Circulating Tumor Cells: A Noninvasive Liquid Biopsy in Cancer

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Introduction

Circulating tumor cells (CTC) were first described by Thomas Ashworth, an Australian physician, in 1869 [1], while in 1889 Steve Paget in the very first historical issue of Lancet described "The Seed and Soil Hypothesis," a hypothesis revisited many years later by Fidler [2]. During the last decade, the critical role that CTC play in the metastatic spread of cancer has been widely recognized [3–5]. The clinical importance of CTC detection and enumeration has been established in several clinical studies, and a correlation with decreased progression-free survival (PFS) and overall survival (OS) has been shown in many types of solid cancers.

CTC analysis provides a unique source of cancer cells that can be used as a noninvasive liquid biopsy for the continuous follow-up of cancer patients, when the primary tumor is already surgically removed. CTC are outstanding tools for understanding tumor biology and tumor cell dissemination and their molecular characterization offers an exciting approach to better understand the biology of metastasis and resistance to established therapies [6, 7]. However, since CTC are circulating in peripheral blood at low concentrations in most cases, the amount of available sample for analysis is very limited and this, together with their heterogeneity, presents a formidable analytical and technical challenge for their isolation, detection, and molecular characterization. The emerging interest in the analysis of CTC is reflected in the growing number of publications in this field (Fig. 9.1).

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Clinical Significance of CTC

Breast Cancer

Very recently, the first comprehensive meta-analysis of published literature on the prognostic relevance of CTC in patients with early-stage and metastatic breast cancer (MBC) clearly indicated that the detection of CTC is a reliable prognostic factor [8].

Metastatic Breast Cancer

In patients with MBC, Cristofanilli and colleagues have clearly shown many years ago by using the CellSearch system (Veridex, USA) that CTC represent an independent prognostic factor for PFS and overall survival (OS) and that a cut-off of 5 CTC/7.5 ml of blood in MBC patients was highly predictive of clinical outcome [9]. This seminal paper led to the FDA clearance of the CellSearch assay that revolutionized the clinical applications of CTC in many types of cancer, since it is standardized, semiautomated, and not subjected to pre-analytical errors.

G.M. Yousef and S. Jothy (eds.), *Molecular Testing in Cancer*,
DOI 10.1007/978-1-4899-8050-2_9,

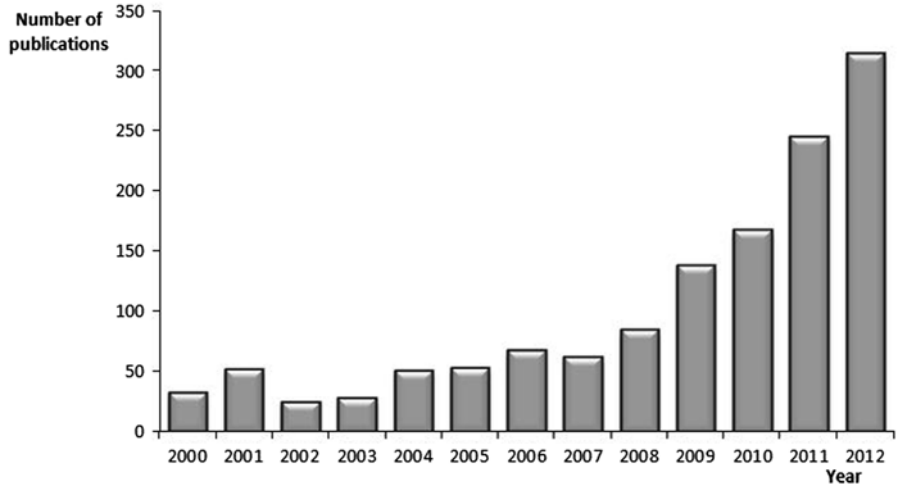

Fig. 9.1 CTC detection and analysis is a fast emerging field that has the potential to contribute to cancer patient diagnosis, prognosis, and response to therapy, as well as for accelerating oncologic drug development

Since then, a plethora of clinical studies have verified the importance of CTC enumeration in MBC [10–14].

