mRNA [25]. The study enrolled 444 patients

with stage I–III breast cancer, with all molecular subtypes represented according to their natural prevalence, and median follow-up of 53.5 months. Using CK-19 mRNA-positivity to isolate CTCs, they were detected in 40.8 % of early stage breast cancer patients. CTCs were isolated in every disease subtype and the difference in incidence among the subtypes was not statistically significant.

CTC detection was generally associated with decreased disease-free survival (DFS) and OS. Subgroup analysis at 5 years revealed that CTC detection predicted poor prognosis for patients with estrogen-receptor (ER)-negative disease, but not for ER-positive disease. This is an interesting distinction since genomic tumor profiling often places patients with ER-positive disease in low recurrence risk categories and patients with ER-negative disease are often higher risk. Therefore, CTCs may provide further information to risk stratify ER-negative patients.

As mentioned previously, the survival curve in the study by Ignatiadis et al. for ER-positive patients with positive CTCs was similar to ER-positive patients without CTCs. However, the survival curves start to separate after 5 years which may coincide with stopping adjuvant endocrine therapy at 5 years. This suggests that monitoring CTC count longitudinally may be helpful in determining which patients would benefit from extended endocrine therapy. In this study, ER status and detection of CK-19 mRNA-positive CTCs were the strongest factors predicting DFS and OS.

There is still debate on the predictive value of CTCs in HER2-positive patients, especially those who have been treated with HER2 targeted therapy. In a pooled analysis of studies which examined CTCs in MBC, patients with HER2-positive cancer had a significantly lower incidence of having ≥5 CTC compared to other subtypes [16]; they also found decreased frequency in patients undergoing treatment with HER2 targeted therapy. However, this difference was not noted in the group of patients who were HER2-positive and had not started any treatment. The lower incidence of CTCs in HER2-positive patients may reflect their previous exposure to HER2 targeted therapies which have been shown to decrease CTCs [26]. A retrospective analysis by Munzone et al. analyzed CTC count among the different molecular subtypes [27]. Again, they found that CTCs were predictive for OS for all subtypes. Generally the group of patients with 0 CTCs had better outcomes than those with 1–4 CTCs or >5 CTCs except this difference was not noted in the HER2+ group. Subsequently, two large prospective trials, with over 200 MBC patients each, found CTCs to be prognostic regardless of molecular subtype or prior therapy [28, 29].

13.2.4 CTCs in Neoadjuvant Treatment of Locally Advanced Breast Cancer

CTCs have been studied in the neoadjuvant setting as well. Pierga et al. conducted a phase II study to see if CTCs could be detected before and after neoadjuvant chemotherapy, and if there was a correlation between CTC detection and response to chemotherapy and prognosis [30]. Using a cutoff value of 1 CTC per 7.5 mL of blood, 23 % of patients had CTCs detected before initiation of chemotherapy and

17 % of patients after chemotherapy. Interestingly, they did not find a correlation between changes in CTC count and pathologic complete response. At a median follow-up of 18 months, they found that CTC detection was associated with early metastatic relapse. The follow-up study with a median follow-up of 36.4 months, demonstrated that CTC detection either before or after neoadjuvant chemotherapy was significantly associated with decreased distant metastasis-free survival (DMFS) and OS [31]. Specifically, CTC detected before initiating neoadjuvant chemotherapy was an independent prognostic factor for OS. The data for CTC detection after neoadjuvant therapy was less robust but consistent with the pretreatment data.

This is an informative study as pathologic complete response is considered a positive prognosticator but this data did not show a correlation with CTC detection and tumor response in the post-neoadjuvant setting. This is in contrast to a previous smaller study by Camara et al. that demonstrated changes in CTC count during neoadjuvant therapy was predictive of tumor response [32]. The study by Pierga, along with other studies [33], suggests a difference in responsiveness to chemotherapy by the CTCs and the primary tumor. This difference may be accounted for by the heterogeneity of CTCs and the complex selection process that occurs during metastasis [34]. This study, however, established a role for CTC evaluation in non-metastatic breast cancer patients in the neoadjuvant and post-neoadjuvant setting.

13.2.5 Biologic Heterogeneity of CTCs

The difference in OS that correlates with detection of CTCs raises the question of whether this difference stems from biologic variation in the CTCs or is it a reflection of the underlying biology of the tumor. A study by Klein et al. which examined DTC in the bone marrow, lymph nodes and blood of patients with breast, prostate and gastrointestinal cancers who were without clinically evident metastatic disease [35]. There was evidence of DTCs in 14 % of patients and these cells were highly heterogeneous, suggesting genomic instability. The authors postulate that one or a few of these DTCs are genetically selected or "fitter," and therefore survive and establish the metastatic clone. Further investigation into the characterization and phenotyping of these cells is warranted to better understand the process of metastasis development.

13.3 Disease Monitoring

13.3.1 Detection of HER2-Positive CTCs

The current standard for establishing HER2 status is by assessing HER2 overexpression/amplification by immunohistochemistry (IHC)/fluorescence in situ hybridization (FISH) in the primary tumor. There is evidence, however, that there can be discordance between the HER2 status of the primary tumor and the

metastatic lesion, indicating that the HER2 status may change during the metastatic process [36]. HER2 expression has been associated with circulating stem cell (CSC) phenotype in absence of gene amplification [37] and particularly in Luminal B disease [38]. It is often impractical to re-biopsy metastatic lesions so alternative methods for HER2 status evaluation and monitoring are necessary.

CTCs are an appealing method for HER2 detection as it is noninvasive and can be easily monitored during treatment. HER2 status can be assessed in CTC either by the AdnaTest Breast Cancer method, which extracts mRNA from isolated from CTCs and evaluates it by PCR, or by anti-HER2 antibody in the CellSearch™ assay [39]. However, a disadvantage of the mRNA analysis is that the mRNA expression levels cannot be quantified, in contrast to the CellSearch method, which quantifies HER2 overexpression in the immunostaining score (0, 1+, 2+, 3+). In a study by Fehm et al., they found 32 % of patients with HER2-negative primary tumors had HER2-positive CTCs via the CellSearch™ assay.

A trial currently being conducted, DETECT III, is a phase III study evaluating patients with HER2-negative metastatic disease who have HER2-positive CTCs. Patients are randomized to standard treatment versus standard treatment plus lapatinib. The results of this study will be informative and further support the need to establish the best method for HER2 assessment in CTCs, and to measure clinical outcomes after HER2 targeted therapy is initiated based on HER2 detection on CTCs.

13.4 Current Clinical Impact

There is collective evidence for the clinical impact of CTC detection for prognostication, both at baseline and during treatment [1, 16, 21]. After initiating a new treatment, small changes in CTC count, especially if baseline is ≥5 and follow-up is <5, could reflect response to therapy at an earlier time point than is otherwise available. The reverse is also true, a change from <5 at baseline to ≥5 indicates lack of response to therapy and suggests changing therapy at an earlier time. The authors of a pooled analysis of 1944 MBC patients created a clinicopathological prognostic model to determine the added impact of CTCs for PFS and OS [16]. They found that adding CTC count (<5 or ≥5) to their predictive model significantly increased the prognostication for OS and PFS. This is in contrast to serum tumor markers, CEA and CA15-3, when checked at 3–5 or 6–8 weeks which did not add significant prognostic value in their model. The prognostic value of CTCs was consistent across all subtypes of disease.

13.5 Ongoing Studies and Future Impact of CTCs

The role of CTCs continues to evolve from a prognostic marker to a tool used to aid in treatment decisions. CTC are being investigated as a determining factor for initiating chemotherapy. A phase III study (STIC trial NCT01710605) is randomizing

hormone-receptor positive MBC patients to a standard arm versus CTC arm for first line therapy [22]. The standard arm patients will be treated according to physician preference. In the CTC arm, if <5 CTCs the patient will be treated with hormone therapy. If ≥5 CTCs the patient will receive chemotherapy. The primary end point is to show noninferiority of CTC arm for PFS. This study will also evaluate the medico-economics of utilizing CTCs by comparing the cost per progression-free life years gained by the two approaches.

Another ongoing trial is the *Treat CTC* trial which is phase II study investigating the use of trastuzumab in patients whose tumors are HER2-negative but have ≥1 CTCs (regardless of HER2 overexpression) [22]. Patients who have completed neoadjuvant therapy and surgery will be randomized to observation versus trastuzumab every 3 weeks for six cycles. The primary endpoint will be CTC detection at 18 weeks with secondary endpoint of recurrence-free survival.

The results of the ongoing trials are eagerly anticipated as they may change the role of CTCs from a prognostic marker to a sensitive tool that can guide clinical decisions. CTCs may broaden the pool of patients eligible for targeted therapy by detecting HER2-positive cells in patients whose primary tumors may be HER2-negative. And with the advent of DNA mutation analysis from CTCs, they will play an increasingly important role in molecular monitoring of metastatic disease.

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Chapter 14
Evolution of Metastatic Disease: The Need for Monitoring and Emerging Therapeutic Opportunities

Bernhard Polzer and Christoph A. Klein

Abstract During the last few years tailored therapies have improved overall survival of patients with metastatic disease. However, iatrogenic selection pressure continues to drive the evolution of systemically spread cancer cells, resulting in the generation of aggressive and therapy-resistant tumor cells. The outcome is cancer relapse and death in the majority of patients. In early disease stages, (neo) adjuvant targeted therapies often fail for unknown reasons. Therefore, diagnostic and therapeutic strategies have to be reevaluated on the basis of an evolutionary concept of disease. This obviously implies the ability to monitor the molecular evolution of the disease. To this end, a novel diagnostic pathology for systemic cancer has to be developed that will enable precision medicine for cancer patients.

Keywords Tumor cell heterogeneity • Parallel progression model • HER2 • Disseminated cancer cells • Targeted therapy • Therapy resistance • Single cell analysis

14.1 Introduction

Between 10 and 90 % (depending on cancer (sub)type) of cancer patients will relapse with metastatic disease after initial curative treatment. Despite multiple lines of systemic therapies most of them will eventually die from metastasis [1, 2],

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indicating a great medical need for improvement. During the last two decades the identification of molecular subtypes characterized by specific mutations and activated signaling pathways has led to the identification of numerous new drug targets. Tailored therapies against specific targets are currently tested in many clinical trials and have shown effect in metastatic cancer. For example, targeting HER2, EGFR, or BRAF has improved survival in subsets of patients with breast, lung or colorectal cancer and patients with melanoma [3–6]. However, the initial benefit usually lasts only a few months, as some metastatic cancer cells acquire or already carry mutations leading to resistance against the specific drug. Even more disappointing are the observations that—with the exception of HER2 targeting drugs—several targeted therapies including those targeting EGFR mostly fail to improve survival of patients in earlier stages of cancer [7, 8].

In this chapter, we discuss underlying mechanisms behind these clinical observations and indicate how direct molecular analysis of systemic cancer could improve therapy selection and outcome in metastatic and adjuvant cancer patients.

14.2 Cellular Heterogeneity and Cancer Progression

In 1976, Peter Nowell described leukemia as an evolutionary disease driven by stepwise accumulation of somatic mutations and selection for the fittest tumor cell subclone [9]. Together with the seminal paper of J. Cairns [10] this laid ground for the current understanding of cancer as an evolutionary process based on clonal selection [11–13]. Thirty-five years later, the advent of new sequencing technologies has opened a new window to the complexity of cancer cell genomics and evolution [14]. Several studies have shown an extensive genetic heterogeneity of tumor cells at the primary tumor site [15–17], one prerequisite for Darwinian evolution. Surprisingly, this heterogeneity on a single cell level had been observed much earlier in early systemic cancer, when single disseminated cancer cells (DCCs; also termed disseminated tumor cells, DTCs) had been genetically analyzed [18]. Based on the assumption that cancer originates from one single cell and that mutations detected in all or many cells of a tumor represent early events, an evolutionary tree of individual cancers can be reconstructed. In a recent study, this rationale was tested by single nucleus genome sequencing of primary breast cancer cells and mathematical modelling. The authors could show that while aneuploidy rearrangements occurred early in individual tumor evolution and remained highly stable during clonal expansion, point mutations evolved gradually. Many of the detected mutations occurred at low frequencies (<10 % of cells) in the bulk of tumor and thus were responsible for extensive clonal diversity [19].

