



Review

Applications of circulating tumor cells for prostate cancer



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Abstract One of the major challenges that clinicians face is in the difficulties of accurately monitoring disease progression. Prostate cancer is among these diseases and greatly affects the health of men globally. Circulating tumor cells (CTCs) are a rare population of cancer cells that have shed from the primary tumor and entered the peripheral circulation. Not until recently, clinical applications of CTCs have been limited to using enumeration as a prognostic tool in Oncology. However, advances in emerging CTC technologies point toward new applications that could revolutionize the field of prostate cancer. It is now possible to study CTCs as components of a liquid biopsy based on morphological phenotypes, biochemical analyses, and genomic profiling. These advances allow us to gain insight into the heterogeneity and dynamics of cancer biology and to further study the mechanisms behind the evolution of therapeutic resistance. These recent developments utilizing CTCs for clinical applications will greatly

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impact the future of prostate cancer research and pave the way towards personalized care for men.

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1. Introduction

Prostate cancer (PCa) remains one of the most prevalent cancers affecting men across the globe. In 2012, PCa was the second most common cancer in men with more than 1.1 million new cases, accounting for around 8% of all new cancer cases and 15% in men. It was also noted to be the fifth prominent cause of cancer-related mortalities in men globally with more than 307,000 deaths [1].

The understanding of PCa biology has been advanced with tremendous amount of data from molecular studies, generating new classification schema [2,3], and prognostication tools [4] that go beyond the Gleason grading and TNM staging systems. It is anticipated that these advancements will reshape our therapeutic approaches for PCa. Tissue samples taken during prostatectomy and core needle biopsies are now being utilized for molecular profiling, driving many of these advances. These specimens provide information representing historic and often untreated cancers. There have also been autopsy series that have shown a very different molecular portrait of PCa and in particular, metastatic-castration resistant PCa (mCRPC). Profiling from these autopsies has provided further insight into the temporospatial heterogeneity that underlies advanced cancer. This has raised questions about the dynamic and evolving biology of mCRPC in the interval between diagnosis and death, which is currently under investigation in centers where tissue-oriented studies have been conducted. The typical clinical practice in caring for men with PCa has not involved routine tissue biopsies. This poses a challenge for translational research efforts as such material is needed to conduct molecular characterizations over time. The challenge in obtaining metastatic tissue samples in mCRPC mainly lies in its typically osteotropic nature resulting in the formation of osteoblastic lesions. The vast majority of patients are not inclined toward having serial bone biopsies which may require specialized instrumentation such as drills to cut through osseous metastases. Additionally, sampling viable tissue samples from osteoblastic lesions requires experienced interventional radiologists with specialized expertise in obtaining samples from bone and other sites that are useful for analysis. Even with the highest levels of expertise, these methods will tend to yield usable samples in only 60%–70% of cases. Finally, the process of removing or decalcifying pieces of bone from biopsies or bone marrow aspirates, places relevant and important information about the tumor at risk for loss or deterioration.

In contrast to this, blood analysis has been a standard of practice in this clinical setting. Clinical phlebotomy is simple enough that frequent blood draws can be used to measure changes in serum prostate specific antigen (PSA) concentration. For decades, serum PSA concentration has been utilized to monitor the therapeutic efficacy of various

interventions including surgery, chemotherapy, androgen-receptor (AR) inhibition, and radiotherapy. It is known, however, that changes in PSA do not predict clinical benefit for other types of treatments such as immunotherapy and targeted radionuclide therapy. Decisions made solely based on serum PSA concentration changes early on in therapy when patients are clinically well are cautioned against in the recommendations provided by Prostate Cancer Working Group 3 [5]. Nonetheless, it is apparent that blood-based observations of PCa patients can be readily obtained, which has led to greater interest in developing similar tests that could complement PSA measurements and overcome some tumor biopsy limitations [6,7].

The unmet need for new blood-based tests also stems from the restrictions in monitoring anaplastic or atypical carcinomas in the prostate gland, which have absent or low levels of PSA production. A distinctive feature common to these aggressive variants, is the potential for developing visceral metastases (VM), which often times progresses rapidly and lead to end organ failure and death. It is widely recognized that serum PSA measurements lacks predictive capacity in many mCRPC cases with VM, which tend to be less dependent on AR activation and are more resistant to conventional AR-targeted therapies [8–10]. In addition to this limitation, routine monitoring of metastases in soft tissue organs is conducted far less frequently in PCa patients, which leads to later diagnoses of VM events. This is further complicated by the observation of an increasing incidence of VM events that coincides with the more frequent and early use of potent AR-inhibitors [8]. Thus, a blood-based tool capable of monitoring the behavior of these cancers would be an ideal way to address this unmet need.

