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Molecular characterization of circulating tumor cells in breast cancer: challenges and promises for individualized cancer treatment

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Abstract Blood testing using Circulating Tumor Cells (CTCs) has emerged as one of the hottest fields in cancer diagnosis. Research on CTCs present nowadays a challenge, as these cells are well defined targets for understanding tumour biology and improving cancer treatment. The presence of tumor cells in patient's bone marrow or peripheral blood is an early indicator of metastasis and may signal tumor spread sooner than clinical symptoms appear and imaging results confirm a poor prognosis. CTC enumeration can serve as a "liquid biopsy" and an early marker to assess response to systemic therapy. Definition of biomarkers based on comprehensive characterization of CTCs has a strong potential to be translated to individualized targeted treatments and spare breast cancer patients unnecessary and ineffective therapies but also to reduce the costs for the health system and to downsize the extent and length of clinical studies. In this review, we briefly summarize recent studies on the molecular characterization of circulating tumor cells in breast cancer and discuss challenges and promises of CTCs for individualized cancer treatment.

Keywords Breast cancer · Cancer treatment · CTC · Molecular characterization · Individualized treatment · Liquid biopsy

1 Introduction

The identification of prognostic and predictive biomarkers that will help in selecting the optimal therapeutic strategy for each cancer patient according to individual's relapse risk is essential for avoiding both over-treatment and under-treatment. At present, the success or failure of anti-cancer therapies is only assessed retrospectively by the absence or presence of overt metastases during the post-operative follow-up period. Current research on circulating tumor cells (CTCs) is focusing on their molecular characterization and on the identification of potential diagnostic and therapeutic biomarkers expressed in these cells. CTC are well-defined targets for understanding tumour biology and tumour cell dissemination and hold the promise of playing a role of "liquid biopsy" which may allow physicians to follow cancer changes over time and tailor treatment [1].

European groups have firstly shown the prognostic impact of disseminated tumor cells (DTC) in the bone marrow of breast cancer patients [2] and that CTC detection and enumeration is correlated with decreased progression-free survival and overall survival in operable [3–7] and advanced breast cancer [8]. Detection of post-chemotherapy CTCs in breast cancer patients was also shown to be of prognostic significance [9]. Nowadays, CTCs are associated with prognosis in many human cancers such as breast, lung, and prostate, and their enumeration is used for repeated follow-up examinations [10, 11]. Most of these studies have been based on the epithelial properties of CTC and their isolation and detection through epithelial markers like EpCAM and CK-19 [1–11].

Molecular characterization of CTC presents a very hot topic in cancer research nowadays [12] since it is important not only to confirm their malignant origin but also to identify diagnostically and therapeutically relevant targets expressed in these cells and help stratifying cancer patients for individual therapies [10–14]. Characterization of these cells might contribute to the identification of metastatic stem cells among CTC with important implications for the

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development of improved therapies in the near future [15]. Also motivating the interest in CTCs has been the recent development of molecularly targeted cancer therapies that work best on patients whose tumors have a particular mutation [16]. Complete genomic profiles and expression patterns have to be considered in order to understand the biological properties and the molecular characteristics of CTCs, as well as their connection to cancer stem cells [15]. Molecular characterization of CTCs, while important for the identification of diagnostically and therapeutically relevant targets that could help stratifying cancer patients for individual therapies, is difficult to address since they are very rare and the amount of available sample is very limited.

Currently there is a plethora of analytical methodologies for isolating and detecting CTCs. However, there are still a lot of analytical challenges to be solved. Since CTCs are very rare in most cases, they are specifically detected by using a combination of two steps: (a) isolation enrichment and (b) detection. Detection strategies include detection at the cellular and protein level through immunological approaches and imaging systems, and molecular assays like RT-PCR and multiplex RT-PCR through the detection of gene expression in CTCs. The most important limitation of all available methodologies for CTC analysis is the required amount of sample and the small number of gene targets that can be analyzed [17–19]. Recent technical advancements in CTCs detection and characterization include highly sensitive RT-qPCR methods [20–22], image-based approaches like the FDA cleared CellSearch system [8, 23], or a combination of molecular and imaging methods [24]. Many different new devices have been developed and are now commercially available for CTC isolation from blood. A membrane microfilter device was introduced for single-stage capture and electrolysis of circulating tumor cells [25], and a microchip for CTC isolation and analysis was developed [26].