Could CTC clearance be used as a "surrogate" marker for potentially improved survival of breast cancer patients? In the official website of the National Institutes of Health, a search (Nov 2012) on clinical studies, based on the key word "Circulating Tumor Cells," revealed 479 ongoing clinical studies, while the combination "Circulating Tumor Cells and breast cancer" revealed 116 ongoing clinical studies. These trials have different designs in various patient populations but are expected to be the pivotal trials for CTC implementation in the routine management of breast cancer patients [15].

For all these reasons, the American Society of Clinical Oncology (ASCO) cited CTC and disseminated tumor cells (DTC) for the first time in its 2007 recommendations on tumor markers, but in the category of insufficient evidence to support routine use in clinical practice [16]. Very recently, the American Joint Committee on Cancer has proposed a new category, M0(i+), for TNM staging in BC defined as "*no clinical or radiographic evidence of distant metastases*, *but deposits of molecularly or microscopically detected tumor cells* (*no larger than 0.2 mm*) *in blood*, *bone marrow, or other non-regional nodal tissue in a patient without symptoms or signs of metastases*."

Early Breast Cancer

In bone marrow the detection of DTC in breast cancer patients at the time of primary diagnosis has been confirmed to be of prognostic significance by a large pooled analysis by Braun and colleagues already in 2005 [17]. The heterogeneity of DTC has already been shown many years ago [18, 19]. Since then, the prognostic impact of DTC in breast cancer patients has been shown in numerous studies [20, 21]. According to the results recently reported by the Norwegian group in Oslo, the presence of DTC after neoadjuvant chemotherapy indicated a high risk for disease relapse and death, irrespective of the DTC status before treatment. These findings support the potential use of DTC analysis as a monitoring tool during follow-up, for the selection of patients who are candidates for secondary treatment intervention within clinical trials [22]. Janni and colleagues recently reported that the persistence of DTC after adjuvant therapy in breast cancer patients significantly predicted an increased risk for subsequent relapse and death and can serve as a clinically useful monitoring tool [23]. However, bone marrow sampling is very invasive and patients do not easily accept repeated follow-up

examinations. The analysis of CTC in peripheral blood represents a noninvasive alternative to bone marrow analysis of DTC.

In peripheral blood the prognostic value of *CK-19* mRNA-positive CTC in axillary lymph node-negative breast cancer patients, based on a nested RT-PCR, was already shown in 2002 [24]. Later on, by using a real-time RT-qPCR assay for *CK-19* RNA [25, 26], CTC detection was shown to be an independent prognostic factor for reduced disease-free and overall survival before [27], during [28], and after [29] chemotherapy in early breast cancer. Detection of *CK-19* mRNA-positive CTC before adjuvant chemotherapy predicted poor clinical outcome mainly in patients with ER-negative, triple-negative, and HER-2-positive early-stage breast cancer [30]. When the prognostic significance of *CK-19* mRNA-positive CTC in peripheral blood of women with early-stage breast cancer after the completion of adjuvant chemotherapy was evaluated, it was found that the detection of *CK-19* mRNA-positive CTC in the blood after adjuvant chemotherapy was an independent risk factor indicating the presence of chemotherapy-resistant residual disease [29]. However, when the prognostic value of DTC and CTC was compared in early breast cancer by using another method, it was reported that only the presence of DTC was highly predictive for OS [31]. When CTC were prospectively detected before and after neoadjuvant chemotherapy in a phase II trial it was found that detection of one or more CTC in 7.5 ml of blood before neoadjuvant chemotherapy can accurately predict OS [32]. A more recent study investigating the value of CTC detection during the first 5 years of follow-up in predicting late disease relapse has shown that persistent detection of *CK-19* mRNA-positive CTC during the first 5 years of follow-up was associated with an increased risk of late disease relapse and death in patients with operable breast cancer and indicated the presence of chemo- and hormonotherapy-resistant residual disease. This may be useful when deciding on subsequent adjuvant systemic therapy [33]. Lucci et al. prospectively collected data on CTC at the time of definitive surgery from chemonaïve patients with stage 1–3 breast cancer. They enumerated CTC and assessed outcomes at a median follow-up of 35 months. According to their findings, the presence of one or more CTC predicted early recurrence and decreased overall survival in chemonaïve patients with non-metastatic breast cancer [34].