Circulating tumor cells (CTCs) may provide an important means of addressing the limitations facing clinicians caring for men with PCa. CTCs are a rare population of tumor cells that circulate in the blood stream after detaching from a primary tumor and/or metastatic lesions. Compared to the 10 hematologic cells found in 1 mL of whole blood [9], there are usually less than 100 CTCs found in the same blood volume [11–13]. This creates challenges in conducting molecular characterization of CTCs. In order to address this issue, numerous methods and technologies have been developed for isolating and studying CTCs. These initiatives are uniting the basic and clinical sciences together allowing for the possibility of real-time dynamic profiling of disease progression and better care for PCa patients.

2. Early clinical application of CTCs: enumeration

In 2008, the FDA cleared the CellSearch™ System (Veridex) for the enrichment and enumeration of CTCs in mCRPC

patients. This system separates CTCs from other circulating blood cells based on the expression of the epithelial-cell adhesion molecule (EpCAM) by most epithelial cells, including malignant epithelial cells. The EpCAM-expressing hematologic cells are fixed and then mixed with magnetic beads conjugated with anti-EpCAM. Thus, any cell expressing EpCAM attached to the beads could be isolated and harvested via magnetism. The captured cells are subjected to immunofluorescent antibody labeling for cytokeratin (CK)-8, CK-18, CK-19, and the common leukocyte antigen (CD45), and identify CTCs as CK+/CD45-nucleated events.

The IMMC38 study [14] evaluated the performance of this CTC enumeration assay in mCRPC patients starting a new line of cytotoxic chemotherapy. Prior to starting therapy, patients with unfavorable CTC counts (>5 CTCs/7.5 mL) had shorter overall survival (OS) (11.5 vs. 21.7 months; hazard ratio (HR) 3.3; $p < 0.0001$). Post-treatment CTC counts that were unfavorable also predicted shorter OS (6.7–9.5 vs. 19.6–20.7 months; HR 3.6–6.5; $p < 0.0001$). At baseline, patients with CTC counts, which converted from unfavorable to favorable, had improved OS from 6.8 to 21.3 months. Patients with CTC counts that converted from favorable to unfavorable had poorer OS than those who remained with favorable CTC counts (9.3 vs. 26 months).

Therefore, it is shown that CTC enumeration can provide prognostic insight to disease progression in patients and is a better predictor of OS than serum PSA changes. While these efforts have been important in establishing the clinical importance and utility of CTCs, the field continues to evolve with greater needs and possibilities. Newer CTC-technologies are being developed that address emerging concepts resulting from the evolution of technology and cancer biology creating newer applications for CTCs in PCa.

3. New applications of emerging CTC technologies

Improvements in CTC-capture methods have created opportunities to advance our understanding of dynamic cancer biology. Along with the constant process of shedding and invasion/extrusion into the vasculature, CTCs exist in a dynamic equilibrium with the underlying tumor. This association between tumor and CTCs may provide a minimally invasive way of characterizing cancers and may make CTCs an important alternate source of cellular information.

3.1. Morphologic phenotyping of CTCs

Several of the emerging CTC identification systems utilize more advanced microscopy and imaging techniques to record variations in cellular morphology. In classical cytopathology, differences in morphology are related to functional and biological differences among cells which make these studies informative for characterizing CTCs. One group reported that CTCs not only range in size, but they also form clusters ranging from 2 to 50 cells which were shown to be precursors of metastases in breast cancer [15]. These CTC clusters were also found to contain tumor cells mixed with not only other tumor cells but other

hematopoietic cells such as leukocytes. A retrospective analysis was conducted by our group on a range of patients with clinical PCa that have a variety of metastatic burdens and disease sites. Upon analysis on the distribution of cellular features, nuclear sizes were associated with the behavior of mCRPC. In one particular case, the presence of VM in mCRPC patients was associated with CTCs that have nuclei smaller than 9 μm [16]. Several groups are now describing variations in cellular and nuclear morphology in CTCs using modern shape characterizations going beyond simple diameter descriptions. As histopathology has repeatedly linked both cellular and nuclear shape to function and biology, it is anticipated that several of these studies will provide insight into the biology present within the CTC compartment.