The detection, enumeration, and isolation of CTCs have considerable potential to influence the clinical management of cancer patients. However, there is still a lot to be done for the automation, standardization, quality control, and accreditation of analytical methodologies used for CTC detection and molecular characterization [27, 28]. There is a substantial variability in the rates of positive samples using existing isolation and detection techniques. Different detection methods lead to different results as shown by the comparative analysis of the same patient samples with different technologies. Thus, the clinical results largely depend on the technology used to isolate and detect CTCs. Despite the fact that most of these methods are highly specific and sensitive, there are not so far extensive studies especially designed to compare their efficacy when using the same clinical samples. This is an important issue for using CTC in the clinic, since especially in early disease differences in analytical sensitivity between these methods can play a very critical role. The lack of standardization and validation of technology hampers the implementation of CTC measurement in clinical routine practice [27, 28].

2 Molecular characterization of CTC in breast cancer

CTCs are highly heterogeneous as has already been shown both through imaging and molecular methods. This is highly important especially in the case that therapeutic targets are expressed in CTCs and not in the primary tumor. However, the importance of CTC heterogeneity has not been fully exploited clinically as yet. Here we summarize the recent progress on the molecular characterization of CTC in breast cancer (Fig. 1).

2.1 HER-2

There is now a growing body of evidence that human epidermal growth factor receptor (HER-2) status can change during disease recurrence or progression in breast cancer patients. Based on this, re-evaluation of HER-2 status by assessment of HER-2 expression on CTCs is a strategy with potential clinical application. HER-2 analysis in CTCs may have clinical significance for HER-2-targeted therapy as HER2-positive CTCs and DTCs can be detected in patients with HER2-negative primary tumors who currently do not have access to HER-2-targeted therapy. A quantitative analysis by confocal microscopy assay for evaluation of HER-2 expression in individual tumor cells has shown that there was a significant positive correlation between HER-2 overexpression and gene amplification in individual CTCs [29].

Many studies and different groups have evaluated HER-2 expression on CTC in breast cancer patients [13, 14, 30–35]. Early on as in 2004, it was shown that therapy-resistant CK-19 mRNA-positive cells in peripheral blood could be effectively targeted by trastuzumab administration [13, 14]. HER-2-positive CTCs have been detected in patients with HER-2-negative tumors; nevertheless, their presence was more common in women with HER-2-positive disease [34]. According to a recent prospective multicenter trial, HER-2-positive CTCs can be detected in a relevant number of patients with HER-2 negative primary tumors [31].

This finding has been confirmed by other groups as well [36, 37]. Punnoose *et al.* report that in the majority of patients (89 %), there was a concordance between HER-2 status in CTC, and in the primary tumor tissue, though in a subset of patients (11 %), HER-2 status in CTCs differed from that observed in the primary tumor [38].