In addition to the mutational repertoire, different phenotypes of tumor cells in an individual cancer are regulated by distinct epigenetic states. For example, in primary glioblastoma gene expression studies have defined four molecular subtypes: proneural, neural, classical, and mesenchymal [20]. These subtypes could be linked to genomic profiles of glioblastomas and more importantly to therapy response, showing greatest benefit for the “classical subtype” and worst outcome in the

"proneural subtype." In a recent study, single-cell RNA-seq revealed the phenotypic heterogeneity in 430 single cells of five primary glioblastomas [17]. Not surprisingly, the authors uncovered an extensive heterogeneity in gene expression of individual cells within the same glioblastoma. However, the expression profiles corresponded to the different molecular subtypes and all five cancers were found to harbor cells of the proneural subtype. When the different phenotypes were analyzed in an existing data set of proneural tumors from the Cancer Genome Atlas [20], it was found that increased subtype heterogeneity was associated with decreased survival, emphasizing the clinical importance of intratumoral heterogeneity [17].

Therefore, such emerging molecular data strengthen the concept that cellular diversity or evolutionary state could serve as a novel form of biomarker [21, 22]. It seems plausible that the clinical relevance is directly related to the diversity and evolutionary state of disseminated cancer cells and metastasis as systemic therapies specifically attempt to target these cells. Here, it is noteworthy that the cellular diversity may be significantly higher in metastases than in primary tumor cells [23].

Heterogeneity within primary tumors and metastases may reflect not only the different phenotypes generated during aberrant organogenesis, which already Rudolf Virchow considered to be the underlying process of tumor formation and metastasis, but also the complexity of events that are imposed on metastasizing cancer cells [24]. This sequence of events has been frequently termed the "metastasis cascade." Briefly, tumor cells have to (1) invade locally through surrounding tumor stroma, (2) intravasate into blood or lymph vessels, (3) survive the mechanical pressure by the circulation, (4) arrest and extravasate at the distant organ, (5) adapt to the foreign microenvironment to colonize the distant organ (micrometastasis), and finally (6) reinitiate proliferative programs to form solid tumors again (macrometastasis). As for the primary tumor, metastatic outgrowth follows the principles of adaptation and selection. During the metastatic cascade, considerable environmental pressures are acting on the tumor cells and cells have to revert to different underlying cell-biological and molecular mechanisms, which have been repeatedly reviewed [25–27]. To survive these selection forces tumor cells must be genetically equipped or phenotypically very plastic. Thus, it is hardly surprising that metastasis is a highly inefficient process [27, 28]. In summary, one may wonder how malignant evolution and cancer cell heterogeneity impacts on the clinical situation and how we should integrate this knowledge in our therapeutic algorithms.

14.3 The Implications of Linear and Parallel Progression

14.3.1 Selection of Systemic Therapies Based on the Linear Progression Model

Our current clinical thinking is based on the linear progression model of cancer. Here, the stepwise progression of cancer-associated morphological changes [29] was initially linked to the accumulation of genetic and epigenetic changes in

primary tumor cells [30]. As a consequence, cancer evolution was thought to be reflected by the size of a primary tumor and tumor cell dissemination to occur from the most advanced and hence largest population of tumor clones. Furthermore, dissemination would start after clonal expansion of highly malignant cells that will found the distant metastases. This model of progression is supported by the clinical observation that the frequency of metastasis increases with tumor size (which is reflected in the TNM staging system). Most importantly it lead to the general concept that most systemic therapies are selected according to characteristics of primary tumor cells, although the target cells to be eradicated lie detached from the primary site. Currently, many target proteins are usually encoded by genetically activated oncogenes that upregulate pathways linked to increased cell survival and proliferation. As a consequence, the tumor cells strongly rely on these pathways, a phenomenon termed oncogene addiction, which is the rationale behind most targeted therapy approaches [31].

From an evolutionary perspective, however, the rationale behind this concept may raise concerns, which are discussed further below. First, we will take a look on the results of targeted therapies that have been applied based on the linear progression model so far. During the last decade, treatment of metastatic cancer with drugs against genetically activated HER2, EGFR, or BRAF (as determined in resected primary tumor specimens) has without doubt improved survival in subsets of patients with breast, lung, or colorectal cancer and patients with melanoma [3–6]. However, this benefit usually is transient and lasts only a few months before disease progresses again. While it is disappointing that resistance develops so quickly in metastatic patients, the data are in line with an evolutionary concept in which therapies select for genetic variants from a rather large pool of metastatic cancer cells. The linear progression model would also hold that the resistant cancer cells were preexisting within the primary tumor, and more specifically among the predominant clones of a cancer.

14.3.2 Clinical Data Inconsistent with a Linear Progression Model

In the adjuvant setting (with metastatic cancer undetectable by current clinical imaging) only a latent seed of cancer cells may be left in the body of the patient after resection of the primary tumor. As a consequence, expectations for targeted therapies in the adjuvant setting were high but unfortunately, could hardly be met until today. The only promising results so far have been achieved for HER2-targeted therapies. Whereas adjuvant treatment of HER2-positive breast cancer with trastuzumab leads to a substantial increase of disease-free survival, no positive effect on overall survival could be observed [32]. However, this could be explained by the high number of patients in the observation cohort crossing over to trastuzumab treatment, after the drug was found to be effective. In contrast, recent studies using small-molecular inhibitors or antibodies against EGFR have completely failed in

adjuvant lung and colorectal cancer [7, 8] despite therapeutic success in patients with metastatic disease [4, 33]. Surprisingly, not even a slight initial response could be observed, as judged from the failure to induce increased progression free survival. This indicates that the targeted cells were resistant a priori and did not acquire resistance for EGFR-targeted therapy later on.

Based on the concept of linear progression these findings are hard to understand. If the disseminated tumor cells display the mutational profile of the primary tumor and the number of surviving tumor cells in the adjuvant setting is significantly lower as compared to metastatic disease, preexisting therapy-resistant variants should be exceptionally rare. However, clinical observations imply that an already existing intrinsic resistance of disseminated cancer cells plays a major role in early systemic cancer. The mechanisms behind this primary resistance may be different from the resistance acquired during multiple lines of treatment of metastatic cancer patients. First, the targeted pathways may not be active in DCCs before metastatic outgrowth, counteracting the principle of oncogene-addiction. For example, it has been shown in head and neck cancer that although EGFR is highly expressed in dormant cancer cells, it is not activated by phosphorylation, thus rendering the pathway inactive [34].

Intrinsic resistance may also result from the absence of the target or the sensitizing mutation from early DCCs. The paired analysis of primary tumor and corresponding metastases shed some light on this question. Interestingly, most studies published until today report considerable cellular heterogeneity for mutations in classical oncogenes or tumor suppressor genes as for example *KRAS* or *TP53* (as reviewed in Ref. [35]). For the example of non-small-cell lung cancer where the EGFR inhibitor gefitinib failed to improve outcome of early stage patients [8], a disparity of primary tumors and metastases for EGFR mutations had previously been reported in more than 75 % of paired samples [36]. These findings suggest that cancer cells often leave the primary tumor before the acquisition of the alteration that is targeted, rebutting the linear progression model.

14.3.3 Understanding Clinical Findings on the Basis of the Parallel Progression Model

Apparently, therapeutic predictions made from a linear progression model do not hold water upon clinical testing and our concepts are in need of revision. It may be asked what underlies these unexpected and disappointing clinical observations. As indicated, tumor cells may disseminate before acquisition of mutations within the primary tumor that are used for treatment selection. Under this premise, disseminated cancer cells would need to undergo malignant progression (selection and adaptation) at the distant site in parallel to the primary tumor. Hence, this model was termed parallel progression model [37].

Several studies have shown that DCCs can already be detected in the bone marrow of breast cancer patients at the stage of carcinoma in situ [38–40]. This surprising observation was further investigated in mouse models of breast cancer, for

which it could be shown that individual tumor cells break through the basement membrane during pre-invasive atypical hyperplasia by transmission electron microscopy [39]. Moreover, molecular studies demonstrated that most DCCs in early cancer patients are genomically in a less advanced stage (compared to the primary tumor) in breast, prostate and esophageal cancer [41–44].

In breast cancer, nearly 50 % of isolated early DCCs even showed balanced karyotypes in metaphase CGH. Their malignant origin could be proven by detection of small, subchromosomal deletions that have been frequently described in breast cancer, indicating that the cells left the primary tumor before the onset of genome-wide instability in the primary tumor [41]. For prostate cancer, genetic heterogeneity for different DCCs of the same patient was extensive and higher than in patients with metastatic disease. More important, intra-patient heterogeneity between DCCs isolated from early cancer patients and tumor cells isolated from the primary tumor is substantial. For example, in a study on prostate cancer only 1 of 11 patients showed chromosomal aberrations shared by the main clone of the primary tumor and isolated bone marrow-derived DCCs. Furthermore, even in this patient only 8.0 % of total detected aberrations were shared [44]. Strikingly, while chromosomal alterations frequently described in primary prostate cancer (loss of 8p, 13q, and 16q, gain of 8q) could be detected in many DCCs of metastatic patients, paired analysis showed that in most cases DCCs and primary tumor tissue of an individual patient did rarely match even for these regions [44], indicating that these selected changes were acquired independently within and outside the primary tumor.

The early time-point of dissemination and extensive heterogeneity between primary tumor, single DCCs and metastases contradict the linear progression model and instead favor a parallel progression of local and systemically spread tumor cells [37]. It would also explain why disseminated cancer cells are not necessarily addicted to oncogenes found in the primary tumor. Dissemination with an immature genome may further enable higher flexibility for tumor cells to adapt to various "hostile" microenvironments of distant organs and at the same time may cause many DCCs to enter a state of cellular dormancy. The model predicts that strong driver mutations leading to extensive tumor growth may be acquired *independently* for colonization of the distant organ.

14.3.4 Acquisition of Alterations outside the Primary Tumor Irrespective of Linear vs. Parallel Progression

The discussion of linear vs. parallel progression, though fundamental, may appear largely academic. However, it is clinically relevant which tissue is to be used for molecular characterization and therapy decisions. Here, it should be considered that during the last years, disease courses have become longer and longer, partially because of early diagnosis, partially because of better treatment [45]. Thus, prolonged disease courses as a consequence of several lines of treatment, inevitably drive the evolution of cancer cells that are increasingly disparate from the primary

tumor that was surgically removed long time ago. Hence, primary tumor tissue increasingly becomes a less relevant source of molecular information on systemically spread cancer cells and the evolutionary forces of selection and adaptation acting on metastases become the major determinant for therapy resistance [13, 46].

As a consequence, monitoring the progression of systemic disease on a molecular level over time is one of the paramount challenges to improve benefit from the emerging therapeutic options in the near future. However, monitoring of systemic cancer is rarely performed. The major reason for this is that extracting information from metastatic tissue is a challenge itself as (1) metastatic tissue is frequently inaccessible for biopsy and (2) multiple metastases are usually present in an individual patient and (3) repeated intralesional bioptic sampling is hardly tolerable and last but not least (4) there is no metastatic tissue for biopsy in early systemic cancer. To overcome these limitations alternative means to monitor systemic cancer have to be established.

14.4 CTCs and DCCs as Monitoring Tools in Systemic Disease

The potential to detect solid cancer cells in the vascular system was already recognized in 1869 by Thomas Ashworth, who first described circulating tumor cells (CTCs) in the blood of a deceased cancer patient [47]. Peripheral blood would be an ideal organ to monitor systemic cancer, as it is (1) easily accessible and repeated sampling is well tolerated by patients; and (2) the cellular composition of blood is relatively homogeneous and well defined compared to other organs. However, as CTCs are very rare events in the bloodstream (1–10 CTC per ml of blood) [48, 49], the breakthrough for DCC and CTC analysis occurred after Schlimok and Riethmüller identified antibodies that opened the door By using the histogenetic markers EpCAM and cytokeratin they could specifically detect epithelial cells in the mesenchymal organ bone marrow [50], which was subsequently complemented by blood and lymph node.

Two decades later, the systematic analysis of systemic tumor load started with the advent of highly sensitive technologies for CTC enrichment, described extensively in previous chapters of this book. Despite the development of numerous technologies currently the only FDA-cleared method for detection and quantification of CTCs in whole blood is the CellSearch® system (Janssen Diagnostics LLC). This platform enriches CTCs using immunomagnetic EpCAM-beads and tumor cells are then identified by an experienced operator judging (1) positivity for cytokeratin 9, 18, and 19 expression, (2) negativity for CD45, (3) an intact nucleus (DAPI-staining), and (4) size and morphology [51]. Although, the immunomagnetic enrichment strategy applied by CellSearch® was demonstrated to be highly specific for CTC detection, it has been shown that $EpCAM^+/CK^+/CD45^-$ cells can be detected in low numbers in the blood of healthy individuals [52] and in even higher numbers in patients with benign chronic diseases [53]. To avoid ambiguous results, cutoff levels have been defined ($\geq$5 CTCs for breast and prostate cancer, $\geq$ 3 CTCs for colorectal

cancer) that were found to be predictive for decreased survival [54–56]. However, it should be noted that in early breast cancer the current threshold of a single positive cell is sufficient to assign an increased risk for disease progression [57].

