Circulating Tumor Cells in Prostate Cancer: Beyond Enumeration

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Abstract: Circulating tumor cells (CTCs) are a population of rare cancer cells that have detached from the primary tumor and/or metastatic lesions and entered the peripheral circulation. Enumeration of CTCs has demonstrated value as a prognostic biomarker, and newer studies have pointed to information beyond enumeration that is of critical importance in prostate cancer. Technologic advances that permit examination of the morphology, function, and molecular content of CTCs have made it possible to measure these factors as part of liquid biopsy. These advances provide a way to study tumor evolution and the development of resistance to therapy. Recent breakthroughs have created new applications for CTCs that will affect the care of patients with prostate cancer.

Introduction

Prostate cancer (PC) continues to be the most common cancer affecting men in the United States and was the second leading cause of cancer death in 2016. This translates to 220,000 new diagnoses and 27,500 deaths[1]—figures that are unacceptable. Tremendous advances have been made in our understanding of this disease, and recent data are leading to new classification schema[2,3] and prognostication tools[4] that are based on molecular characteristics. These advances ultimately will result in the development and implementation of therapeutic strategies that will further reshape our approach to prostate cancer. Nonetheless, the clinical classification of PC in 2016 has not evolved significantly beyond Gleason grading and TNM (tumor, node, metastasis) staging.

Most of the advances in PC research stem from molecular profiling of tissue samples obtained from prostatectomy and core needle biopsies. As such, the bulk of the information is derived from historical and relatively untreated tissue. Even now, the current standard of practice in PC (in contrast to that for other solid and liquid malignancies) does not involve regular tissue biopsy for the molecular characterization of disease over time. Through carefully planned clinical studies involving tissue biopsies and a limited number of autopsy series, scientists in the field have gained biological insight into metastatic castration-resistant PC (mCRPC) at the time of death. However, few centers conduct tissue-oriented studies during the interval between diagnosis and death to investigate the dynamic and evolving biology of mCRPC in the face of newer therapies. Organizations such as the National Cancer Institute, the US Department of Defense, and the Prostate Cancer Foundation have supported important efforts to address this need.

Obtaining tumor tissues from patients with mCRPC is particularly challenging because most bone lesions are osteoblastic. First, most patients do not wish to undergo serial bone biopsies, especially those requiring significant instrumentation, such as drills for cutting through osseous metastases. Second, many interventional radiologists lack experience in sampling viable tumor cells from osteoblastic lesions. These procedures yield useful samples in approximately 60% to 70% of cases. Third, bone biopsies or bone marrow aspirates include solid pieces of bone that must be removed or decalcified, putting the valuable information within tumor cells at risk for loss or degradation during processing.

In contrast to the difficulties with tissue biopsy, blood-based characterization of disease behavior is fairly simple and extremely common in PC. Frequent measurement of serum prostate-specific antigen (PSA) levels is standard. Measurement of PSA has been used for decades to monitor responses to a variety of therapies, including surgery, radiation, androgen receptor (AR) inhibition, and even taxane-based chemotherapy. For certain forms of therapy, such as radionuclide therapy and immunotherapy, PSA changes have been less useful to predict therapeutic benefit. The Prostate Cancer Working Group 3 cautions against making decisions based solely on changes in serum PSA concentration early in therapy, when patients are clinically well.[5] Use of serum PSA exemplifies the ease of blood-based monitoring. This generates interest in developing other blood tests to overcome the limitations of tumor biopsies.[6,7]

Another important limitation of conventional approaches based on blood PSA is the inability to monitor atypical or anaplastic carcinomas of the prostate gland that cause diminished or absent PSA production. An important feature of these aggressive variants is the potential for the development of visceral metastases (VMs). VMs often progress rapidly and culminate in end-organ failure and death. In some cases, mCRPC with VMs is less dependent on AR activation and therefore more resistant to conventional AR-targeted therapies.[8-10] It is recognized that serum PSA cannot predict the onset of these lethal events. Moreover, soft-tissue organs (eg, liver and lungs) are routinely monitored far less frequently in PC than in other malignancies. Without imaging of the viscera, VMs typically are found late in the course of the disease. With the increasing use of potent AR inhibitors, the frequency of VM events has also increased.[8] Thus, a blood-based tool for monitoring these cancers is an unmet need.

