Circulating tumour cells—monitoring treatment response in prostate cancer

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Abstract | The availability of new therapeutic options for the treatment of metastatic castration-resistant prostate cancer (mCRPC) has heightened the importance of monitoring and assessing treatment response. Accordingly, there is an unmet clinical need for reliable biomarkers that can be used to guide therapy. Circulating tumour cells (CTCs) are rare cells that are shed from primary and metastatic tumour deposits into the peripheral circulation, and represent a means of performing noninvasive tumour sampling. Indeed, enumeration of CTCs before and after therapy has shown that CTC burden correlates with prognosis in patients with mCRPC. Moreover, studies have demonstrated the potential of molecular analysis of CTCs in monitoring and predicting response to therapy in patients. This Review describes the challenges associated with monitoring treatment response in mCRPC, and the advancements in CTC-analysis technologies applied to such assessments and, ultimately, guiding prostate cancer treatment.

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Introduction

In the USA, prostate cancer is the most common cancer in men and second most common cause of cancer-related death, with an estimated 29,480 deaths likely to be attributed to this disease in 2014.[1] In the past 3 years, the therapeutic landscape in metastatic castration-resistant prostate cancer (mCRPC) has changed substantially, with the FDA approval of five therapies associated with improved overall survival.[2–7] Monitoring the effectiveness of individual therapies in patients with mCRPC is a complex problem because of the high prevalence of bone metastases, which are difficult to quantitate. Furthermore, the currently available biomarkers and imaging assessments of clinical response do not enable optimal management of individual patients, owing to insufficient specificity for clinically relevant outcomes.[8] Additionally, the increasing number of treatment options available in mCRPC has created new challenges with regard to the design of clinical trials investigating novel therapies: whereas overall survival was a reasonable clinical trial end point in an earlier era, the availability of effective therapies that patients might receive after an experimental treatment confounds the ability to measure any survival benefit attributable to the new therapy. Although serum prostate-specific antigen (PSA) serves as a useful biomarker of treatment response and disease progression in the earlier stages of prostate cancer, this protein has been shown to be an unreliable biomarker in the setting of mCRPC and fails to meet the strict definitions of surrogacy for overall survival.[9] Thus, for both the clinical management of an individual patient and the assessment of novel therapies in development, new biomarkers in the metastatic setting represent an unmet clinical need.[8]

Circulating tumour cells (CTCs) are rare cancer cells that have been shed from primary or metastatic tumour deposits and have entered into the peripheral blood.[10,11] Studies have demonstrated that CTCs are genetically representative of the main tumour deposit and, therefore, might serve as a readily accessible source of tumour cells for various analyses.[12,13] In other types of cancers, tumour biopsies performed before and after initiation of therapy can enable molecular evaluation of the cancer during treatment and provide the opportunity to tailor the use of molecularly targeted therapies. However, as prostate cancer frequently metastasizes to the bone and bone tumour biopsies are relatively challenging to reliably obtain, this approach is not always feasible in patients with mCRPC. Thus, CTCs could serve as a 'liquid biopsy' that might provide the opportunity to noninvasively and repeatedly sample representative tumour cells before and during therapy, and thus provide information concerning not only tumour burden, but also the molecular characteristics of tumour cells as they evolve during treatment.[14,15] However, CTCs are rare, with an estimated abundance of one cell per billion normal blood cells, and reliable isolation and detection of these cells from peripheral blood has proven extremely challenging. This Review provides an overview of the challenges associated with monitoring therapeutic responses in prostate cancer and summarizes developments in technologies that enable the detection and analysis of CTCs associated with prostate cancer. In addition, the available data supporting the potential for CTC analysis to provide prognostic information that could be used to guide therapy in mCRPC are examined.

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Key points

- Reliable biomarkers that can guide the treatment of metastatic prostate cancer in the clinic remain an unmet need
- Circulating tumour cells (CTCs) are rare cells shed by tumours into the peripheral circulation, and might represent a means of noninvasive tumour sampling
- Technological advances have improved the isolation and analysis of rare CTCs from patients with cancer
- CTC enumeration has been shown to be predictive of prognosis in patients with metastatic castration-resistant prostate cancer
- Molecular analyses of CTCs have the potential to enable real-time monitoring and predictions of response to therapy in patients with metastatic prostate cancer

Methods of evaluating prostate cancer

The most common sites of prostate cancer metastasis are bone and lymph nodes. Bone metastases are present in 90% of men with terminal prostate cancer and represent the major cause of morbidity and mortality associated with this disease. Skeletal-related events, including pathological fractures and spinal cord compression in particular, have substantial effects on health and quality of life, and contribute to mCRPC mortality.[16] Standard imaging modalities for assessment of prostate cancer and associated metastases include CT of the abdomen and pelvis, largely to evaluate lymph nodes, and bone scan using ^{99m}Tc-methylene diphosphonate (^{99m}Tc-MDP) as the imaging agent. Although lymph nodes or other visceral metastases constitute measurable disease using the modified Response Evaluation Criteria in Solid Tumours (mRECIST) criteria,[17] bone lesions change slowly over time and are considered unmeasurable sites of disease according to these criteria. The Prostate Cancer Working Group 2 (PCWG2) guidelines defined progression of metastatic disease as the identification of at least two new bone lesions on two consecutive bone scans;[18] however, improvement in disease according to information from bone scans is often not defined. PET scans were not recommended for the assessment of bone metastases by the PCWG2.[18] More recently, however, the use of ^{18}F-fluorodeoxyglucose (^{18}F-FDG), ^{18}F sodium fluoride (^{18}F-NaF), and ^{11}C-based tracers (such as choline and acetate) has shown promise in PET-based monitoring of prostate cancer in small studies,[19–22] but, to date, these investigations have not resulted in a widely available, clinically useful PET tracer. Hence, PET remains an investigational imaging modality in patients with prostate cancer at present.