The utility of CTC enumeration to assess prognosis and response to therapies has been shown in numerous studies and is discussed in a previous chapter of this book. Systemic tumor cell load in blood, however, is significantly lower in patients without evidence of distant metastasis (stage M0), which significantly raises the hurdles for CTC detection. In a recent study, Fischer et al. increased the blood volume screened by applying diagnostic leukapheresis (DLA) for cell-density based enrichment of mononuclear cells (including CTC). By this approach, they investigated 2.3 ml DLA product by the CellSearch system—equivalent to ~60 ml of peripheral blood—and detected CTCs in the majority of screened M0 patients. Additionally, they found that the CTC count correlated with UICC stage, indicating that the number of CTCs is an indicator of systemic tumor burden [48]. In another study on early breast cancer, Rack et al. applied CellSearch® analysis to samples from 2026 early breast cancer patients. Circulating tumor cells were detected in 21.5 % of patients before adjuvant treatment and the presence of CTCs was correlated with disease-free and overall survival [57]. However, the percentage of positive patients cannot be directly compared to metastatic patients for two reasons (1) for each patient, mononuclear cells of 30 ml of peripheral blood were enriched by density gradient prior to application of the CellSearch® method; (2) every positive CTC event was counted, i.e., patients below the threshold of 5 CTCs as defined in metastatic patients were also considered as positive. In fact, most patients harbored very low numbers of CTCs in 30 ml of blood and only 63 patients (3.1 %) $\geq$ 5 CTCs. Importantly, the impact on survival for this small subset of patients as well as for patients with persisting CTCs after adjuvant therapy was significantly higher than for patients with a single positive cell [57]. Unfortunately, the prevalence of single positive events in healthy individuals using their approach and evaluation was not reported. This would have been helpful as it has been shown that 23/344 (6.7 %) of healthy patients or patients with benign diseases may harbor 1–3 $EpCAM^+/CK^+/CD45^-$ cells in 7.5 ml of blood [52]. Extrapolating this number to the 30 ml used in the study by Rack et al., overlap between true cancer cells and confounding events detected by CellSearch® has to be considered. As Rack et al. found a prognostic impact of a single CTC on survival, their data suggest that morphological criteria may exist to differentiate normal and malignant $EpCAM^+/CK^+/CD45^-$ cells.

The low CTC numbers in early disease reduce the utility of peripheral blood as the organ of choice to detect systemic cancer in adjuvant disease and additional approaches may be helpful. Fortunately, systemic tumor spread can also be detected in other organs. For example, DCCs are detected in bone marrow of 20–60 % of patients without metastasis by the expression of cytokeratins but only very rarely in control patients [58, 59]. However, the cellular composition of bone marrow is more heterogeneous than of blood, which considerably complicates method development for routine clinical application. Nevertheless, standardized protocols for enrichment and detection of DCCs exist [60] and have been applied in numerous studies showing that the detection of only one DCC in 10^6 bone marrow or lymph node cells is correlated with survival in many types of cancer (reviewed in Ref. [61]). Another

drawback of bone marrow analysis is that patients are reluctant to consent to repeated (potentially painful) biopsies during follow-up. For this reason, most studies have been performed with samples drawn at the time of surgery. However, there are informative and notable exceptions. In breast cancer, the persistence of DCCs after surgery was associated with increased risk of relapse [62]. Additionally, the eradication of DCCs after adjuvant treatment with bisphosphonates [63] and docetaxel [64] has been identified as a means to predict therapy response. These results in breast cancer are contrasted by a study on prostate cancer, in which prostate cancer patients were followed up to 8 years by annual bone marrow sampling. Surprisingly, while DCCs predicted progression to biochemical relapse (postsurgical PSA increase) and metastasis, the persistence of cytokeratin-positive DCCs in patients with prostate cancer could not be associated with an increased risk for relapse. Moreover, DCCs were detected in a similar frequency (~20 %) throughout the whole time-course of the study in patients showing no signs of progression [44].

Another organ that can be used for an initial assessment of systemic tumor load at the time of surgery are (sentinel) lymph nodes. In most solid cancers, lymph nodes are surgically removed together with the primary tumor in a very systematic approach, and as for bone marrow, DCCs have been described in many malignancies and associated with poor prognosis of the patient, e.g., for non-small-cell lung cancer [58, 65, 66]. In melanoma, one single tumor cell detected in the sentinel lymph node suffices to stage the patient with N1 disease according to the recommendations of the AJCC [67]. We recently have shown for a cohort of 1027 patients that precisely quantifying disseminated melanoma cells in the sentinel lymph node level enables a superior prognostic model for melanoma survival [66], indicating that at least for some cancers DCCs in lymph node could represent an elegant way to assess systemic cancer already in early stages and provide a baseline at the time of surgery.

In summary, to monitor systemic disease we should explore different strategies for different types of cancer and in different stages of disease. While monitoring of metastatic disease on the basis of CTCs in the blood might soon enter clinical routine, at least for early cancer strategies involving diagnostic leukapheresis, bone marrow and lymph node sampling may be needed to complement assays based on puncture of a peripheral vein. However, for all sources of cancer cells enrichment methods need to be improved.

14.5 Molecular Single Cell Analysis and Its Therapeutic Implications

14.5.1 CTCs for Guidance of Treatment Decisions in Metastatic Cancer

While the detection and enumeration of CTCs and DCCs itself is valuable to assess prognosis and therapy response in cancer patients, molecular analysis could significantly increase the impact of liquid biopsies and may be the only way to guide treatment decisions. For example, identification of known druggable targets on CTCs

may open therapy options for metastatic cancer patients that are precluded based on primary tumor characteristics.

The most prominent example is HER2 in patients with breast cancer. Different assays to assess HER2 status in CTCs are in place, including immunofluorescence, fluorescence in situ hybridization (FISH), and PCR-based approaches. Although the different assays use different technologies [68], most studies show a remarkable heterogeneity between HER2 status of the primary tumor and systemic disease as measured on CTCs in the peripheral blood. For example, a HER2 immunostaining score which ranges from 0 (no expression) to 3 (high expression) has been implemented to the FDA-approved CellSearch® system and validated on cell lines with known HER2 amplification status [69]. Applying this assay, in 25 of 78 (32 %) patients with HER2-negative primary breast cancer and more than 5 circulating tumor cells, HER2 expression was detected on CTCs [70]. Heterogeneity between primary tumor, CTCs and manifest metastasis has also been observed in other drug targets, such as EGFR in colorectal cancer [71].

This reasoning has driven the initiation of six clinical trials that currently explore the power of molecularly defined subsets of CTCs to guide clinicians in selecting tailored therapies for metastatic cancer patients (Table 14.1). For example, CTCs in peripheral blood in metastatic breast cancer patients with first to third line therapy and HER2-negative primary tumor are evaluated in the German multicenter trial DETECT III using the CellSearch® system (NCT0161911, available from www.clinicaltrials.gov). Patients harboring CTCs expressing the HER2 protein are subsequently randomized to two therapeutic arms one half of the patients receiving standard chemotherapy only, the other half lapatinib, and a small molecule inhibitor of HER2. Expectations to observe a significant effect on the primary endpoint of progression-free survival are high.

14.5.2 CTCs to Explore and Monitor Mechanisms of Drug Resistance in Metastatic Disease

In addition to the molecular assessment of drug targets, CTCs could help to identify mechanisms of drug resistance. For example, Maheswaran and coworkers could identify a specific point mutation in the EGFR gene which confers to the drug resistance against EGFR inhibitors by genotyping of pools of CTCs [74]. Following these lines, it has been described that activating ESR1 mutations can be frequently observed in metastatic breast cancer previously treated with antiestrogens or estrogen deprivation [75]. The underlying mechanism was suggested as a drug resistance mechanism in estrogen receptor (ER)-positive breast cancer treated with ER-targeted therapy. Recent functional studies supported the use of CTC analysis for assessment of drug resistance. The group of Daniel Haber succeeded in establishing six cell lines from CTCs of metastatic breast cancer patients, which were shown to be tumorigenic in mice [76]. Interestingly, three of these cell lines harbored mutations in ESR1. In vitro drug sensitivity tests not only confirmed resistance against

Table 14.1 Clinical trials utilizing molecular characterization of circulating tumor cells

Identifier	Tumor type	Molecular CTC profile	Tested drug	Clinical phase	State of the study
NCT00694252	Metastatic breast cancer	HER2-positive *or* EGFR-positive CTCs	Lapatinib	Phase II	Complete, no results reported
NCT00820924	Metastatic breast cancer	HER2-positive CTCs	Lapatinib	Phase II	Terminated, no benefit for patients [72, 73]
NCT01185509	Metastatic breast cancer with HER2-negative primary tumor	HER2-positive CTCs	Trastuzumab and Vinorelbine	Phase II	Recruiting completed
NCT01619111	Metastatic breast cancer with HER2-negative primary tumor	HER2-positive CTCs	Standard-Chemo or endocrine +/− Lapatinib	Phase III	Recruiting
NCT01975142	Metastatic breast cancer with HER2-negative primary tumor	HER2-positive CTCs (FISH and ICC)	Trastuzumab emtansine	Phase II	Recruiting
NCT02035813	Metastatic breast cancer with HER2-negative and hormone-receptor positive primary tumor	HER2-negative CTCs	Standard -Endocrine +Evorolimus	Phase II	Recruiting

ER-targeted therapies but also suggested that HSP90 inhibitors could be a novel approach to target ESR1-mutated metastatic breast cancer [76]. The study suggests that resistance mechanisms may be similarly identified from CTCs as from metastasis and that monitoring emergence of known resistance mechanisms by molecular profiling of CTCs may be feasible.

14.5.3 The Need to Screen for Novel Drug Targets in Early Systemic Cancer

CTC analysis in metastatic cancer seems to be straightforward, however, we face a fundamentally different scenario in early systemic cancer. As described, genomic studies have shown that inter- and intra-patient heterogeneity is far more pronounced in single DCCs of non-metastasized patients as compared to DCCs of metastasized patients [42–44] as tumor cells start to disseminate very early in cancer progression and have to evolve to a fully malignant clone outside the primary lesion [13]. This implies that early disseminated cells may not express classical therapeutic targets like EGFR (either by lacking underlying mutations or pathway inactivation by epigenetic regulation) which could explain the failures of tailored therapies.

These observations indicate that the current approach of transferring conclusions from metastatic cancer to the adjuvant setting has limitations as evidenced by the failure of EGFR-targeted therapies in colorectal and lung cancer [7, 8]. Instead of focusing on known drug targets we are in need to identify common early genetic hits in DCCs of non-metastasized patients. For this, we have to invest some effort to analyze the genomes of early DCCs and CTCs on a more global level (e.g., whole genome analysis) to identify those aberrations that are present in all cancer cells (early and late clones) of an individual patient.

14.5.4 HER2 as a Drug Target in Adjuvant Cancer

As paradigm for this thinking we may consider HER2-targeted therapy that confers significant benefit in early breast cancer patients [32, 77].

As for metastatic breast cancer, considerable heterogeneity has been demonstrated when comparing the HER2 status of the primary tumor and the expression of HER2 on CTCs or DCCs in early breast cancer. In non-metastatic breast cancer patients, HER2 amplification could already be detected in single CTCs and DCCs, showing considerable discordance between local and systemic disease [41, 78]. Moreover, a subpopulation of DCCs showed HER2 amplification even in the absence of gross chromosomal aberrations, suggesting that it manifests as one of the earliest genetic events in these patients [41].

Interestingly, retrospective studies suggest that even breast cancer patients without DNA copy number gain of HER2 in the primary tumor could benefit from HER2-targeted therapies [79, 80]. These results are currently tested in the prospective phase III trial NSABP B47, in which women with HER2-negative breast cancer are treated by adjuvant trastuzumab. It is therefore important to understand the underlying mechanism of the clinical efficacy of trastuzumab in these patients. One explanation suggests HER2 as an important regulator for breast cancer stem cells (CSCs). It was shown that HER2 overexpression increases and HER2 inhibition decreases the CSC population in breast cancer cell lines and mouse xenografts [81].