In contrast to that in many other common solid tumors, the DNA mutation rate in PC is relatively low.[2,11] One of the most frequently mutated genes in PC is *SPOP*, which has a mutation rate of less than 15%. Much of the research in PC, particularly in mCRPC, has focused on the transcriptome. Therefore, finding ways to characterize delicate and disease-related alterations in RNA remains important to the field.

Circulating tumor cells (CTCs) may provide a way to address limitations in the care of men with PC. CTCs, which are a population of rare cancer cells in the bloodstream, are either shed by or detached from the primary tumor and/or metastatic lesions. There are usually fewer than 100 CTCs in 1 mL of whole blood, compared with 10^9 hematologic cells in the same volume of blood.[12-14] Their extremely low abundance has made the detection and characterization of CTCs technically challenging. To address this issue, numerous technologies have been developed. These new methods for isolating and studying CTCs bring with them the hope of uniting the basic and clinical sciences to allow the real-time dynamic profiling of relevant biological information.

Enumeration: the Initial Clinical Application of CTCs

In 2004, the US Food and Drug Administration (FDA) cleared the first device for CTC enrichment and enumeration.[15] This system uses cell surface expression of epithelial cell adhesion molecule (EpCAM) to achieve cell separation. Magnetic beads conjugated with anti-EpCAM interact with fixed blood cells such that any EpCAM-expressing cells are coated with the beads and can later be separated out. Captured cells are released and stained for CK8, CK18, and CK19, as well as for leukocyte common antigen (CD45). CTCs are defined as CK+/CD45- nucleated objects. This device and approach have been used as an embedded biomarker in several therapeutic clinical trials (Table 1).

The first large-scale trial involving CTC enumeration was conducted in 2004 in patients with metastatic breast cancer. Here, enumeration of CTCs before and during therapy predicted both progression-free survival (PFS) and overall survival (OS).[16] In a very short period, similar studies were conducted in other cancer types, including colorectal cancer,[17,18] melanoma,[17] lung cancer,[18] and prostate cancer,[19,20] with similar findings.

In the IMMC-38 study (Circulating Tumor Cells and Survival in Hormone Refractory Prostate Cancer Patients Receiving Chemotherapy),[21] CTC enumerations were performed in patients with mCRPC who were starting cytotoxic chemotherapy. In this study, 231 patients were evaluated by serial CTC collection. CTC enumerations were defined as "favorable" if fewer than 5 CTCs were identified in 7.5 mL of blood, and as "unfavorable" if at least 5 CTCs were identified. The investigators found that patients with an unfavorable pretreatment CTC count had shorter OS (median OS, 11.5 vs 21.7 months; Cox hazard ratio [HR], 3.3; $P<.001$). Unfavorable CTC counts after treatment also predicted shorter OS (median OS, 6.7-9.5 vs 19.6-20.7 months; HR, 3.6-6.5; $P<.001$).

Table 1. Therapeutic Clinical Trials With CTC Enumeration by CellSearch Platform

Study/Publication	Trial Design	Interpretation of CTC Results
Prostate cancer		
Attard et al[106]	A phase 1/2 trial assessing abiraterone in chemotherapy-naive CRPC	CTC count was among the exploratory endpoints. The study showed that abiraterone can result in a CTC count decline.
TOPARP[23]	A phase 2 trial assessing response to olaparib in patients with mCRPC	Conversion of CTC count from ≥5/7.5 mL blood at baseline to <5/7.5 mL blood was one of the composite primary outcomes.
SWOG S0925[24]	A phase 2 trial comparing androgen deprivation + cixutumumab vs androgen deprivation alone in metastatic hormone-sensitive disease	CTC count was measured as a secondary outcome. The study showed that a lower baseline CTC count was associated with a higher rate of PSA decline.
Smith et al[107]	A phase 2 trial assessing cabozantinib in patients with chemotherapy-pretreated mCRPC	CTC count was one of the efficacy outcomes. The study showed that cabozantinib resulted in a lower CTC count.
SWOG S0421[108]	A phase 3 trial of docetaxel +/- atrasentan in mCRPC	The prognostic value of CTC was assessed in mCRPC patients receiving docetaxel. The study showed that a high baseline CTC count and a rise in CTC count by week 3 were associated with a worse outcome after docetaxel.
Scher et al[109]	A phase 3 trial assessing abiraterone vs placebo in patients with chemotherapy-pretreated mCRPC	A secondary objective was to evaluate CTC count as a surrogate marker of survival. The study showed that a biomarker panel containing CTC counts and LDH levels served as an effective surrogate marker of survival.
Other cancer types		
NeoALTTO[110]	A phase 3 trial comparing the efficacy of neoadjuvant lapatinib + paclitaxel, vs trastuzumab + paclitaxel, vs concomitant lapatinib/trastuzumab + paclitaxel in breast cancer with *HER2* overexpression and/or amplification	CTC count was among the secondary outcomes. The study showed a nonstatistically significant trend indicating that detectable CTCs may be associated with a lower likelihood of pathologic complete remission.
LAP 07[111]	A phase 3 study comparing gemcitabine +/- chemoradiation and +/- erlotinib in patients with locally advanced pancreatic adenocarcinoma	The CTC counts of a group of the trial patients were measured. Detectable CTCs were associated with poor tumor differentiation and shorter overall survival.