Ductal Carcinoma In Situ (DCIS)

Despite the general belief that only invasive cancers are assumed to shed isolated tumor cells into the bloodstream and infiltrate lymph nodes, latest studies indicated that tumor cell dissemination may occur before stromal invasion, i.e., in DCIS. This can be explained by that these cells have started already to disseminate from preinvasive mammary lesions or represent the earliest step of microinvasion in a preinvasive lesion [35, 36]. The clinical relevance of these cells has to be further evaluated.

Colorectal Cancer

A very recent systematic review and meta-analysis that investigated the prognostic value of CTC and DTC in patients with resectable colorectal liver metastases or widespread metastatic colorectal cancer (mCRC), and was based on data reported in 12 studies representing 1,329 patients, showed that the detection of CTC in peripheral blood of patients with resectable colorectal liver metastases or widespread mCRC is associated with disease progression and poor survival [37].

Metastatic Colorectal Cancer

Cohen and colleagues were the first to show in 2008 that the number of CTC before and during treatment was an independent predictor of PFS and OS in patients with mCRC. In this prospective multicenter study, CTC were enumerated in the peripheral blood of 430 patients with mCRC at baseline and after starting first-, second-, or third-line therapy by using the CellSearch system. This study led to the FDA clearance of the CellSearch assay for mCRC [38].

CTC enumeration before and during treatment independently predicts PFS and OS in advanced colorectal cancer (CRC) patients treated with chemotherapy plus targeted agents and provides additional information to CT imaging [39]. Recent data support the clinical utility of CTC enumeration in improving the clinician's ability to accurately assess oxaliplatin-based chemotherapy treatment benefit and in expediting the identification of effective treatment regimens for individual patients [40]. By using immunomagnetic enrichment for CTC followed by real-time RT-qPCR analysis of the tumor-associated genes *KRT19*, *MUC1*, *EPCAM*, *CEACAM5*, and *BIRC5*, de Albuquerque and colleagues have shown that CTC detection during treatment was significantly correlated with radiographic findings at the 6-month staging and patients with CTC positivity at baseline had a significant shorter median PFS compared with patients with no CTC. This study showed a strong correlation between CTC detection and radiographic disease progression in patients receiving chemotherapy for CRC [41].

Jiao and colleagues found that surgical resection of metastases, but not radiofrequency ablation, immediately decreases CTC levels. In patients with colorectal liver metastases, CTC appear localized to the hepatic (and pulmonary) macrocirculations. This may explain why metastases in sites other than the liver and lungs are infrequently observed in cancer [42]. The qualitative and quantitative detection of CTC in the central and mesenteric venous blood compartments was investigated to elucidate the patterns of hematogenous tumor cell dissemination in patients with CRC. This study has shown that qualitative and quantitative detection of CTC is higher in the mesenteric venous blood compartments of patients with CRC [43].

Non-metastatic Colorectal Cancer

Currently one of the challenges facing medical oncologists is the identification of patients at higher risk of recurrence after primary CRC resection. CTC may represent a surrogate marker of an early disease spread in patients without overt metastases. However, the prognostic significance of CTC in non-metastatic CRC is less clear than in mCRC. A recent review examined the possible clinical significance of CTC in non-metastatic CRC (TNM stage I–III) with the primary focus on detection methods and prognosis. According to the findings reported in this review, the presence of CTC in peripheral blood is a potential marker of poor disease-free survival in patients with non-metastatic CRC. The low abundance of CTC in non-metastatic CRC requires very sensitive and specific detection methods. An international consensus on choice of detection method and markers is needed before incorporating CTC into risk stratification in the clinical setting [44].

Gazzaniga and colleagues have recently come to the conclusion that CTC detection might help in the selection of high-risk stage II CRC patient candidates for adjuvant chemotherapy, after enumerating CTC with the FDA-cleared CellSearch system. They detected CTC in 22 % of patients with a significant correlation with regional lymph nodes involvement and stage of disease [45].