This reasoning could recently be extended to luminal breast cancers where HER2 expression was upregulated by NFkB in the absence of HER2 DNA amplification. The effect of HER2 targeting in HER2 non-amplified cancers may therefore be a consequence of CSC eradication [82].

Moreover, the expression of HER2 on systemically spread cancer cells might be important not only in breast cancer but also in other tumor entities. In a study on esophageal cancer, we could show that HER2 amplification in DCCs isolated from bone marrow or lymph node is more frequent in DCCs of patients with adenocarcinoma of the esophagus than in DCCs of breast cancer patients. Moreover, the detection of HER2 amplification by qPCR in only one single DCC was correlated with significantly reduced overall survival with all affected patients dying within the first 24 months. Strikingly, HER2 amplification in primary tumor tissue had no prognostic relevance for the survival of the patients [43]. Therefore, it would be interesting to investigate in a prospective clinical study, if early disseminated tumor cells harboring HER2 amplification are vulnerable to HER2-targeted therapies.

14.6 Developing a Diagnostic Pathology for Systemic Cancer and Perspectives

Based on recent findings [83], some authors suggest that the analysis of CTCs may be complemented if not replaced by the analysis of circulating tumor DNA (ctDNA). In metastatic cancer, ctDNA can be detected in more than 75 % of patients and in most of these cases allows assessment of clinically relevant point mutations (e.g., *KRAS* in colorectal cancer) with high sensitivity and specificity [84]. Similar to CTCs, the detection of ctDNA is correlated with the overall tumor load and thus significantly more difficult in non-metastatic cancer patients. As a consequence, mutational profiling by ctDNA holds great promise in targeted assays for metastatic cancer. However, it is currently open whether and how mutations may be identified that are present only in subclones of CTCs or solid tumor masses (most ctDNA is derived from apoptotic or necrotic tumor material). A great advantage of CTC analysis in a cell-by-cell approach therefore is clonal purity. Cell-by-cell analysis enables to monitor the mutational profiles over disease courses and track lineages to the unit of selection—the individual tumor cell. The downside of this approach, however, is that it is unclear how many CTCs are needed to draw clinically relevant conclusions.

Cell-by-cell analysis also enables to uncover combinations of mutations that may underlie resistance to specific drugs. For this reliable whole genome amplification methods (WGA) for single cells are needed. The task is daunting with the low amount of 6–7 pg genomic DNA in a single human diploid cell [85] being an obvious obstacle. Although single cell analysis is feasible with targeted PCR or FISH, these assays are only suited for a limited number of targets. However, to assess the heterogeneity and retrieve the mutational profile of single cells a genome represen-

tation is needed. For this, multiple WGA technologies have been developed during the last two decades, including PCR based amplification using degenerate oligonucleotides as primers [86, 87], adaptor-linker mediated PCR [88], multiple strand displacement amplification [89, 90], as well as methods based on combinations of these general methodologies [91].

In principle, all WGA methods aim (1) for high genomic coverage, i.e., ideally all 3×10^9 nucleotides comprising the human genome have to be amplified; (2) for reliable quantification of copy number variation, i.e., all regions of the genome have to be amplified homogeneously; and (3) for low allelic dropout rate, i.e., avoid the artificial loss of one or even both inherited gene copies (maternal and paternal) to reliably assess sequence variation. Besides performance indicators of a given WGA technology, the origin of the single cell sample impacts on the result, as the DNA quality of clinical samples is usually lower compared to single cells isolated directly from cell culture. Sample fixation necessary for shipment and enrichment or detection methods can have a significant impact on DNA quality, as most fixative agents lead to DNA crosslinking and thus fragmentation. Additionally, the cells of interest may contain damaged DNA even before isolation and fixation. For example, it was shown that a substantial proportion of CTCs is apoptotic [92] harboring fragmented DNA. DNA fragmentation or degradation of DNA irrespective if caused by fixation or apoptosis will specifically reduce the performance of WGA methods that rely on the amplification of long DNA sequences.

To introduce single cell analysis in a clinical diagnostic setting, protocols and methods have to be standardized and be broadly available. Several WGA technologies have recently been commercialized (Table 14.2). In addition, the resulting sin-

Table 14.2 Commercially available methods for whole genome amplification of single cells

WGA technology	OmniPlex® (Rubicon Genomics) / GenomePlex® (Sigma)	*Ampli1*™ WGA (Silicon Biosystems)	Repli-G (Qiagen) / GenomiPhi (GE Healthcare)	PicoPlex™ (Rubicon Genomics) / SurePlex™ (BlueGnome) / EasyAmp™ (PerkinElmer)	MALBAC (Yikon Genomics)
Mechanism	PCR	PCR	MDA[a]	MDA[a] + PCR	MDA[a] + PCR
Primer design	Defined	Defined	Random	Hybrid[b]	Hybrid[b]
Priming pattern	Random	Defined	Random	Random	Random
Whole genome representation of single cells	up to 38.7 % [93]	up to 74 % [94]	up to 72 % [91]	up to 36 % [95]	up to 93 %[c] [91]
Allelic drop out in single cells	<30 %[d]	5–10 % [41]	10–30 % [96, 97]	<10 %[d]	<10 %[c,d]

[a]Multiple displacement amplification
[b]Primers contain random and defined sequences
[c]Significantly lower when analyzing single CTCs [98, 99]
[d]Information as provided by the manufacturer of commercial kit

gle cell WGA has to provide sufficient yield of DNA to support test repetitions and long-term storage without quality loss. Here, it is important that treatment of samples prior to single cell isolation has significant impact on single cell WGA quality. For example, it could be shown by whole genome sequencing that the MALBAC WGA method is able to cover up to 93 % of the human genome after amplifying a freshly isolated single human SW480 cancer cell [91]. However, analyzing single CTCs of lung cancer patients after CellSearch® enrichment and MALBAC [99] resulted in a dramatically decreased genomic coverage in a direct comparison of the two datasets [98]. Similarly, the allelic representation was reduced in single cell WGA products from clinical samples [99]. It is of utmost importance to develop assays that not only overcome these technical difficulties but meticulously assess the quality of each single cell WGA product to generate high-quality genomic data.

Therefore, we devised a semi-automated workflow for enrichment, isolation, and molecular analysis of breast cancer CTCs [100]. For this, we applied CellSearch® for CTC enrichment and detection, the DEPArray™ system for pure single cell isolation and Ampli1™ for single cell whole genome amplification. Due to the strictly deterministic approach of Ampli1™ WGA (based on usage of one defined restriction site for DNA fragmentation, ligation of a primer binding site and one primer for amplification) we could develop a quality control assay that is able to assess the genome integrity of isolated cells by a simple multiplex PCR assay. This assay identified high-quality WGA samples that can be subjected to multiple molecular downstream analysis (e.g., sequencing, qPCR, and array CGH) with a high diagnostic reliability of >90 % (Polzer et al., EMBO Molecular Medicine in press). Application of this approach unraveled the extent of cellular heterogeneity among CTCs, uncovered the existence of preexisting therapy escape variants and linked oncogene mutations to mechanisms of genome rearrangement (Polzer et al., EMBO Molecular Medicine 2014).

Therefore, the development of a diagnostic pathology of systemic cancer is within reach. Such diagnostic pathology of systemic cancer will embrace cancer as a dynamic disease and enable monitoring and therapy adjustment. It may also provide a direct answer to the lesson learned from the SWOG 0500 trial, where CTC enumeration failed to inform about alternative chemotherapeutic treatments [101]. As soon as the community succeeds to overcome the remaining technically and regulatory hurdles to include molecular characterization, single DCC and CTC analysis will be applicable for the benefit of cancer patients.

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Chapter 15
CTCs for Biomarker and Companion Diagnostic Development

Shih-Min A. Huang and Mark R. Lackner

Abstract Circulating tumor cells (CTCs) were first recognized in the blood of cancer patients by Thomas Ashworth in 1869. Recent advances have propelled CTCs to center stage in diagnostic and translational research, largely owing to advances in isolation technology and increasing sensitivity of the various genomic, transcriptomic, and proteomic platforms. As we enter the era of personalized medicine, it is foreseeable that CTCs will become an integral component of clinical practice to not only understand disease evolution but also match patients with appropriate therapeutics.

The current field is inundated with an enormous amount of data studying diverse aspects of CTC biology. In this chapter, we focus on recent developments in the incorporation of CTCs in oncology clinical studies that suggest promising utility for CTCs as pharmacodynamic and predictive biomarkers that can fundamentally aid decision making in clinical trials. In light of the complexity inherent in codeveloping in vitro diagnostic assays with therapeutic agents, we highlight key challenges and suggest solutions to enable the ultimate goal of developing CTCs as companion diagnostic assays for targeted oncology therapeutics.

Keywords CTC • Molecular characterization • Genomic profiling • Transcriptomic profiling • Proteomic profiling • Pharmacodynamic biomarker • Predictive biomarker • Companion diagnostic

15.1 Introduction: Circulating Tumor Cells (CTCs) as a Game-Changing Medium for Biomarker Assessment

The past 20 years of cancer research have yielded a formidable amount of information on the underlying molecular mechanisms and key drivers in human cancers. Many of these basic research findings have been translated to oncology drug

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development, and have shifted the paradigm in this arena from broad spectrum cytotoxic agents prescribed to "all comer" patient populations, to focused development of "targeted" therapies that are designed to specifically inhibit key molecular drivers of cancer. Early examples of this paradigm include development of antiestrogen signaling agents in breast cancer, as well as the anti-HER2 therapeutic antibody trastuzumab in a distinct subset of breast cancer patients [1–4]. These examples underscore the concept that targeted agents cannot be effectively prescribed without a means of identifying patients that have cancers driven by aberrant activation of the target in question. Thus, only women with estrogen receptor positive breast cancer are selected for antiestrogen targeted therapies, while only women with HER2 positive breast cancers are treated with agents such as trastuzumab. This paradigm has now been repeated successfully in cancers as diverse as EGFR mutated lung cancers (treated with erlotinib, gefitinib), and BRAF (V600E) driven melanoma (treated with dabrafenib, vemurafenib). In each of these cases, expression, mutation, or amplification of the target has been harnessed as a predictive biomarker that can be used to efficiently select the patients most likely to respond by means of a Companion Diagnostic (CDx) test. A biomarker has been defined by the 1998 NIH biomarkers definition working group as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [5]. In this chapter, we primarily consider how CTCs have the power to transform the development and clinical application of predictive biomarkers (i.e., patient selection biomarkers) and pharmacodynamic biomarker (i.e., biomarkers of pharmacological response or target modulation in response to therapeutics), with special attention to the CDx path for CTC evaluation in the final section.

Despite the manifest success of tumor-tissue based biomarker assessments in enabling the first generation of CDx tests and ushering in the era of personalized medicine, such approaches have several inherent limitations. Specifically, archival tumor tissue collected at diagnosis offers only a fixed snapshot of the cancer, often taken from the primary tumor and hence not representative of later stages of disease. Moreover, recent research has suggested that cancers undergo a process of clonal evolution, with different metastatic lesions showing marked heterogeneity such that a single sample is unlikely to be representative of the overall disease. Fresh biopsies collected serially prior to new lines of therapy have the potential to offer more representative information. However, such samples are difficult to collect (typically one biopsy from a single site), and are not without risk of adverse events to patients, so are unlikely to be part of routine clinical practice [6]. In addition, despite the success of targeted therapies, resistance has been well documented and arises in almost all patients receiving even the most efficacious targeted therapies [7]. A long-term aspirational goal for the field of oncology should be to shift metastatic cancer from a nearly universally fatal disease to one where longitudinal monitoring of molecular resistance mechanisms provides a basis for rational switching of therapies. Such a vision will be very difficult to realize with tumor-tissue based biomarker assessments, given the limitations described above.

It is into this gap that circulating tumor cells and cell free tumor DNA (ctDNA) have emerged and shown so much promise in terms of the potential to enable real-time, comprehensive molecular biomarker assessment from a minimally invasive blood draw. As an added benefit, blood can be collected serially over time and treatment, potentially enabling longitudinal biomarker assessments in a manner not practical from tumor tissue. Others have discussed the promise of ctDNA extensively [8–10]. We only touch briefly on literature that has compared the technologies head to head, and focus primarily on the potential of CTCs as a game changing technology for biomarker development in this chapter. We start with a discussion of some of the highlights from the recent literature that demonstrate the feasibility of characterizing CTCs at the DNA, RNA, and proteomic level. While promising, the field is in a state of rapid evolution and most of the work to date has been conducted using research grade assays and a variety of platforms. In the final section, we turn to discussion of a theoretical path to bridge from these promising research-based efforts to the strictly regulated realm of formal CDx devices, which will be required if the field is to deliver on the full promise of CTCs and change real-world medical practice.