CRPC, castration-resistant prostate cancer; CTC, circulating tumor cell; HER2, human epidermal growth factor receptor 2; LDH, lactate dehydrogenase; mCRPC, metastatic CRPC; PSA, prostate-specific antigen.

CTC enumeration provided prognostic insight for various groups of patients; those who converted from an unfavorable CTC count at baseline to a favorable CTC count had better OS, at 21.3 vs 6.8 months. The OS of patients who converted from a favorable to an unfavorable CTC count was poorer than the OS of those whose CTC count remained favorable, at 9.3 vs 26 months. The investigators concluded that CTC count predicted OS. This study led to the FDA clearance of the CellSearch Circulating Tumor Cell Kit for clinical use in PC in 2008.

A subgroup analysis of the IMMC-38 study[22] focused on men starting docetaxel. Baseline CTC count was one of several factors correlated with risk for death (HR, 1.58; P<.001). Changes in CTC number were also strongly associated with risk for death (P≤.001). Changes in PSA level were only modestly associated with risk for death (P=.04-.8). Two additional studies have confirmed that a favorable CTC count at 3 weeks after treatment is predictive of improved OS for patients with mCRPC receiving docetaxel-based chemotherapy. Studies with this platform have continued to be performed in contemporary investigations, including the TOPARP study (A Phase II Trial of Olaparib in Patients With Advanced Castration Resistant Prostate Cancer), in which conversion of CTC status was part of the composite measurement of benefit from poly(adenosine diphosphate-ribose) polymerase (PARP) inhibitor therapy.[23]

In contrast to studies of mCRPC or metastatic

castration-sensitive disease,[24] those of localized PC have generated fewer data, possibly owing to insufficient detection of events with the CellSearch approach. One study using a modification of the CellSearch protocol reported on CTC enumerations in 35 patients with clinically high-risk localized PC as defined by National Comprehensive Cancer Network criteria (disease stage ≥cT3a, Gleason score of 8-10, and/or PSA level >20 ng/mL).[25] The investigators were able to detect CTCs in 49% of patients. There was no correlation between CTC count and biochemical recurrence after surgery. Using additional immunofluorescence, the same investigators found that the percentages of CD133-negative and E-cadherin–positive CTC fragments correlated with biochemical recurrence at 1 year after surgery.

As the technology in the CTC field has evolved, a larger number of needs and possibilities are being addressed. Many of the new systems (Table 2) offer technical advantages that create additional clinical opportunities. First, increased sensitivity has been reported with many of the developing systems, which remains important for clinical application.[26] Second, several of the developing systems are less dependent on EpCAM expression, making it possible to identify aggressive biological alterations such as cells undergoing an epithelial-to-mesenchymal transition (EMT). It is believed that EMT plays a key role in the generation of CTCs.[27,28] Third, several newer systems incorporate methods of identifying various CTC subpopulations. In recent studies, CTCs with specific morphologic or biochemical features have been associated with metastatic pattern[29] and drug sensitivity.[11,12] These findings point toward the use of CTC biomarkers beyond enumeration. Fourth, many developing systems avoid fixation that can restrict the types of molecular analyses of isolated CTCs.

Technologies for CTC Enrichment and Isolation

Flow cytometry historically has been one of the most powerful technologies for the detection and isolation of subpopulations of cells.[12] Many groups have used this approach to detect CTCs. The introduction of multicolor flow cytometry allows the simultaneous analysis and/or sorting of CTCs on the basis of protein expression.[30,31] The low abundance of CTCs, particularly in patients with lower-volume disease or atypical biology, has been a major challenge in the use of flow cytometry, prompting the development of alternative methods of CTC isolation.