Owing to the high prevalence of bone metastases in patients with mCRPC, improved assessment of tumour burden on bone scans might provide a clinically relevant tool for both individual patient management and imaging end points for clinical trials. An automated computer-aided detection (CAD) assessment system has been described that could provide objective, reproducible, and quantifiable measurements of ^{99m}Tc-MDP uptake in bone.[23] The CAD system integrates image intensity normalization, lesion identification and segmentation according to anatomical-region-specific intensity thresholds, and quantitation of disease burden, as well as independent review by a nuclear-medicine physician.[23] Using this assessment system, 'Bone Scan Lesion Area' (BSLA) was found to be the most informative metric in differentiating between patients with mCRPC who were treated with cabozantinib, an investigational drug that inhibits c-Met and VEGFR, and those patients who did not receive this agent.[23] BSLA might, therefore, represent a promising new indicator of disease response in mCRPC. At present, validation of BSLA as an objective measure of post-treatment response in comparison with other clinically relevant outcome measures is required; the results of small studies have indicated the potential utility of this approach.[24]

Assays of serum PSA levels are widely available, and this biomarker is generally considered to reflect tumour burden in patients with prostate cancer; however, post-treatment changes in serum PSA levels have not been proven as a surrogate measure of clinical benefit.[9,25] Indeed, no therapy for prostate cancer has been approved solely based on an observed post-treatment decline in serum PSA levels. Furthermore, several FDA-approved and experimental therapies have demonstrated beneficial therapeutic effects that were not concordant with decreased serum PSA levels.[26] Thus, there is a critical unmet need for improved biomarkers of therapeutic response in patients with prostate cancer.

Cell-free circulating tumour DNA and RNA have been detected in plasma and serum from patients with prostate cancer, and studies have observed a correlation between circulating tumour nucleic acid burden and prognosis in men with metastatic prostate cancer.[27,28] These cell-free nucleic acids might originate from necrotic tumour tissues, exosomes, oncosomes, or dead tumour cells that enter the circulation.[28,29] A principle advantage of assessments based on the detection of circulating nucleic acids is the high sensitivity potentially obtainable using PCR-based amplification techniques; a chief disadvantage is that separation of tumour-derived nucleic acids from other circulating nucleic acids is not possible and, therefore, only the detection of tumour-specific gene mutations can prove the presence of DNA or RNA released from tumour cells. In addition, as individual tumour cells themselves are not identified using this approach, potentially useful information on intercellular heterogeneity and intracellular signalling pathway activity is lost. Thus, assays for circulating tumour-derived nucleic acid and CTC might have complementary uses, with the former providing information regarding gene mutations and genetic translocations, and the latter providing specific information regarding CTC numbers, cell morphology, and intracellular signalling events in response to therapy.

Technologies for the detection of CTCs

The presence of CTCs in a patient with metastatic cancer was first reported in 1869,[30] but these cells have been extremely difficult to isolate and study because of their rarity (abundance of approximately one CTC per billion normal blood cells). Although considerable challenges remain in the development of robust technologies that enable detection of CTC with high accuracy, sensitivity, and specificity, owing to the rarity, fragility, and biological heterogeneity of CTCs, improved methods for CTC detection have been developed over the past

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Table 1 | Selected CTC-detection technologies that have been tested in patients with prostate cancer

CTC-detection technology or process	Basis of CTC enrichment and detection	Assay examples (manufacturer)
Positive selection using cell-surface antigen(s)		
Immunomagnetic beads	EpCAM-based immunomagnetic selection; immunofluorescence for $CK^+/CD45^-$ cells or RT-PCR for a panel of genes (*MUC1, HER2, EPCAM*)	CellSearch® (Veridex, USA),[37] AdnaTest (AdnaGen, Germany)[46]
Microfluidic microposts chip	EpCAM-based or PSMA-based selection; immunofluorescence for $CK^+/CD45^-$, $PSA^+/CD45^-$, or $PSMA^+/CD45^-$ cells, or RT-PCR for selected genes	$^{\mu p}$CTC-Chip (MGH, USA),[51] GEDI (Cornell University, USA)[54]
Microfluidic mixing chip	Selection based on EpCAM or other tumour-specific markers; immunofluorescence for selected tumour markers (such as CK, PSA, and PMSA), or RT-PCR for selected genes	^{HB}CTC-Chip (MGH, USA)[52]
Microfluidic inertial focusing chip	EpCAM-based selection; immunofluorescence for selected tumour markers (CK, PSA, and PSMA)	^{pos}CTC-iChip (MGH, USA)[36]
Patterned silicon nanowire microfluidic chip	EpCAM-based selection; immunofluorescence for $CK^+/CD45^-$ cells	NanoVelcro (UCLA, USA)[53]
Immunomagnetic sweeper	EpCAM-based immunomagnetic selection; immunofluorescence for $CK^+/CD45^-$ cells, or RT-PCR for selected genes	MagSweeper (Stanford University, USA)[47]
Immiscible phase filtration	EpCAM-based immunomagnetic selection; immunofluorescence for $CK^+/CD45^-$ cells	VerIFAST (University of Wisconsin, USA)[50]
Negative selection using cell-surface antigen(s)		
Microfluidic inertial focusing chip	Depletion of $CD45^+$ cells; immunofluorescence for selected tumour markers (CK, PSA, and PSMA); RT-PCR for selected genes	^{neg}CTC-iChip (MGH, USA)[36]
Microfluidic negative selection	Bulk haematopoietic-cell removal, followed by depletion of $CD45^+$ cells; immunofluorescence for $CK^+/CD45^-$ cells	Microfluidic Cell Concentrator[55]
Other biological approaches		
Detection of proteins shed from viable CTCs	Short-term cell culture after $CD45^+$-cell depletion; immunofluorescence for MUC1, PSA, or CK-19	EPISPOT (CHU, France & UKE, Germany)[56]
CAM ingestion	Density-gradient centrifugation, short-term culture; immunofluorescence for cell-surface markers	CAM Vita-Assay™ (Vitatex, USA)[57]
RT-PCR in whole-blood nucleated cells	RT-PCR for gene panels (such as *KLK3, KLK2, HOXB13, GRHL2,* and *FOXA1*)	PAXgene Blood RNA tube and RT-PCR[69]
Physical selection methods		
Size-based separation	Filtration based on cell size; immunofluorescence or FISH	ISET® (RARECELLS, France),[61] CTC Membrane Microfilter (University of Miami, USA)[63]
Dielectric field flow fractionation (DFFF)	Application of electric field to isolate cells; immunofluorescence for tumour-specific markers	ApoStream® (ApoCell, USA)[60]
Other approaches		
Fibre-optic array scanning technology (FAST) cytometry	RBC lysis and density-gradient centrifugation; immunofluorescence for CK, PSMA, or other tumour-cell markers	Epic HD-CTC Assay (Epic Sciences, USA)[71]
Laser-scanning cytometry	RBC lysis; immunofluorescence for $EpCAM^+/CD45^-$ cells	Maintrac® (Simfo, Germany)[72]
Functionalized nanodetector inserted into patient's vein	EpCAM-based selection; immunofluorescence for EpCAM or CK	CellCollector™ (GILUPI, Germany)[73]