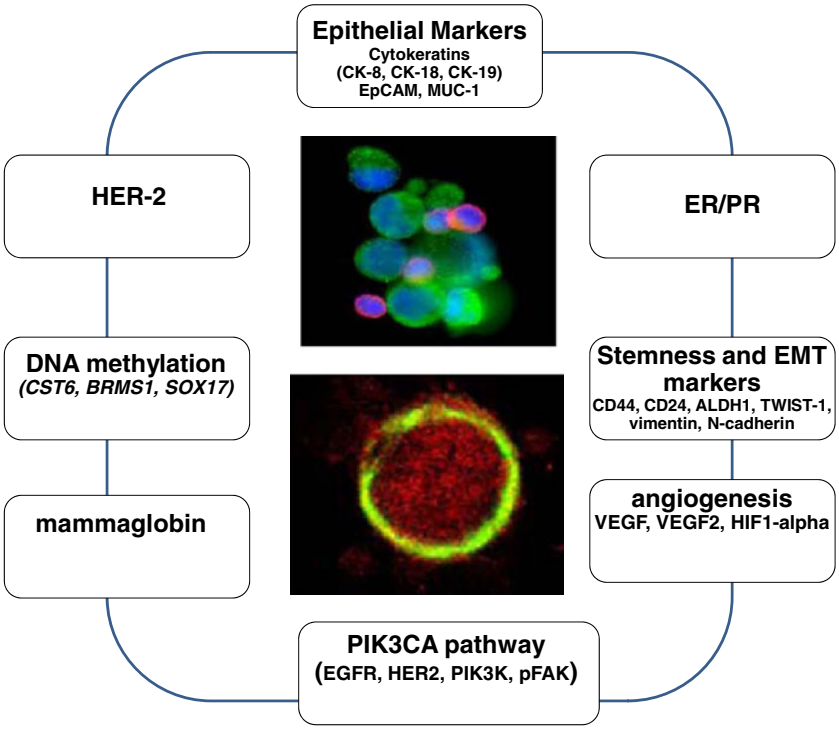

Fig. 1 Recent progress on the molecular characterization of CTC in breast cancer

2.2 ER/PR

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. When the expression of ER and PR was assessed in CTCs by RT-PCR, Fehm *et al.* report that interestingly, the spread of CTCs was mostly found in triple-negative tumors and CTCs in general were mostly found to be triple-negative regardless of the ER, PR, and HER-2 status of the primary tumor [30]. They state that (a) due to the weak concordance between CTCs and DTCs, the clinical relevance may be different; (b) the biology of the primary tumor seems to direct the spread of CTCs; and (c) since the expression profile between CTCs and the primary tumor differs, the consequence for the selection of adjuvant treatment has to be evaluated. By evaluating the expression of ER and PR receptors on CTC in blood of metastatic breast cancer patients, Tewes *et al.* could predict therapy response in 78 % of cases [32].

Aktas *et al.* compared the hormone receptor status expression profile of CTCs with the primary tumor in metastatic breast cancer patients. Most of the CTCs were ER/PR-negative despite the presence of an ER/PR-positive primary tumor. According to their findings, in the metastatic setting, the phenotype of CTC reflects the phenotype of metastatic disease; therefore, palliative treatment selected based on the expression profile may not be effective since the phenotype has changed during disease progression [39]. According to another very recent study aimed to investigate the influence of removal of the primary tumor on incidence and phenotype of circulating tumor cells in primary breast cancer, the most common CTC phenotype was triple negative followed by HER2+/ER−/PR− subtype and ER and/or PR positive, while 95 % of the corresponding primary tumors were ER and PR positive. They found that CTC phenotype before and after the surgery generally remains identical but may differ from that of the primary tumor [40]. The same was confirmed by another study, comparing transcript levels in CTCs with those measured in corresponding primary tumors that showed clinically relevant discrepancies in estrogen receptor and HER-2 levels [37].

2.3 Stemness and EMT markers

The persistence of CTC in breast cancer patients might be associated with stem cell-like tumor cells which have been suggested to be the active source of metastatic spread in primary tumors. Furthermore, these cells also may undergo phenotypic changes, known as epithelial–mesenchymal transition (EMT), which allows them to travel to the site of metastasis formation without getting affected by conventional treatment [41, 42]. During cancer progression, malignant cells undergo EMT and mesenchymal–epithelial transitions as part of a broad invasion and metastasis program. EMT is characterized by upregulation of vimentin, Twist, Snail, Slug, and Sip1 among others. Recently, it was shown that the induction of EMT program not only allows cancer cells to disseminate from the primary tumor but also

promotes their self-renewal capability [43]. Furthermore, the expression of stemness and EMT markers in CTCs was associated with resistance to conventional anti-cancer therapies and treatment failure, highlighting the urgency of improving tools for detecting and eliminating minimal residual disease [43]. Though the relationships between EMT and CTCs remains largely unexplored, data validating the implication of EMT processes in CTC formation and animal models with transplantable human breast tumor cells to help characterizing EMT/CTC relationships have been recently reviewed [43].

The detection of cells in mesenchymal transition, retaining EMT and stemness features, may contribute to discover additional therapeutic targets useful to eradicate micrometastatic disease in breast cancer. However, most currently used methods to detect and enumerate CTCs rely on the expression of EpCAM and cytokeratins, and this selection may exclude cells that have undergone intrinsic modifications of their phenotype, as EMT. Because of the frequent loss of epithelial antigens by CTC, assays targeting epithelial antigens may miss the most invasive cell population; thus, there is an urgent need for optimizing CTCs detection methods through the inclusion of EMT markers [44]. Many groups are now working on elucidating the connection between CTC and cancer stem cells as well as EMT markers on CTC. Many different recent studies have shown that subsets of CTCs have a putative breast cancer stem-cell phenotype and express EMT markers. Balic *et al.* were the first to show that the majority of occult metastases in the bone marrow are cancer stem cells [45]. Aktas *et al.* studied the expression of the stem cell marker ALDH1 and markers for EMT in CTC of metastatic breast cancer patients and correlated these findings with the presence of CTC and response to therapy. Their data indicate that a major proportion of CTC of metastatic breast cancer patients shows EMT and tumor stem cell characteristics [46].

