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14. ABSTRACT This Physician Research Training Award supports a mentored training program integrated with a research project focused on the study of circulating tumor cells (CTCs) in prostate cancer patients using a novel microfluidic "CTC-chip". During this award, we have successfully developed a quantitative immunofluorescence assay to measure AR activity in single CTCs, and have piloted this assay in men with metastatic prostate cancer receiving treatment with hormonal therapy. We have shown that this novel assay can effectively measure AR activity levels in CTCs, and that CTC AR activity at baseline and after therapy may be correlated with treatment outcomes in prostate cancer. In addition, we have made progress in single CTC digital gene expression profiling to better understand heterogeneity of metastatic precursor cells, and have identified differences in transcriptional profiles in castration-resistant prostate cancer cells that have acquired resistance to AR targeting therapies. Finally, this Award has enabled the protection of time for research and mentored training of the PI to continue his development towards a productive career in translational prostate cancer research.					
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INTRODUCTION

Castration-resistant prostate cancer (CRPC) is thought to arise from the persistence of androgen receptor (AR) signaling in cancer cells despite castrate levels of testosterone (1). In the Research Project supported by this Physician Research Training Award, we use a novel microfluidic technology (the “CTC-chip”) to interrogate the status of AR signaling and other signaling pathways in circulating tumor cells (CTCs) isolated from metastatic prostate cancer patients. An AR activity signature developed in prostate cancer cell lines is being applied to CTCs in patients with castration-resistant prostate cancer (CRPC) before and after secondary hormonal therapies to test the hypothesis that effective suppression of AR signaling in CTCs correlates with clinical response to hormonal therapy. To identify novel genes and pathways involved in the evolution of treatment resistant disease, digital gene expression profiling of single CTCs is being performed. These studies are aimed at providing initial validation of a novel molecular biomarker that can monitor and predict responses to second-line hormonal therapy in patients with CRPC, as well as revealing fundamental insights into the mechanisms underlying treatment resistance in prostate cancer. Progress during Year 1 of this Research Project included development of a single cell immunofluorescence-based assay for measurement of AR activity; demonstration of the feasibility of using AR activity in CTCs as a biomarker to monitor and potentially predict response to second line hormonal therapy in a small cohort of patients with CRPC; and the demonstration of feasibility of performing high throughput qRT-PCR and whole transcriptome RNA-sequencing of single prostate CTCs. Progress during Year 2 of this Project included recruitment of additional patients for AR signaling analysis, transitioning to a novel 3rd generation CTC isolation platform, and initial analysis of single CTCs for digital gene expression analysis to identify pathways that correlate with treatment resistance. These studies have so far resulted in two published manuscripts during Year 1, and one critical review published during Year 2. Ongoing studies are aimed at further validation of the CTC AR activity assay in additional patients, and on continued transcriptional profiling analysis of single CTCs to provide insights into the molecular mechanisms of the development of treatment resistance in CRPC. In combination with this integrated research project, this Physician Research Training Award has enabled the protection of time for research and mentored training of the PI towards his development into a productive translational prostate cancer researcher.

SPECIFIC AIMS

1. Define an AR activity score in CTCs and test the hypothesis that AR signaling activity in prostate CTCs correlates with response to second-line hormonal therapy in metastatic prostate cancer patients.
2. Perform digital gene expression (DGE) profiling of prostate CTCs to identify novel pathways that promote castration resistance.

BODY

Progress on Statement of Work (SOW).

This Research Project uses a novel microfluidic technology called the “CTC-chip” to interrogate cellular signaling activity in circulating tumor cells (CTCs) isolated from metastatic prostate cancer patients in an effort to monitor and predict treatment response, as well as gain a deeper understanding of molecular mechanisms of treatment resistance (2, 3). An AR activity signature developed in prostate cancer cell lines is being applied to CTCs in patients with castration-resistant prostate cancer (CRPC) before and after secondary hormonal therapy to test the hypothesis that effective suppression of AR signaling in CTCs correlates with clinical response to hormonal therapy. To identify novel genes and pathways involved in the evolution of castration-resistant prostate cancer, digital gene expression profiling of CTCs is being performed. Progress on Tasks related to the Research Project are outlined below.

Task 1. Regulatory review and approval of clinical protocol.