Affinity-Based CTC Enrichment/Isolation Technologies

Affinity-based enrichment relies on the interaction between capture agents (antibodies in most cases) and molecules on the cellular surface to either capture CTCs (positive selection) or deplete hematologic cells (negative selection). Typically, anti-EpCAM antibody is used for positive selection[32,33] and anti-CD45 antibody for the depletion of unwanted leukocytes. Although this interaction is highly specific, issues of sensitivity arise with many of the platforms in the setting of limited or absent EpCAM expression.

Immunomagnetic approaches to separation use the expression of molecules on the cell surface to achieve effective enrichment. Capture agents are conjugated onto magnetic beads for either positive or negative selection.[16] The CellSearch, MACS (magnetic-activated cell sorting),[34] and AdnaTest[35] assays fall into this category. Several other groups have combined immunomagnetics with either flow cytometry[37] or microfluidic devices, such as MagSweeper,[36] IsoFlux,[37] VerIFAST,[38] and CTC-iChip,[39] to improve sensitivity and specificity. To improve accuracy, an immunofluorescence staining process targeting cancer-specific markers is often needed after CTC enrichment.

Non–Affinity-Based Technologies

A number of groups have taken the approach of using non-affinity mechanisms to capture CTCs. This approach inherently avoids the limitations that may be caused by the loss of EpCAM in cells undergoing EMT, which may be at risk for failed capture. Non–affinity-based technologies instead are based on features such as cellular size, invasive capacity, and protein expression and typically use immunofluorescent staining to identify CTCs. Filtration-based methods, such as ISET (isolation by size of epithelial tumor cells),[40] take advantage of size differences between CTCs and hematopoietic cells. A large collection of kits/systems are now commercially available to support research utility. A similar approach was adopted to develop a microfluidic device specifically for isolating CTC clusters.[41] Another physical property–based technology uses a dielectrophoretic field to separate CTCs (ApoStream),[42] given the fact that the dielectric properties of CTCs are different from those of the other cells in circulation.

Aside from physical properties, the biological activities of cancer cells can also be used to enrich CTCs. The CAM (cell adhesion matrix) Vita-Assay isolates CTCs on the basis of the biological proclivity of metastatic tumor cells to invade collagenous matrices.[43]

Microfluidic Devices for CTC Enrichment/Isolation

Microfluidic devices facilitate the capture of CTCs by increasing the contact interactions between CTCs and capture agents. Typically, CTCs are immobilized when flowing through microfluidic channels coated with capture agents (usually anti-EpCAM). The nonselected blood components are washed through the channel and excluded.[44-48] Variations on the physical device configuration have been

Table 2. CTC Technologies Used in Clinical Studies of Prostate Cancer

CTC Technology	CTC Capture Mechanism
Affinity-based separation	
MACS[34]	Immunomagnetic beads used for CTC separation
MagSweeper[112]	Specifically designed immunomagnetic cell separator
AdnaTest[113]	Combined immunomagnetic enrichment and polymerase chain reaction–based detection
CTC-Chip[44] ^{HB}CTC-Chip[46]	Microfluidic devices using antibody-coated microposts and/or an additional herringbone mixing device for enrichment
NanoVelcro Chip[114,115]	Microfluidic device using patterned nanowires and a chaotic mixer for antibody-mediated enrichment
IsoFlux[116]	Microfluidic device using immunomagnetic selection for CTC isolation
VerIFAST[38] Versa[74]	Microfluidic devices combining immiscible phase filtration and antibody-based immunomagnetic selection
GEDI[117]	Microfluidic device with an anti-PSMA antibody–coated 3D geometry that captures CTCs
CTC-iChip[118]	Microfluidic device using size-based deflection to remove non-nucleated cells and magnetophoresis after inertia focusing to remove antibody-labeled hematopoietic cells
CellCollector[119]	Functionalized structured medical Seldinger guidewire coated with anti-EpCAM for in vivo CTC capture
Non-affinity CTC capture	
ISET[120] and CellSieve[121]	Size-based micropore filtration
Acoustophoresis[52,53]	Size-based acoustic sorting
ApoStream[42]	CTC separation by dielectrophoretic field
Vortex[50] Spiral microfluidics[51]	Microfluidic devices using inertial force to separate CTCs from hematopoietic cells
No Cell Left Behind[57,122] AccuCyte – CyteFinder[58]	All nucleated cells stained by immunofluorescence and imaged in an automatic scanner
CAM Vita-Assay[43]	Enrichment based on epithelial cells' ability to invade extracellular matrix

