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TITLE: Probing Androgen Receptor Signaling in Circulating Tumor Cells in Prostate Cancer

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**Title:** Probing Androgen Receptor Signaling in Circulating Tumor Cells in Prostate Cancer

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**Dates Covered:** 1 July 2012 – 30 June 2013

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**Abstract:**
This Award focuses on using a novel microfluidic “CTC-chip” to capture and measure androgen receptor (AR) activity in circulating tumor cells (CTCs) in prostate cancer patients before and after hormonal therapy. During the first year of 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. Preliminary results suggest 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. Thus, this CTC-based assay may serve as a non-invasive biomarker that can potentially help guide therapy in prostate cancer. These initial results were published during this term in Cancer Discovery. Ongoing efforts include single CTC transcriptional profiling to better understand heterogeneity of metastatic precursor cells and uncover alternative signaling pathways that may be activated in castration-resistant prostate cancer.
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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 this Research Project, we use a novel microfluidic technology (the “CTC-chip”) to interrogate the status of AR signaling activity 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 castration-resistant disease, digital gene expression profiling of 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 castration-resistance in prostate cancer. Progress during Year 1 of this Research Project includes 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. These pilot studies have resulted in two published manuscripts so far, attached in the Appendix to this report. Ongoing studies are aimed at further validation of the CTC AR activity assay in additional patients, and on transcriptional profiling analysis of single CTCs to provide insights into the molecular mechanisms of the development of CRPC.

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.
Progress on Statement of Work (SOW) During this Reporting Period.

This Research Project uses a novel microfluidic technology called the “CTC-chip” to interrogate the status of androgen receptor (AR) signaling activity in circulating tumor cells (CTCs) isolated from metastatic prostate cancer patients (2). 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 disease, 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 was in turn approved by the HRPO on 19 July 2013. This approval is due for continuing review by the DFCI IRB on 30 May 2014.

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

Thus far, 17 patients with CRPC have been recruited for the purposes of AR activity analysis in CTCs through the DF/HCC 05-300 clinical protocol described above. Interim results from CTC AR activity analysis from these patients are described below.

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

As second line AR targeting therapies enter clinical care for CRPC (e.g. abiraterone acetate and enzalutamide) in addition to cytotoxic therapeutics (e.g. docetaxel, cabazitaxel, Ra-223), no reliable biomarkers exist to target appropriate therapies to individual patients (3, 4). 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). While developing and testing our two color assay using androgen responsive prostate cancer cell lines, we effectively identified PSA+/PSMA- cells as androgen activated (AR-on) and PSA-/PSMA+ cells as androgen suppressed (AR-off). In transition between AR-on and AR-off states, we identified PSA+/PSMA+ (AR-mixed) cells.

Application of this CTC-based assay in a small cohort of men with prostate cancer revealed several interesting findings. In treatment-naïve men with metastatic prostate cancer, the majority of CTCs were AR-on. Within 4 weeks of initiation of androgen deprivation therapy, these CTCs turned predominantly to AR-off, and CTC counts thereafter dropped below
detection. In contrast, striking heterogeneity was evident in CRPC where some patients had only AR-on cells, some had only AR-off cells, and others had large numbers of dual positive AR-mixed cells. In a cohort of 17 patients with CRPC treated with abiraterone acetate, the presence of AR-mixed cells prior to treatment or an increase in AR-on cells despite therapy were associated with decreased overall survival. Together, these studies point to a novel approach to measure the fraction of CTCs that are AR-driven and likely to be sensitive to hormonal therapy. These preliminary results are described in detail in the manuscript attached in the appendix (Miyamoto et al., Cancer Discovery 2012), which was published during this reporting period (5).

In our future plans, we plan to initiate a larger-scale clinical trial 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, 17 patients with CRPC and 3 patients with CSPC have been recruited for the purposes of digital gene expression profiling of CTCs through the DF/HCC 05-300 clinical protocol described above. Interim results from digital gene expression profiling of these patients is described below.

**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 castration-resistance 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 immunophenotyping described above (5). 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 (6).