15.2 Emerging Platforms for CTC Molecular Characterization

Several platforms have been established and tested for their sensitivity and specificity in capturing CTCs in the past few years. The first generation of CTC platforms used cellular markers that distinguish epithelial cells from blood cells in circulation as the basis to capture and enrich CTCs from whole blood. In this instance, the CTC population in blood is usually defined as CD45 negative, epithelial cell adhesion molecule (EpCAM) positive, and cytokeratins 8, 18, and/or 19 positive. More often than not, CTC population is isolated by immunomagnetic capture of EpCAM expressing cells followed by manual screening for an intact nucleus and negativity for the lymphocyte marker CD45. The Veridex CELLSEARCH® platform is one such system that is based on the aforementioned markers and is approved by FDA for the purpose of CTC enumeration and early prediction of progression-free survival and overall survival in patients with metastatic breast, colorectal, or prostate cancers [11]. The CELLSEARCH® system has some utility in molecular characterization, but the manual nature of the scoring coupled with relatively lower sensitivity compared to newer platforms, suggest that additional platform development is required [12, 13]. In order to improve efficiency of capturing EpCAM expressing cells, Stott et al. designed a high-throughout microfluidic mixing device named the herringbone-chip, or “HB-Chip,” that significantly improves the physical interactions between CTCs and antibody-coated surface through microvortex mixing [14]. To further enhance capturing of all potential CTCs, a non-sorting methodology was recently offered by Epic Science. This platform requires spreading of all nucleated

cells onto several microscope slides, staining with suitable antibodies to detect CTCs and relevant biomarkers, and analyzing image files with a proprietary algorithm to identify CTCs [15]. However, using EpCAM or other cellular markers to isolate CTCs in blood neglects tumor cells that lose EpCAM expression due to epithelial–mesenchymal transition (EMT)or are from non-epithelial origin. To overcome this deficiency, Karabacak et al. applied the principle of "negative depletion" and developed a tumor antigen-independent methodology to achieve an average of 3.8-log depletion of white blood cells by the combinatorial utility of CD45 and CD66b antibodies and capture approximately 97 % of tumor cells in blood [16]. A variety of size-based filtration method have also been developed that exploit the size differential between CTCs and lymphocytes, and some of these have shown promise as a capture methodology that allows downstream molecular characterization of CTCs [17]. In the foreseeable future, technologies isolating rare CTCs in blood will likely continue to become more sensitive and specific, paving the way for incorporating CTC collection as a routine procedure in the clinical setting. As discussed later in this chapter, the key to taking any of these platforms along a CDx path will require stringent attention to technical and analytical validation of the platform, combined with careful codevelopment with a partner therapeutic. Inevitably, part of this challenge will be the extraction of the most clinically relevant biological information from CTCs.

As discussed earlier, CTCs could become an invaluable source of material that can be obtained from patients in a noninvasive manner, thus overcoming logistic hurdles in studying of tumor clonal evolution in the absence or presence of pharmaceutical intervention. Concordance of genetic variants between CTCs and primary or metastatic tumors have been described previously. Deep sequencing on primary or metastatic tumor samples showed that most mutations detected in CTCs from the same patient were also present in tumors [18]. Nevertheless, the limited number of evaluable tumor cells in circulation does impose technical challenges to execute a comprehensive interrogation of biological information harbored within CTCs. Although it is now becoming increasingly attainable to culture and expand CTCs ex vivo for a limited duration to amass number of CTCs and improve the feasibility of downstream analyses [19], comparability of these derivatives to the original parental cells requires further validation. CTCs could hypothetically represent the heterogeneity of all clones of metastatic tumors in a given patient in contrast to the traditional tumor biopsies, in which it would be nearly impossible to access a sufficient number of lesions representing all tumor clones. Given this, the field has collectively invested significant effort in improving specificity, sensitivity, reproducibility, and practicality of platforms that allow faithful detection of genomic, transcriptomic, and proteomic modulations in CTCs. Ultimately, it is hoped that recent breakthroughs in technology should advance the feasibility of routinely assessing analytes within a single circulating tumor cell to capitalize on the possibility of understanding tumor heterogeneity. The ability to dissect biological information from patient-derived CTCs real-time during the course of therapeutic intervention would be invaluable to predict response, inform next line of treatment, and monitor disease progression.

The following sections seek to emphasize the most recent achievements in extracting and accurately dissecting genomic, transcriptomic, and proteomic information in CTCs. Rather than providing a comprehensive historical recounting of development of CTC capture technologies, here we highlight recent breakthroughs that are most relevant to the development of robust biomarker assays and CDx tests that can be conducted on captured CTCs.

15.3 Single-Nucleotide Variant (SNV), Insertion/Deletion (INDEL), and Copy Number Variant (CNV) Profiling in CTCs

In most cases, minute quantities of DNA extracted from circulating tumor cells requires whole-genome amplification (WGA) before being subjected to downstream profiling applications such as array genomic hybridization (aCGH) and whole-exome sequencing. Early application of these technologies to CTCs has focused on examining general feasibility rather than confident determination of genomic variants [18, 20–22]. The accumulated evidence suggests that DNA from even one CTC can provide adequate material for genomic characterization. The major issue seems to lie in the ability to distinguish differences accurately and reproducibly among various samples. The major roadblock appears to be polymerase bias and errors imposed by the amplification process that undercut the accuracy of variant calls. Approaches such as multiple annealing and looping-based amplification cycles (MALBAC) or multiple displacement amplification (MDA) have been employed to improve consistency throughout the amplification procedure [23, 24]. While Ni et al. demonstrated reproducible copy number variation (CNV) patterns among CTCs purified from a given patient with exome sequencing after MALBAC-based WGA, reproducibility was more problematic when attempting to call single-nucleotide variations (SNV) [24]. Similar observations were described by Lohr et al., who showed that improving the amplification procedure alone did not seem to sufficiently overcome the polymerase bias and that the rates of success in amplification of single prostate CTCs varied widely (11–100 %). To further address this issue, Lohr et al. employed "census-based sequencing," which involves combining sequencing data from independent CTC libraries in order to improve sensitivity. Specifically, the authors found that the total coverage of both alleles among independent CTC libraries compared well to a representative bulk library from the primary tumor, with only 0.005 % of sites improperly genotyped. Similar findings were demonstrated using independent CTC libraries from different patients. Importantly, the authors also found that amplifying a single pool of CTCs was sensitive to the same allelic distortion as any other individual library. Subsequently, using carefully designed experimental and analytical protocols, the authors were able to successfully demonstrate detection of evolution of CTC mutations utilizing samples obtained from early and metastatic stages of prostate cancer. Importantly, 90 % and

73 % of mutations detected in early stage and metastatic stage tumor tissues, respectively, were also identified in CTC exomes [23]. The aforementioned results seem to suggest that by overcoming technical challenges in accurately identifying genetic variants in CTCs, it is possible to identify the majority of corresponding tumor clones in tissue samples regardless of tumor staging, thus providing a potential path to the utilization of CTCs for molecular biomarker assessments that may be used to guide treatment decisions.

15.4 Transcriptome Profiling in CTCs

Conventional technologies to detect limited number of transcripts, such as RNA in situ hybridization (ISH) and real-time PCR (qRT-PCR) seem to be applicable to CTCs in general, even at the single-cell level [25–29]. However, profiling the whole transcriptome of a single CTC still proves to be challenging. Similar to the WGA procedure in amplifying minute DNA, starting with small amount of mRNA also requires amplification of cDNA for downstream applications. The technical bias that is introduced during amplification stage makes it rather difficult to discern true biological variations among samples. Therefore, the real issue rests within the degree of confidence regarding fidelity of single-cell transcriptomes when compared to pre-amplification RNA populations. Recently, Ramsköld et al. described the "Smart-Seq" technology, which improves read coverage and enhances detailed analyses of alternative transcript isoforms and identification of single-nucleotide polymorphisms. Specifically, the authors introduced a single-cell RNA-seq protocol that samples cDNAs from more than just the ends of mRNA and improved full-length coverage of all transcripts longer than 1 kb. The subsequent assessment of sensitivity and reproducibility contributes to the apparent superiority of the Smart-Seq technology. Most importantly, the authors demonstrated that transcriptome analyses from a single cell maintained relative differences in the detected transcripts. The methodologies published in this paper for cDNA generation and amplification are now available in the manual of SMARTer Ultra Low RNA Kit (Clontech) for Illumina sequencing [30].

In contrast to genetic variant profiling, the true utility of transcriptome profiling of CTCs may lie more in enabling assessment of dynamic expression changes that accompany and underlie the metastatic process rather than providing a cross-sectional snapshot that is concordant to tumor tissue. Understandably, the architecture of gene expression is influenced by multiple biological cues taken from the surrounding microenvironment and various signal pathways that may be switched on or off in tumor cells. For example, Yu et al. utilized CTCs isolated from genetically engineered mouse pancreatic cancer models to extract RNA and perform subsequent digital gene expression sample prepping and analysis on the HeliScope Single Molecule Sequencer. The authors identified transcripts that were increased more than twofold compared to controlled samples and absent in blood of non-tumor-bearing mice. After confirmation with dual staining of cytokeratin and candidate

genes using RNA-ISH methodology, Yu et al. found significant increase in *Wnt2* expression in the majority of CTCs or metastatic cells from ascites, but not in primary tumor specimens, thus implicating WNT signaling in metastasis. The authors then determined that the noncanonical WNT pathway activated by increased WNT2 expression could contribute to metastasis [29]. Subsequently, Yu et al. followed up with the observation that circulating breast tumor cells express epithelial and mesenchymal markers (measured by quantitative RNA-ISH assays) concurrently, whereas tumor cells in pre-invasive ductal carcinoma in situ lesions express epithelial markers exclusively. By cataloging CTCs into subgroups along a spectrum of the EMT process, the authors were able to reveal an intriguing pattern that the presence of an increasing mesenchymal CTC population is associated with disease progression and dissemination of breast cancer. Yu et al. again attempted to apply RNA sequencing technology in mesenchymal CTC populations using a single-molecule platform to avoid amplification bias associated with rare transcripts and found evidence of TGF-β pathway activation that is well described for EMT [28]. Other data indicate this may be a general mechanism across different indications. For example, through RNA-ISH and Fluidigm Single Cell Expression Analysis, Sullivan et al. reported that CTCs isolated from glioblastoma patients are also enriched for mesenchymal over neuronal differentiation markers [27].

The clinical potential suggested by the above findings is enormous if it can be translated to practical assays. Metastasis is the major cause of mortality in cancer, and is difficult to predict or detect without overt clinical manifestation. By monitoring the percentage of mesenchymal CTC population, one can perhaps evaluate the level of EMT and predict the likelihood of metastasis, though more correlative clinical studies will need to be conducted to solidify the relationship.

15.5 Proteomic Profiling of CTCs

Protein detection in CTCs is mostly accomplished at a limited scale using immunofluorescence- and antibody-based methodologies with approaches such as proximity-mediated immunoassay to amplify signal and facilitate downstream quantification [31–39]. In most cases, protein detection in CTCs is restricted to assessment of protein abundance, with rare success in evaluating protein phosphorylation events [31, 40, 41]. It seems rather unlikely that profiling protein abundance and phosphorylation at a broader scale would be possible in the near future. Recently, Ullal et al. described an antibody-based DNA barcoded technology, named antibody barcoding with photocleavable DNA (ABCD) platform, capable of multiplexing protein abundance or protein phosphorylation detection for up to 90 epitopes, with minimum material input approaching single cell quantities [42]. Specifically, antibodies were conjugated to DNA sequences (70-mer) derived from potato genome and inoculated with permeabilized cells. After washing, DNA probes were released from cells by photocleavage, hybridized to fluorescent barcodes, and subjected to imaging on a cartridge using the nCounter Analysis System

(NanoString). Most significantly, the authors were able to apply this technology to fine needle aspirates (FNA) collected pre- or post-treatment in clinical trials and confirmed biomarkers that were expected to be modulated by the therapeutic agent (PI3K inhibitors). Moreover, by assessing treatment-naïve samples from patients who either responded or were refractory to PI3K inhibitor treatment, the authors derived a set of protein biomarkers that could potentially predict response to PI3K inhibitor therapy [42]. Since FNA samples usually contain much smaller number of cells than core biopsies, the accurate depiction of differential protein expression levels in various patient samples upon treatment is particularly encouraging. Specifically, the authors were already able to demonstrate proteomics profiling feasibility using single cells from FNA samples. It may be that this technology can be utilized routinely in evaluating signaling pathway modulations in CTCs in the near future.