Abbreviations: $^{\mu p}$CTC-Chip, micropost CTC-Chip; CAM, cell-adhesion molecule; CHU, Centre Hospitaliers Universitaires; CK, cytokeratin; CTC, circulating tumour cell; EpCAM, epithelial cell-adhesion molecule; EPISPOT, epithelial immunospot; FAST, fibre-optic array scanning technology; FISH, fluorescence *in situ* hybridization; GEDI, geometrically enhanced differential immunocapture; ^{HB}CTC-Chip, herringbone CTC-chip; HD-CTC, high-definition-CTC (assay); ISET, isolation by size of epithelial tumour cells; MGH, Massachusetts General Hospital; ^{neg}CTC-iChip, negative selection CTC-inertial-focusing-chip; ^{pos}CTC-iChip, positive selection CTC-inertial-focusing-chip; PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigen; RBC, red blood cell; RT-PCR, reverse transcription polymerase chain reaction; UCLA, University College of Los Angeles; UKE, Universitätsklinikum Hamburg–Eppendorf.

two decades.[11,14,31] A number of these technologies have been applied to the detection and analysis of CTCs in patients with prostate cancer in pilot studies. However, translation of any of these technologies into routine clinical practice will require extensive analytical and clinical validation in prospective trials. We provide an overview of currently available CTC-detection technologies, with a particular emphasis on technologies that have shown promise in the study of CTCs in patients with prostate cancer. These technologies can be stratified into methods that rely on either biological or physical cellular characteristics for detection of CTCs (Table 1; Figure 1).

Surface-antigen-based enrichment of CTCs

Two general approaches to surface-antigen-based enrichment of CTCs have been developed: positive selection, in which CTC-specific cell-surface markers are used to purify CTCs away from normal blood cells; and negative selection, which uses leukocyte-specific cell-surface markers to remove immune cells from blood, thus leaving behind other cells, including CTCs. Epithelial cell-adhesion molecule (EpCAM) has been widely used for positive selection of CTCs (Table 1), as this transmembrane glycoprotein is consistently expressed by epithelial-derived tumour cells, but is not found on

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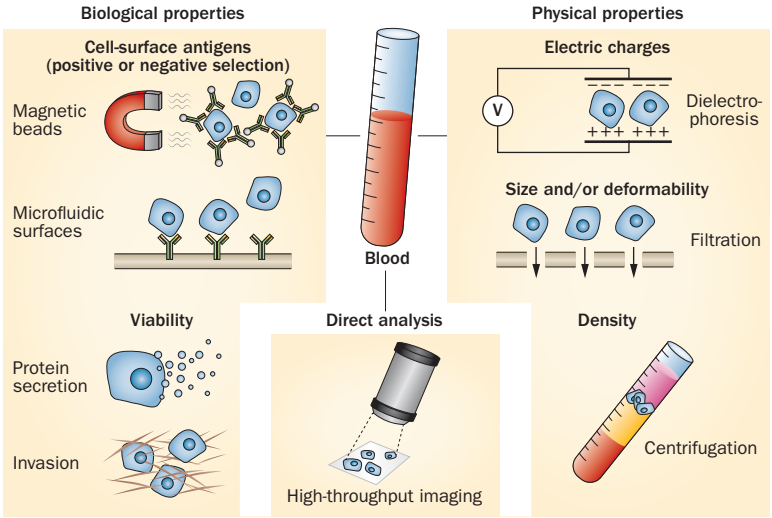

Figure 1 | Approaches to detection of CTC. CTCs can be enriched from whole-blood samples based on biological or physical properties, or can be detected directly after lysis of red blood cells through high-throughput imaging approaches. Enrichment of CTCs based on biological properties can be achieved through positive or negative selection for tumour-specific cell-surface antigens, and assays for cell viability and phenotype. Approaches to enrichment of these cells based on physical properties exploit tumour-specific differences in density, size, deformability, and electric charges. Abbreviation: CTCs, circulating tumour cells.

normal leukocytes. Indeed, EpCAM is expressed highly in a variety of carcinomas, including prostate cancers, and has an important role in cell adhesion, signalling, migration, proliferation, and differentiation.[32] Although EpCAM-based positive selection has been successfully used as a strategy to isolate CTCs in a variety of cancer types, EpCAM expression might decrease in cells undergoing epithelial–mesenchymal transition (EMT), a potential key process in tumour metastasis (Figure 2).[33,34] Thus, interest in positive selection using alternate tumour-cell markers that enable capture of CTCs with low EpCAM expression is increasing.[34,35] Alternatively, CTCs expressing low levels of EpCAM have been identified using negative selection strategies that deplete blood samples of normal haematopoietic cells and, therefore, leave behind enriched populations of all CTCs.[36] The two broad categories of technologies that have been used for surface-antigen-based enrichment of CTCs are methods based on immunomagnetic beads and approaches using microfluidic devices.

Immunomagnetic-bead-based enrichment of CTCs

The CellSearch® assay (Veridex, USA), the only FDA-cleared CTC-detection technology,[37] relies on anti-EpCAM-antibody-coated magnetic beads for capture of CTCs, which are subsequently identified as cells positive for cytokeratin (CK)-8, CK-18, and CK-19 expression, and negative for common leucocyte antigen (CD45) expression by immunofluorescence staining (Table 1).[38–40] As the CellSearch® platform has undergone extensive analytical validation and clinical qualification,[38–40] leading to its FDA clearance,[37] this CTC-detection assay is used widely among the prostate cancer research community. Several clinical studies have demonstrated a relationship between patient prognosis and CellSearch®-determined CTC abundance before and after treatment of prostate cancer.[38–41] However, several limitations of the CellSearch® system have stimulated the development of new technologies for CTC enrichment and detection. For example, performing informative molecular analyses in CTCs isolated using the CellSearch® technology is relatively difficult because of the low purity of the cell populations obtained, the requirement for fixation of cells in preparation for immunofluorescence-based detection, and the nature of the processing conditions. Nevertheless, studies have demonstrated the feasibility of molecular characterization of CellSearch®-derived CTCs.[13,42] The requirement for operator review and interpretation of the CellSearch® data has been shown to contribute to variability in CTC counts;[43] therefore, an automated algorithm has been developed to provide unbiased counts of CTCs in the recorded CellSearch® images.[44] This automated algorithm has also been used to extract data on the morphological features of CTCs, including cell size, roundness, and apoptotic features, which were found to be closely correlated with overall survival in univariate analysis, although not in multivariate analysis.[45]

To address the problem of capturing cells that are undergoing EMT (Figure 2), a cadherin-11-based capture method has been developed by investigators at Duke University, NC, USA, to complement the EpCAM-based CellSearch® platform.[35] Cadherin-11 (also known as osteoblast cadherin) is a cell-adhesion molecule expressed in osteoblasts and prostate cancer cells.[35] Mesenchymal cells are immunomagnetically enriched using anti-cadherin-11-antibody-conjugated magnetic particles, and potential CTCs are identified by immunofluorescence analysis according to expression of β-catenin, after exclusion of contaminating CD45-positive leukocytes.[35] A pilot study using this method detected potential mesenchymal CTCs in a subset of patients with mCRPC at an increased frequency compared with healthy volunteers,[35] although further studies will be required to define the clinical relevance of these findings.