A clinical research protocol for the collection of blood from patients with solid tumors for CTC analysis (DF/HCC 05-300) was initially received by the US Army Medical Research and Materiel Command (USAMRMC), Office of Research Protections (ORP), Human Research Protection Office (HRPO) on 9 May 2012, and reviewed for compliance with human subject protection requirements. A revised research consent form and clinical research protocol was approved by the HRPO on 7 June 2012, and this revised protocol was approved by the Dana-Farber Cancer Institute Institutional Review Board (DFCI IRB) on 12 July 2012. This final protocol then received approval by the HRPO on 30 July 2012. Continuation of the subject protocol was approved by the DFCI IRB on 30 May 2013, and most recently on 9 May 2014. This approval is due for continuing review by the DFCI IRB on 9 May 2015.

Task 2. (Aim 1) Recruitment of patients with castration-resistant prostate cancer for CTC AR activity analysis.

Thus far, 27 patients with CRPC have been recruited for the purposes of AR activity analysis in CTCs. Accrual was slowed down during this reporting period due to a transition in CTC isolation technology in the laboratory from the 2nd generation HB CTC-chip technology (2) to the 3rd generation CTC iChip technology (4). It is anticipated that subject accrual will increase during the next reporting period now that the technology has been properly established and tested in the laboratory.

Task 3. (Aim 1) CTC AR signature activity analysis in patients.

As second line AR targeting therapies have entered clinical care for CRPC (e.g. abiraterone acetate and enzalutamide) in addition to cytotoxic therapeutics (e.g. docetaxel, cabazitaxel, R-223), no reliable biomarkers exist to target appropriate therapies to individual patients (5, 6). To address this challenge, we developed a single cell immunophenotyping approach to measure AR activity in CTCs using two genes that we identified as most consistently upregulated and downregulated following AR modulation in prostate cancer cells: PSA (androgen driven) and PSMA (androgen suppressed). Application of this CTC-based assay in a small cohort of men with prostate cancer revealed that the AR activity in CTCs as measured by PSA and PSMA expression levels could be used to monitor the treatment of patients undergoing AR targeting therapies, as well as potentially predict the outcomes of therapy. We describe these results in detail in a manuscript published in *Cancer Discovery* in 2012 (7).

Since the publication of these results, which were based on CTC isolation using the 2nd generation HB CTC-chip technology (2), we have transitioned the laboratory to the 3rd generation CTC iChip technology (4), and have adapted the PSA/PSMA assay for use with this new CTC isolation platform. We are now continuing our efforts to validate this assay in additional castration-resistant prostate cancer patients receiving second-line hormonal therapy, with the hope that it will prove useful in directing individualized therapies for castration-resistant disease.

Task 4. (Aim 2) Recruitment of patients with metastatic castration-resistant prostate cancer and metastatic castration-sensitive prostate cancer for digital gene expression profiling.

Thus far, 18 patients with metastatic prostate cancer and 4 patients with localized untreated prostate cancer have been recruited for the purposes of digital gene expression profiling of CTCs. Accrual slowed down during this reporting period due to a transition in CTC isolation technology in the laboratory from the 2nd generation HB CTC-chip technology (2) to the 3rd generation CTC iChip technology (4). Patient samples that had been previously processed using the early HB CTC-chip could not be utilized for the purposes of digital gene expression profiling because of excessive leukocyte contamination on the surface of the HB CTC-chip, which interfered with tumor specific RNA sequencing. Thus, additional blood samples have been obtained from patients (in accordance with the clinical protocol which allows for multiple blood draws from patients who give informed consent), and these have been processed for single cell CTC isolation using the CTC iChip, followed by RNA sequencing and digital gene expression profiling.

Task 5. (Aim 2) Digital gene expression profiling of CTCs including sample preparation and RNA sequencing.

One of our primary goals is to identify cellular pathways that underlie the acquisition of treatment resistance in prostate cancer by dissecting the transcriptome of CTCs. Reactivation of AR signaling by itself cannot fully explain castration resistant disease, given the relatively modest clinical benefit of second line hormonal therapies, as well as the evident heterogeneity of androgen signaling readouts that we have uncovered through quantitative immunofluorescence PSA/PSMA single cell analysis described above (7). However, given this intercellular heterogeneity, defining the relevant signaling pathways will require RNA sequencing and digital gene expression profiling at the level of individual single CTCs. The use of single CTC analysis also obviates the need for digital subtraction of contaminating leukocyte signal that has been necessary with RNA sequencing of preparations of whole CTC/leukocyte mixtures (8).