15.6 Using CTCs for Predictive and Pharmacodynamic Biomarker Analysis

The growing arsenal of targeted therapies is driving the advancement of the realization of personalized medicine. Theoretically, targeted therapies with exquisite cellular specificity to cancer specific alterations should have relatively minimal toxicity and a wider therapeutic index than traditional chemotherapies. Nevertheless, at high dosages, many drugs will have off-target effects, and in some cases on-target toxicity can occur from targeting oncogenes that have a role in regulating normal cellular processes. To take full advantage of benefits of targeted therapies, it is crucial to evaluate target engagement or pathway modulation and ascertain the minimal dosage that achieves the intended specific biological effects. Pharmacodynamic (PD) biomarkers serve as an essential means to attain this goal. Ideally, evaluation of alterations of a group of diverse PD biomarkers best help construct a complete picture consists of proximate target engagement and pathway inhibition, leading to changes in distal proliferative and apoptotic cellular biomarkers, or glucose uptake in case of fluorodeoxyglucose positron emission tomography (FDG-PET), and the eventual antitumor activity measured by response evaluation criteria in solid tumors (RECIST).

While PD biomarkers reveal information in regard to the optimal dosage of a given targeted therapy, predictive biomarkers pinpoint patient populations that will most likely benefit from a given treatment. As confidence of a given predictive biomarker accumulates along phases of clinical trials, an eventual companion diagnostic codevelopment paradigm can be conducted in parallel with drug development to ensure assay availability upon drug approval. This process is described in detail later in this chapter.

In the following two sections, we summarize highlights from the recent literature that underscore the potential for in which CTCs to provide valuable information on PD and predictive biomarkers.

15.7 Harnessing the Potential of CTCs for PD Biomarker Evaluation

Several published reports have exemplified the detection of signaling, apoptotic, or DNA damage regulatory protein complexes in CTCs as PD biomarkers [31, 34, 43–46]. Particularly, Wang et al. were able to reliably measure changes in the drug induced γH2AX as a pharmacodynamic biomarker in CTCs obtained from trials involving topotecan, topotecan plus PARP inhibitor, and cyclophosphamide plus PARP inhibitor. The authors convincingly demonstrated that the percentage of γH2AX-positive CTCs increased at day 2 of cycle 1 compared to that in pre-dose samples in all the above mentioned trials [46]. In a different utility, enumeration of CTCs may serve as a surrogate to observe early signs of RECIST response. In this instance, the number of CTCs represents a "distal" biomarker that could reflect the summation of local tumor responses to a given therapy, as demonstrated by Luo et al. in a publication that describes enumerating CTCs from metastatic melanoma patients who are treated with BRAF inhibitors and correlating CTC numbers with radiographic tumor measurement [47]. To offer more specificity, one can imagine a scenario where a therapeutic target can be incorporated as a marker into enumeration of CTCs. Numerous publications have exemplified the utility exploiting HER2 expression in evaluating efficacy of anti-HER2 therapy [35, 36, 44, 48–51]. In yet another example assessing the efficacy of an anti-IGF-IR antibody, de Bono et al. attempted to monitor the fluctuation of the number of IGF-IR positive CTCs during the course of anti-IGF-IR treatment. The authors found that anti-IGF-IR antibody, when administered alone or with cytotoxic chemotherapy, could decrease CTCs and IGF-IR-positive CTCs. The authors also observed a relationship between sustained decreases in CTC counts and prostate-specific antigen declines [43].

Understandably, a major pitfall of studying "proximal" or "distal" PD biomarker modulations in CTCs is that they may not represent the actual extent of pathway/biological perturbation in local tumor due to differential exposure to therapeutic agents, since plasma pharmacokinetics (PK) is usually distinct from tumor tissue PK. As such, CTC based PD analyses must, for the time being, be considered as a surrogate PD assay, and further experiments with matched serial biopsies alongside parallel CTC collection are required in order to demonstrate the utility of CTC PD assessments in clinical decision making.

15.8 Utility of CTCs for Predictive Biomarker Evaluation

Evaluating status of tumor-associated predictive biomarkers before subjecting patients to a particular treatment regimen has become increasingly common practice in clinical trials. However, due to operational hurdles, more often than not, biopsies from local tumors sites are not readily available for predictive biomarker assessment and the biomarker assessment is typically performed on archival tissue

from the original diagnosis. As such, CTCs provide a valuable alternative that could offer insight into genetic content of primary and/or metastatic tumors. Published reports have indicated that circulating tumor DNA (ctDNA) can provide information on genetic alterations with perhaps greater sensitivity than CTC-based analyses [52, 53]. For example, in a recent paper published by Bettegowda et al., the authors attempted to quantify mutant DNA isolated from either CTCs or ctDNA of the same patients. The subsequent comparison revealed that ctDNA is more sensitive than CTCs in revealing tumor genetic alterations [52]. However, the comparison may not be fair in that PCR was used to detect mutations from cell pellets that contain various blood cells in addition to CTCs. Without upfront enrichment of CTCs, it is possible to lose sensitivity even with the most cutting-edge amplification and sequencing technologies. Interestingly, the authors also observed that ctDNA was often present in patients without detectable circulating tumor cells, suggesting that these two substrates for biomarker evaluation are distinct.

While ctDNA may provide advantages for assessment of the mutational landscape in circulation, predictive biomarkers consisting of mRNA transcripts, fusion genes, splice variants, or cellular proteins will more likely be feasible using CTCs. In the earlier section, we described how measuring mesenchymal genes expression in CTCs could potentially facilitate early detection of metastasis and could be used to measure efficacy of potential therapeutic agents targeting metastasis. Such analyses would not be possible from ctDNA-based approaches as they involve a cellular phenotype.

The detection of fusion genes in CTCs was illustrated by detection of TMPRSS2:ERG in CTCs from prostate cancer patients. This biomarker is being tested as a candidate predictive biomarker of sensitivity in castration-resistant prostate cancer patients treated with abiraterone acetate [54, 55]. Strikingly, a recent example published by Antonarakis et al. unequivocally demonstrated the value of CTCs in detecting splicing variants of oncogenic driver genes during the course of treatment in prostate cancer. Briefly, the authors prospectively collected CTCs from patients with metastatic castration-resistant prostate cancer before receiving treatments with enzalutamide or abiraterone and evaluated the expression of androgen receptor splice variant 7 (AR-V7) in CTCs. The results demonstrated that AR-V7-positive patients receiving either enzalutamide or abiraterone had lower PSA response rates than AR-V7-negative patients and shorter PSA progression-free survival, progression-free survival, and overall survival [56]. It should be noted that these promising findings are from a small, retrospective study using a research grade assay, and hence will require further prospective validation. In a later section, we consider how such initial studies can pave the way for the ultimate development of companion diagnostics.

In addition to measuring expression of cellular targets of given therapies, such as HER2 and IGF-IR expression mentioned in the earlier section, it would be of great interest to assess the application of antibody barcoding with photocleavable DNA (ABCD) platform in clinical trials to profile proteomes of CTCs and evaluate a collection of biomarkers that are associated with efficacy of a given targeted therapy from a pathway perspective. Already, using this prototypic platform, Ullal et al. was

able to demonstrate that the baseline protein expression of a cluster of genes (H3K79me2, PARP, pS6RP, pH2A.X, and 4EBP1) best predicts response of drug-naïve patients receiving PI3K inhibitors using FNA [42]. It would be of profound interest to assess the feasibility of such an approach using CTCs.

To conclude, although the value of revealing genetic alterations in CTCs may overlap with that obtained from ctDNA, the totality of biological information, including at the transcriptome and proteome level, that can be extracted from CTCs suggests that the two can have complementary roles in enabling biomarker assessments. With the continued advancement of technologies to capture and characterize CTCs, one can hope for an era of routinely incorporating CTCs as a means for predictive and PD biomarker assessments.

15.9 The CDx Development Path for CTC-Based Biomarkers

Molecular biomarker characterization of CTCs has shown great scientific promise as a potentially revolutionary strategy for developing CDx tests in oncology. As described throughout this chapter, numerous studies have suggested utility for CTCs in providing a real time liquid biopsy that can be used to assess the status of a variety of biomarkers, and to do so in a minimally invasive and dynamic manner. However, to date these promising studies have generally relied on retrospective analyses of relatively small datasets using research grade assays, and hence have not provided a clear path to registration of a CDx test. Here we discuss some of the considerations that must be accounted for if CTC based-technologies will realize their full potential and proceed down the carefully regulated path from exploratory research biomarkers to ultimate companion diagnostic approval.

As discussed in the introduction, CTC-based analyses have dual promise as biomarkers of disease prognosis and also as a substrate on which to carry out PD or predictive biomarker assessments. In terms of the former, CTC counts or dynamic changes in CTC numbers over time and treatment can be considered biomarkers, and indeed have been prospectively validated across several oncology indications as having substantial utility as a prognostic biomarker associated with disease recurrence [57–59]. In contemplating utility of CTCs as a tool for predictive biomarker assessments, one may think of the CTCs as a surrogate tissue source for the usual archival tumor sample, and the biomarker itself as some aspect of biology that can be assayed in the isolated CTCs. For instance, one might wish to assess HER2 status, a well validated tumor biomarker with both predictive and prognostic significance, in CTCs and determine whether this status was predictive of benefit to HER2-targeting agents such as trastuzumab or lapatinib [60–62]. A first step would be establishing a research grade assay that would allow retrospective analysis of HER2 status in patient samples. Ideally, careful attention would be paid to technical and analytical validation of the assay to ensure the results were interpretable and

repeatable. In the case of this example, a number of groups have shown that HER2 protein expression and underlying DNA amplification can be assessed in CTCs captured by a variety of means [13, 49, 63, 64]. The next and most challenging step in the process is to clinically qualify the biomarker assay in CTCs. A qualified biomarker is one where the results can be reliably interpreted for a specific context of use in medical decision making [54]. Qualification must be accomplished through the use of an analytically validated assay in well-designed prospective clinical studies that show the utility of the biomarker assessment in predicting prespecified clinical outcomes. Such a paradigm requires a high degree of coordination between the drug developer and the device manufacturer.

An important component of the process will be moving from research applications that may be carried out in a diverse manner across different labs to a highly validated medical device that is used to select or exclude patients for therapy. Specifically, the FDA regulates medical devices based on the perceived risk and benefit to patients and classifies them into risk categories based on intended use (http://www.fda.gov/medicaldevices/deviceregulationandguidance/guidancedocuments/ucm262292.htm). A challenge for the use of CTCs in this regard is that currently the field is in a state of flux and rapid development, and there is no single definition of a CTC, coupled with a lack of clarity as to which biomarkers can be robustly evaluated in CTCs. Such a situation creates challenges in terms of determining (i.e., standardizing all aspects of design and accompanying protocols) a device for CTC evaluation that can be brought forward as an in vitro diagnostic (IVD) test. An IVD companion diagnostic is a device that provides information that is essential for the safe and effective use of a corresponding therapeutic product (http://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/UCM262327.pdf). Based on recent FDA guidance to industry, a therapeutic product and its corresponding IVD companion diagnostic device should be developed contemporaneously, with the clinical performance and clinical significance of the IVD companion diagnostic device established using data from the clinical development program of the corresponding therapeutic product [65]. The device can be thought of as the entire system including the instrument, apparatus, in vitro reagent, and any component that is intended for use in the diagnosis. Most oncology companion diagnostics are considered high risk (Class III) devices because their use results in clinical decisions that can subject patients to agents with substantial potential for adverse events. Inaccurate results from a poorly or incompletely validated test can result in the drug being used improperly and subjecting patients to exposure to a nonefficacious therapy in the case of false positives, or conversely failure to treat a patient likely to benefit in the case of false negatives. Thus the approval of companion diagnostic tests is tightly regulated by the FDA and other health authorities. It should be noted that the FDA generally recognizes two types of IVD devices [66]. IVD kits can be manufactured, distributed and used for analysis at any clinical laboratory with appropriate instrumentation. In contrast, a laboratory developed test (LDT) refers to a test that is developed and run by a single clinical testing site. Regulation of LDTs has not been consistently enforced in the past, but recent guidance from the FDA suggests that such tests require the same

level of validation and regulatory oversight as kit-based IVDs (http://www.fda.gov/downloads/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm407409.pdf). Given the rapid flux and emergence of new technologies in the CTC space, the LDT IVD model might offer an appealing model for development of CTC-based diagnostics, since early partnership on a promising platform could be tightly integrated with early phases of clinical development and allow time for optimization and validation of the platform.