CAM, collagen adhesion matrix; CTC, circulating tumor cell; 3D, 3-dimensional; EpCAM, epithelial cell adhesion molecule; GEDI, geometrically enhanced differential immunocapture; HB, herringbone; ISET, isolation by size of epithelial tumor cells; MACS, magnetic-activated cell sorting; PSMA, prostate-specific membrane antigen.

used since the introduction of microfluidic devices, resulting in altered mechanical properties that have affected capture efficiency. Typical examples include herring bone structure (^{HB}CTC-Chip)[46] and patterned silicon nanowires (NanoVelcro Chip).[49] Microfluidic devices can also be combined with other affinity-based or non–affinity-based CTC isolation mechanisms, such as immunomagnetic separation (CTC-iChip[39] and VerIFAST[38]), size-based inertial focusing (Vortex[50] and spiral microfluidics[51]), and acoustic separation.[52,53] Recent advances in surface chemistry further allow efficient CTC release after capture.[54-56]

Direct CTC Identification

With the advances in high-throughput slide-scanning technologies and image-analyzing computer algorithms, it is now possible to identify CTCs with minimal enrichment. Platforms such as No Cell Left Behind[57] and AccuCyte – CyteFinder[58] use direct visualization and identification of CTCs after immunofluorescence staining of a blood smear. A variety of PC-related markers, including AR/AR-V7, PSA, and prostate-specific membrane antigen (PSMA), have been used to increase the chance of finding CTCs.[59] Following the identification of CTCs, many of these platforms are now capable of recovering individual CTCs for further characterization.

Molecular and Phenotypic Profiling of CTCs

Improved methods of CTC capture have advanced our understanding of CTC biology. Several studies describe CTCs as a pool of tumor cells that are heterogeneous by morphology and biochemistry. Although enumeration of

CTCs has already proved useful, additional information can be extracted from them. CTCs and the underlying tumor exist in a dynamic homeostasis based on the constant process of shedding and/or invasion/extrusion into the vasculature. Because of this balance between tumor and CTCs, they may be usable as an alternate source of cellular information to allow the minimally invasive and dynamic characterization of a cancer.

CTC Morphology

One study reported that in addition to ranging in size, CTCs cluster in groups of 2 to 50 cells. At times, these clusters contain tumor cells admixed not only with other tumor cells but also hematopoietic cells, such as leukocytes. CTC clusters were shown to be the precursor of metastases in breast cancer.[60] As in classic cytopathology, these variations in morphology likely reflect functional and biological differences. Our group conducted a retrospective analysis of patients across a range of clinical PCs with varied metastatic burdens and sites of disease. In examining the distribution of cellular features, nuclear size correlated with metastatic behavior in mCRPC. In particular, CTCs with nuclei smaller than 9 μm were associated with VMs in mCRPC.[29] In another study focusing on CTC morphology, Beltran and colleagues[58] used the CTC platform from Epic Sciences and identified distinct features of CTCs from patients with neuroendocrine PC. These CTCs often present in clusters, exhibit reduced AR and cytokeratin (CK) expression, and are smaller than those from CRPC patients without atypical features, such as radiographic progression in the setting of a serum PSA of less than 1 ng/mL, VMs in the absence of PSA progression (defined by the Prostate Cancer Working Group 2 criteria), and/or elevated serum chromogranin A greater than 3-fold the upper limit of normal. These studies cast light on change in CTC morphology, which likely reflects the biology of the underlying PC and potentially can be a biomarker for the disease.

CTC Molecular Biology and Markers

Although much effort has been directed at obtaining pure CTC samples, some investigators have focused on the identification of key signals from CTCs to facilitate clinical translation. Several attempts have been made to detect genetic alterations commonly found in PC tumors. A typical example is the detection of *TMPRSS2-ERG* fusion in CTCs. This fusion event, the most common one among the ETS rearrangements, can be found in 27% to 79% of radical prostatectomy tissues[61] and approximately 56% of biopsies from advanced PC.[2] It leads to androgen-dependent expression of the *ERG* oncogene and exhibits prognostic value.[62] In a study focusing on patients with mCRPC treated with abiraterone acetate (Zytiga, Janssen), Attard and colleagues demonstrated the detection of *ERG* rearrangements in CTCs by fluorescence in situ hybridization (FISH).[63] The presence of *ERG* rearrangements in tumors and CTCs was associated with the magnitude of PSA decline in the patients receiving abiraterone. However, Danila and colleagues, in a later study of 48 patients, reported that *TMPRSS2-ERG* fusion in CTC alone does not predict the magnitude of PSA decline or clinical response to abiraterone.[64] The prognostic value of CTC-based *ERG* rearrangements in clinical use is still unclear.