During this reporting period, we have piloted the use of a third generation CTC-chip (CTC-iChip) recently designed at the MGH, which is capable of generating high purity CTC preparations in solution that can be micromanipulated for single cell analysis (7). As proof of principle to demonstrate our ability to micromanipulate single CTCs and extract high quality RNA for molecular analyses, we used the CTC-iChip to isolate 15 CTCs from a patient with metastatic CRPC and performed microfluidic qRT-PCR for a panel of 43 genes. These results are described in detail in the manuscript attached in the appendix (Ozkumur et al., Science Translational Medicine 2013), specifically in Figure 7 of the manuscript, which was published during this reporting period (7). This initial analysis has set the stage for our subsequent analyses using RNA sequencing of individual prostate CTCs.

We have subsequently established protocols in our lab for single CTC RNA-sequencing by adapting methods published previously for single cell RNA-sequencing of single mouse blastomeres (8). 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 (8). To date, we have piloted this approach with 30 single CTCs from 6 CRPC patients and have derived RNA sequencing reads that are comparable to those with control cancer cell lines, with an average of 9,508,135 reads per cell and 5,005,694 reads aligned to the known transcriptome per cell, providing approximately 10-20x coverage (Fig. 1). We plan to continue applying this single cell RNA sequencing protocol to 100 individual CTCs from patients with castration resistant compared to castration sensitive prostate cancer.

**Figure 1:** (A) Heat map of whole transcriptome RNA-seq digital gene expression profiles for 30 single prostate CTCs isolated from 6 different CRPC patients. (B) Close up of selected key gene groupings, demonstrating high expression of prostate-specific and epithelial genes, and minimal expression of hematopoietic genes. (Miyamoto et al., unpublished)

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

Using the above strategy for single cell RNA-sequencing, we will analyze the digital gene expression profiles of single CTCs isolated from patients with castration resistant compared to castration sensitive prostate cancer. We will attempt to address two primary questions: 1. What is the heterogeneity of CTCs, and is there a subset with expression profiles that may be consistent with distinct metastatic precursors/cancer stem cell phenotypes? 2. What are the signaling pathways activated in individual cells from patients with castration resistant prostate cancer, and do they identify potential drug targets that could be combined with hormonal therapies to suppress metastasis in advanced prostate cancer? These analyses are currently in the preliminary stages, and progress will be described in the next reporting period.
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 castration-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 have published a manuscript in Cancer Discovery during this reporting period describing our preliminary results analyzing AR signaling activity in CTCs using the PSA/PSMA immunofluorescence-based assay (attached in the Appendix) (5). 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 during this reporting period (attached in the Appendix) (7). Finally, several presentations were delivered during this reporting period, including a departmental seminar at the MGH Cancer Center in December 2012 and an oral presentation at the BIO International Convention in Chicago, IL in April 2013. It is anticipated that several additional manuscripts will be published during the term of this Research Project.
KEY RESEARCH ACCOMPLISHMENTS

- Development of an androgen receptor (AR) signaling assay in CTCs based on a two-color PSA/PSMA immunofluorescence assay.
- Preliminary 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.

REPORTABLE OUTCOMES

- Manuscripts:

- 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.

CONCLUSION

During this reporting period, we have made considerable progress in demonstrating the use of 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 includes 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. 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 castration-resistant and castration-sensitive 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.
REFERENCES


**APPENDICES**

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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

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)

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<td>Harvard Radiation Oncology Program, Boston, MA</td>
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<td>Resident, Radiation Oncology</td>
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A. Personal Statement

My multidisciplinary training in molecular cell biology, clinical radiation oncology, and prostate cancer biology has enabled me to become a translational physician scientist with a strong clinical and research interest in prostate cancer. I received my MD-PhD working in the laboratory of Dr. Timothy Mitchison studying the mechanisms of mitosis. I began postdoctoral research in circulating tumor cells in prostate cancer with Dr. Daniel Haber while completing my radiation oncology clinical training. I am currently an Instructor in Radiation Oncology at Harvard Medical School, devoting 80% of my effort to translational research in prostate cancer and 20% to clinical activities treating patients with genitourinary malignancies.