Other immunomagnetic-bead-based systems, such as the AdnaTest (AdnaGen, Germany; Table 1), enable molecular characterization of CTCs, including reverse transcription-PCR (RT-PCR) analysis of prostate-specific gene transcripts.[46] The MagSweeper device, developed by researchers at Stanford University, CA, USA, is an immunomagnetic cell separator that uses magnetic rods to collect CTCs that are bound to anti-EpCAM-antibody-coated magnetic beads from diluted blood samples (Table 1);[47] nonspecifically bound blood cells are released through a controlled shear force produced by movement of the magnetic rods in wash buffer.[47] The isolated cells have been demonstrated to contain RNA of sufficient quality to perform multiplex quantitative RT-PCR and RNA sequencing of single CTCs, although the RNA from many of the CTCs showed signs of degradation consistent with apoptosis.[48,49] Another promising

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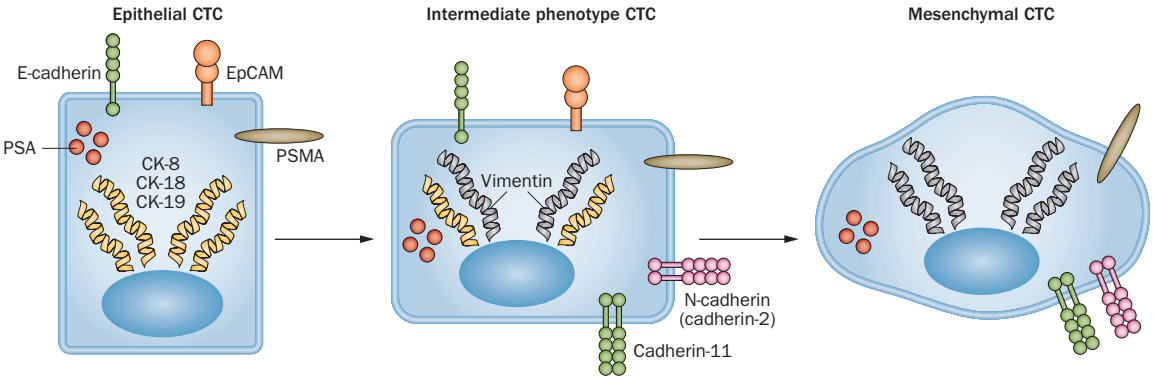

Figure 2 | Molecular markers used to detect prostate CTCs undergoing epithelial–mesenchymal transition. Epithelial–mesenchymal transition is characterized by the gain and loss of specific molecular markers, and the exclusive use of epithelial markers for the isolation and detection of CTCs could result in lack of detection of the mesenchymal subpopulation of these cells. For example, since EpCAM is often downregulated in mesenchymal cells, the use of EpCAM as a selection marker is probably not sufficient to detect mesenchymal CTCs. Abbreviations: CK, cytokeratin; CTC, circulating tumour cell; EpCAM, epithelial cell-adhesion molecule; PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigen.

immunomagnetic approach to isolation of CTC from blood samples is the immiscible phase filtration platform VerIFAST, developed by a team at the University of Wisconsin, WI, USA.[50] The VerIFAST technique uses magnets to selectively move the desired cells between immiscible liquids, relying upon the high interfacial energy between the immiscible liquids to ensure that only the cells bound to immunomagnetic beads can cross between phases (Table 1), and enables rapid isolation and processing of CTCs.[50]

Microfluidic devices for enrichment of CTCs

Improvements in microfluidic engineering over the past decade have enabled the development of innovative microfluidic devices for efficient and gentle isolation of CTCs from whole-blood samples. Our group at the Massachusetts General Hospital (MGH), MA, USA, has developed a series of microfluidic devices that enrich CTCs from whole blood using cell-surface antigens. The first generation $^{\mu P}$CTC-Chip consisted of 78,000 microposts coated with anti-EpCAM antibodies, which capture EpCAM-expressing CTCs that come into contact with the microposts as blood flows through the microfluidic chip (Table 1).[51] A second generation version of the CTC-Chip, the ^{HB}CTC-Chip, consists of microfluidic channels etched with herringbone patterns, inducing the formation of microvortices as blood flows through the chip, thus increasing the contact time between cells and the walls of the channel coated with anti-EpCAM antibodies (Figure 3).[52] The capture antibodies used to functionalize the microfluidic channels can be tailored based on the biological characteristics of the cells of interest, such as in the use of antibodies against nonepithelial tumour antigens to capture CTCs undergoing EMT.[34]

Other microfluidic technologies have also been developed based on the concept of positive selection. Developed by researchers at the University of California Los Angeles (UCLA), CA, USA, the NanoVelcro microfluidic device incorporates anti-EpCAM-antibody-coated silicon nanowires integrated with an overlaid polydimethylsiloxane (PDMS) chaotic mixer, which generates vertical flows and enhances contacts between CTCs and the capture substrate (Table 1).[53] This technology has been piloted in patients with CRPC, and produced data that suggested a correlation exists between changes in CTC numbers and response to therapy.[53] To specifically capture prostate-cancer-associated CTCs, a platform with microposts coated with antibodies targeting prostate-specific membrane antigen (PSMA), the 'geometrically enhanced differential immunocapture' (GEDI) device, has been developed by a team at Cornell University, NY, USA.[54] A pilot study of the GEDI device showed that PSMA-expressing CTCs were more abundant in samples from patients with CRPC compared with blood from healthy donors, and that on-chip monitoring of effective drug-target engagement to predict treatment response might be feasible.[54]