Another commonly tested genetic alteration in PC is the loss of *PTEN*, which has been associated with progression to CRPC. *PTEN* loss was often found coexisting with *ERG* rearrangements in both tumor tissues[61] and CTCs.[63] Although *PTEN* loss in CTCs seems to be associated with *PTEN* loss in tumor tissues in patients with mCRPC,[65] the clinical significance of *PTEN* status in CTCs remains to be determined.

The search for CTC-derived biomarkers also puts substantial emphasis on the AR and its variants. In the field of AR biology, interest has developed in splice variants of AR that are of functional importance.[66-68] AR splice variants (particularly the AR-V7 variant) have been associated with reduced efficacy of contemporary AR therapies, such as abiraterone and enzalutamide (Xtandi, Astellas/Medivation), in preclinical models.[69,70] Given the highly focused nature of the molecular signal being pursued, Antonarakis and colleagues characterized AR-V7 expression in CTCs from men undergoing therapy with abiraterone and enzalutamide.[35] By using a modification of the AdnaTest, they were able to measure AR-V7 expression in patients with mCRPC and found that AR-V7 expression was associated with a greater likelihood of resistance to AR-targeted therapy. This series has been expanded to 200 patients who are classified as positive or negative for CTCs; CTC-positive patients are further classified as positive or negative for AR-V7.[71] The evolution is significant in understanding the importance of enrichment because CTC-negative patients are essentially unclassifiable with regard to AR-V7 status. This CTC-based biomarker may help physicians to select between AR-targeted therapy and taxanes because AR-V7 expression does not predict resistance to taxane therapy.[72,73]

The AR-V7 CTC assay was incorporated as part of ARMOR (A Study of Galeterone Compared to Enzalutamide in Men Expressing Androgen Receptor Splice Variant-7 mRNA Metastatic CRPC; NCT02438007), an attempted phase 3 study in a population expressing AR-V7 that its data-monitoring committee recently closed because of futility. The study was designed to measure the efficacy of galeterone in a population of patients with AR-V7 expression. Given the difficulty of establishing a

molecular diagnosis in patients with mCRPC, ARMOR allowed the use of a CTC-based assay for AR-V7. The assay itself combined immunomagnetic separation and polymerase chain reaction (PCR)–based amplification of both AR-V7 and full-length AR (AR-FL). For the assay result to be read as positive, sufficient CTCs were required in the initial purification. Second, both AR-FL and AR-V7 had to be detected by PCR within a prespecified ratio. As such, a concern in the earlier-stage disease group was the number of CTCs detectable with this methodology and so the capacity to detect AR transcripts. Had the study been successful, it would have, as an additional aim, validated the AR-V7 CTC assay. Although the assay is now available through The Johns Hopkins Hospital, its usefulness in the clinical setting remains unclear. This experience highlights an unfulfilled need that may be met in the future by assays with better sensitivity in the detection of disease-relevant markers in CTCs. Studies also have been conducted to explore other AR variants in CTCs and their clinical significance.[74]

Recently, with the rapid development of immuno-oncology and immune checkpoint inhibitors, the expression of programmed death ligand 1 (PD-L1) on CTCs has been tested for its clinical significance. Several groups have demonstrated a high level of PD-L1 expression detected in CTCs from patients with PC that is resistant to enzalutamide,[75] and it may be associated with shorter survival.[76] However, these findings and the association with response or resistance to checkpoint blockade still require further investigation in larger studies.

The Hopkins studies previously described, along with other findings in the field, point to the utility of CTC-based biomarkers and biology in the clinical setting. Within the pool of CTCs are subsets of cells with specific biological properties. These actively metastasizing cells have the capacity to invade and colonize secondary sites, driving the metastatic cascade via hematogenous dissemination. Chu and colleagues have proposed that within the population of CTCs exist metastasis-initiating cells, which possess not only metastatic potential but also the ability to recruit and reprogram dormant cells to become active metastatic cells.[77-79] These rare events and molecular findings, such as metastasis-initiating cells and AR-splice variants in the pool of CTCs, point to the need to further investigate the genomic and transcriptomic content of CTCs.