During this Award, we have demonstrated that the third generation CTC-chip technology (CTC-iChip) can be used to generate high purity CTC preparations in solution that can be micromanipulated for single cell analysis (4). We have also demonstrated our ability to micromanipulate single CTCs and extract high quality RNA for molecular analyses including microfluidic qRT-PCR for a panel of multiple genes, and this work was published in part in *Science Translational Medicine* in 2013 (4). Subsequently, we have established protocols in our lab for single CTC RNA-sequencing by adapting methods published previously for single cell RNA-sequencing of single mouse blastomeres (9). In the single cell context (as opposed to CTC/leukocyte mixtures), PCR amplification of signal is essential and does not carry the risk of loss of CTC signal, hence the single molecule Helicos sequencing platform is not required. We are using the ABI SOLiD 5500 library preparation procedures, including reverse transcription,

sonication to fragments of 80-130bp, end repair, blunt-end ligation and emulsion PCR, followed by Next Generation Sequencing using the ABI SOLiD 5500 machines provided by the MGH Center for Cancer Research Core facility (9).

We have applied the CTC iChip platform to efficiently deplete normal hematopoietic cells from whole blood specimens, revealing untaged CTCs that can be individually selected for single cell RNA sequencing analysis. Cell surface staining of unfixed cells for either epithelial (EpCAM) or mesenchymal (CDH11) markers, together with the absence of staining for the common leukocyte marker CD45, is being used to confirm the identity of selected tumor cells (Fig 1A-C). During this reporting period, we have isolated 236 single prostate CTCs from 18 patients with metastatic prostate cancer and 4 patients with localized prostate cancer. Of these, 130 (55%) had RNA of sufficient quality for next generation RNA sequencing. While many cancer cells in the circulation appear to undergo apoptosis, the presence of intact RNA identifies the subset enriched for viable cells. In addition to candidate CTCs, we also obtained comprehensive transcriptomes for 12 primary tumor prostatectomy specimens, 30 single cells derived from four different prostate cancer cell lines and 5 patient-derived leukocytes.

Figure 1: (A) Schematic of CTC iChip platform for isolation of CTCs from patient blood. (B) Merged image (brightfield and fluorescence) of a single CTC labeled with Alexa Fluor 488 tagged EpCAM, harvesting by micromanipulation for subsequent RNA sequencing. (C) Immunofluorescence labeling of live CTCs with EpCAM and CDH11 in green and CD45 in red to identify CTCs.

Task 6. (Aim 2) Data analysis of CTC digital gene expression profiles.

We have made considerable progress in the analysis of the whole transcriptome digital gene expression profiles of the CTCs that have been sequenced thus far. Of the 130 candidate single prostate CTCs that were successfully analyzed by RNA sequencing, 77 were identified to be bonafide CTCs based on expression of prostate-specific genes and low expression of hematopoietic genes (Figure 2A). Unsupervised hierarchical clustering analysis of these 77

Figure 2: (A) Scatter plot used to identify bona fide CTCs that have high expression of cytokeratin or prostate-specific genes, and low expression of hematopoietic genes, based on thresholds determined by WBC and cell line controls. (B) Heatmap of unsupervised hierarchical clustering of transcriptional profiles of 77 single CTCs, 30 single prostate cancer cell line samples, and 12 prostate tumors. (C) Gene panels demonstrating expression of lineage-specific markers and other phenotypes of interest.

3A). Deeper analysis of these genes and the pathways that they represent are currently ongoing.

Figure 3: (A) Volcano plot demonstrating differential expression of genes in CTCs from patients with prostate cancer that is enzalutamide-resistant or enzalutamide-naïve.

A

Task 7. (Aim 2) Validation and follow-up studies of promising genes and pathways identified through CTC digital gene expression profiles.

Progress on this Task will be reported after we have identified candidate genes and pathways involved in treatment-resistant prostate cancer through analysis of CTC digital gene expression profiles, obtained through the methods described above.

Task 8. Preparation of results, presentations, and manuscripts.

We published a manuscript in *Cancer Discovery* in 2012 describing our results analyzing AR signaling activity in CTCs using the PSA/PSMA immunofluorescence-based assay (7). In addition, pilot data demonstrating the isolation of single CTCs using the CTC-iChip followed by microfluidic multigene qRT-PCR was published as part of a manuscript in *Science Translational Medicine* in 2013 (4). A critical review on the potential of CTCs for monitoring and predicting treatment response in prostate cancer was published in *Nature Reviews Clinical Oncology* in 2014 (Attached in Appendix) (3). Finally, the PI has delivered several oral presentations, including at the MGH Cancer Center departmental seminar in December 2012, the BIO International Convention in Chicago, IL in April 2013, the NextGen Diagnostics Summit in Washington DC in August 2013, the World CTC Summit in Boston, MA in November 2013, and the SELECTBIO Circulating Biomarkers Conference in Boston, MA in May 2014. It is anticipated that several additional manuscripts will be published during the term of this Research Project.

