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Circulating Tumor Cells in Prostate Cancer Diagnosis and Monitoring: An Appraisal of Clinical Potential

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Abstract

Circulating tumor cells (CTCs) have emerged as a viable solution to the lack of tumor tissue availability for patients with a variety of solid tumors, including prostate cancer. Different approaches have been used to capture this tumor cell population and several of these techniques have been used to assess the potential role of CTCs as a biological marker to predict treatment efficacy and clinical outcome. CTCs are now considered a strong tool to understand the molecular

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characteristics of prostate cancer, and to be used and analyzed as a ‘liquid biopsy’ in the attempt to grasp the biological portrait of the disease in the individual patient.

1 Introduction

Prostate cancer (PC) is the most prevalent malignancy and the second leading cause of cancer death in men in the USA. Radical prostatectomy and radiation therapy are established treatments for men with clinically localized PC, and androgen-deprivation therapy (ADT) represents the mainstay for the management of locally advanced, recurrent, or metastatic PC [1]. Although chemical castration is temporarily effective in most patients who develop advanced disease, nearly all men eventually progress to castration-resistant PC (CRPC). Recent studies show that, even in the setting of castrate levels of testosterone, CRPC is frequently reliant on androgen receptor (AR)-mediated growth pathways that facilitate growth and survival [2]. This has led to a number of effective therapies that target the androgen axis, including abiraterone and enzalutamide [3–6]. Men with metastatic CRPC are also commonly treated with taxane-based chemotherapy (docetaxel and cabazitaxel), and approximately half of patients experience a clinical response [7–9]. Thus, in contrast to 10 years ago, today there are many effective therapies for men with CRPC that significantly improve survival. Nevertheless, there are currently no validated biomarkers that can predict response to a specific therapy, with post-therapy decline in prostate-specific antigen (PSA) in patients remaining the most commonly used biomarker to measure clinical benefit [10–13].

Concomitantly with an increased number of available treatments, there has been a greater understanding of the molecular abnormalities that lead to the development and progression of PC. Nevertheless, the molecular basis of why patients respond differently to, and in some cases are resistant to, specific treatments remains elusive. In particular, the lack of PC tissue to analyze before and during therapy, and at the time of relapse, has significantly hampered the ability to assess the mechanisms of response and resistance to treatment. Recently, a number of investigators have begun performing biopsies of metastatic lesions before and after therapy, but, clearly, using this approach outside of a research setting is problematic.

Circulating tumor cells (CTCs) have emerged as a viable solution to the problem whereby patients with a variety of solid tumors, including PC, often do not have recent tumor tissue available for analysis. CTCs isolated from the peripheral blood of cancer patients may represent a valid and readily accessible source of tumor tissue in the form of ‘liquid biopsy’. However, the clinical use of this cell population is limited by the rarity of viable CTCs in the peripheral blood (1 CTC/ 1×10^6 – 9 blood cells). Today, the CellSearch® Circulating Tumor Cell Test (Janssen Diagnostics) remains the only US FDA-cleared method to isolate and enumerate CTCs from the peripheral blood of cancer patients, in which tumor cells are captured based on an epithelial cell adhesion molecule (EpCAM)-dependent immunomagnetic principle, and higher counts have been associated with poor prognosis in many studies [14]. However, several studies have shown that EpCAM-based immunocapture identifies only a subpopulation of CTCs, owing to their high molecular heterogeneity [15]. In this review, we describe recent advances in the CTC field in PC,

ranging from their isolation to clinical significance as a biomarker that optimizes diagnostic and therapeutic decision making.

2 Methods to Isolate Circulating Tumor Cells (CTCs) from Prostate Cancer (PC) Patients

Tremendous effort has focused on optimizing the technical aspects of CTC isolation and enrichment from the peripheral blood of cancer patients. These efforts are mainly directed to avoid the qualitative and quantitative underestimation of the total CTC population, as well as to minimize CTC loss caused by excessive sample handling during blood processing. Most of the available approaches to capture CTCs exploit the physical properties (e.g. size, density, membrane capacitance) and molecular characteristics (e.g. surface marker expression, invasion potentials) that are considered tumor-selective and that separate tumor circulating cells from the normal cellular components found in blood (Table 1).

2.1 PC CTC Isolation Based on Physical Properties

Physical isolation strategies have been developed based on characteristics in size, density, compliance, and membrane capacitance that potentially differentiate CTCs from the other cells in the circulation. These approaches have a theoretical advantage in that they do not rely on CTC molecular characteristics such as antigen expression, as loss of tumor-selective markers may occur during tumor progression owing to phenotypic changes exemplified by epithelial-mesenchymal transition (EMT). One of the most commonly used physical separation methods is density-gradient centrifugation, in which CTCs are separated from the other blood cells in a density-dependent manner. Using a density-gradient solution (i.e. Ficoll-Paque®, GE Healthcare Life Sciences; OncoQuick®, Greiner bio-one), blood cells and CTCs divide into distinct layers (plasma, mononuclear cells and anucleate cells). Because of their size and shape, CTCs separate into the mononuclear cell layer (peripheral blood mononuclear cells [PBMCs]). The sorted CTCs can subsequently be identified by reverse transcriptase polymerase chain reaction (RT-PCR) or immunofluorescence. With this approach, CTCs were identified in 79 % of CRPC patients [16]; however, the principal disadvantage of this approach is the low purity of the final sample, which is due in part to cross-contamination between the different cell layers [17].