Prostate Cancer

Although the metastatic cascade in prostate cancer is yet to be fully understood, monitoring CTC and quantifying the load of tumor cell dissemination can be used for estimating prognosis and monitoring treatment success [46]. Unmet needs in prostate cancer drug development and patient management are the ability to monitor treatment effects and to identify therapeutic targets at the time treatment is being considered. CTC enumeration at baseline and posttreatment is of prognostic value, with no threshold effect, and the shedding of cells into the circulation represents an intrinsic property of the tumor, distinct from extent of disease. The clinical utility of monitoring CTC changes with treatment, as an efficacy-response surrogate biomarker of survival, is currently being tested in large phase III trials, with the novel antiandrogen therapies abiraterone acetate (AA) and MDV3100. Molecular determinants can be

identified and characterized in CTC as potential predictive biomarkers of tumor sensitivity to a therapeutic modality [47].

Metastatic Prostate Cancer

In 2001, Moreno and colleagues investigated the diurnal variations in CTC in metastatic carcinoma of the prostate and concluded that CTC levels can be quantified in the circulation of patients with metastatic prostate cancer and that the change in the number of CTC correlates with disease progression with no diurnal variations [48]. In 2007, Danila and colleagues evaluated the association of baseline CTC number with clinical characteristics and survival in patients with castrate metastatic disease considered for different hormonal and cytotoxic therapies. Baseline CTC was predictive of survival, with no threshold effect. The shedding of cells into the circulation represents an intrinsic property of the tumor, distinct from extent of disease, and provides unique information relative to prognosis [49].

In 2008, de Bono and colleagues showed that CTC enumeration by using the CellSearch platform has prognostic and predictive value in patients with metastatic castration-resistant prostate cancer (CRPC) and is an independent predictor of overall survival. Their data led to the FDA clearance of this assay for the evaluation of CRPC [50]. CTC numbers, analyzed as a continuous variable, predict OS and provide independent prognostic information to time to disease progression; CTC dynamics following therapy need to be evaluated as an intermediate end point of outcome in randomized phase III trials and can be used to monitor disease status [51, 52].

Real-time PCR assays of Kallikrein gene mRNAs are highly concordant with CellSearch CTC results in patients with CRPC. *KLK2/3* (KLK3 is also known as PSA)-expressing CTC are common in men with CRPC and bone metastases but are rare in patients with metastases diagnosed only in soft tissues and patients with localized cancer [53].

Resel and colleagues analyzed the correlation between CTC and PSA level, Gleason score, and TNM stage in patients with metastatic hormone-sensitive prostate cancer and reported that CTC count in peripheral blood could provide a method for correctly staging prostate cancer and for assessing the prognosis of metastatic hormone-sensitive cancer [54]. Combination of CTC and PSA velocity or doubling-time assessments may offer insights into the prognosis and management of advanced prostate cancer [55].

Early-Stage Prostate Cancer

Within 10 years of radical prostatectomy up to 30 % of prostate cancer patients will have a rise in PSA, requiring radiation therapy. However, with current technology, distinction between local and distant recurrent prostate cancer is not possible. This lack of an accurate test constrains the decision whether to offer systemic or local treatment. CTC and DTC have been detected in prostate cancer and may be new surrogate candidates. The current prognostic significance of CTC/DTC in prostate cancer patients has been recently extensively reviewed [56]. Lowes and colleagues hypothesized that tests for detecting CTC in the blood may assist with clinical decision-making and investigated in a pilot study whether CTC could be detected in early-stage prostate cancer patients receiving salvage radiotherapy using the CellSearch system. Their results demonstrated that CTC can be detected in early-stage cancer and suggest the possibility that posttreatment reduction in CTC levels may be indicative of radiation therapy response [57].