Progress in Training Plan for PI.

This Physician Research Training Award combines a training plan for the PI consisting of mentorship, coursework, conferences, seminars, and patient care, together with an integrated research project to enable the PI to become an effective translational investigator in prostate cancer. With regard to the Research portion of my training award, I have continued to attend weekly formal mentorship meetings with Dr. Daniel Haber and Dr. Shyamala Maheswaran to discuss my research progress and future research directions. I have continued to meet with Dr. Matthew Smith on a monthly basis for continued guidance regarding clinical aspects of my research. I have also continued to receive scientific guidance from key collaborators and advisors, including Dr. Mehmet Toner and Dr. Sridhar Ramaswamy.

With regard to coursework, conferences, and seminars, I have taken several formal courses in clinical trial design, biostatistics, informed consent, and data safety and monitoring through the MGH Clinical Research Program Education Unit. I have also been attending several relevant regularly scheduled conferences, including the biweekly MGH multi-disciplinary urologic oncology conference, the MGH Cancer Center Grand Rounds, and the MGH Radiation Oncology chart rounds. I also attended the 2013 and 2014 Annual Meetings of the American Association for Cancer Research (AACR).

Finally, as a component of my training as a physician scientist specialized in prostate cancer, I have engaged in the clinical care of patients as a radiation oncologist specializing in genitourinary malignancies. I devote approximately 1 day a week to the care of patients who are undergoing or who have completed radiation therapy, and a half day per week providing new consultations for patients presenting to the MGH Multidisciplinary GU Oncology clinic together with Urology and Medical Oncology colleagues. My clinical responsibilities have been limited to 20% to 30% of my total effort. Together, this training plan has been effective in enabling my growth as a translational prostate cancer investigator.

KEY RESEARCH ACCOMPLISHMENTS

- Development of an androgen receptor (AR) signaling assay in CTCs based on a two-color PSA/PSMA immunofluorescence assay.
- Demonstration of the ability of the immunofluorescence-based AR signaling assay to measure AR activity in CTCs in patients with prostate cancer, and potentially predict patient outcomes after abiraterone acetate treatment.
- Development of a methodology to isolate single CTCs for RNA analysis using the microfluidic CTC-iChip.
- Demonstration of the feasibility of microfluidic qRT-PCR to measure mRNA transcript levels in single prostate CTCs.
- Demonstration of feasibility of RNA-sequencing and digital gene expression profiling of whole transcriptomes in single prostate CTCs.
- Successful RNA-sequencing and digital gene expression profiling of 130 single prostate CTCs.
- Preliminary analysis of genes upregulated in CTCs from patients with metastatic CRPC and acquired resistance to enzalutamide.

REPORTABLE OUTCOMES

- Manuscripts:
 - **Miyamoto, D.T.**, Lee, R.J., Stott, S.L., Ting, D.T., Wittner, B.S., Ulman, M., Smas, M.E., Lord, J.B., Brannigan, B.W., Trautwein, J., Bander, N.H., Wu, C.L., Sequist, L.V., Smith, M.R., Ramaswamy, S., Toner, M., Maheswaran, S., Haber, D.A. (2012). Androgen receptor signaling in circulating tumor cells as a marker of hormonally responsive prostate cancer. *Cancer Discovery*, 2:995-1003.
 - Ozkumur, E., Shah, A.M., Ciciliano, J.C., Emmink, B.L., **Miyamoto, D.T.**, Brachtel, E., Yu, M., Chen, P., Morgan, B., Trautwein, J., Kimura, A., Sengupta, S., Stott, S.L., Karabacak, N.M., Barber, T.A., Walsh, J.R., Smith, K., Spuhler, P., Sullivan, J., Lee, R., Ting, D.T., Luo, X., Shaw, A.T., Bardia, A., Sequist, L.V., Louis, D.N., Maheswaran, S., Kapur, R., Haber, D.A., Toner, M. (2013). Inertial Focusing for Positive and Negative Sorting of Rare Circulating Tumor Cells. *Science Translational Medicine*, 5:179ra47.
 - **Miyamoto, D.T.**, Sequist, L.V., Lee, R.J. (2014). Circulating tumour cells – monitoring treatment response in prostate cancer. *Nature Reviews Clinical Oncology*, 11(7):401-12.
- Presentations:
 - **Miyamoto, D.T.** “Monitoring Androgen Receptor Signaling in Circulating Tumor Cells in Prostate Cancer.” Departmental Seminar, MGH Cancer Center, Massachusetts General Hospital, Boston, MA 12 December 2012.
 - **Miyamoto, D.T.** “Microfluidic Isolation and Molecular Analysis of Circulating Tumor Cells.” BIO International Convention, Chicago, IL, 22 April 2013.
 - **Miyamoto, D.T.** “Microfluidic Isolation and Molecular Analysis of Circulating Tumor Cells.” Next Generation Diagnostics Summit, Washington DC, August 2013.
 - **Miyamoto, D.T.** “Microfluidic Isolation and Molecular Analysis of Circulating Tumor Cells.” World CTC Summit, November 2013.
 - **Miyamoto, D.T.** “Microfluidic Isolation and Molecular Characterization of Circulating Tumor Cells.” SELECTBIO Circulating Biomarkers Conference, May 2014.