Other techniques based on physical properties include microfiltration devices, in which CTCs are purified by size [18, 19]. Tumor cells have, on average, a larger diameter and cell area (15–25 μm and 396–796 μm^2 , respectively) than PBMCs (less than 12 μm and 140 μm^2 , respectively) [20]. One method to isolate CTCs from the PC patient is ISET® (Isolation by Size of Epithelial Tumor cells, Rare cells Diagnostics), in which CTCs are isolated by passing whole blood through a filter with 8 μm -diameter pores that allow the passage of blood cells but retain CTCs. In one study, ISET proved effective in isolating CTCs from the peripheral blood of PC patients with a detection rate superior to that of CellSearch® (75 vs. 60 %) [21]. The major limitation of this approach is the lack of capture of smaller CTCs, which hampers some clinical applications. CTCs in PC patients are highly heterogeneous and, in some cases, can be smaller than expected, with some metastatic PC CTCs demonstrating a cross-section area as low as 89 μm^2 , similar to that of leukocytes [22].

Size is also the key characteristic used in the high definition (HD)-CTC assay (Epic Sciences), in which after red blood cells lysis, nucleated cells are plated on custom glass slides for standard immunofluorescent staining for an extremely detailed morphologic interpretation. This platform was able to identify CTCs in 80 % of the metastatic PC patient population examined, as single cells or CTC clusters [23–25]. Recently, exciting results have been reported using this approach to monitor CTC counts and interrogate the molecular profile of the disease, assessing CTC status of biomarkers like AR, PTEN, and ERG in CRPC patients [26, 27].

An exciting developing approach for CTC isolation discriminates CTCs from circulating leukocytes based on electrical properties [28]. Dielectrophoresis (DEP) has been extensively used to enrich cancer cells from peripheral blood [29, 30]. Applying external electric fields, DEP-guided cancer cell isolation can minimize leukocyte contamination in immunocapture systems [31]. Preliminary reports with PC cell lines indicate a recovery rate of 76–83 % with a striking high purity of 74–82 % [32]; however, this method still needs to be clinically validated.

2.2 PC CTC Isolation Based on Biological Properties

Isolation methods based on the biological properties of CTCs are usually related to the expression of selected cell-surface markers recognized through an antibody-antigen interaction. In these antibody-dependent capture methodologies, an antibody (or cocktail of antibodies) identifies one or more antigens expressed either on the plasma membrane of cancer cells (positive selection) or on leukocytes (negative selection), leading to specific and sensitive CTC isolation. Following enrichment, CTCs can be visually detected by immunostaining for epithelial (cytokeratin [CK]) and pan-leukocyte (CD-45) markers with CTCs identified as CK⁺/CD-45⁻/DAPI⁺ events. CTC-negative selection relies on leukocyte depletion from the blood sample using monoclonal antibodies targeting leukocyte-specific antigens (commonly CD-45). Immunomagnetic separation with anti CD-45 ferrobeads or centrifugation with the RosetteSep kit have been used for PBMC removal. Enriched CTCs can thus be identified and further analyzed using this approach. He et al. [33] were able to isolate CTCs this way and observed that 10 of 13 PC patients had a significantly higher CTC count than healthy donors.

The most widely used, and the only FDA-cleared, system for CTC-positive selection is the CellSearch[®] CTC Test system. This highly validated antibody-dependent system positively isolates EpCAM-expressing CTCs using anti-EpCAM-coated magnetic beads starting from 7.5 ml of peripheral blood. CellSearch[®] isolation has been extensively studied in PC patients. Using a cut-off of 5 CTCs/7.5 ml of blood, Danila et al. [34] identified CTCs in 57 % of 120 metastatic PC patients evaluated. PC patients with fewer than 5 CTCs in 7.5 ml of blood have a greater overall survival (OS) than patients with 5 or more CTCs/ 7.5 ml. Several studies have also shown that changes in CTC counts during therapy are prognostic of CRPC patient survival [34–36]. Furthermore, using a CTC count cut-off of 3 CTCs/7.5 ml of blood predicts the magnitude and duration of response to ADT in patients with hormone-sensitive PC [37]. Despite its clinical success, CellSearch[®] also has several limitations. Being dependent on EpCAM expression on tumor cells, this technique likely

underestimates actual CTC count as EpCAM-negative or low expressing cancer cells are missed. Moreover, the reported low purity of the recovered CTC population hampers any further reliable analysis of the cells [38].

Similar to CellSearch[®], MagSweeper isolates CTCs through an EpCAM-dependent immunomagnetic approach. Cann and colleagues [39] demonstrated that this technique reached capture efficiency of 63 ± 25 % with purity of the isolated cell population of 10 ± 6 % when tested with PC cell lines. In PC patients, MagSweeper performed similarly to CellSearch[®], with a non-significant trend for a higher detection rate in patients with low CTC numbers.