B. Positions and Honors

Positions 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
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
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/IGH
2012 - 2017 Physician Research Training Award, Department of Defense
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
2003 – present  Member, American Society for Cell Biology
2013 – present  Member, American Association for Cancer Research
2008 – present  Member, American Society for Radiation Oncology
2008 – present  Member, Radiological Society of North America
2009 – present  Member, American Society of Clinical Oncology

C. Selected Peer-reviewed Publications

Peer-reviewed articles


Reviews and Book Chapters


Abstracts (within the last 3 years)


D. Research Support

Ongoing Research Support

Dana Farber/Harvard Cancer Center Miyamoto (PI) 08/01/2011-07/31/2013
A. David Mazzone Career Development Award
Analysis of androgen receptor signaling in circulating tumor cells in prostate cancer.

Department of Defense Miyamoto (PI) 07/01/2012 – 06/30/2017
Physician Research Training Award, Prostate Cancer Research Program
Probing androgen receptor signaling in circulating tumor cells in prostate cancer.

NCI/MGH
Federal Share of the Proton Beam Program, Spiro Award Miyamoto (PI) 04/15/2012 – 04/14/2014
Evaluation of Multigene Expression Signatures as Predictors of Outcome After Radiation Therapy for Prostate Cancer

Pending Research Support
None

Completed Research Support

Howard Hughes Medical Institute Predoctoral Fellowship Miyamoto (PI) 07/01/2001-11/30/2004
Probing the functions of kinesins in mitosis.

NCI/MGH Miyamoto (PI) 07/01/2011-06/30/2012
Federal Share of the Proton Beam Program, Research Career Development Award
Molecular characterization of circulating tumor cells in prostate cancer.

Overlap
None
Androgen Receptor Signaling in Circulating Tumor Cells as a Marker of Hormonally Responsive Prostate Cancer


ABSTRACT

Androgen deprivation therapy (ADT) is initially effective in treating metastatic prostate cancer, and secondary hormonal therapies are being tested to suppress androgen receptor (AR) reactivation in castration-resistant prostate cancer (CRPC). Despite variable responses to AR pathway inhibitors in CRPC, there are no reliable biomarkers to guide their application. Here, we used microfluidic capture of circulating tumor cells (CTC) to measure AR signaling readouts before and after therapeutic interventions. Single-cell immunofluorescence analysis revealed predominantly "AR-on" CTC signatures in untreated patients, compared with heterogeneous ("AR-on, AR-off, and AR-mixed") CTC populations in patients with CRPC. Initiation of first-line ADT induced a profound switch from "AR-on" to "AR-off" CTCs, whereas secondary hormonal therapy in CRPC resulted in variable responses. Presence of "AR-mixed" CTCs and increasing "AR-on" cells despite treatment with abiraterone acetate were associated with an adverse treatment outcome. Measuring treatment-induced signaling responses within CTCs may help guide therapy in prostate cancer.

SIGNIFICANCE: Acquired resistance to first-line hormonal therapy in prostate cancer is heterogeneous in the extent of AR pathway reactivation. Measurement of pre- and posttreatment AR signaling within CTCs may help target such treatments to patients most likely to respond to second-line therapies.