In addition to the positive selection strategy used by earlier microfluidic technologies, a third generation CTC-Chip technology developed at the MGH, the CTC-iChip, also enables a negative selection strategy that purifies CTCs independent of antigens present on the tumour-cell surface (Table 1).[36] The CTC-iChip consists of three integrated components: a hydrodynamic sorting step that results in size-based removal of red blood cells and platelets; an inertial focusing step that aligns the remaining cells in a single file in the flow channel; and a subsequent magnetophoresis step that removes cells that have been labelled with antibody-coated magnetic beads, which are CTCs in the case of positive selection or leukocytes in the case of negative selection.[36] The negative selection mode (^{neg}CTC-iChip) yields a gently isolated population of CTCs that have not been labelled with antibodies or magnetic beads, thus enabling subsequent molecular analyses, including single-cell transcriptional profiling.[36] Moreover, the CTC population obtained using the ^{neg}CTC-iChip is unselected and, therefore, CTCs with a range of phenotypes, including epithelial and mesenchymal cells, can potentially be detected and analysed for molecular

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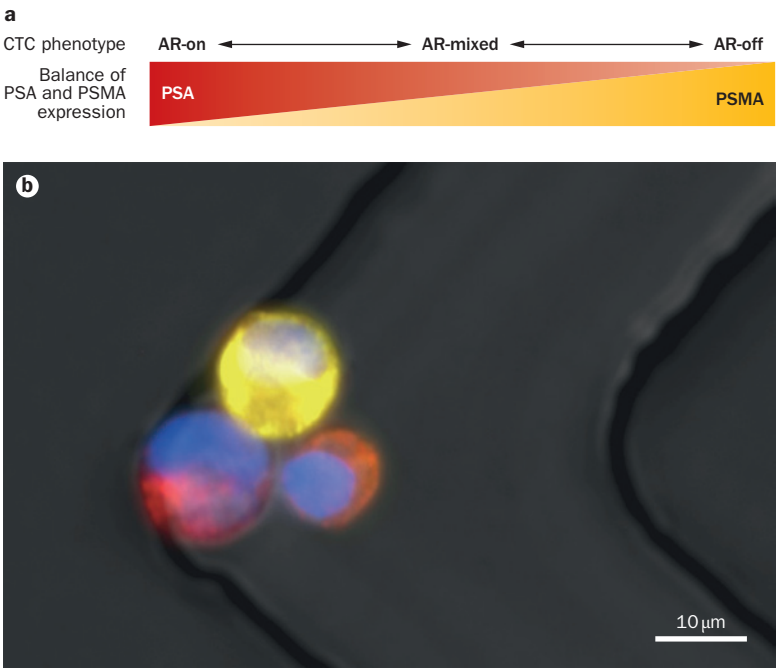

Figure 3 | Assay for measuring signalling activity of the AR in prostate CTCs.[70] **a** | Schematic shows the relative expression levels of PSA and PSMA in 'AR-on', 'AR-off', and 'AR-mixed' signalling states. **b** | CTCs from a patient with mCRPC captured on the ^{HB}CTC-Chip, a microfluidics-based assay that enables anti-EpCAM-antibody-mediated capture of CTCs from whole-blood samples. The image is a composite of fluorescence micrographs that visualize immunostaining of PSA (red) and PSMA (yellow) expression, and DAPI staining of DNA (cell nuclei; blue), merged with a phase contrast microscopy image. Heterogeneity in AR signalling activity between mCRPC-associated CTCs is evident, as demonstrated by the presence of a red cell (AR-on), a yellow cell (AR-off), and an orange cell (AR-mixed). Herringbone grooves on the ^{HB}CTC-Chip, which generate microvortices within the microfluidics channels that direct the cells towards the antibody-coated surfaces to increase the efficiency of CTC capture, are visible (dark angled lines). Abbreviations: AR, androgen receptor; CTC, circulating tumour cell; DAPI, 4',6-diamidino-2-phenylindole; EpCAM, epithelial cell-adhesion molecule; ^{HB}CTC-Chip, herringbone CTC-Chip; mCRPC, metastatic castration-resistant prostate cancer; PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigen.

variation. Other groups have also developed methodologies based on negative selection that have been applied to the isolation of CTCs associated with prostate cancer, including a microfluidic device called the Microfluidic Cell Concentrator (MCC), which performs gentle negative selection of CTCs after bulk erythrocyte and haematopoietic-cell removal.[55]

CTC isolation using other biological properties

Alternative approaches to the isolation of CTCs rely on biological characteristics of viable CTCs, such as invasiveness and secretion of specific proteins. These approaches are not based on assumptions regarding the physical properties of CTCs or differential expression of cell-surface antigens, and thus have the potential advantage of capturing subsets of CTCs that would not be otherwise identified. However, such methods necessitate the assumption that CTCs will remain viable under the *in vitro* cell-culture conditions used, and that these specific culture conditions are sufficient to recapitulate the *in vivo* biological behaviour of CTCs.

A functional enzyme-linked immunosorbent spot (EPISPOT) assay, for example, can detect the presence of viable CTCs based on proteins released during short-term cell culture (24–48 h), such as PSA secreted by CTCs associated with prostate cancer (Table 1).[56] Similarly, the cell-adhesion matrix (CAM)-based Vita-Assay™ platform (Vitatex, USA) enables viable invasive CTCs to be isolated by virtue of the propensity of tumour cells to invade into collagenous matrices (Figure 1).[57] Thus, the Vita-Assay™ can be used for identification of CTCs independent of EpCAM status, and enables CTC enumeration and analysis of CTC DNA.[57] These approaches have been used in several pilot analyses of CTCs from patients with mCRPC, including immunocytochemistry for PSMA, and markers of EMT and stemness, array comparative genomic hybridisation (CGH), and whole-genome methylation array analysis.[58] Follow-up studies are required to clarify the potential utility of these methodologies in the isolation and assessment of CTCs.

Physical-property-based enrichment of CTCs

Several physical properties seem to distinguish CTCs from most normal peripheral blood cells, and many of these have been exploited to isolate CTCs from blood (Figure 1). The characteristics that can differ between CTCs and other blood-borne cells include density, size, deformability, and electrical properties.[14,59,60] After enrichment based on these physical properties, CTCs can be detected using immunohistochemistry, immunofluorescence, or molecular techniques such as PCR. In patients with prostate cancer, microfiltration methods have been used according to the assumption that CTCs are larger than leukocytes, and thus pores of varying geometries can retain CTCs while allowing leukocytes to pass through.[61–65] For example, the ISET® (Isolation by Size of Epithelial Tumour cells) system (RARECELLS, France) enriches for CTCs by filtering blood through membranes with pores 8 μm in diameter, followed by staining of cells retained on the filter for cytomorphological examination or immunocytochemistry (Table 1).[61] Although most prostate CTCs do seem to be larger than leukocytes, they exhibit wide variation in size, and a subset of these cells might be smaller than leukocytes.[36,66] A direct comparison between the CellSearch® assay and the ISET® microfiltration assay demonstrated only 60% concordance in the results obtained using samples from patients with prostate cancer, suggesting that these two cell-isolation techniques can identify different subpopulations of CTCs;[67] however, different criteria were used to validate and characterize CTCs isolated using each of these two platforms, which might account for some of the discordance observed. Specifically, CTCs detected using the ISET® assay were identified by a cytopathologist according to morphological criteria, whereas CTCs detected according to the CellSearch® methodology were identified based on the intensity of cytokeratin immunofluorescence signals and location of the 4',6-diamidino-2-phenylindole (DAPI) nuclear stain in the cell.[67] Thus, the ISET® protocol might have identified a subset of CTCs that do not stain for epithelial markers and,

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therefore, were not detected using the CellSearch® assay, whereas the CellSearch® could potentially have identified smaller CTCs that were lost during the ISET® procedure.