15.10 Roadmap to CDx Approval for CTCs

A key consideration to thinking about the path to CDx development is whether the test is being codeveloped with a therapeutic from the outset, or whether the CTC CDx test is being developed to identify a new indication for an already approved agent (Fig. 15.1). As an example of the latter, we consider again the utility of HER2 positive CTCs in identifying patients who might benefit from targeted therapies such as trastuzumab or lapatinib. Currently, identification of such patients relies on CDx IVD tests for HER2 protein status (immunohistochemistry, IHC) or DNA copy number (fluorescence in situ hybridization, FISH) that are conducted on archival tumor tissue [67]. Several studies have shown that HER2 positive CTCs can be detected in patients whose primary tumor tested negative for HER2 with the approved CDx assays [13, 49]. This observation could be due to tumor evolution over time and treatment, or alternatively due to heterogeneity in the original clone that resulted in an inaccurate diagnosis. In either case, this provocative finding suggests that detecting HER2 status in CTCs might have clinical utility in the diagnosis and treatment of patients with HER2-targeted therapies, who otherwise would not be treated. Further supporting this notion, a study of 76 patients with matched tissue and analysis of HER2 in CTCs showed that patients with HER2 overexpression in CTCs had poorer progression-free survival compared with those without CTCs or with HER2− CTCs, suggesting the prognostic nature of this biomarker is conserved between tissue and CTCs [63]. Other studies have reported similar findings [68, 69]. Based on these observations, investigators designed a prospective clinical study, DETECT III, in order to determine whether the HER2 inhibitor lapatinib shows efficacy in patients with metastatic breast cancer who exhibit HER2-positive CTCs despite having a HER2 negative primary tumor or biopsy from metastatic sites (NCT0161911). Following standard HER2 tissue testing, negative patients are screened for the presence of any CTCs using the CELLSEARCH® platform. Patients with detectable CTCs that are HER2 positive by IHC or FISH are then randomized to receive standard treatment or standard treatment plus lapatinib, with a primary endpoint of progression free survival. This study provides a good model for how biomarker analysis of CTCs can be brought along a CDx pathway, but highlights some of the challenges and questions inherent in such an approach. First, when using the Veridex CELLSEARCH® platform up to 30–40 % of metastatic breast cancer patients do not have any CTCs, hence would not be eligible for this study or

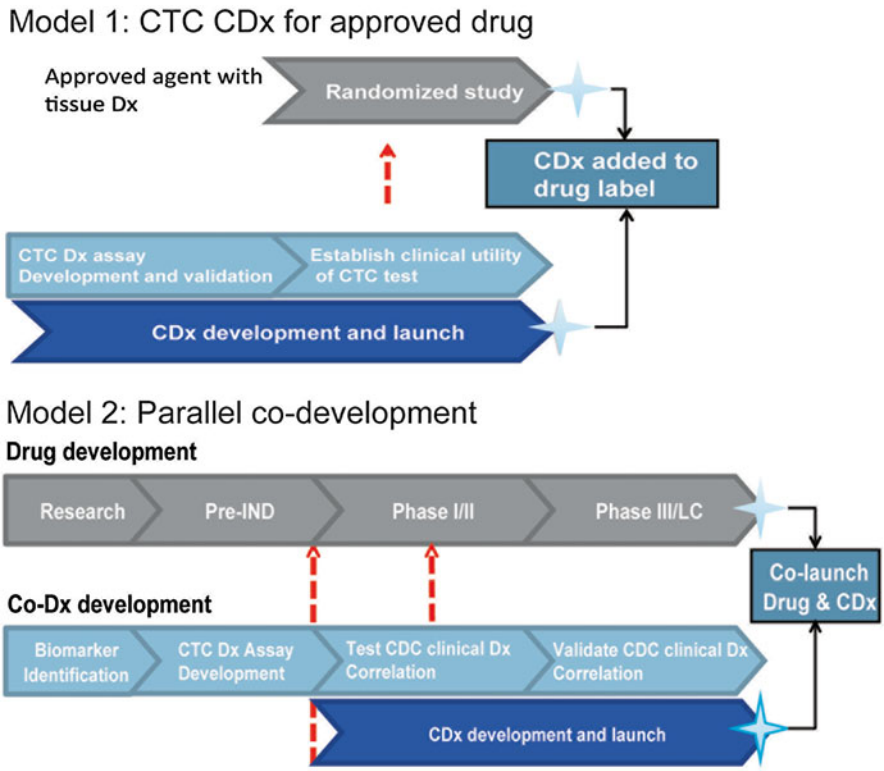

Fig. 15.1 Codevelopment of drugs and CTC companion diagnostics. Conceptual models for the codevelopment of CTC-based diagnostic assays with therapeutic products. In the first model, technical and analytical validation of a surrogate CTC assay for an approved tissue precedes prospective incorporation in a confirmatory study. In the second model pertaining to unapproved therapeutic agents, validation of the CTC-based assay starts in phase I or earlier, and qualification of the CTC-based biomarker is synchronized with overall therapeutic development

ultimately to be diagnosed with a CTC-based IVD assay [13, 70]. Other technologies have reported a higher prevalence of CTCs in the major indications, but these technologies are mostly still at the research stage and have not progressed to formal platforms that can proceed down a regulatory path [12, 71]. Second, very little data is available as to the technical and analytical validation of the biomarker assays that are being used in this study, and the assay requires a complex system involving both CellSearch as well as downstream molecular assays. According to guidance from the FDA, analytical validation components including preanalytical variables, post-analytic variables, and assay characteristics must all be documented and reported as part of the approval process (http://www.fda.gov/downloads/regulatoryinformation/guidances/ucm126957.pdf). Moreover, the "device" in this case would include the instruments (i.e., both CellSearch and the downstream platforms), as well as reagents and any accessory that is required to obtain the diagnosis, and there are

attendant legal issues around the platform technologies, intended use, and freedom to operate. Clearly a high degree of coordination will be required to gain regulatory approval even if the clinical study establishes utility. Since this approach would consist of "after market" approval for an already approved therapeutic, the likely path would require a premarket approval (PMA) submission, with Center for Devices and Radiological Health (CDRH) conducting the primary review and test approval, along with a consultation to Center for Drug Evaluation and Research (CDER) for approval of any drug label changes specifying the new CTC test as the basis for therapeutic decision making.

An alternative avenue for projects that are in earlier stage development would be to follow the traditional drug–diagnostic codevelopment paradigm (Fig. 15.1). Such an approach might be feasible for many of the emerging candidate biomarkers that have been described and linked to candidate therapeutics that have yet to be approved. Ideally, candidate biomarker hypotheses could be identified in early phases of research based on the mechanism of action of the therapeutic in question, allowing time to develop robust assays to enable quantitation of the biomarker in CTCs, hopefully also with comparison and benchmarking to tissue. Careful thought should be given to the most appropriate platform based on both technical considerations in terms of the analyte being quantitated, as well as the potential for the CTC capture and analysis system to ultimately gain approval as a device. Initial phases of the study could include "spike-in" cell line experiments to validate the assay, followed by comparison of biomarker status in CTCs with status in matched tumor tissue. An excellent example of this is the work of Attard and colleagues to show that ERG, AR, and PTEN gene status in CTCs from patients with castration resistant prostate cancer in general faithfully reflects the status in match tumor tissue [72]. Such studies provide confidence that CTC-based analysis can accurately provide relevant biomarker data and can advance to clinical testing. The next key question is how the assay will be deployed in the context of clinical development. Will it be a stand-alone assay, or initially an adjunct or back-up assay to a tissue-based assay? A conceptually appealing approach would be to aggressively evaluate both a tissue and CTC-based assay, perhaps in a phase I expansion cohort following identification of a recommended phase II dose. Such trials can often be an important component of showing early proof of concept by demonstrating activity in a defined patient population (e.g., vemurafenib, crizotinib, both employed this strategy) [73]. Careful testing of the CTC assay alongside the tissue assay could be used to demonstrate equivalence, or potentially even superiority in these early phase clinical studies, and a decision could be made as to whether to take one or both assays forward. In such a scenario, early and close partnership between the drug development sponsor and the CTC diagnostic company would be essential to navigate the regulatory landscape through phase II and III clinical studies and allow for synchronized review of the drug by CDER and the CTC-based diagnostic by CDRH, followed by a joint approval decision (Fig. 15.1).

CTCs offer tremendous potential to enable real-time precision medicine and overcome the limitations of using archival tissue to make treatment decisions in

oncology. Successful realization of this goal will require careful consideration of biology, overcoming a host of technical and analytical challenges, and successful navigation of a complex regulatory landscape.

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Chapter 16
Perspectives on Clinical Applications of CTCs

Rajan P. Kulkarni and Stefanie S. Jeffrey

Abstract Though technologies for isolating and analyzing CTCs have advanced rapidly, there has been little clinical uptake of these technologies, despite the potential to assist medical decision-making. The first clinical studies examined the enumeration of CTCs and there were differing outcomes of utility; many of these studies were hampered by small sample sizes. More recent clinical studies have focused on molecular and genetic analysis of CTCs, rather than mere enumeration, to be utilized as a companion diagnostic to determine if a druggable mutation is present or absent (such as the T790M EGFR or V600E BRAF mutations). The rise of genetically targeted therapies has increased interest in CTC analysis in a clinical setting, particularly to obtain actionable information. CTCs hold the potential to assist with clinical decision-making, though further controlled trials will be necessary to better realize this promise.

Keywords Circulating tumor cell (CTC) • Breast cancer • Prostate cancer • Lung cancer • EGFR • AR-V7 • BRAF • Melanoma • Companion diagnostics

16.1 Perspectives on Clinical Applications

Circulating tumor cells (CTCs) hold the potential to assist medical decision-making through both prognostic and diagnostic capabilities. However, this potential has largely been unrealized in a clinical setting. Several clinical trials involving CTC identification have noted some prognostic benefit in enumerating CTCs but little or no overall change in patient outcome, and the current medical consensus is that CTCs are of unclear utility and that there is "insufficient evidence to support routine

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use in clinical practice" [1]. Despite these findings, several newer small studies have suggested potentially powerful clinical applications, particularly regarding isolation of genetic information from CTCs that may predict drug response. Such findings, when further validated, may allow CTCs to become more clinically utilized. In this chapter, we discuss some recent clinical trials employing CTC isolation and analysis and discuss future clinical possibilities.

16.2 Status of CTCs in the Clinic

The first clinical studies primarily focused on enumeration of CTCs to provide prognostic information and reached differing conclusions regarding utility [2–4]. However, several were limited by small sample sizes. In response, larger studies were begun and were published more recently. The SWOG trial in breast cancer represented an effort to utilize CTC count to directly assist decision-making [5]. In this study, the investigators measured CTC count at baseline and included those who had >5 CTC per 7.5 mL of blood; all were started on cytotoxic chemotherapy. They resampled the patients at day 21; those who still had >5 CTC were randomized to either continue with the current therapy or switch cytotoxic chemotherapies. The authors found no benefit in overall survival by switching chemotherapies in response to CTC count. For those with >5 CTCs per 7.5 mL of blood, they found some prognostic significance of CTC enumeration, yet no benefit in overall survival by switching chemotherapy early in response to CTC number. This suggested that not only did early detection of poor outcome not change this clinical result compared to later detection by imaging, but probably more importantly, switching empirically from one ineffective therapy to another equally ineffective therapy does not provide benefit, suggesting that some form of real-time molecular guidance will be necessary for improved therapy selection.

Similarly, trials in prostate cancer have shown some modest prognostic significance of CTC enumeration for predicting outcome. In many cases, there may be a prognostic benefit of CTCs, yet this has not translated into significant clinical benefit vis-à-vis overall survival or progression free survival. One reason for this may be due to the chemotherapy regimens utilized; most trials incorporating CTC enumeration have been in the setting of non-targeted therapies. With more recent development of molecularly targeted agents, this may change.

As mentioned above, current American Society for Clinical Oncology (ASCO) guidelines do not recommend the use of CTC isolation or enumeration for any cancer type [1]. In addition, insurance companies will generally not reimburse for the cost of CellSearch analysis, or any non-FDA approved test, drastically limiting clinical use. Many of the ASCO guidelines are several years old and it is possible that future revised guidelines may call for consideration of CTC analysis. However, for CTC isolation and analysis to be clinically relevant, there need to be additional well-designed clinical trials to demonstrate utility.