Molecular Characterization of CTC and Individualized Treatment

Molecular characterization of CTC is very important to increase the diagnostic specificity of CTC assays and to investigate therapeutic targets and their downstream pathways in CTC [58]. Molecular characterization of CTC is now a hot research topic and a lot of interesting information is exponentially accumulating in a number of cancers. As an example, to improve patient

selection, assessing mutation status in CTC, which possibly better represent metastases than the primary tumor, could be advantageous [59]. We strongly believe that this information will have a great impact on the clinical management of patients, hopefully sooner than anticipated.

Breast Cancer

HER-2 and ER/PR Status in CTC

According to accumulating data CTC may have a different hormone receptor and HER-2 status than the primary tumor. There is a growing body of evidence that the HER-2 status can change during disease recurrence or progression in breast cancer patients. Based on this, it is clear that reevaluation of HER-2 status by assessment of HER-2 expression on CTC is a strategy with potential clinical application. Monitoring of HER-2 expression on CTC might be useful in trials with anti-HER-2 therapies. An optimal individualized treatment could then be selected by characterizing ERα and HER-2 status in CTC and comparing it to the primary tumor [60].

It was shown in 2004 that HER-2 gene amplification by FISH is present in CTC and that administration of trastuzumab could eliminate CTC since a high proportion of these cells expressed the HER-2 receptor [61, 62]. Recently, Georgoulias and colleagues have shown in a pilot randomized study that the administration of trastuzumab can eliminate chemotherapy-resistant *CK19* mRNA-positive CTC, reduce the risk of disease recurrence, and prolong the DFS [63].

The existence of tumor-initiating cells in breast cancer has profound implications for cancer therapy. Magnifico and colleagues investigated the sensitivity of tumor-initiating cells isolated from HER-2 overexpressing carcinoma cell lines to trastuzumab and they provided evidence for the therapeutic efficacy of trastuzumab in debulking and targeting tumor-initiating cells of HER-2 overexpressing tumors [64]. HER-2-positive CTC were detected in DCIS/LCIS or M0 breast cancer irrespective of the primary tumor HER-2 status. Nevertheless, their presence was more common in women with HER-2-positive disease [65].

In a prospective study, Fehm and colleagues reported that HER-2-positive CTC could be detected in a relevant number of patients with HER-2-negative primary tumors [66]. The same investigators reported that most of the CTC were "triple-negative." Since the expression profile between CTC and the primary tumor differs, the consequence for the selection of adjuvant treatment has to be evaluated [67].

According to findings reported by Rack and colleagues, trastuzumab is effective in clearing HER-2-positive cells from bone marrow during recurrence-free follow-up of breast cancer patients. Given the heterogeneity of minimal residual disease, these patients might benefit from a combination of targeted treatment approaches [60].

Estrogen receptor (ER)-positive breast cancer often recurs many years after the initial diagnosis, and understanding the patterns of timing of relapse could identify patients who need more aggressive treatment. Reliable prediction of early treatment failure may identify patients who require adjuvant therapy to prevent the early onset of distant metastases. When *CK-19* mRNA-positive cells were prospectively and longitudinally detected in 119 patients with estrogen and/or progesterone receptor-positive tumors during the period of tamoxifen administration, multivariate analysis revealed that the detection of *CK-19* mRNA-positive cells during the administration of tamoxifen was associated with an increased risk of relapse [28]. Exploiting the molecular differences between early versus late recurrences may also guide the development of effective novel drug combinations in this group of patients. Towards this goal, a recent study by Liu and colleagues provided clear evidence that robust molecular differences exist between ER-positive breast cancers that recur early on versus much later, despite adjuvant tamoxifen; this group analyzed gene-expression data from breast tumor biopsies, and then correlated them with the development of distant metastases. What emerged was a 91-gene classifier that reliably separates early recurrences (distant relapse $\leq$3 years from diagnosis) from late recurrences ($\geq$10 years) [68].