Enrichment of CTCs using methods based on other biophysical properties include dielectrophoresis to separate CTCs from peripheral blood cells based on intrinsic differences in the polarizability (that is, the electrical properties) of these cell types (Table 1), thus avoiding the necessity for antibody labelling and enabling the isolation of minimally modified CTCs for subsequent analysis.[59,60] Application of a nonuniform electric field generated by electrodes causes the attraction of tumour cells by positive dielectrophoretic forces while other cells flow past, and subsequent removal of the electric field enables the captured tumour cells to be collected.[59,60] Nevertheless, evidence indicates that dielectrophoresis and microfluidic immunocapture using the J591 anti-PSMA antibody can be used synergistically to improve the performance of CTC capture modalities.[68] These physical-property-based CTC enrichment technologies require further evaluation and clinical validation in patients with prostate cancer.

Other innovative approaches to CTC detection

Other approaches to CTC detection have been developed that avoid enrichment biases that might arise from the assumptions made regarding the physical or biological differences between CTCs and normal blood cells that often form the basis of cell selection. One method relies on RT-PCR-based detection of transcripts specific to prostate cancer cells in whole-blood nucleated-cell populations, and was shown to enable prediction of overall survival in patients with mCRPC in a pilot study.[69] However, this bulk RNA-assessment technique does not provide morphological data for the cells from which the prostate-cancer-related RNA transcripts are derived.

A technique that provides extensive morphological data is the high-throughput fibre-optic array scanning technology (FAST), which involves imaging every nucleated cell contained in a whole-blood sample spun onto a microscope slide (Table 1; Figure 1), thus avoiding the biases that might occur when CTC enrichment technologies are used.[66,70,71] However, the FAST approach remains limited by the choice of antibodies used for the immunofluorescence-based detection of CTC, as only cells expressing cytokeratin or other selected markers can be detected at present, which re-introduces a source of potential bias that is also encountered using other isolation methodologies. Another technology based on laser-scanning cytometry, a technique that combines flow cytometry with microscopy-based imaging, similarly avoids an enrichment step to maximize the detection of CTCs, but also relies on anti-EpCAM antibodies for visualization of these cells.[72] An additional comprehensive approach has been developed that enables the detection of CTCs directly in the blood *in vivo* using a medical wire functionalized with EpCAM antibodies that is placed into the patient's peripheral arm vein;[73] however, this unique method is again restricted by the limited number of available markers that are known to distinguish CTCs.

Standardization and validation of technologies

The development of innovative CTC-detection technologies has been driven largely by a desired ability to perform more sensitive and comprehensive analyses of CTCs, and many of the novel modalities have shown increased sensitivity of CTC detection in single-arm pilot studies. However, comparisons of sensitivity of cell detection across different platforms and validation of results have been hampered by a lack of standardization in the definition of CTCs, as well as differences in the clinical characteristics of the patient cohorts studied. At present, considerable disagreement regarding the classification of CTCs remains, depending on the isolation technique used, ranging from cytomorphological criteria, to the presence of specific protein markers (epithelial and/or mesenchymal), to measures of cell viability or invasiveness. Of note, in a comparison between the CellSearch® and ISET® systems, certain cells isolated by ISET® and identified as CTCs by an expert cytopathologist would not be identified using the CellSearch® assay, owing to the absence of immunostaining of these cells by specific antibodies.[67] In the development of our own microfluidic devices, definitions of CTCs have evolved with the use of different detection antibodies and increasingly sophisticated semi-automated image analysis technologies, necessitating recalibration of scoring parameters based on frequencies and intensities of the signals measured in healthy donor controls and cell-spiking experiments.[52,74] Thus, standardized comparisons of sensitivity of CTC detection between platforms and clinical validation are difficult to achieve, as a result of the wide-ranging, varied, and rapidly evolving definitions and criteria used for CTC classification and enumeration.

Standardization of the criteria that define CTCs will require coordination and consensus among pathologists, biologists, clinical investigators, and bioengineers from different institutions. Key issues that need to be addressed include the development of clear guidelines for the biological markers and cytomorphological characteristics that define CTCs, and whether different sets of criteria will be necessary to define specific subsets of CTCs (for example, epithelial versus mesenchymal). Standardized classification criteria for CTCs will be necessary not only for meaningful comparisons of sensitivity and specificity across CTC-detection platforms, but also as a prerequisite for analytical validation of CTC-related biomarkers before routine clinical use.

CTC enumeration in prostate cancer

CTC enumeration and prognosis

Although numerous pilot studies have been conducted assessing CTCs in patients with prostate cancer using a variety of cell-detection platforms, limited data from large clinical trials have been reported. The largest datasets relating to CTCs in prostate cancer were obtained using the CellSearch® system. Indeed, the FDA has cleared CellSearch®-based assessment of CTCs as a prognostic indicator in patients with metastatic breast, colon, and prostate cancers.[8,37] The prospective study