The expression of CD44, CD24, and ALDH1 on CTCs of patients with metastatic breast cancer was also verified by using triple-marker immunofluorescence microscopy [47]. Raimondi *et al.* investigated the expression of EMT and stemness markers in CTCs from breast cancer patients in all stages of disease. They found that the expression of ALDH1 on CTCs correlated to the stage of disease and to the expression of vimentin and fibronectin [48]. The gain of mesenchymal markers in CTC was correlated to prognosis of patients and predicted more accurately worse prognosis than the expression of cytokeratins alone [49]. Armstrong *et al.* found that more than 75 % of CTCs from women with metastatic breast cancer coexpress CK, vimentin, and N-cadherin [50]. TWIST-1 expression on CTC was also shown in patients with early and metastatic breast cancer by quantitative RT-PCR and liquid bead array [51, 52]. When the expression of Twist and vimentin in CTCs of metastatic and early breast cancer patients was investigated by using double-immunofluorescence experiments in isolated peripheral blood mononuclear cell cytospins using anti-cytokeratin (anti-CK) anti-mouse (A45-B/B3) and anti-Twist or anti-vimentin anti-rabbit antibodies, a significant correlation was found between the number of CTCs expressing Twist and vimentin within the same setting [53]. The high incidence of these cells in metastatic disease compared to early stage breast cancer strongly supports the notion that EMT is involved in the metastatic potential of CTCs [53]. Further studies are needed to prove whether these markers might serve as an indicator for therapy resistant tumor cell populations and, therefore, an inferior prognosis.

2.4 Angiogenesis

Using double staining experiments and confocal laser scanning microscopy, Kallergi *et al.* have shown that the expression of pFAK, HIF-1alpha, VEGF, and VEGF2 in CTCs of patients with metastatic breast cancer could explain the metastatic potential of these cells and may provide a novel therapeutic target for their elimination [24].

2.5 EGFR and phosphoinositide-3 kinase/AKT pathway markers in CTC

Studies of epidermal growth factor receptor (EGFR) expression in breast cancer have shown inconsistent results due in part to a large range of methods used. Anti-EGFR therapy trials have often not used patient selection because of this. Measurement of EGFR on the surface of CTCs, derived from individuals with metastatic breast cancer patients, is possible using the CellSearch system. Payne *et al.* have used this system to enumerate and measure EGFR expression on the surface of CTCs, derived from the peripheral blood of individuals with metastatic breast cancer over time [54]. Although a proof for the clinical significance of EGFR-positive circulating tumor cells is currently lacking, expression of EGFR may predict response to lapatinib-based treatments as in a case recently presented by Liu *et al.* [55]. The results of these studies should be validated in prospective studies aiming to identify patients for anti-EGFR therapy based on the expression profile of CTCs.

The phosphoinositide-3 kinase (PI3K)/Akt pathway, operating downstream of EGFR and HER2, is implicated in cell migration and survival. EGFR and HER2 are expressed in circulating tumor cells, but the activation status of downstream signaling molecules has been addressed in just a few studies up to now. In the first study performed, Kallergi *et al.* focused on the phenotypic profile of micrometastatic

cells in peripheral blood mononuclear cells preparations from breast cancer patients. In these samples, they studied the expression of phosphorylated FAK (p-FAK), phosphorylated PI-3 kinase (p-PI-3K), and HER2 using confocal laser scanning microscopy. The expression of p-FAK was documented in all CK-positive samples, while all CK-negative samples were tested negative for p-FAK. p-PI-3K was documented in a high percentage (88 %) of CK- and p-FAK-positive samples. Immunoblot analysis of micrometastatic cells in co-culture with PBMC confirmed the specific expression of both p-FAK and p-PI-3K. Finally, impaired actin organization was apparent in CK- and p-FAK/p-PI-3K-positive samples, comparable to that observed in MCF-7 human breast cancer cells. These findings provide strong evidence that micrometastatic cells express activated signaling kinases, which may regulate migration mechanisms, supporting the presumption of their malignant and metastatic nature [56]. The same group has further investigated the expression levels of EGFR, HER2, PI3K, and Akt in CTC. Their findings demonstrated that circulating tumor cells express receptors and activated signaling kinases of the EGFR/HER2/PI3K/Akt pathway, which could be used as targets for their effective elimination [57]. In a very recent paper, Kasimir-Bauer *et al.* studied the expression of the stem cell marker ALDH1 and markers of the PI3K/AKT pathway in CTCs of 502 patients and found that a subset of primary breast cancer patients shows EMT and stem cell characteristics. They conclude that the currently used detection methods for CTCs are not efficient to identify a subtype of CTCs which underwent EMT [58].