CONCLUSIONS

In the course of this project, we have made considerable progress in demonstrating the use a novel microfluidic technology (the “CTC-chip”) to isolate circulating tumor cells (CTCs) from metastatic prostate cancer patients, interrogate their AR signaling activity, and perform RNA-sequencing to identify genes and pathways involved in the evolution of castration-resistant prostate cancer (CRPC). Progress during Year 1 of this Research Project included development of a single cell immunofluorescence-based assay to measure AR activity; demonstration of feasibility of using AR activity in CTCs as a biomarker to monitor and

potentially predict response to second line hormonal therapy in patients with CRPC; and demonstration of feasibility of performing high throughput qRT-PCR and whole transcriptome RNA-sequencing of single prostate CTCs. Progress during Year 2 of this Project included recruitment of additional patients for AR signaling analysis, transitioning to a novel 3rd generation CTC isolation platform, and initial analysis of single CTCs for digital gene expression analysis and identification of upregulated genes that correlate with treatment resistance. Ongoing efforts are aimed at further validation of the CTC AR activity assay in additional patients, and transcriptional profiling analysis of single CTCs from patients with prostate cancer. If successful, this Research Project will provide initial validation of a novel molecular biomarker that can monitor and predict responses to second-line hormonal therapy in patients with CRPC, as well as reveal fundamental insights into the mechanisms underlying castration-resistance in prostate cancer. Finally, this Award has enabled the protection of time for research and mentored training of the PI to continue his development towards a productive career in translational prostate cancer research.

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3. Miyamoto DT, Sequist LV, Lee RJ. Circulating tumour cells-monitoring treatment response in prostate cancer. *Nat Rev Clin Oncol* 2014; 11: 401-12.
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APPENDICES

Includes the following:

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Curriculum vitae, David T. Miyamoto, July 2014**15**

Miyamoto, D.T., et al. (2014). Circulating tumour cells – monitoring treatment response in prostate cancer. *Nature Reviews Clinical Oncology*, 11:401-412**19**

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Miyamoto, David T.	POSITION TITLE Instructor in Radiation Oncology, Harvard Medical School Assistant Physician, Massachusetts General Hospital		
eRA COMMONS USER NAME (credential, e.g., agency login)			
EDUCATION/TRAINING <i>(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)</i>			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	MM/YY	FIELD OF STUDY
Harvard College, Cambridge, MA	A.B.	06/1997	Chemistry
Harvard University, Cambridge, MA	Ph.D.	11/2004	Cell & Developmental Biology
Harvard Medical School, Boston, MA	M.D.	06/2006	Medicine
Brigham & Women's Hospital, Boston, MA		06/2007	Health Sciences & Technology
Harvard Radiation Oncology Program, Boston, MA		06/2011	Intern, Internal Medicine
			Resident, Radiation Oncology

A. Personal Statement

My multidisciplinary training in molecular cell biology, clinical radiation oncology, and cancer biology has enabled me to become a translational physician scientist with a strong clinical and research interest in genitourinary malignancies. I received my MD-PhD working in the laboratory of Dr. Timothy Mitchison studying the mechanisms of cell division. My postdoctoral research involves the study of circulating tumor cells (CTCs) in prostate cancer with Dr. Daniel Haber. I am currently an Instructor in Radiation Oncology at Harvard Medical School, devoting 70% of my effort to translational cancer research and 30% to clinical activities treating patients with genitourinary malignancies. My research focuses on the development of novel microfluidic bioengineering technologies for the detection and molecular analysis of CTCs, the development of new genomic-based biomarkers, and the application of these technologies to guide the treatment of patients with genitourinary malignancies.