Further evolution of antibody-based CTC isolation led to the development of microfluidic devices (CTC chips) that selectively isolate cancer cells through collisions of CTCs with antibody-coated microposts, resulting in cancer cell capture; Nagrath et al. [40] tested an EpCAM-coated CTC chip to isolate CTCs from the peripheral blood of cancer patients and were able to isolate tumor cells in 100 % of the PC patients, with an average purity of 49 %. A structural modification of this microfluidic device led to the development of the 'herringbone chip' in which CTCs are forced to collide with antibody-coated grooves by vortical flow. Stott and colleagues [41] assessed the performance of this device and reported a capture efficiency of 91 % in spiking experiments with PC cell lines and a CTC detection rate of 93 % in PC patients, with a purity of the isolated cell population of 14 %; using this device, simple molecular analysis of CTCs for PSA and prostate-specific membrane antigen (PSMA) expression prior to treatment with abiraterone was prognostic in a small group of patients [35]. Further evolution of these devices has led to the CTC iChip, a microfluidic device that uses a series of inertial focusing and magnetic separation steps to sort viable CTCs. This approach demonstrated high efficiency in recovering cancer cell lines with extremely heterogeneous EpCAM expression levels, proving to be potentially independent from any EMT molecular changes. Ozkumur et al. [42] showed that this platform could identify CTCs in 90 % of CRPC patients evaluated, with a mean purity of 7.8 %.

Another microfluidic device is the geometrically enhanced differential immunocapture (GEDI) chip (Fig. 1a, b), in which CTC capture is based on both physical properties (larger diameter than leukocytes) and molecular characteristics of CTCs (overexpression of tumor-specific surface antigen) [43, 44]. The PC-specific GEDI chip uses an antibody that recognizes PSMA, which is a non-secreted cell surface membrane protein expressed primarily in prostatic tissue and at low levels in other cell types (e.g. renal tubular cells and intestinal epithelial cells). PSMA is upregulated in PC cells and is not affected by the molecular and phenotypical changes that occur during EMT. The levels of enrichment achievable by the PSMA-coated GEDI chip is quite high, 97 ± 3 % for cells spiked in phosphate buffered saline (PBS) and in 90 % of CRPC patients, with a reported purity of 62 % [44]. In another study, Kirby et al. [45] used the PSMA-GEDI device to capture PC cells spiked in 1 ml whole blood and used them for molecular characterization. The investigators were able to extract RNA from the captured cells and analyze them for the presence of AR point mutations as well as for expression of TMPRSS2-ERG fusion protein by immunofluorescence. Moreover, the authors developed a predictive drug sensitivity assay to evaluate whether CTC sensitivity following ex vivo drug treatment on the GEDI device

correlates with actual patient clinical response [45]. This assay relies on the viability of captured CTCs and can be used to investigate whether CTC drug response can serve as a surrogate for the patient's clinical response, thus enabling customization of therapy for the individual. Prospective validation of this assay is still needed and is currently ongoing within a phase II clinical trial (clinicaltrials.gov NCT01718353) [46]. Several differences can be promptly spotted. As shown in Table 1, the amount of blood processed through each of these devices is different and ranges from 1 ml (GEDI chip) to 10 ml (CTC-iChip). This characteristic can be relevant in the clinical setting especially where various devices must be used to perform different assays. Conversely, the Hb-chip has the convenience to be cast in transparent plastic, guaranteeing the possibility of performing a pathologic evaluation of the captured potential CTCs through hematoxylin/eosin staining, thus increasing the confidence in the identification of real CTCs from the analyzed sample. Unfortunately, a direct comparison among the different microfluidic devices reported in the literature has not been conducted; therefore it is not possible to determine clinical superiority of one over the other.

Flow cytometry has also been used for CTC identification in PC patients [47]. Using a fluorescently labeled PSMA antibody, Wu et al. [48] were able to detect PC cells spiked in peripheral blood. In a clinical study, Danila et al. [49] isolated patient CTCs by fluorescence-activated cell sorting (FACS) and analyzed them by RT-PCR, assessing the expression of several important PC factors such as AR, PSA, and the TMPRSS2-ERG fusion protein. Cell adhesion on blood vessels has been exploited as a biological approach to isolate PC CTCs. Cell attachment, rolling, and binding were induced using selectin-coated microtubes. Using this method, Gakhar et al. [50] identified CTCs in 23 of 27 PC patients, while Hughes and colleagues [51] detected CTC in 14 of 14 tested PC patients, with a purity of 66 %.

The ability of viable cancer cells to secrete proteins like mucins or CKs has been proposed as an alternative assay to identify and enumerate CTCs from patients with solid tumors, and the ELISPOT (enzyme-linked SPOT) and EPISPOT (EPithelial Immuno SPOT) assays were developed based on this approach [52, 53]. After gradient centrifugation, mononuclear cells are incubated on anti-PSA-coated membranes, and PSA secretion is detected through a second fluorescently labeled anti-PSA antibody. Alix-Panabieres et al. [54] used the EPISPOT assay to isolate PSA-secreting CTCs from 24 men with metastatic PC and were able to identify tumor cells in 20 (83 %) of them. The same group confirmed these results in a second analysis where mucin (MUC)-1-secreting CTCs and PSA-secreting CTCs were identified in 83 and 65 % of the population analyzed, respectively [55].