2.6 Mammaglobin

Mammaglobin A is a highly specific molecular marker for the detection of circulating tumor cells in breast cancer, since it is specifically expressed in the mammary tissue. Mammaglobin expression has been reported in CTCs by many groups [6, 59–63]. According to Ignatiadis *et al.*, the detection of peripheral blood CK19mRNA+ and MGB1mRNA+ cells before adjuvant chemotherapy predicts poor DFS in women with early breast cancer [6]. Study of the expression of mammaglobin in CTC offers specificity and could be a valuable tool for monitoring breast cancer patients during and after therapy [60, 61]. Since the relative expression of this gene in CTC is very low, very low percentages for mammaglobin expression have been reported in CTC [51, 52]. According to Marques *et al.*, MAM mRNA detection at diagnosis or during follow-up does not predict breast cancer recurrence [62]. On the contrary, according to a very recent study by Reinholz *et al.*, a decrease in MGB1+ mRNA CTCs may help predict response to therapy of MBC patients [63].

2.7 DNA methylation in CTC

Very recently, Chimonidou *et al.* have shown for the first time that tumor suppressor and metastasis suppressor genes are epigenetically silenced in CTCs isolated from peripheral blood of breast cancer patients [64]. They tested DNA extracted from the EpCAM-positive immunomagnetically selected CTC fraction and found by methylation-specific PCR that the promoter sequences of (a) cystatin M (*CST6*), an endogenous inhibitor of cathepsins B and L that is postulated to be a tumor suppressor in breast cancer [65] and its promoter methylation provides important prognostic information in patients with operable breast cancer [66]; (b) breast cancer metastasis suppressor 1 (*BRMS1*), a predominantly nuclear protein that differentially regulates expression of multiple genes, leading to suppression of metastasis without blocking orthotopic tumor growth [67], and coordinately regulates expression of multiple metastasis-associated miRNAs [68]; and (c) SRY-box containing gene 17 (*SOX17*) that plays a tumor suppressor role through suppression of Wnt signaling [69] are highly methylated. These findings add a new dimension to the molecular characterization of CTCs and may underlie the acquisition of malignant properties, including their stem-like phenotype [64].

3 Molecular characterization of CTC and individualized targeted therapies

Definition of biomarkers based on comprehensive characterization of CTCs has a strong potential to be translated to individualized targeted treatments and spare breast cancer patients unnecessary and ineffective therapies but also to reduce the costs for the health system and to downsize the extent and length of clinical studies. At present, the success or failure of anti-cancer therapies is only assessed retrospectively by the absence or presence of overt metastases during the post-operative follow-up period. However, overt metastases are, in general, incurable by most current therapies. Molecular characterization of CTCs may identify patients most likely to be cured with aggressive therapy, as well as patients with a propensity for systemic failure. This information may be used to match patients with the most appropriate treatment strategy including combinations of local and systemic therapy.

In metastatic breast cancer, the prognostic value associated with the detection of CTCs raise additional issues regarding the biological value of this information. A drug-resistance profile of CTCs, which is predictive of response to chemotherapy, independent of tumor type and stage of disease was recently identified and therefore could be used for patient selection [70]. Drug-resistant CTCs have

predictive value in metastatic breast cancer and possibly retain stem-like properties. Very recently it was found that in metastatic breast cancer, the presence of CTCs expressing multidrug-resistance-related proteins, and ALDH1, is predictive of response to chemotherapy [71]. In metastatic breast cancer, the change in the number of CTCs was highly correlated with results from imaging before and after therapy. Based on these findings, CTCs were proposed as a biomarker that may predict the effect of treatment earlier than imaging modalities [72]. The differential prognostic and overall survival showed between patients with and without elevated CTCs before and, at the end of chemotherapy, is of special interest in patients without clinical evidence of metastasis [73].