Cancer Discov; 2(11); 1–9. © 2012 AACR.
INTRODUCTION

Prostate cancer is highly dependent upon androgen receptor (AR) signaling for cell proliferation and survival. Androgen deprivation therapy (ADT) results in high rates of initial response in most patients with metastatic prostate cancer. However, disease progression is invariably observed with tumor cells resuming proliferation despite continued treatment (termed castration-resistant prostate cancer or CRPC; ref. 1). The propensity of metastatic prostate cancer to spread to bone has limited repeated sampling of tumor deposits that have acquired castration resistance, but insights into resistance mechanisms have emerged through bone marrow biopsy and autopsy studies, as well as mouse modeling experiments (2). The concept that CRPC results from reactivation of AR signaling despite low levels of serum testosterone is consistent with a frequently observed rise in serum prostate-specific antigen (PSA), an androgen-responsive gene product secreted into blood by prostate cancer cells (1, 2). Potential mechanisms by which AR reactivation occurs in CRPC include variable levels of AR gene amplification (~30% of cases), activating AR mutations, alternative mRNA splicing (~10%), increased expression or activation of AR transcriptional coactivators, activation of modulatory kinase pathways (e.g., Ras, phosphoinositide-3 kinase [PI3K], tyrosine phosphorylation of AR, and increased intratumoral androgen synthesis (see ref. 2 for review). The functional significance of reactivated AR signaling in CRPC has been inferred from mouse xenograft models of prostate cancer, in which even modest increases in AR gene expression cause tumors to become resistant to castration (3). The concept of AR reactivation in CRPC has become therapeutically relevant with the development of potent novel inhibitors of AR signaling (4, 5). The demonstration that abiraterone acetate, a CYP17A1 inhibitor that potently suppresses adrenal and intratumoral steroid biosynthesis, increases overall survival in men with metastatic CRPC who have previously received chemotherapy lends support to the rationale of suppressing AR reactivation in CRPC (5). Notably, there is a wide variation in patient response to abiraterone acetate as measured by serum PSA (5), and there is an unmet need for reliable biomarkers that can predict treatment response to abiraterone acetate and other potent inhibitors of AR signaling under development. Taking advantage of recent technological advances in the capture, imaging, and molecular characterization of rare circulating tumor cells (CTCs) shed into blood from otherwise poorly accessible metastatic tumor deposits (6, 7), we established a noninvasive “real-time” measure of intratumoral AR signaling before and after initial- or second-line hormonal therapy in patients with metastatic prostate cancer.

RESULTS

Single-Cell Measurement of AR Signaling Parameters in Prostate CTCs

To measure the status of AR signaling within individual cells, we established a quantitative immunofluorescence assay based on the expression of AR-regulated genes. We reasoned that such a readout would be independent of mechanisms of AR reactivation in CRPC (e.g., AR amplification or mutation, ligand overexpression, or AR cofactor misregulation) and would therefore provide a clear measure of whether the AR pathway has been reactivated during the acquisition of resistance to ADT. To identify optimal downstream readouts of AR signaling, we subjected a prostate cancer cell line (LNCaP) to androgen deprivation or stimulation, and used digital gene expression profiling to identify transcripts that are differentially regulated in response to changes in AR signaling (Supplementary Fig. S1). Among candidate gene products that are prostate cancer-specific and for which reliable antibodies are available, we selected PSA (KLK3) and prostate-specific membrane antigen (PSMA; FOLH1) as most consistently upregulated following AR activation and AR suppression, respectively (Fig. 1A and B; Supplementary Fig. S1A and S1B). Selection of PSMA as a marker of AR suppression was also recently described by Evans and colleagues while this work was in progress (8).

Our assay for quantitative measurement of signal intensity profiles for cells stained with antibodies against PSA and PSMA was developed using a model cell system (LNCaP). Treatment of androgen-starved LNCaP cells with the androgen R1881 and measurement of immunofluorescence signals using an automated fluorescence microscopy scanning platform revealed time-dependent progression from an initial “AR-off” (PSA-/PSMA-) to an intermediate “ARmixed” (PSA+/PSMA-) phenotype, and finally to an “AR-on” (PSA+/PSMA+) pattern (Fig. 1C; Supplementary Fig. S2A). The reverse progression was observed upon treatment with the AR inhibitor bicalutamide (Fig. 1D; Supplementary Fig. S2B). Similar results were observed using VCaP cells, another androgen-responsive prostate cancer cell line (Supplementary Fig. S3).