Epithelial–Mesenchymal Transition and Stem Cell Markers

The persistence of CTC in breast cancer patients might be associated with stem cell-like tumor cells which have been proposed to be the active source of metastatic spread in primary tumors [69]. Current models suggest that the invasive phenotype appears to be associated with an epithelial–mesenchymal transition (EMT), which enables detachment of tumor cells from a primary site and migration. The reverse process of mesenchymal–epithelial transition (MET) might play a crucial role in the further steps of metastasis when CTC settle down in distant organs and establish metastasis. Nevertheless, the exact mechanisms and interplay of EMT and MET are only partially understood and their relevance in cancer patients is unclear. A subset of CTC shows EMT and stem cell characteristics. Research groups have just started to apply EMT-related markers in their studies of CTC in cancer patients. In a recent review, the current state of investigations on CTC in the context of research on EMT/MET is discussed in detail [70].

Aktas and colleagues reported that a major proportion of CTC of MBC patients show EMT and tumor stem cell characteristics [71]. Moreover CTC co-expressing TWIST and vimentin, suggestive of EMT, were identified in patients with metastatic and early breast cancer patients. The high incidence of these cells in patients with metastatic compared to early-stage breast cancer strongly supports the hypothesis that EMT is involved in the metastatic potential of CTC [72]. A recent study showed that a subset of primary breast cancer patients shows EMT and stem cell characteristics but the currently used detection methods for CTC are not efficient to identify the subgroup of CTC which underwent EMT [73].

Activated Kinases and Angiogenic Molecules

It was also shown by immunofluorescence that CTC express receptors and activated signaling kinases of the EGFR/HER-2/PI3K/Akt pathway, which could be used as targets for their effective elimination [74] as well as pFAK, HIF-1 alpha, VEGF, and VEGF2 [75]. These data could explain the metastatic potential of these cells and may provide a therapeutic target for their elimination.

Colorectal Cancer

Molecular characterization of CTC could provide important information for improving the management of CRC patients. In mCRC, the presence of *KRAS* and *BRAF* mutations is currently assessed in the primary tumor, since it has been shown to reflect anti-EGFR therapy efficacy. The mutation status of *KRAS* and *BRAF* in CRC patients matching primary tumors, liver metastasis, and CTC was very recently investigated, and it was interesting to find discordance between primary tumors, CTC, and metastatic tumors [76].

Gasch and colleagues isolated CTC from patients with metastatic and non-metastatic CRC and further assessed EGFR expression, *EGFR* gene amplification, and *KRAS*, *BRAF*, and *PIK3CA* mutations in single CTC. They demonstrated a considerable intra- and interpatient heterogeneity of EGFR expression and genetic alterations in *EGFR*, *KRAS*, and *PIK3CA* in CTC, possibly explaining the variable response rates to EGFR inhibition in patients with CRC [77]. Barbazán and colleagues isolated CTC by EpCAM-based immunobeads and performed whole transcriptome amplification and hybridization onto cDNA microarrays. They found 410 genes that characterized the CTC population, that were related to cell movement and adhesion, cell death and proliferation, and cell signaling and interaction. When the expression of genes related to the main cellular functions characterizing the CTC population was evaluated by RT-qPCR in an independent series of mCRC patients, controls showed a correlation of CTC-gene expression with clinical parameters and prognosis significance [78].

A very recent and interesting study by the group of M. Mori has shown that Plastin3 is a novel marker for CTC undergoing EMT and is associated with CRC prognosis. They found that PLS3 was expressed in mCRC cells but not in

normal circulation and by using fluorescent immune-cytochemistry, they clearly showed that PLS3 was expressed in EMT-induced CTC in peripheral blood from patients with CRC with distant metastasis. PLS3-expressing cells were detected in the peripheral blood of approximately one-third of an independent set of 711 Japanese patients with CRC. Multivariate analysis showed that PLS3-positive CTC was independently associated with prognosis and that the association between PLS3-positive CTC and prognosis was particularly strong in patients with Duke B and Duke C [79].

Prostate Cancer

To improve future drug development and patient management for patients with CRPC, surrogate biomarkers that are linked to relevant outcomes are urgently needed. This area is rapidly evolving, with recent trials incorporating the detection of CTC, imaging, and patient-reported outcome biomarkers [80].