3.2. Biochemical analysis of CTCs: CTC-based biomarkers

Molecular classification of tumors has become the focus of strategies geared toward personalization of oncologic care. Access to contemporary tissue with the ability to perform repeated sampling limits the ability to conduct dynamic classification of cancers that evolve over time and in the face of therapy. This is particularly important in PCa where natural histories can run as long as 10–15 years. Many have begun to look to CTCs as a means of obtaining a liquid biopsy that can yield information equivalent or possibly superior to traditional core needle or surgical biopsies.

An example of success in studying biology specific to CRPC lies in the work done looking at AR splice variants. These alterations of the AR hold functional importance has evoked the interests of many in the field of AR biology. In preclinical models investigating the efficacy of contemporary AR-targeted therapies such as abiraterone, enzalutamide, galeterone, apalutamide and others, certain AR splice variants such as AR-V7 have been associated with altered or diminished anti-cancer effect [17,18]. In a prospective clinical study, Antonarakis et al. [19] characterized AR-V7 expression in CTCs from patients receiving abiraterone and enzalutamide therapies. By using an adapted AdnaGen assay, AR-V7 expression levels were measured in mCRPC patients and AR-V7 expression was found to be associated with a greater likelihood of developing AR-targeted therapy resistance. Since then, the study has expanded to 200 patients classified as CTC (+) or (–); and CTC (+), AR-V7 (+) or (–) [20]. The emergence of a new CTC-based biomarker could significantly aid physicians in selecting between taxanes [21] and AR-targeted therapy. The AR-V7 CTC assay was part of the ARMOR study intended to measure the activity of galeterone in an AR-V7 expressing population prospectively. This trial was closed, however, due to poor accrual. It was hoped that this study would prospectively validate this biomarker, but as it stands alternative validations will be required.

The Johns Hopkins studies described above and other studies following in their footsteps, have started to bring to light the potential clinical applications of CTC-based biomarkers. Among the pool of CTCs are subsets of actively metastasizing cells that have the capacity to re-invade and colonize secondary sites. Chu and Chung et al. [22–24]

identified a subpopulation of CTCs they have called metastasis initiating cells (MICs), which possess the ability to recruit and reprogram dormant cells to become active metastasizers. These rare findings of MICs and AR-splice variants in CTCs, point toward the need to investigate the genomic and transcriptomic content of these cells.

Recently, many modern genomic tools have been adapted to study CTCs. Among these techniques are next-generation sequencing technologies which now use picograms of genomic material appropriate for CTC analysis [25]. Additionally, there are contemporary RNA characterization methods which include fluorescence-labeled oligonucleotide hybridization [26], whole transcriptome RNA microarrays [27], and single cell RNA sequencing [28]. Lastly, genome amplification and single cell DNA sequencing is now possible [29,30] although it has the potential for creating PCR-related errors and preferential amplification of specific regions [31].

Despite the growing field for the biochemical analysis of CTCs, there remains a need to determine the degree to which CTC biology reflects the underlying tumor biology. In one study, Magbanua et al. [32,33] demonstrated that CTCs possess similar copy number variations (CNVs) reflective of the primary tumor. However, CNV measurements in CTCs is not accurate enough to provide definitive proof of biological relatedness in disease progression. Studies have also point towards targeted sequencing to compare single nucleotide variances in CTCs and tumor tissues, detecting mutations in oncogenes such as *TP53* and *BRAF* [34,35]. Our group set out to explore the relationship between CTCs and tumor tissue samples by performing whole exome sequencing on single CTCs and corresponding tissue biopsies [36]. With this approach, our group [37] and Lohr et al. [38] independently reported many genomic similarities between PCa tissue samples and single CTCs.

As more biochemical features of CTCs grow, more studies are showing strong molecular similarities between CTCs and tumor tissues. Therefore, it is proposed that CTCs may provide another dimension for studying cancer biology: dynamics. The ability to obtain temporally discrete and frequent information will pave the way for us to understand the mechanisms underlying PCa in terms of heterogeneity and drug resistance.