For isolation of CTCs, we made use of our recently developed “second-generation” microfluidic chip, in which herringbone (HB) grooves in the ceiling of the channel create anisotropic flow conditions, generating microvortices that direct cells toward the anti–epithelial cell adhesion molecule (EpCAM) antibody-coated walls of the device (18CTC-Chip; ref. 9). 18CTC-Chip parameters for single-cell AR signaling analysis were first established by modeling LNCaP cells treated with R1881 or bicalutamide, spiked into control blood specimens, captured on the 18CTC-Chip, and stained with antibodies against PSA and PSMA (AR signaling) along with anti-CD45 (to exclude contaminating leukocytes) and 4′, 6-diamidino-2-phenylindole (DAPI; for nuclear morphology; Supplementary Fig. S4A and S4B). To achieve multiparameter single-cell analysis of AR activity, an automated fluorescence microscopy scanning platform was adapted to distinctly and specifically measure 4 fluorescent emission spectra simultaneously. We carefully selected the choice of secondary fluorophores and optical band pass filters to avoid “cross-talk” between the multiple fluorescent signals that are closely located on the electromagnetic spectrum while maximizing signal intensity (see Methods). The 4-color immunofluorescence imaging parameters established using LNCaP cells were then applied to accurately enumerate patient-derived CTCs (Fig. 2A). To minimize the risk of counting false-positive events as CTCs, we adopted a previously reported strategy (6), calibrating threshold signal intensity and setting a cut-off value for detection of CTCs based on the number of positive events detected in healthy donor samples. Using the newly optimized 4-color
Figure 1. Multiparameter single-cell immunofluorescence assay to measure changes in AR activity in cultured prostate cancer cells. A, Western blot analysis for PSA, PSMA, and α-tubulin in LNCaP cells treated with 1 nmol/L R1881 after being cultured under androgen-deprived conditions (left), or treated with 10 µmol/L bicalutamide after being cultured under standard conditions (right). B, merged immunofluorescence images of LNCaP cells dual stained with antibodies against PSA (green) and PSMA (red) after treatment with 1 nmol/L R1881 or 10 µmol/L bicalutamide. C, pseudocolor density plots of multiparameter immunofluorescence profiles of LNCaP cells treated with 1 nmol/L R1881, imaged using an automated fluorescence microscopy scanning system. x- and y-axes represent “area-pixel” single-cell signal intensity measurements for PSMA and PSA, respectively. The total fraction of PSA+/PSMA− (AR-on), PSA−/PSMA+ (AR-off), and PSA+/PSMA+ (AR-mixed) cells is shown in the bar graph. D, comparable analysis for LNCaP cells treated with 10 µmol/L bicalutamide after being cultured under standard conditions.
immunofluorescence imaging parameters, we established a threshold value for positive CTC detection of more than 4 PSA+/PSMA−/CD45− cells/mL, which was higher than any count noted in any healthy donor sample (Supplementary Fig. S5). The expansion of our CTC characterization to include new organic fluorophores, narrow band fluorescent filter cubes, and a new automated imaging platform (see Methods). The result was a more specific assay with less background signal in our healthy donors compared with our previous papers (6, 7). We tested the validity of this threshold cut-off value in a separate cohort of age-matched male patients with no known diagnosis of cancer. Using this threshold, CTCs were not detectable in any patients without a diagnosis of prostate cancer (n = 0/21; Supplementary Fig. S5). In contrast, subsequent analysis of pretreatment blood samples from patients with metastatic prostate cancer revealed detectable CTCs above the predetermined threshold cut-off in 72% of patients (n = 18/25; Supplementary Fig. S5 and Supplementary Table S1).

Active AR Signaling in CTCs from Untreated Patients with Metastatic Prostate Cancer

Having standardized CTC detection using 4-color imaging criteria, we applied the PSA/PSMA dual immunophenotyping assay to patients with newly diagnosed metastatic prostate cancer. CTCs were detectable in 4 of 5 (80%) patients with newly diagnosed metastatic prostate cancer before the initiation of ADT. AR activity was predominantly positive among the patients with detectable CTCs, with the vast majority (median 99.1%; range, 75%–100%) of isolated CTCs from each patient showing the “AR-on” (PSA+/PSMA+) phenotype (Figs. 2B and C; Supplementary Table S2). The initiation of ADT in treatment-naïve patients with metastatic prostate cancer with detectable CTCs resulted in transformation of the majority of CTCs from the “AR-on” to the “AR-off” phenotype within 1 month, followed by the complete disappearance of CTCs by 3 months after initiation of therapy (Fig. 3A–C; Supplementary Table S2).
Heterogeneous AR Signaling in CTCs from Patients with CRPC