For breast cancer patients, treatment decisions based on the molecular profile of the primary tumor have been used for many years. In HER2-overexpressing tumors, trastuzumab is a key component of therapy. According to the GeparQuattro trial, aimed at detecting and characterizing CTCs before and after neoadjuvant therapy in the peripheral blood of patients with breast cancer, information on the HER2 status of CTC might be helpful for stratification and monitoring of HER2-directed therapies [33].

This rationale is strengthened by a very recent study conducted in women with early stage HER2-negative breast cancer who were at high risk of relapse because they had detectable CK19mRNA-positive CTCs. Georgoulias *et al.* demonstrated that in these patients, treatment with "secondary adjuvant" trastuzumab resulted in a significantly reduced probability of disease relapse and increased disease-free interval compared to patients receiving only standard treatment [74]. Moreover, monitoring of circulating epithelial tumor cells (CETC) was used as a timely control of trastuzumab therapy in patients with HER2/neu-positive breast cancer. Pachmann *et al.* report that patients treated with trastuzumab had a better relapse-free survival than patients without trastuzumab treatment during the first 2–4 years of follow-up. Decrease in numbers of CETC or no change *versus* highly variable numbers or increase (fivefold or more) allowed to discriminate highly significantly and clearly between patients with a low or high risk of relapse. An increase in CETC was accompanied by an increasing portion of cells containing a very high number of HER2/neu gene amplificate [75]. In metastatic breast cancer, Hayashi *et al.* prospectively assessed the prognostic value of HER2 status in CTCs from patients with MBC who started a new line of systemic therapy and showed that HER2 status in CTCs may be a prognostic factor [76].

However, despite persistent expression of HER2, most tumors eventually become resistant to trastuzumab. When this happens, the patients benefit from a regime containing lapatinib, a dual EGFR and HER2 tyrosine kinase inhibitor. Liu *et al.* have recently reported on a patient affected by chemo-refractory metastatic HER2-positive breast cancer enrolled in a translational research program for the detection and characterization of CTCs. Depletion of the EGFR-positive CTC pool in the blood was associated with tumor response, whereas disease progression was related to a recurrence in CTCs, which were both EGFR and HER2 negative. Although a proof for the clinical significance of EGFR-positive circulating tumor cells is currently lacking, expression of EGFR may predict response to lapatinib-based treatments as in the case presented [55]. Well-powered prospective studies are necessary to determine the potential role of HER2-targeted therapies for patients with HER2-positive CTCs and HER2-negative primary tumors.

Molecular characterization of CTCs could also help to identify novel targets for biological therapies aimed to prevent metastatic relapse. In addition, understanding tumor "dormancy" and identifying metastatic stem cells might result in the development of new therapeutic concepts [77]. The role of CTCs in treatment failure and disease progression can be explained by their relation to biological processes, including the EMT and "self seeding," defined as reinfiltration of the primary tumor or established metastasis by more aggressive CTCs [78]. Interruption of the metastatic cascade *via* the targeting of CTCs might be a promising therapeutic strategy [79]. Molecular CTC analysis will provide insights into the selection of tumor cells and resistance mechanisms in patients undergoing systemic therapies. This information might support assessing individual prognosis, stratifying patients at risk to systemic therapies, and monitoring therapeutic efficacy [80, 81].

4 Conclusions—future prospects

The future of CTCs lies in the molecular characterization of these cells. Molecular characterization of CTC is absolutely necessary, simple enumeration is just not enough. Molecular characterization of CTCs can provide valuable information on the expression of cancer specific genes in these cells as well as mutations of oncogenes, and tumor suppressor genes, or epigenetic silencing of tumor suppressor genes and metastasis suppressors as well as FISH-based detection of numerical chromosomal aberrations. This will enable the identification of novel therapeutics that will target micrometastatic spread and elucidate CTC connection to cancer stem cells. CTC technologies that are complementary, like advanced imaging and molecular characterization, should be used in combination in order to have a complete view of the malignant nature of these cells. Moreover, an agreement on the standardization of protocols for isolation and detection of CTCs as well as cross validation of findings between labs and a universal internal and external quality control system both for CTC detection and enumeration is nowadays

absolutely necessary [82]. In the near future, application of modern powerful technologies such as next generation sequencing and proteomics will enable the elucidation of molecular pathways in CTCs and lead to the design of novel molecular therapies targeting specifically these cells.

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