3 Role of CTCs in the Clinical Management of PC Patients

3.1 Implications of CTCs in PC Diagnosis

PC screening is intended to result in early detection and treatment of early-stage cancers that should theoretically result in improved survival and fewer deaths from PC. Currently, the strategy used to screen a population at risk for PC combines digital rectal examination (DRE) and measurement of PSA blood levels [56–58]. Despite widespread use and many case-controlled and prospective randomized trials, there is still significant controversy on the actual benefit of PSA testing as a screening biomarker [59–61]. PC is characterized by

clinical heterogeneity, as many PC patients have an indolent course but others develop an aggressive and fast-progressing PC. Consequently, a screening program that can accurately identify the subpopulation of patients with a higher risk of developing aggressive and potentially lethal PC could significantly improve PC-related mortality [62].

The recent advances in CTC detection and downstream molecular characterization may provide a more sensitive and reliable methodology to detect early-stage disease and differentiate indolent from aggressive PC, with the potential to be integrated into PC screening programs. Allard and colleagues [38] used CellSearch® to quantify CTCs in healthy subjects and patients with benign or nonneoplastic diseases in comparison with patients with metastatic carcinomas. They found that 1 CTC/7.5 ml of peripheral blood was detected in only 5.5 and 7.5 % of the healthy population and in patients with benign or nonneoplastic prostate biopsies, respectively, with a mean of 0.1 CTC/7.5 ml of blood, far below the accepted threshold of 5 CTCs/7.5 ml used for metastatic PC patients. However, controversial findings concern the sensitivity of CTC counts for the diagnosis of malignant prostate disease, as no difference in CTC counts was identified between locally advanced PC and healthy controls using CellSearch® [63]. In contrast, Stott et al. [64] detected CTCs in 42 % of patients with localized PC using a CTC chip microfluidic device. Murray et al. [65, 66] investigated the potential role of circulating prostate cells in the differential diagnosis of malignant versus non-malignant prostate disease in subjects undergoing a prostate biopsy. The authors used P504S expression as a PC-specific marker and showed that PSA⁺/P504S⁻ cells found in the peripheral blood of the screened subjects specifically correlated with benign conditions [65, 66]. Although these findings are provocative, evaluation of this marker requires more extensive evaluation in prospective clinical trials to assess its potential usefulness.

Overall, the possibility of identifying CTCs in early-stage PC appears to be achievable; nevertheless, more sensitive and specific techniques need to be more extensively tested in this clinical scenario. Thus, the involvement of CTC counts into the screening program for PC detection together with the current procedures seems a very fascinating, even though still premature, perspective that deserves to be further investigated.

Another potential utility of CTC detection is in early diagnosis of metastasis in PC patients. CTC detection may anticipate metastatic progression and allow prompt treatment before metastatic foci become clinically detectable with standard radiographic imaging. CTCs have been found in the peripheral blood of patients with localized or locally advanced PC without clinical evidence of metastatic disease, suggesting that these cells may act as the seeds for the development of metastases [67]. In addition, the presence of tumor cells in the bone marrow (disseminated tumor cells [DTCs]) of patients with non-metastatic PC has been documented and correlated with a higher PSA relapse rate [68]. The presence of CTCs and DTCs after treatment of localized PC represents minimal residual disease, and correlates with negative recurrence-free survival [68]. Alix-Panabieres et al. [55] detected CTCs in patients with localized PC, reporting that a subset of these identified tumor cells were characterized by the secretion of FGF2, underlining their metastatic potential. However, no correlations were done between these findings and the actual metastatic progression in the analyzed patient cohort. Interestingly, Nagrath et al. [40] reported similar CTC counts in

patients with either locally advanced or metastatic PC, suggesting that the clinical impact of CTC enumeration alone may be limited and that downstream molecular characterization of CTCs is required to inform clinical decision making.

3.2 Prognostic and Predictive Role of CTCs in Castrate-Resistant PC (CRPC)

Detection of CTCs in the peripheral blood of cancer patients does not in itself inform the clinical characteristics of the disease and its response to the treatment. Since their earliest clinical application, CTC enumeration has been studied for clinical relevance as a prognostic marker of disease progression and survival. In PC, a consistent correlation has been reported between high CTC number, disease progression, and OS using the FDA-cleared CellSearch®-based approach [14, 69, 70]. High CTC counts at baseline have been associated with an unfavorable prognosis and a worse clinical outcome. Even when different cut-off values were evaluated to define high CTC counts, its prognostic role proved consistent. Most studies in PC use the standard CellSearch®-validated threshold of 5 CTCs/7.5 ml or, in some cases, an even lower CTC count [37, 71, 72]. CTC numbers retain their prognostic relevance even considering them as a continuum variable without a specific cut-off [34]. Importantly, CTC counts correlate with clinical outcome independently of other prognostic factors and are superior to PSA kinetics in predicting OS. The prognostic significance of CTC enumeration is retained not only if assessed as an absolute value at baseline but also when assessing the conversion between favorable and unfavorable risk groups (from >5 to <5 CTCs/7.5 ml) or fold-change in CTC numbers after treatment relative to baseline (>50 % decline or >30 % increase) [36, 71, 73]. Higher CTC numbers are detected most commonly in PC patients with bone metastases [74]; however, CTC enumeration is independent of disease burden [36]. The association of CTC numbers and prognosis led to the FDA-approval of the CellSearch® test as an indicator to monitor PC patients [75].