In marked contrast, patients with CRPC with detectable CTCs pretreatment (n = 14/20; 70%) displayed both intrapatient and interpatient heterogeneity in CTC AR activity (Fig. 2; Supplementary Table S2). Most remarkable was the abundance within each patient of CTCs with the “AR-off” (PSA−/PSMA+) signature (median 51.9%), as well as CTCs with an “AR-mixed” (PSA+/PSMA−) phenotype (median 17.6%). Despite the expected reactivation of AR signaling in CTCs, only a relatively small fraction of CTCs in these patients had the “AR-on” (PSA+/PSMA+) phenotype (median 11.1%). In contrast to the consistent treatment-induced changes in AR signaling patterns seen within CTCs of patients with castration-sensitive prostate cancer (CSPC), second-line hormonal treatment in patients with CRPC had varying effects on CTC numbers and AR phenotypes (Fig. 4; Supplementary Tables S1 and S2). This included patients treated with the relatively weak hormonal agents ketoconazole (n = 1) and bicalutamide (n = 2), as well as the potent CYP17A1 inhibitor abiraterone acetate (n = 17; Supplementary Table S2). Four of 17 (24%) patients with CRPC treated with abiraterone acetate had a 50% or more decline in the percentage of “AR-on” CTCs within 2 to 5 weeks of therapy, suggesting that the reduction in systemic androgen levels may have suppressed a subset of metastatic tumor cells with reactivated AR signaling (Fig. 4A–C; Supplementary Tables S1 and S2). In contrast, 2 of 17 (12%) patients with CRPC had a 2-fold or more increase in the percentage of “AR-on” CTCs within the first 2 to 5 weeks of therapy with abiraterone acetate, suggesting increased AR signaling despite therapy (Fig. 4D–F; Supplementary Tables S1 and S2). Eleven of 17 (65%) patients with CRPC had a stable percentage of “AR-on” CTCs after therapy. Analysis of baseline CTC AR signaling before the initiation of abiraterone acetate therapy suggested that the presence of a more than 10% component of “AR-mixed” CTCs was associated with decreased overall survival (log-rank P < 0.05; Fig. 4G). In addition, an increase in the percentage of “AR-on” CTCs despite abiraterone acetate therapy was correlated with decreased overall survival (Supplementary Fig. S6).

DISCUSSION

Cancer cells circulating in the peripheral blood provide a uniquely accessible source of tumor-derived material for molecular analyses. In metastatic prostate cancer, which primarily spreads to bone, the inability to noninvasively sample metastatic lesions has limited the ability to individualize second-line therapies according to the mechanism of drug resistance. Thus, while potent new inhibitors of the AR pathway are under active development, their clinical deployment still remains empiric. Given the interpatient variation in outcome, there is an unmet clinical need for biomarkers that may enable prediction of treatment response for individual patients. Here, we show that the activity of the AR pathway may be monitored in CTCs. Although the trends we observe need confirmation in subsequent analysis with additional patients, our results support the relevance of CTCs as dynamic tumor-derived biomarkers, reflecting “real time” effects of cancer drugs on their therapeutic targets, and the potential of CTC signaling analysis to identify the early emergence of resistance to therapy.

Although CTC enumeration using immunomagnetic bead capture has previously been shown to be a potential prognostic biomarker in patients with metastatic prostate cancer (9), enumeration alone does not yield direct insight into the effects of drugs on their molecular targets and may simply reflect relative tumor burden or leakiness of tumor-associated vasculature. In contrast, interrogation of the activity of specific signaling pathways within CTCs may reveal whether targeted therapies are effectively hitting their target in vivo, thus providing information that may be useful in guiding therapeutic decisions. Reverse transcription–PCR of transcripts from CTC-enriched cell populations may provide an alternative method for detecting CTCs (10), with the potential for measuring changes in relevant transcriptional output in CTCs. However, the need for quantitative analysis of signal within the heterogeneous cell populations, as documented here, supports the importance of single-cell measurements based on CTC imaging.