B. Positions and HonorsPositions and Employment

2009 - Postdoctoral Research Fellow, Massachusetts General Hospital Cancer Center / Howard Hughes Medical Institute, Boston, MA (Professor Daniel A. Haber)
2011 - Instructor in Radiation Oncology, Harvard Medical School
2011 - Assistant Physician in Radiation Oncology, Massachusetts General Hospital

Other Professional Positions

1999 - 2006 Resident Tutor in Medicine, Leverett House, Harvard College, Cambridge, MA
2007 Visiting Physician, National Cancer Center Hospital, Tokyo, Japan
2011 - Harvard-MIT Health Sciences and Technology MD Board of Advisors, Harvard Medical School

Academic and Professional Honors

1993 National Merit Scholarship
1993 – 1997 John Harvard Scholarship
1996 Merck Undergraduate Research Fellow
1997 *Magna cum laude*, Harvard College
1997 High Honors in Chemistry, Harvard College
2001 – 2004 Howard Hughes Medical Institute Predoctoral Fellow
2009 – 2011 B. Leonard Holman Research Pathway, American Board of Radiology
2010 ASTRO Annual Meeting Scientific Abstract Award

Principal Investigator (Last, First, Middle): Miyamoto, David T.

2011 – 2013	A. David Mazzone Career Development Award, Dana-Farber/Harvard Cancer Center
2011 – 2012	Research Career Development Award, Federal Share of the Proton Beam, NCI/MGH
2012	Ira J. Spiro Translational Research Award
2012 – 2017	Physician Research Training Award, Department of Defense
2014	Ira J. Spiro Translational Research Award

Licensure and Certification

2008 – present	Massachusetts Full License, Board of Registration in Medicine
2012 – present	Board Certification, Radiation Oncology, American Board of Radiology

Membership in Professional Societies

2013 – present	Member, American Association for Cancer Research
2008 – 2012	Member, American Society for Radiation Oncology
2003 – 2011	Member, American Society for Cell Biology
2008 – 2011	Member, Radiological Society of North America
2009 – 2011	Member, American Society of Clinical Oncology

C. Selected Publications

Peer-reviewed original articles

1. Haggarty, S.J., Mayer, T.U., **Miyamoto, D.T.**, Fathi, R., King, R.W., Mitchison, T.J., and Schreiber, S.L. (2000). Dissecting cellular processes using small molecules: identification of colchicine-, taxol-like, and other small molecules that perturb mitosis. *Chemistry & Biology*, 7:275-86.
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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 ¹⁸F-fluorodeoxyglucose (¹⁸F-FDG), ¹⁸F sodium fluoride (¹⁸F-NaF), and ¹¹C-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

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 (<i>MUC1</i> , <i>HER2</i> , <i>EPCAM</i>)	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	¹⁰ PCTC-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 PSMA), or RT-PCR for selected genes	¹⁰ PCTC-Chip (MGH, USA) ⁵²
Microfluidic inertial focusing chip	EpCAM-based selection; immunofluorescence for selected tumour markers (CK, PSA, and PSMA)	¹⁰⁰ sCTC-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	¹⁰⁰ gCTC-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 <i>KLK3</i> , <i>KLK2</i> , <i>HOXB13</i> , <i>GRHL2</i> , and <i>FOXA1</i>)	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: ¹⁰ PCTC-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 <i>in situ</i> hybridization; GEDI, geometrically enhanced differential immunocapture; ¹⁰⁰ PCTC-Chip, herringbone CTC-chip; HD-CTC, high-definition-CTC (assay); ISET, isolation by size of epithelial tumour cells; MGH, Massachusetts General Hospital; ¹⁰⁰ gCTC-iChip, negative selection CTC-inertial-focusing-chip; ¹⁰⁰ sCTC-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

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

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 ^{HP}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

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 ^hCTC-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 ^hCTC-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; ^hCTC-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 (ELISPOT) 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 (Vitatest, 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,

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

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 trial³⁹ 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-301⁴ 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.

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

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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Author contributions

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