CTC enumeration has been studied as a potential surrogate marker of OS and prognosis and incorporated as either a primary or a secondary endpoint in a number of clinical trials assessing therapeutic efficacy of anti-cancer drugs in PC (Table 2). Vaishampayan et al. [76] tested the potential role of CTCs as an alternative endpoint for survival in heavily pretreated CRPC patients receiving a combination of the anti-angiogenic immunotherapy bevacizumab and the oral platinum compound satraplatin in a phase II clinical trial. CellSearch®-assessed post-therapy CTC numbers >5 were associated with a worse OS and time to progression, with a progression hazard ratio (HR) of 1:1.91; unfortunately, CTC assessment was limited to the post-treatment time point and was not compared with a baseline analysis, precluding any evaluation of possible CTC count drops. Moreover, CTC examination was available for only 17 (seven with <5 CTCs and ten with >5 CTCs) of the 31 individuals clinically evaluated in the study, thus hampering the statistical evaluation of the data and limiting the clinical implications of these results. Armstrong et al. [77] used CTC enumeration as primary endpoint to assess the clinical efficacy of the mammalian target of rapamycin (mTOR) inhibitor temsirolimus in patients with previously treated CRPC. The authors evaluated CTC count using CellSearch® at baseline and after 8 weeks of treatment and found that 73 % of the enrolled subjects did not show any conversion to favorable CTC values. This result paralleled the lack of antitumor activity evaluated by PSA and radiographic response and OS [77]; even in this case, the exiguous number of subjects

analyzed (11 patients) did not allow a comparison in terms of survival between patients with and without a CTC response. Lee et al. [78] performed a phase I trial testing the MET/vascular endothelial growth factor receptor (VEGFR)-2 inhibitor cabozantinib in CRPC and measured CTCs as a marker to monitor clinical benefit as their secondary endpoint; CTCs were evaluated every 3 weeks through week 15 of treatment. The authors reported that 11 of 12 patients with unfavorable baseline CTC numbers (92 %) experienced a >30 % reduction in CTC counts, and 58 % of them converted from unfavorable to favorable CTC levels. Subjects with conversion experienced a higher response rate and a longer median time on study; the reported results strongly suggest the prognostic role of CTC assessment. Shamash and colleagues [79] evaluated the clinical benefit of high doses of the alkylating agent melphalan in CRPC patients and found that patients who experienced a CTC count drop below 5/7.5 ml had a significantly longer OS (30.6 vs. 15.3 months; $p = 0.03$); very interestingly, the CTC conversion happened within 2 weeks from the beginning of the therapy and faster than a concomitant PSA decline, suggesting that CTC evaluation could be helpful to promptly target such a toxic approach only to CTC-responding patients. Most recently, Goldkorn et al. [80] prospectively analyzed the prognostic value of CTC enumeration in 212 evaluable CRPC patients receiving first-line docetaxel-based therapy within a large phase III clinical trial (SWOG S0421). The authors showed that unfavorable CTC numbers correlated with a shorter median OS, with an HR of 2.74 for 2-year survival ($p < 0.001$); furthermore, a rise in CTC numbers after only one cycle of chemotherapy was associated with a significantly worse OS. The reported data are enlightening, as changes in CTC counts could lead to an early change in the management strategy. Overall, these results are strongly promising, underlining the prognostic role of CTC counts in CRPC patients, as previously indicated in other studies; further analyses need to be carried out to statistically evaluate the role of CTC numbers as a surrogate endpoint for OS.

CTC enumeration has also been evaluated as a treatment-efficacy indicator and a surrogate marker of response to treatment. Scher et al. [81] assessed the antitumor activity of the AR antagonist enzalutamide in a phase I–II clinical trial and monitored CTCs before and during treatment. The authors noted a conversion from unfavorable CTC counts to favorable in 75 % of the chemotherapy-naïve and in 37 % of chemotherapy-pretreated CRPC patients, with a concurrent but not always consistent decrease in PSA levels. Anand et al. [82] evaluated CTC numbers in chemotherapy-naïve CRPC patients receiving enzalutamide and reported that CTC count at 12 weeks was a significant predictor of time to radiographic progression. Reid et al. [83] analyzed the role of CTC numbers as a marker of treatment response in a phase II clinical trial evaluating the cytochrome P450 (CYP)-17 inhibitor abiraterone in docetaxel-pretreated CRPC patients. The authors observed a decrease in CTC counts to <5/7.5 ml in 41 % of the patients and a significant correlation between CTC decrease and PSA level decline in the ERG-gene rearranged tumors. However, a significant correlation between the CTC numbers and traditional markers of response was not observed. Recently, Scher and colleagues [84] evaluated the potential role of CTC enumeration as a biomarker predictive of response after abiraterone treatment in the context of the COU-AA-30 phase III clinical trial; the authors assessed CTC counts after 12 weeks of treatment and reported that individuals with <5 CTCs/ 7.5 ml in conjunction with normal levels of lactate

dehydrogenase (LDH) had a 2-year survival of 46 % compared with 2 % for patients with >5 CTCs and high levels of LDH.