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that led to FDA clearance of the prognostic use of the CellSearch® assay in prostate cancer, IMMC38,[39] enrolled 276 patients with progressive mCRPC who were starting a new chemotherapy regimen, 231 of whom were evaluable. Similar to prior studies in breast cancer,[75,76] CTC numbers were evaluated in blood samples taken before treatment and monthly after initiation of therapy using the CellSearch® assay, and patients were categorized as having 'unfavourable' (five or more CTCs in 7.5 ml blood) or 'favourable' (fewer than five CTCs in 7.5 ml of blood) CTC counts.[39] The primary outcome of the IMMC38 trial[39] was that median overall survival in patients with unfavourable CTC counts at 2–5 weeks after initiation of the new chemotherapy regimen was >50% shorter than in the individuals with favourable CTC counts at this time point (9.5 months versus 20.7 months; HR 4.5; P <0.0001). Pretreatment CTC enumeration was also shown to have prognostic value, as patients with unfavourable numbers of CTCs before induction of the new therapy had shorter median overall survival compared with individuals with favourable CTC counts (11.5 months versus 21.7 months, respectively; HR 3.3; P <0.0001).[39] Unfavourable post-treatment CTC numbers were also significantly associated with shorter median overall survival regardless of the time point at which CTCs were assessed (6.7–9.5 months versus 19.6–20.7 months; HR 3.6–6.5; P <0.0001).[39] Furthermore, patients who converted from unfavourable CTC numbers at baseline to favourable CTC counts after treatment had a corresponding improvement in median overall survival (from 6.8 months to 21.3 months);[39] conversely, those who converted from favourable to unfavourable CTC levels had reduced median overall survival (from >26 months to 9.3 months).[39] Importantly, CTC count was a better predictor of overall survival than post-treatment changes in serum PSA levels at all time points.[39] Together, these findings indicated that CTC count was a useful biomarker of treatment response and overall survival in patients with mCRPC, with better performance than assessment of serum PSA.

A re-analysis of the IMMC38 trial data was performed,[40] focusing on the patients included who were receiving first-line chemotherapy, and evaluating CTC count as a continuous variable (rather than according to favourable versus unfavourable risk categories), as well as other pretreatment and post-treatment variables. Patients with bone metastases had higher CTC counts in general than individuals with visceral metastases, although this difference was not statistically significant.[40] Higher lactate dehydrogenase (LDH) concentrations (HR 6.44, 95% CI 4.24–9.79; P <0.0001), CTC counts (HR 1.58, 95% CI 1.41–1.77; P <0.0001), and serum PSA (HR 1.26, 95% CI 1.10–1.45; P = 0.0008) in baseline samples were associated with increased risk of death.[40] CTCs were also evaluated as an intermediate end point, by examining the relationship between changes in CTC numbers and survival. An increase in CTC count was moderately associated with decreased survival, whereas increased serum PSA levels were weakly or not associated with risk of death, suggesting that change in CTC counts could be a more accurate intermediate end point for clinical trials than variation in post-treatment PSA titres.[40]

Other studies have confirmed the prognostic value of CTC enumeration in mCRPC using the CellSearch® assay. A study in 120 patients with progressive mCRPC initiating treatment with a variety of hormonal or cytotoxic therapies found that baseline CTC count was strongly associated with overall survival, without a threshold 'unfavourable' effect.[38] The optimal cutoff point designation for favourable and unfavourable CTC counts has been evaluated in a single-institution study involving 100 patients with CRPC, with or without metastatic disease;[41] threshold analysis identified four CTCs in 7.5 ml of blood as the optimal cutoff point for correlation of CTC numbers with overall survival, and this threshold was 100% specific for the presence of radiographically evident metastatic disease.[41] Multivariate analysis also identified serum LDH concentration and CTC counts as independent prognostic factors.[41]

CTC enumeration as an intermediate end point

COU-AA-301[4] was a phase III, double-blind, randomized placebo-controlled trial that evaluated abiraterone acetate in 1,195 men with mCRPC who had previously received chemotherapy with docetaxel. Patients were randomized 2:1 to receive 1,000 mg of abiraterone acetate daily or placebo, together with 5 mg of prednisone twice daily.[4] At a median follow-up period of 12.8 months, this study met its primary end point of significantly improved overall survival in the abiraterone cohort versus the placebo cohort (14.8 months versus 10.9 months; P <0.001).[4] Of note, this trial was the first phase III trial to prospectively define a secondary end point evaluating whether CTC enumeration could be used as a surrogate efficacy-response biomarker of overall survival. In the planned final analysis of COU-AA-301 at a median follow-up duration of 20.2 months,[77] abiraterone treatment (HR 0.70, 95% CI 0.59–0.828; P <0.0001), baseline LDH concentration (HR 2.98, 95% CI 2.496–3.565; P <0.0001), and CTC count (HR 1.19, 95% CI 1.137–1.245 ; P <0.0001) were prognostic for survival, although PSA was not (HR 1.04, 95% CI 0.983–1.093; P = 0.1797). Interestingly, a change in CTC numbers from unfavourable (five or more CTCs in 7.5 ml of blood) to favourable (fewer than five CTC in the 7.5 ml of blood), or vice versa, was predictive of overall survival at the earliest post-treatment time point assessed (4 weeks), and conversion to an unfavourable CTC count substantially reduced the abiraterone-related treatment effect at all post-treatment time points.[77] A combined biomarker panel including CTC number conversion (from unfavourable to favourable) and baseline LDH level was developed using the trial data, and the treatment effect on survival was found to be correlated with this biomarker panel.[77] Further development of CTC assessments, possibly in the context of a biomarker panel including baseline LDH concentrations, are needed to clarify the potential role of CTC enumeration as an intermediate end-point surrogate for overall survival.

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Molecular analysis of prostate CTCs

Genetic alterations in CTCs

Several studies have demonstrated the feasibility of molecular characterization of prostate-cancer-related CTCs, which might provide prognostic information beyond CTC enumeration alone. Several groups have detected chromosomal translocations resulting in *TMPRSS2–ERG* gene fusion in CTCs isolated from patients with prostate cancer using RT-PCR and fluorescence *in situ* hybridization (FISH), with around 70% concordance between the presence of this genetic aberration in CTCs and the primary tumour.[74,78] The predictive value of *TMPRSS2–ERG* fusion status in CTCs was evaluated in patients with mCRPC who were treated with abiraterone acetate, but the presence of this gene fusion was not predictive of treatment response.[79] Other genetic alterations have been identified in CTCs, including loss of *PTEN* and *MYC* amplification,[78,80] as well as both amplification[78,80] and point mutation of the *AR* gene,[81] which encodes the androgen receptor. Other studies have extended the scope of molecular analyses to genome-wide copy number analysis in prostate CTCs.[82] The potential clinical relevance of these molecular analyses, however, remains limited at this time, given the paucity of effective molecularly targeted therapies targeting factors relating to specific genetic mutations in prostate cancer.