In CRPC persistence of ligand-mediated androgen receptor signaling has been documented. Abiraterone acetate (AA) is an androgen biosynthesis inhibitor shown to prolong life in patients with CRPC already treated with chemotherapy. AA treatment resulted in dramatic declines in PSA only in a subset of patients and no declines in others, suggesting the presence of molecular determinants of sensitivity in tumors. Androgen deprivation therapy is initially effective in treating metastatic prostate cancer, and secondary hormonal therapies are being tested to suppress androgen receptor (AR) reactivation in CRPC.

Danila and colleagues studied the role of transmembrane protease, serine 2 (TMPRSS2)-v-ets erythroblastosis virus E26 oncogene homolog (ERG) fusion, an androgen-dependent growth factor, in CTC as a biomarker of sensitivity to AA. Molecular profiles of CTC with an analytically valid assay identified the presence of the prostate cancer-specific TMPRSS2-ERG fusion but did not predict for response to AA treatment. This finding demonstrates the role of CTC as surrogate tissue that can be obtained in a routine practice setting [81].

Miyamoto and colleagues presented data that prostate-specific antigen/prostate-specific membrane antigen (PSA/PSMA)-based measurements of AR signaling in CTC enable real-time quantitative monitoring of intra-tumoral AR signaling. This finding indicates that measuring AR signaling within CTC may help to guide therapy in metastatic prostate cancer and highlights the use of CTC as liquid biopsy [82].

FISH analysis of CTC has been shown to be a valuable, noninvasive surrogate for routine tumor profiling. Leversha and colleagues assessed the feasibility of characterizing gene copy number alteration by FISH in CTC in patients with progressive metastatic CRPC. They have shown that FISH analysis of CTC can be a valuable, noninvasive surrogate for routine tumor profiling. The finding that as many as 50 % of these patients have substantial amplification of the AR locus indicates that androgen signaling continues to play an important role in late-stage prostate cancer [83]. Recent results by Darshan and colleagues suggest that monitoring AR subcellular localization in the CTC of CRPC patients might predict clinical responses to taxane chemotherapy [84].

Coding mutations in the AR gene have been identified in tissue samples from patients with advanced prostate cancer and represent a possible mechanism underlying the development of CRPC. AR mutations have been identified in CTC-enriched peripheral blood samples from CRPC patients. This approach has the potential to open new perspectives in understanding CTC and the mechanisms for tumor progression and metastasis in CRPC [85]. It was also recently shown that the majority (>80 %) of CTC in patients with metastatic CRPC co-express epithelial proteins such as EpCAM, cytokeratins, and E-cadherin, with mesenchymal proteins including vimentin, N-cadherin, and O-cadherin, and the stem cell marker CD133 [86].

BRCA1 allelic imbalances were detected among CTC in multifocal prostate cancer. By using FISH analysis of primary tumors and lymph node sections, and CTC from peripheral

blood Bednarz and colleagues found that 14 % of 133 tested patients carried monoallelic BRCA1 loss in at least one tumor focus. BRCA1 losses appeared in a minute fraction of cytokeratin- and vimentin-positive CTC. Small subpopulations of prostate cancer cells bearing BRCA1 losses might be one confounding factor initiating tumor dissemination and might provide an early indicator of shortened DFS [87].

Hormone-driven expression of the ERG oncogene after fusion with TMPRSS2 occurs in 30–70 % of therapy-naive prostate cancers. Attard et al. have used multicolor FISH to show that CRPC CTC, metastases, and prostate tissue invariably had the same ERG gene status as therapy-naive tumors and reported a significant association between ERG rearrangements in therapy-naive tumors, CRPC, and CTC and magnitude of PSA decline ($P = 0.007$) in CRPC patients treated with abiraterone acetate [88].

Little information exists regarding the utility of CTC enumeration in hormone-sensitive prostate cancer. Goodman and colleagues enumerated CTC in 33 consecutive patients undergoing androgen deprivation therapy and their data revealed that initial CTC values predict the duration and magnitude of response to hormonal therapy. CTC enumeration may identify patients at risk of progression to CRPC before initiation of androgen deprivation therapy [89].