One limitation of the studies described above is the use of CellSearch® to isolate CTCs. This technology may not detect a fraction of EpCAM-negative circulating cells possibly relevant for tumor response to therapy and disease progression. An evaluation of the entire CTC population may be more informative; thus, implementation of newer technologies able to isolate CTCs based not only on Ep-CAM expression is warranted. Yu et al. [15] reported that CTCs can show an epithelial or a mesenchymal profile in metastatic breast cancer patients and that the fluctuation of numbers of each specific subgroup correlated with response to treatment, with CTCs harboring a mesenchymal molecular profile associated with disease progression. Therefore, the presence and the clinical value of CTCs with EMT characteristics in PC needs to be extensively investigated and several evidence has been reported in the literature supporting the presence of this subpopulation of CTCs in PC patients. Armstrong and colleagues [85] showed that CTCs with mesenchymal and stem cell-like characteristics can be isolated from 100 % of CRPC patients and that 84 % of the isolated CTCs co-express epithelial and mesenchymal markers; however, the authors used CellSearch® to isolate CTCs in their analysis and the EpCAM-based approach could have significantly limited the reported evaluation, reducing the identification of the mesenchymal fraction of CTCs. In addition, Chen and colleagues [86] demonstrated that CTCs isolated from CRPC patients express EMT-related genes at a significantly higher rate than castrate-sensitive PC patients; nevertheless, the patient sample analyzed in this study was very small and only a minor fraction of the isolated CTCs was subsequently evaluated for EMT-related gene expression profile. Despite these preliminary observations supporting the evidence of a mesenchymal subpopulation of CTCs in PC, its clinical relevance and prognostic role is still to be elucidated. The biological and clinical significance of this subclass of mesenchymal CTCs cannot be disregarded and should be investigated in CRPC patients in order to validate all CTCs as prognostic and predictive markers in clinical practice.

4 CTC Molecular Interrogation to Draw the Biological Portrait of the Disease

Although simple CTC enumeration has been identified as a potential surrogate marker for prognosis in PC, using CTCs to characterize and evaluate the expression, mutation, and dysregulation of key molecular drivers for PC is expected to add useful information for clinical practice. A list of some examples follows.

4.1 The Androgen Receptor (AR)

The androgen signaling axis is one of the major drivers in PC development and progression, and it is often activated in men with CRPC [2]. Consequently, AR is the final target of several FDA-approved treatments for hormone-sensitive and hormone-resistant PC patients (e.g. abiraterone and enzalutamide). The possibility of closely monitoring AR expression levels, mutations, and signaling activity may help us to better select patients to treat with AR-targeted therapies and monitor the molecular effect of the drug. CTCs offer a unique source of patient-derived tumor cells to investigate these aspects, by both functional and molecular biology assays.

In an initial attempt to evaluate AR status in CTCs, Shaffer et al. [87] assessed AR gene copy number by fluorescence in situ hybridization (FISH) in CTCs isolated from CRPC patients using the CellSearch system and found AR amplification in 56 % (5/9) of the patients with >5 CTCs/7.5 ml of blood. Similarly, Leversha and colleagues [88] found AR amplification in CellSearch®-isolated CTCs from 30 of 49 PC patients evaluated, confirming Shaffer's observation. Overall, these results proved the feasibility of AR status analysis in CTCs, even though high heterogeneity of AR gene copy gain among CTCs from the same individual has been recently reported [89]. The biological role of AR amplification in CTCs, its correlation with AR status in the primary tumor and the correlation with response to anti-androgen treatment still need to be clinically evaluated.

Recently, Miyamoto et al. [35] analyzed AR signaling activity in CTCs isolated from PC patients using EpCAM-based CTC chip to isolate CTCs and immunofluorescence to molecularly characterize them. They found that the AR pathway is active in treatment-naïve PC patients, becomes inactive following ADT, and may be reactivated at the time of progression to CRPC, when high heterogeneity in AR signaling was observed. This suggests that other pathways besides AR reactivation may also contribute to CRPC progression. These results support the importance of CTCs as dynamic tumor-derived biomarker that can reflect 'real time' effects of cancer drugs on their therapeutic targets. In this regard, Tagawa et al. [90] are capturing CTCs in PC patients undergoing taxane treatment in the context of an ongoing prospective, randomized phase II clinical study (TAXYENERGY, NCT01718353). As it has been previously shown, AR utilizes microtubules (MTs) to traffic into the nucleus, and its signaling is impaired by MT-targeting drugs such as docetaxel, which act by keeping AR inactive in the cytoplasm downstream of MT stabilization [91–93]. Along these lines, Darshan et al. [92] showed significant correlation between AR cytoplasmic sequestration in CTCs isolated from CRPC patients and clinical response to taxane treatment in a small pilot study. In addition, recent data have shown that expression of the clinically relevant AR splice variant ARv7, which lacks the ligand-binding domain (LBD) and the MT-binding domain, confers taxane-resistance in both in vitro and in vivo models [94]. The TAXYENERGY trial will isolate CTCs at several time points during taxane treatment and will assess AR subcellular localization and MT network pattern in CTCs as a readout of taxane activity. CTC molecular response to treatment will be monitored and correlated with clinical benefit (Fig. 1c). Moreover, RNA will be extracted from the isolated CTCs to perform AR sequencing and dissect the clinical role of AR variants at the onset of taxane resistance. At the completion of the study, the results should give us important information on the clinical relevance of the AR variants and the interaction with taxane-based treatment outcome. Further clinical validation of these biomarkers within larger prospective clinical trials will eventually clarify their clinical application.