Next-generation sequencing (NGS) technologies are now capable of using picograms of genomic material, so that they can be adapted for CTC analysis.[80] Contemporary methods of RNA characterization have also been found to be adaptable for CTC analysis, including fluorescently labeled oligonucleotide hybridization,[81] whole-transcriptome RNA microarrays,[82] and single-cell RNA sequencing.[83] Single-cell DNA sequencing after whole-genome amplification is now possible,[84,85] although genomic amplification creates the potential for PCR-related errors and the preferential amplification of specific regions.[86]

There remains a need to characterize the degree to which CTC biology reflects underlying tumor biology. Several studies have compared CTCs and tumor tissues. Magbanua and colleagues demonstrated that CTCs possess copy number variations (CNVs) that mirror those of the associated primary tumor.[87,88] Targeted sequencing studies also have been conducted to compare single-nucleotide variances in CTCs and those in tumor tissues. Studies have detected mutations in known oncogenes, including *TP53* and *BRAF,* in single CTCs.[89,90] To further demonstrate the similarity between CTCs and tumor tissues, both our group[91,92] and Lohr and colleagues[93] performed whole-genome and whole-exome sequencing on single CTCs as well as temporally related biopsy specimens, and they independently reported similarities between single CTCs and PC tumor tissues at the genomic level. These studies provide evidence supporting the use of EpCAM-based approaches to isolate CTCs and suggest the possibility of using CTCs as a window to obtain critical, disease-relevant information. Although NGS has been increasingly used for precision medicine,[94-96] there are still barriers to be overcome before the use of NGS for CTCs is ready for clinical application. Technical challenges include the isolation of high-quality CTCs, the development of methods for high-uniformity and high-coverage whole-genome amplification, and the creation of strategies for accurate mutation identification. Another challenge is the heterogeneous nature of cancer and the CTC population.

As the number of CTC characterization studies grows, increasing data are showing discrepancies between CTCs and tumors, in addition to substantial heterogeneity within the pool of CTCs. Genomic studies have revealed that 14% to 30% of the mutations found in CTCs are CTC-specific.[92,93] Discrepancies were also observed beyond the genomes as several groups made attempts to compare the transcriptomes of CTCs and of tumor tissues. Miyamoto and colleagues recently reported the results of RNA sequencing for single CTCs from patients with PC.[83] Although the CTCs strongly clustered according to patient of origin, substantial heterogeneity was observed in signaling pathways, as well as transcriptional alterations involving resistance to antiandrogen therapies. These observations may have been related to the complex molecular evolution of metastatic PC, which has yet to be comprehensively studied,[97] and to the technical difficulties encountered in verifying findings in single cells.[90,91] However, the studies also clearly indicated molecular

heterogeneity within the pool of CTCs from each patient and brought up several important issues to be addressed before CTCs can be used for liquid biopsy. These include (but are not limited to) the following: (1) the methodology required to effectively distinguish disease-related information from sequencing errors; (2) the number of CTCs required to obtain clinically relevant information; (3) the timing of CTC sampling in relation to the evolution of cancer and/or the effect of therapeutics.

Studies that appropriately address these issues will serve as the foundation for future studies designed to use CTCs as a means of describing and measuring the heterogeneity and dynamic biology of cancer.

Heterogeneity and Dynamic Biology: Future Applications of CTCs

Heterogeneity, a classic hallmark of cancer, has been described at the phenotypic and molecular levels in PC. It exists between patients (interpatient), between tumors in one patient (intertumoral), and among the cells making up a tumor (intratumoral).[98,99] Heterogeneity also evolves over time and physical space (temporospatial). It has been proposed that heterogeneity may be a driver of drug resistance and disease progression.[100]

Many historical studies in this area have relied on tumor samplings, including multiple-site samplings of freshly resected tissue. This is feasible in certain cancers, given that lesions may appear in soft-tissue areas that are amenable to safe and high-yield biopsies. PC and CRPC in particular tend to be less amenable to needle biopsies for the reasons previously noted. In the case of PC, serial liquid biopsy may provide an approach to study the dynamic aspects of tumor heterogeneity.[29,90,100]