CTCs are in a state of flux; given the contradictory findings of utility of the cumulative studies, there is no clear consensus on their isolation or use. From a clinical perspective, there need to be clear guidelines encouraging their use as well as additional validated and FDA-approved platforms for reproducible isolation, before wider clinical acceptance will occur. This can only come about with an improved understanding of the biology of CTCs and additional studies to determine how CTCs reflect the bulk tumor. In this chapter, we describe several promising studies that could help to bring CTCs closer to clinical use.

16.3 Rationale for CTC Use Clinically

With the advent of molecularly guided therapies (such as vemurafenib for V600E/K mutant cancers and afatinib for first line treatment against activating exon 19 EGFR deletions or L858R EGFR mutations), there has been increased need for genetic analysis of tumor cells to ascertain the mutational landscape of actionable target genes. As sequencing costs are rapidly decreasing, it may be possible in short time to complete whole exome, transcriptome, and/or genome sequencing of an entire tumor in order to ascertain the most appropriate therapies and to potentially switch treatments in response to newer genetic or epigenetic changes.

However, one limiting factor is still obtaining sufficient tumor material to complete such studies. In many instances, a patient has a single biopsy from one tumor region; in cases where the entire tumor has been resected, tissue is analyzed from a limited number of regions due to time and cost issues. Furthermore, it is often difficult or impractical to obtain serial biopsies over time, yet such information could be vital to assess tumor cell resistance profiles. Circulating tumor cells may be an important adjunct test to provide actionable tumor information, particularly when repeated tissue sampling may not be feasible.

16.4 CTC Clinical Utility in Specific Cancers

Though there have been some clinical studies assessing CTC characteristics, with increased interest, there are now many more clinically oriented studies underway and likely to yield additional information in the coming years. Here, we provide a brief overview of selected efforts in melanoma, prostate, and lung cancers. While the studies described are not a comprehensive listing, they provide initial promising data and hopefully an impetus for additional work to further characterize the utility of CTC collection.

16.4.1 Prostate Cancer

One of the first applications for CTC analysis in prostate cancer was in analyzing CTCs for the presence of the ERG (TMPRSS2-ERG) fusion, as well as androgen receptor and PTEN gene status [6]. The ERG fusion is present in approximately 50 % of all prostate cancers and is an early event in pathogenesis when it occurs. The authors successfully confirmed presence of the ERG fusion in CTCs from patients with the same mutation in their tumor tissues and thus concluded the CTCs were of prostatic origin (as the fusion is not seen in any other known context). This study had limited clinical utility but provided one of the first attempts to analyze genetic changes in any cancer using CTCs. Because ERG fusion is an androgen-dependent growth factor, its presence in CTCs has also been studied with regard to androgen sensitivity. Again, while its presence verified that the cells studied were prostate cancer-derived CTCs and CTC counts were prognostic, the ERG fusion did not serve as a biomarker of response to androgen treatment [7]. Recently, in a phase III trial studying patients with metastatic castration-resistant prostate cancer treated with abiraterone acetate plus prednisone versus prednisone alone, a biomarker panel of CTC number and LDH was shown to be a surrogate for survival at the individual-patient level [8].

Other recent studies have utilized exome and transcriptome sequencing to assay for the presence of mutations and splice variants. Lohr et al. utilized whole exome sequencing of single prostate CTCs and compared these findings to exome sequencing of matched tumor tissues. They developed a methodology for pooling the data to reduce individual sequencing errors and found that they could identify greater than 70 % of mutations that were also present in the matched tumor tissues [9]. Antonarakis utilized CTC isolation followed by qPCR of the androgen receptor to identify the relative percentages of androgen receptor splice variant 7 (AR-V7) mRNA present among CTCs and utilized this information to determine whether its presence could predict resistance to novel agents enzalutamide and abiraterone in patients with known metastatic castration resistant prostate cancer (mCRPC) [10]. As the splice variant AR-V7 lacks the ligand-binding domain of the androgen receptor, it was hypothesized that the presence of this splice variant would predict a lack of response to drugs that target the androgen receptor directly (enzalutamide) or indirectly (abiraterone). They indeed found that those patients with CTCs positive for AR-V7 had a significantly reduced response to either enzalutamide or abiraterone versus those who were negative for AR-V7, which affected overall survival. Presence of the AR-V7 splice variant may thus predict non-responsiveness to either enzalutamide or abiraterone in patients with mCRPC, although these findings will need to be validated in a larger prospective trial [10].

16.4.2 Lung Cancer

A number of studies have specifically sought to isolate CTCs from patients with stage I–IV lung cancer. Hofman et al. analyzed the prognostic significance of CTC count before and after lung cancer resection surgery and determined that a level of

greater than 50 (per 10 mL of blood collected) portended worse outcome as measured by overall survival and disease free survival times [11]. Punnoose et al. utilized CellSearch to isolate CTCs in the context of a clinical trial of pertuzumab and erlotinib and found that decreased CTC counts had significant association with response and progression-free survival [12]. They found that those with higher baseline CTC counts had better response to treatment by RECIST (Response Evaluation Criteria in Solid Tumors) and that declines in CTC count were correlated with PET or RECIST measurements. They also assayed for EGFR expression in CTCs using immunofluorescence and mutational status of EGFR from the CTCs using qPCR but found that they could only identify one of eight EGFR mutations found in the original tumor specimens, concluding that white blood cell contamination from remnant cells may have obscured the qPCR results. Several other studies have yielded similar results [13].

Caroline Dive's group in the UK has used CTCs to develop models of small-cell lung cancer (SCLC), a neuroendocrine tumor which is associated with large numbers of CTCs, in contrast to non-small cell lung cancer (NSCLC). CTC-derived explants (CDXs) were grown in immune-compromised mice and shown to be genomically similar to original CTCs and to reflect the patient's drug response to platinum and etoposide therapies [14].

16.4.3 Melanoma

Melanoma was the first solid cancer in which the evidence of CTCs was discerned, using RT-PCR for melanoma-specific transcripts, although the authors did not isolate distinct CTCs [14]. Despite this head start, subsequent progress in the field has been slow. Some reasons for this were the lack of good treatment options for melanoma until the advent of vemurafenib and other targeted agents, as well as immunotherapy agents with good efficacy (PD-1/PD-L1 inhibitors and CTLA-4 inhibitor) and the lack of validated studies for analysis of melanoma CTCs.

Only recently have there been validated studies confirming the prognostic utility of isolating CTCs for determining overall survival [15]. Using the CellSearch platform, the authors determined a cutoff of 2 CTCs per 7.5 mL of blood and found a survival benefit in those with less than 2 CTCs (2.6 months vs. 7.2 months). They also determined that those patients who maintained >2 CTCs had decreased survival in response to treatment; however, the treatments were not standardized. In addition, the authors did not attempt to isolate the cells for genetic analysis, and the findings are only of limited clinical utility. One other point is that because melanoma expresses tumor-specific surface markers, use of an EpCAM antibody to capture CTCs (CellSearch platform) may not be optimal.

Furthermore, some clinicians may feel that monitoring melanoma patient progress through CTC count is less useful than performing serial biopsies of cutaneous metastases, which can yield more cancerous cells for downstream analysis. While it is true that most melanoma metastases are cutaneous, melanoma can metastasize to other organs including the liver and brain, without further cutaneous metastases.

Additionally, it may not be feasible to perform serial biopsies due to patient discomfort or cost. If CTC analysis in melanoma can be validated and shown to yield equivalent genetic information to that obtained by serial biopsies, this may prove to be a useful adjunct test for monitoring, particularly in response to novel therapies that have been introduced in the last 5 years including BRAF and MEK inhibitors. In many cases, patients eventually have disease progression while on treatment, but a method to identify treatment failure earlier and to guide the choice for the next therapy may have benefit in helping to change strategies in the hopes of maintaining response.

16.5 Potential of CTCs to Inform Clinical Decision-Making

16.5.1 CTCs as Companion Diagnostics

The advent of molecularly targeted therapies has prompted the need for companion diagnostics to assay for the presence or absence of a particular genetic variant. This is particularly critical for several drugs that have unwanted off-target effects in the absence of the targeted mutation, such as vemurafenib. Furthermore, given the cost of newer agents, it is important to have assays that can reliably confirm the presence of a mutation that is specifically targeted.

As an example, there are efforts to develop companion diagnostics for rociletinib (CO-1686) to assay for the presence of T790M and (R858) mutations in EGFR, using circulating tumor DNA (ctDNA) [16]. CTCs may provide a similar role in helping to determine eligibility for a particular medication, either as a standalone test or as an adjunct diagnostic. CTCs can also serve as a source of cells from different portions of the tumor than those biopsied, and may yield additional information about tumor heterogeneity that can be valuable in formulating treatment regimens.

16.5.2 Tracking Response to Therapy (Including Monitoring for Actionable Information)

CTC isolation may allow clinicians to adjunctively follow tumor response to treatment, both through enumeration as well as having an accessible source of tumor cells for analysis. A drop in CTC count followed by a rise while on particular treatment(s) may indicate early resistance to those treatments and the need to consider alternate therapeutic approaches, possibly even before changes are noted on imaging scans. This could prove especially valuable in reducing treatment failures by allowing for more rapid switching of regimens. As additional molecularly targeted therapies are developed and introduced, such ability to monitor tumor progression and assay for specific genetic changes will be critical.

16.5.3 Epigenetic Information

The ability to capture intact tumor cells is a significant benefit of CTC isolation. While many of the cells may be undergoing apoptosis or already have undergone necrosis, some have clearly not and maintain intact tumor RNA and DNA. These cells are of special interest as they may contain up-to-date information about tumor status from the region that the CTCs were derived from. Analysis of CTC transcriptome can thus yield information about epigenetic changes such as RNA copy number variation and splice variants (that would be missed by DNA or ctDNA analysis), such as the AR-V7 splice variant in prostate cancer.

16.5.4 Propagating CTCs for Downstream Analysis

There has been great interest in developing conditions for culturing CTCs as successful implementation may help enable the goal of molecularly guided and patient-specific treatment regimens. To date, this has proven to be difficult, with success so far reported for long-term (6–24 months) culturing of CTCs from breast, prostate, and colorectal cancers [17–20]. There are several potential reasons for this. A major reason is that many CTCs may already be undergoing apoptosis or necrosis at time of blood collection; another may be the time required for CTC isolation that may compromise cell viability if capture protocols are too long; finally, CTC growth may require initial support from associated cells/extracellular vesicles, or other factors in the circulation. Nonetheless, the success reported for CTC propagation in vitro indicates that some of the captured CTCs have the potential for cultured growth, which is now a highly active area of investigation.

One limitation of culturing CTCs is that the cells may acquire additional genetic or epigenetic changes such that the cells no longer fully reflect the tumor region from which they were originally derived. While this can be mitigated by limited passaging of the cells and only performing DNA-based analyses (including exome or whole genome sequencing), this possibility remains a concern. Furthermore, cells in culture have different growth patterns and sensitivities to drug compounds such that cells that appear sensitive in culture may not be so in a tumor within the human body, or vice versa. Despite these potential limitations, the possibility of culturing CTCs opens up significant prospects for personalized medicine, particularly for analyzing tumor evolution in cases where repeated tumor biopsies are not possible or not desired by the patient, and in potentially testing the cells for drug sensitivity or resistance.

Another possibility for propagating CTCs is utilizing them to generate explants in mice, as described above and reported by Caroline Dive's group for small-cell lung cancer [14]. They were able to generate explants from four out of six tested samples, with successful results from samples with high CTC counts (>400 CTCs per 7.5 mL, measured in a paired sample using the CellSearch platform). The authors found that the CTC-derived explants had similar architecture and morphology,

neuroendocrine markers, and responded similarly to drug treatments, compared to the original patient tumors. The ability to grow explants can allow for more complete tumor microenvironments to be developed and may enable similar environmental conditions (as compared to the original tumor environment in the human), thus yielding better growth characteristics. One caveat is that growing tumor explants or xenografts is feasible primarily in a research setting and may be difficult to utilize on a widespread scale. Nonetheless, explant growth may have utility in the setting of human clinical trials of novel chemotherapeutic agents, particularly to assay for resistance mechanisms.

Conclusions

CTCs hold the potential to significantly assist with clinical decision-making. However, for CTC analysis to achieve more widespread adoption, there need to be standardized instruments and protocols for isolating and analyzing CTCs as well as more relevant and focused prognostic information. This will require additional controlled trials to determine the true benefits of CTC analysis. It is our hope that such studies, several of which are currently underway, will yield actionable information in the coming years such that physicians in the next decade can utilize CTC analysis as an additional piece of information with which to guide prognosis and treatment determinations.

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