4.2 ERG

The v-ets erythroblastosis virus E26 oncogene homolog, ERG, is a transcriptional factor belonging to the proto-oncogene ETS family. ERG is rearranged in approximately half of primary PCs, leading to the formation of a fusion gene between the 5' end region of the androgen-regulated *TMPRSS2* gene and an exon in the *ERG* gene [95, 96]. The resulting fusion gene is androgen-regulated and mediates high-level expression of a transcriptionally

active, N-terminal-truncated ERG protein (amino acids 1–44 being deleted in the most common fusion). This fusion is an early event in PC progression, as it is found in precursor prostatic intraepithelial neoplasia (PIN) lesions and is also highly expressed in CRPC [97, 98]. Attard et al. [89] isolated CTCs from CRPC patients using CellSearch and analyzed the captured cells to investigate the correlation between ERG, AR, and the *PTEN* gene with abiraterone therapy. Reporting a significant correlation between ERG rearrangement assessed in CTCs and PSA decline in abiraterone-treated CRPC patients, the authors reported that CTCs could be used to molecularly identify biomarkers useful to select and target active treatment strategies. Recently, ERG rearrangement has also been correlated to taxane treatment resistance in CRPC [99] and has also been useful in combination with other molecular markers in diagnosing and assessing neuroendocrine PC [100]. Thus, the ability to monitor ERG status in CTCs may allow personalized treatment in an individual patient. Even though provocative and exciting, these results are only hypothesis generating, and further clinical evaluation of ERG assessment in CTCs and its role as predictive biomarker for response to therapy still need to be performed.

4.3 Steroidogenesis Enzymes

Intratumoral androgen production is one of the mechanisms that tumor cells activate in response to ADT and that can mediate progression to CRPC [101, 102]. Recent findings have shown that steroidogenesis enzymes (such as *SRD5A1*, *SRD5A3*, and *AKR1C3*) and AR are overexpressed in PC tissues relative to normal prostate tissue, while the expression of enzymes that inactivate dihydrotestosterone (DHT) (such as *SRD5A2*, *CYP3A4*, *CYP3A5*, and *CYP3A7*), is decreased [103]. Mitsiades et al. [103] showed high interpatient heterogeneity in the expression of these transcripts in CRPC patients, suggesting that the autocrine activation of the androgen synthesis axis may occur by different mechanisms. Using FicolIPlaque followed by FACS, the authors also showed that the levels of these enzymes could be confidently assessed in CTCs, allowing the monitoring of potentially druggable targets, depending on which enzyme/s are responsible for the dysregulation. CTCs could be used to molecularly track disease progression and potentially serve as a basis for individualized therapy as they may help clinicians to choose between different drugs that specifically target the altered enzymes in individual patients.

5 Conclusion

Over the last decade, CTCs have proven a promising tool with the potential to change clinical practice. Numerous technologies have been used to isolate and analyze CTCs from PC patients. Despite much success, each of these approaches has limitations, mostly represented by the difficulty of isolating all CTC subpopulations due to their physical and biological heterogeneity. Thus, the ideal platform for the capture of CTCs in PC is still to be identified. CTC enumeration has been consistently correlated with clinical outcome in PC and is progressively tested and used in clinical practice. Nevertheless, routine CTC incorporation within the PC field has yet to be adopted by most clinicians. CTC evaluation has significant potential as a reliable non-invasive source of PC cells and as an alternative to dangerous and unrealistic serial biopsies. CTCs not only would give us insights into the molecular characteristics of the disease in the single patient but would also allow real-time

monitoring of the biological changes to which the tumor is subjected during progression and selective pressures of therapy. Ultimately, CTCs will likely find their way into clinical practice and provide information to better drive drug selection and eventually improve clinical outcome.

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Key Points

Different approaches have been reported in the literature to isolate and subsequently molecularly analyze circulating tumor cells (CTCs) from prostate cancer (PC) patients, based on selective physical and biological properties that characterize this cell population.

During the last 10 years, the prognostic value of CTC numbers has been widely evaluated and validated in prostate cancer; current efforts are undertaken to assess the role of CTCs as a predictive marker of response to therapy and their potential application in the diagnosis of early-stage PC.

CTC molecular analysis can open new scenarios to study the characteristics of PC in the single patient; being able to follow-up longitudinally the biological changes of the disease during the treatment could give us the tools to better tailor the therapy to the molecular portrait of the disease.