3.3. Heterogeneity and dynamic biology

Encompassing the realms of PCa and other malignancies, heterogeneity is an indicative factor in tumor biology. This existing diversity can be widely observed among tumor cells (intratumoral), within tumors in a single patient (intertumoral) [39], and between patients (interpatient) [40,41]. The evolution of heterogeneity spans over time and physical space (temporospatial) and has been suggested to influence disease progression and promote drug resistance [42].

Many studies of cancer biology heavily rely on tumor specimens including multi-site sampling of fresh resected tissues. Lesions that appear in soft tissues increase the susceptibility of certain cancers, thus PCa and CRPC tend to be less suitable to needle biopsies. As a result, serial liquid biopsies will provide a novel approach to study the dynamic

aspects of tumor heterogeneity. In one particular case, transcriptomic analysis was performed in CTCs using AR gene mutations and splicing variants, showing high degree of heterogeneity among the cells [28].

The addition of temporal information to the relatively rapid blood-based sampling of cancers will supplement CTC studies and provide greater insight to the pathways involved in the evolution of disease and resistance to therapy. Miyamoto et al. [28] demonstrated this when they conducted a CTC profiling study focused on the development of resistance upon initial castration. These investigators found an association between the onset of castration resistance with the activation of noncanonical Wnt signaling. Analysis of serially collected CTCs in other studies also confirm that CTC-derived biological information vary over time [16,37,43].

4. Conclusion

In PCa, CTCs are clinically useful prognostic biomarkers for response and benefit to therapy and may augment information obtained from serum PSA measurements. CTC enumeration is an important application that emerged from the earlier and even more modern developing CTC platforms. Newer technologies are capable of adding to the information that can be obtained by further examining CTCs utilizing methods currently used for tissue biopsies.

As the clinical care of men with PCa become more personalized, the ability to conduct real time and dynamic characterization of the disease will become increasingly important. Both the advancements in the basic sciences and the rising costs of therapies have pointed towards this type of treatment. Thus, the access to a minimally invasive procedure for tissue sampling may revolutionize the care of men with PCa.

Recently, there has been a growing interest in the use of blood components from liquid biopsies. Circulating tumor DNA (ctDNA) extracted from cell-free plasma has been one of the most studied components. However, the nature of PCa suggests that DNA profiling may not provide as useful information as it may in other mutation-derived malignancies. In contrast to other common solid tumors, the DNA mutation rate in PCa is relatively low [3,44]. *SPOP* has been identified as one of the most frequently mutated genes in PCa and still has a mutation rate under 15% [44]. However, research on the transcriptome of PCa, particularly in mCRPC, has shown more promising results. Thus, finding ways of characterizing delicate and disease-related alterations in RNA remain important to the field. That being said, plasma and cellular components of blood samples are not mutually exclusive and may serve as effective supplementary tests in the future.

The future of modern oncology relies greatly on the advancements of genomic medicine and liquid biopsies. More notably, they are critically important in a disease such as PCa where mortality continues to be an issue and where current approaches fail to unite modern biology and clinical practice. However, the timely advances of CTC isolation technologies along with molecular profiling have demonstrated that CTC research can merge these two disciplines together. As a result, this could synergistically allow for

better characterization of the dynamic nature of PCa and other malignancies. By gaining insight into the changes in biological behavior and the heterogeneity in disease progression, a physician may be better able to navigate therapies and understand the emergence of therapeutic resistance.

Newer developments in the area of CTC research in PCa and other cancers have raised important questions and challenges. Due to the rarity of cellular events and the precision of measurements made, standards of collection and analysis will need to be established. Given the wide range of CTC-isolation approaches, it is unlikely that a single cross-platform approach will ever be or should be established. For the optimization of each developing platform, it is important to understand the volume of blood and/or numbers of tumor cells required to conduct comparisons. Moreover, the timing of blood-based biopsies most likely need to vary with the rate of disease progression since the frequency of sample collection may depend on the analyte of interest and the methodology used. While these factors may represent some of the technical issues observed, they will not substantially hinder the progress of this important field of biomedical research.

CTCs are now a part of a repertoire of tools used in the standard practice of modern oncology. Clinical applications for CTCs are now ready to go beyond enumeration and help shape the future of cancer research and personalized oncology. In doing so, CTC-based approaches will certainly have a significant impact on the care of men with PCa.

Conflicts of interest

The authors declare no conflict of interest.

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