Predictive protein markers in CTCs

Several protein markers have been evaluated for their potential prognostic value when measured in CTCs. In a pilot study assessing the marker of proliferation Ki-67, CTCs isolated from different patients with prostate cancer exhibited wide variability in Ki-67 positivity (1–81%), and an increased Ki-67 proliferative index in CTCs was associated with resistance to castration therapy.[74] The AR protein has also been investigated in CTCs derived from patients with prostate cancer, with nuclear versus cytoplasmic localization demonstrated to correlate with clinical response to docetaxel chemotherapy.[83] Other studies have suggested that visualization and measurement of microtubule bundling in CTCs can be used to monitor the drug–target engagement of docetaxel chemotherapy and, therefore, might be useful in predicting the effectiveness of this treatment in individual patients.[54] Further refinement of these CTC-based assays of protein markers and incorporation of such assessments into clinical trials will be required for analytical validation and clinical qualification.

Androgen receptor signalling in CTCs

AR signalling is central to prostate cancer biology, and reactivation of AR signalling despite androgen deprivation therapy represents a fundamental mechanism underlying the emergence of castration-resistant disease.[84] Several effective therapies for mCRPC that target the AR signalling axis, such as abiraterone acetate and enzalutamide, have now been approved by the FDA,[4,5,7] creating an urgent need for biomarkers that can guide the application of these agents in the clinic. Several mechanisms of AR reactivation in mCRPC have been proposed, including *AR* gene amplification and activating mutations in *AR*.[84] *AR* copy number has been studied in prostate-cancer-associated CTCs through the use of FISH, and one study identified amplification of this gene in CTCs from 38% of the men with mCRPC evaluated,[80] similar to the proportion of patients in which *AR* amplification has been observed in bone metastasis biopsy studies.[85] Another study of genomic profiling of CTCs showed high-level copy number gains at the *AR* locus in seven of nine cases (78%), although interestingly these gains in *AR* copy number were not observed in matched archival primary tumour tissues, suggesting the occurrence of genomic evolution during cancer progression.[82] A separate study also demonstrated that mutations in the *AR* gene could be detected in CTCs from patients with prostate cancer using PCR amplification and direct sequencing.[81] Although these approaches can provide a snapshot of *AR* gene status in CTCs, they might have a limited ability to provide a dynamic readout of changes in *AR* signalling in response to therapy, as treatment-induced changes at the genomic level typically take longer to manifest than changes at the protein signalling or transcriptomic levels.

Using the ^{HB}CTC-Chip, our group has developed a single-cell immunophenotyping approach to dynamically measure AR signalling in CTCs.[86] The relative activity of AR signalling in a given cell can be estimated based on the levels of the proteins PSA and PSMA, which are encoded by genes that have been identified as consistently upregulated and downregulated, respectively, following AR activation in prostate cancer cells (Figure 3a); *KLK3* (encoding PSA) is a classic androgen-upregulated gene, whereas *FOLH1* (encoding PSMA) has been demonstrated to be androgen-downregulated in an AR-dependent manner.[87–89] In developing our two-colour immunofluorescence assay using androgen-responsive prostate cancer cell lines, we identified $PSA^+/PSMA^-$ cells as androgen-induced ('AR-on'), $PSA^-/PSMA^+$ cells as androgen-suppressed ('AR-off'), and $PSA^+/PSMA^+$ ('AR-mixed') cells as cells transitioning between AR-off and AR-on states.[86] Applying this assay to CTCs isolated from patients with untreated metastatic prostate cancer, the majority of CTCs initially had an AR-on phenotype, but switched to AR-off phenotype within 1 month of initiation of androgen-deprivation therapy.[86] By contrast, a striking heterogeneity was evident in CTCs derived from patients with mCRPC, with only AR-on or AR-off cells isolated from some patients, whereas AR-mixed cells or CTCs demonstrating all three AR-activity phenotypes were observed in samples from other individuals (Figure 3b).[86] These results suggest that AR reactivation in CRPC could, in some cases, be more modest than expected, pointing to important contributions from other signalling pathways leading to androgen resistance. On the basis of blood samples from patients with mCRPC treated with abiraterone acetate, an increase in the proportion of AR-on CTCs (despite this hormonal therapy) was predictive of decreased overall survival, as was the presence of an AR-mixed population of CTCs at

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baseline.[86] These findings highlight the potential utility of assessing dynamic AR signalling in CTCs in monitoring and predicting treatment responses to AR-targeting therapies, although further evaluation of this approach is required in prospective clinical trials.

Other approaches have also been taken to evaluate the status of AR and its signalling axis in CTCs associated with prostate cancer. The results of one study suggest that AR activity can be inferred based on the subcellular localization of the AR protein in CTCs, with nuclear localization indicative of transcriptionally active AR and cytoplasmic localization reflecting AR inactivity.[83] As introduced earlier, analysis of CTCs isolated from a series of patients with mCRPC revealed a correlation between cytoplasmic sequestration of AR protein and clinical response to docetaxel, leading the authors to postulate that this agent acts in part through inhibition of microtubule trafficking and, therefore, nuclear translocation of the AR.[83] This approach to determining the subcellular localization of AR protein currently requires the use of confocal microscopy and is time intensive; however, advances in semi-automated high-resolution imaging technologies in combination with multispectral imaging might enable the future integration of this assay into a comprehensive panel of biomarker analyses, including assessment of PSA and PSMA expression, that provide data on AR signalling in CTCs.

Conclusions

For both the clinical management of individual patients with prostate cancer and the assessment of therapies in clinical trials, the clinical need for highly informative biomarkers remains unmet. Conventional imaging modalities, serum PSA assays, and biopsy of bone metastases each have important limitations that restrict their utility in the assessment of prostate cancer. New CTC-detection technologies that enable highly sensitive CTC isolation and subsequent detailed molecular analyses of these cell types offer the potential for noninvasive real-time monitoring of disease, although large validation studies are required to clarify the clinical relevance of such approaches. Key advances in CTC isolation made in recent years include: improved sensitivity of CTC detection; increased purity of isolated cell populations, which is important for performing molecular analyses; the development of methodologies for isolating CTCs using cell-surface proteins other than EpCAM, which might enable for capture of cells undergoing EMT; and the use of negative selection to isolate CTCs with minimal manipulation. The ultimate clinical application of CTCs could involve a 'point-of-care' device that provides CTC counts rapidly and reliably, performs molecular characterization of these cells, and assists with therapeutic decision-making. Indeed, the marriage of bioengineering, biology, and medicine that has given rise to CTC isolation technologies promises to propel patient care forward by facilitating the use of rationally targeted therapies based on real-time molecular information obtained from CTC-based liquid biopsies.

Review criteria

We searched PubMed for English-language full-text manuscripts and abstracts published up to November 2013. The search terms used, alone and in various combinations, were "circulating tumour cell", "circulating tumour cells", "circulating tumor cell", "prostate cancer", and "single-cell DNA/RNA". The reference lists of the articles identified were also searched for additional relevant publications.

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