Fig. 1.

a Picture of the GEDI microfluidic device. **b** 10× magnification immunofluorescence mosaic image reconstruction of the GEDI chip where the geometric distribution of the microposts is clearly discernable; bar 2,000 μm. **c** 63× magnification images of CTCs isolated from a representative CRPC patient, before (**a–f**) and after (**g–l**) taxane treatment. **Blue** DAPI (**a, g**); **red** cytokeratin (**b, h**); **yellow** CD-45 (**c, i**); **green** AR (**d, j**); **purple** tubulin (**e, k**); merge (**f, l**). CTCs were defined as CK⁺/CD-45⁻/DAPI⁺ cells. Before treatment, AR localization is mainly nuclear, as expected (**d, f**), and tubulin presents with a diffuse pattern, representative of the typical intricate micro-tubule network (**e**). After taxane treatment, AR is sequestered into the cytoplasm (**j**, *arrowhead*) consequent to drug activity, as shown by the presence of microtubule bundling (**k**, *arrow*). *Dashed line* micropost edge. *Bars* 5 μm. Images courtesy of Matt Sung, Ph.D. *AR* androgen receptor, *CRPC* castration-resistant prostate cancer, *CTC* circulating tumor cells, *GEDI* geometrically enhanced differential immunocapture

Table 1

Principle techniques to isolate circulating tumor cells from prostate cancer patients

Method	Approach	Sample volume (ml)	CTC detection rate	Purity	References
Ficoll-Paque®	Density-based centrifugation	20	79 % (19/24) of aPC pts	NA	Fizazi et al. [16]
ISET®	Size-based microfiltration	7.5	75 % (15/20) of PC pts	NA	Farace et al. [21]
HD-CTC assay	Size-based identification	8	80 % of PC pts (mean: 92.2 CTCs/ml)	NA	Marrinucci et al. [23, 25]
Dielectrophoresis	Electrical property-based separation	0.5–4.5	76–83 % of PC cells spiked in PBS recovered	74–82 %	Gascoyne et al. [30, 32], Huang et al. [32]
Leukocyte depletion	Anti-CD-45 immunomagnetic negative selection	2	77 % (10/13) of PC pts mean: 26 CTCs/ml	NA	He et al. [33]
CellSearch®	Anti-EpCAM immunomagnetic positive selection	7.5	>5 CTCs/7.5 ml in 57 % (69/120) of CRPC pts median: 9 CTCs/7.5 ml	1–10 %	Damila et al. [34], Allard et al. [38]
MagSweeper	Anti-EpCAM immunomagnetic positive selection	7.5	63 % of PC cells spiked in blood recovered	10 %	Cann et al. [39]
CTC chip	Anti-EpCAM microfluidic device	2.7	>5 CTCs in 100 % (19/19) of PC pts, mean: 86 CTCs/ml	49 %	Nagrath et al. [40]
Herringbone chip	Anti-EpCAM microfluidic device	5	CTCs in 93 % (14/15) of PC pts, mean: 63 CTCs/ml	14 %	Stott et al. [41]
CTC iChip	Antibody-independent microfluidic device	10	>0.1 CTC/ml in 90 % (37/40) CRPC pts, mean: 50.3/ml	7.8 %	Ozkumur et al. [42]
GEDi-chip	Anti-PSMA microfluidic device	1	CTCs in 90 % of PC pts, mean: 54 CTCs/ml	62 %	Gleghorn et al. [44], Kirby et al. [45]
Selectine-coated microtubes	CTC adhesion behavior	7.5	85 % (23/27 PC pts)	66 %	Gakhar et al. [50], Hughes et al. [51]
EPISPOT	CTC secretive properties	20	PSA-secreting CTCs in 83 % (20/24) of PC pts; median: 24 CTCs/ml	NA	Alix-Panabieres et al. [54, 55]

aPC advanced prostate cancer, CRPC castrate-resistant prostate cancer, CTC circulating tumor cells, EpCAM epithelial cell adhesion molecule, EPISPOT Epithelial Immuno SPOT assay, GEDI geometrically enhanced differential immunocapture, HD high definition, NA not available, PBS phosphate buffered saline, PC prostate cancer, PSA prostate-specific antigen, PSMA prostate-specific membrane antigen, pts patients

Table 2

Circulating tumor cells as prognostic marker and potential candidate for surrogate of survival in clinical trials

Clinical trial	Drug(s)	Method	Results	References
Phase III	Abiraterone	CellSearch®	CTCs <5 at 12 weeks associated with better 2-year survival	Scher et al. [84]
Phase III	Enzalutamide	CellSearch®	CTC status at 12 weeks predicted time to radiographic response	Anand et al. [82]
Phase III	Docetaxel	CellSearch®	CTCs >5 at baseline associated with better median and 2-year OS	Goldkorn et al. [80]
Phase II	Satraplatin + bevacizumab	CellSearch®	CTCs >5 associated with worse OS	Vaishampayan et al. [76]
Phase II	Temsirolimus	CellSearch®	Lack of CTC conversion to favorable counts during tx matched lack of clinical activity	Armstrong et al. [77]
Phase I	Cabozantinib	CellSearch®	Higher response rate in pts with favorable CTC count conversion	Lee et al. [78]
Phase I	Melphalan	CellSearch®	CTCs <5 after tx associated with longer OS	Shamash et al. [79]

CTC circulating tumor cells, OS overall survival, *pts* patients, *tx* treatment