Interest has been growing in the use of blood-borne components to develop a liquid biopsy. Analysis of circulating tumor DNA (ctDNA) has proved to be a convenient and effective means of detecting and monitoring key mutations in cancer.[101] In addition, ctDNA has been used to study complex DNA alterations.[102,103] The speed of sample acquisition and processing, as well as the relatively simple technology needed to obtain ctDNA for study, is very appealing. These factors have fueled enthusiasm for making ctDNA analysis an active area of research in PC and other solid malignant tumors. A fuller characterization of the biology of PC, especially CRPC, may require greater biological insight than can be obtained from DNA alone. With the advances in single-cell technologies, CTCs may provide additional information on the clonality of cancer because multiple layers of information (eg, DNA mutations, epigenetic alterations, RNA expression, protein levels, morphologic features, functional properties) can be extracted simultaneously.

We and others have proposed that CTCs may provide another dimension in cancer biology: dynamic biology.[104] Serial blood sampling for CTC analysis can rapidly yield temporally discrete data that can provide dynamic insight into disease evolution and therapeutic resistance in PC. Analysis of serially collected CTCs has indicated that variations in CTC-derived biological information over time may reflect changes in the nature of disease and/or response to therapy.[29,49,92,105]

Conclusions

The introduction of technologies capable of capturing and enumerating CTCs is a first and important step in having the field of CTC research progress from theory to clinical practice. In PC, the CTC count serves as a prognostic biomarker for survival and a predictive biomarker. This finding is significant for PC and especially CRPC, in which biomarkers other than the serum PSA concentration may be of importance. Enumeration remains a useful type of data that can be derived from CTCs. Newer technologies that have been developed and introduced are now capable of examining CTCs in a fashion more akin to the way tissue biopsies are examined.

As the clinical care of men with PC becomes more personalized, the ability to conduct a dynamic characterization of the biology of this disease in real time will be increasingly important. This personalization of therapy is made feasible by advances in the basic sciences and pressured by the rising costs of therapy in the face of a long natural history of PC. Minimally invasive access to useful tissue for biological characterization may revolutionize the care of men with PC.

Interest has been growing in the use of blood-borne components to develop a liquid biopsy. Concerns over cost, technologic requirements, and the slow sample processing have led to a decreased interest in CTCs and a greater interest in ctDNA. Although important and informative, because of its biological nature, PC will likely require more than DNA-based analysis. As technology improves, the cost and time required to isolate and analyze CTCs will decrease, making these platforms more accessible to physicians and scientists. Importantly, the study of plasma and the study of the cellular components in blood samples are not mutually exclusive, and they may serve as effective complementary tests in the future as both types of clinical biomarkers are explored.

Genomic medicine and liquid biopsies have been heralded as the future of modern oncology. Advances in both areas are critically important in a disease such as PC, in which mortality continues to be an issue and in which current approaches fail to unite modern biology with clinical practice. With the timely advances in both CTC isolation technology and molecular profiling, CTC research is turning to liquid biopsies to promote genomic medicine.

The union of these disciplines may be supra-additive by allowing a characterization of the dynamic nature of PC and other malignancies, given the existing capabilities to draw blood frequently and even conduct CTC isolation within hours of phlebotomy. By gaining insight into the changes of biological behavior and heterogeneity of malignancies, a physician may be better equipped to navigate therapies and/or prognosticate. Such information may also provide an understanding of emerging resistance to therapy.

Exciting developments in the field of CTC research in PC and other cancers raise new and important questions and challenges. Given the rarity of these cellular events in the bloodstream and the precision of the measurements being made (down to the level of single cells), standards of collection and analysis will need to be established. Given the wide variation in the approaches to CTC isolation, it is unlikely that a single cross-platform approach will ever be established. For the optimization of each advanced platform, understanding the volume of blood and/or numbers of tumor cells needed to conduct the best possible comparisons remains important. Moreover, the timing of blood sampling likely needs to vary with the rate of disease progression. The frequency of sample collection also may depend on the analyte of interest and the methodology used. Although these may represent important issues in technology development, they will not substantially hinder progress in this exciting and important field of biomedical research.

CTCs have become a resource available to oncologists and researchers in the area of PC. Although issues of cost and utility have limited the wide application of their use, an increasing number of studies are showing their potential usefulness in the clinic. With further refinements in technology, their applications will extend beyond enumeration and will help to shape the future of cancer research and personalized oncology. CTC-based approaches will certainly improve the care of men with PC.

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