Stott and colleagues developed a quantitative automated imaging system for analysis of prostate CTC, taking advantage of PSA. The specificity of PSA staining enabled optimization of criteria for baseline image intensity, morphometric measurements, and integration of multiple signals in a three-dimensional microfluidic device. The prostate cancer-specific TMPRSS2-ERG fusion was detectable in RNA extracted from CTC from patients with metastatic disease, and dual staining of captured CTC for PSA and the cell division marker Ki67 indicated a broad range for the proportion of proliferating cells among CTC. This method for analysis of CTC will facilitate the application of noninvasive tumor sampling to direct targeted therapies in advanced prostate cancer and warrants the initiation of long-term clinical studies to test the importance of CTC in invasive local disease [90].

Circulating endothelial cells, CTC, and tissue factor levels alone and combined can predict OS in CRPC patients treated with docetaxel-based therapy [91]. Coumans and colleagues evaluated the association between circulating objects positive for epithelial cell adhesion molecules and cytokeratin (EpCAM+CK+) that are not counted as CTC and survival in patients with prostate cancer and came to the conclusion that each EpCAM+CK+CD45− circulating object showed a strong association with overall survival ($P < 0.001$). This class included small tumor microparticles (S-TMP), which did not require a nucleus and thus are unable to metastasize [92].

Quality Control in CTC Analysis

Since the detection of CTC has been shown to be of considerable utility in the clinical management of patients with solid cancers, a plethora of analytical systems for their isolation and detection have been developed and are still under development and their number is increasing at an exponential rate [93–96]. Since CTC are very rare (1 CTC in 10^6–10^7 leukocytes) [97], in most cases they are specifically detected by using a combination of two steps: (a) isolation-enrichment and (b) detection. The detailed presentation of these systems is beyond the scope of this review.

All these advanced technologies recently developed for CTC isolation and detection are very promising for providing useful assays for oncological drug development, monitoring the course of disease in cancer patients, and in understanding the biology of cancer progression. However, comparison of different methods for CTC enumeration and characterization by using the same samples and quality control is an important issue for the clinical use of CTC analysis as a liquid biopsy. 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.

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) interlaboratory 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 [94, 95].

However, the phenotypic heterogeneity of CTC and their low numbers in the blood stream of patients, together with differences in pre-analytical sample processing, has led to the collection and accumulation of inconsistent data among independent studies [95]. There is still a lot to be done for the automation, standardization, quality control, and accreditation of analytical methodologies used for CTC isolation, detection, and molecular characterization.

Conclusions: Future Directions

The main implication of CTC analysis is based on their unique potential to offer a minimally invasive "liquid biopsy" sample, easily obtainable at multiple time points during the course of the disease which can provide valuable information on the very early assessment of treatment efficacy and can help towards establishing individualized treatment approaches that will improve efficacy with less cost and side effects for cancer patients.

Further research on the molecular characterization of CTC will provide important information for the identification of therapeutic targets and understanding resistance to therapies. The molecular characterization of CTC and DTC at the single cell level is very promising and highly challenging especially in combination with next generation sequencing technologies [98–101]. Even if this is still far from being considered to be applied in a routine clinical setting, it holds a great promise for the future management of cancer patients.

The detection rates of CTC using different analytical systems vary considerably and there is a clear need for an external quality control system for CTC enumeration and validation of findings for the same samples by participating laboratories. Microscopic detection systems used in CTC cytological methods are highly observer-dependent, so the development of international standards for CTC enumeration and characterization is of utmost importance in this case. Cross validation of findings between different labs, using the same or different detection and enumeration platforms, is urgently needed. Especially the application of modern powerful technologies such as next generation sequencing in CTC analysis will enable the elucidation of molecular pathways in CTC and lead to the design of novel molecular therapies targeting specifically CTC.

One of the main clinical issues that are currently being addressed in CTC is to evaluate whether CTC detection can lead to a change in the management of cancer patients and can result in improved clinical outcome. This has not yet been fully proved. Therefore, the challenge of using CTC as novel tumor biomarkers is currently evaluated in clinical trials. In conclusion, the clinical use of CTC as a "liquid biopsy" for selection of patients and real-time monitoring of therapies will have a major impact in personalized medicine.

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