Because PSMA is a cell surface protein, it has been used as an alternative to EpCAM for capture of CTCs from patients with prostate cancer (11). Although more specific to prostate...
AR signaling in CTCs from CRPC patients treated with abiraterone acetate. A, pseudocolor density plots of multiparameter immunofluorescence AR signaling profiles of CTCs in a patient (Prost14) with CRPC, showing a decrease in the proportion of PSA/PSMA “AR-on” CTCs after the initiation of abiraterone acetate. B, Bar graphs depicting the results for this patient at serial time points following treatment. Corresponding CTC numbers and serum PSA levels are shown for pretreatment (pre) and at weeks following therapy. C, Box plots showing composite data for relative proportions of AR signaling phenotypes in CTCs from patients that exhibit stable or declining proportion of “AR-on” CTCs after initiation of therapy \( P = 0.56 \) for PSA only; \( P = 0.12 \) for PSA/PSMA; \( P = 0.14 \) for PSMA only. D, Increase in the proportion of PSA/PSMA “AR-on” CTCs observed in a patient (Prost11) with CRPC after treatment with abiraterone acetate. E, Bar graphs depicting the results for this patient. F, Box plots showing composite data for relative proportions of AR signaling phenotypes in CTCs from patients that exhibit an increasing proportion of “AR-on” CTCs after initiation of therapy \( P = 0.67 \) for PSA only; \( P = 0.67 \) for PSA/PSMA; \( P = 0.67 \) for PSMA only. G, Kaplan–Meier curves for overall survival in patients with CRPC treated with abiraterone acetate, according to baseline percentage of more than 10% “AR-mixed” CTCs (red) versus less than 10% “AR-mixed” CTCs (blue) \( \text{log-rank} \) \( P = 0.048 \).

As such, anti-EpCAM antibody-mediated capture followed by immunophenotyping for PSA versus PSMA expression allows for broad capture of CTCs, followed by characterization of their AR signaling status. Critical for this approach is the optimization of 4-color immunofluorescence staining and imaging parameters, maximizing immunofluorescence signals, while minimizing cross-talk between channels for detection of PSA, PSMA, CD45, and nuclear signals, using a standardized semi-automated microscopy platform. Full automation of this assay will be required for its broad application in the context of clinical trials of novel hormonal agents in CRPC.

Although our study was designed as a “proof-of-concept” for a diagnostic approach, it also provides significant insight into the evolution of initially responsive prostate cancer into castration-resistant disease. We found that profound differences underlie the dramatic response of previously untreated, castration-sensitive disease to ADT, compared with the relatively limited effectiveness of even potent second-line hormonal agents in castration-resistant disease. CSPC is marked by the presence of predominant and strong “AR-on” CTC signals, with rapid switching to “AR-off” upon androgen withdrawal, preceding the disappearance of CTCs from the circulation. In contrast, CRPC is marked by striking heterogeneity among tumor cells from individual patients as well as between different patients with similar clinical histories. Few “AR-on” cells are observed, and instead there is an abundance of both “AR-off” and “AR-mixed” CTCs. Together, these suggest that pathways other than AR signaling contribute to disease progression in CRPC, and that the AR reactivation that...
Androgen Receptor Signaling in CTCs

RESEARCH BRIEF

was associated with a poor outcome, defined as decreased "AR-on" CTCs despite continued abiraterone acetate therapy. Expression in "AR-on" CTC numbers. Indeed, a rising fraction of gene activation would not be expected to show any suppression in "AR-on" CTC numbers. Indeed, a rising fraction of "AR-on" CTCs despite continued abiraterone acetate therapy was associated with a poor outcome, defined as decreased overall survival. In these patients, ligand-independent AR activity may become a driver of tumor cell proliferation, leading to therapeutic failure. Potential mechanisms for the development of resistance to abiraterone acetate in CRPC are the subject of intense investigation (21). Further studies linking such mechanistic insights with the application of novel therapies targeting the relevant pathways may provide critical guidance in molecularly targeted therapy for CRPC.

In summary, in this exploratory study, we show that PSA/PSMA-based AR signaling assay in CTCs may enable real-time quantitative monitoring of intratumoral AR signaling and its potential contribution to disease progression within an individual patient. Although this assay has potential as a promising biomarker, it requires validation in larger prospective studies of patients with prostate cancer undergoing second-line hormonal therapy. While this work was in progress, positron emission tomography imaging using radiolabeled antibodies against PSMA and PSA were reported as biomarkers of AR signaling in prostate cancer mouse xenografts treated with the investigational AR inhibitor MDV 3100 (8, 22). If successful in human tumor imaging, radiisotope scanning for AR activity may complement single-cell CTC assays in providing ongoing monitoring for second-line hormonal agents in CRPC. Such individualization of second-line treatments in metastatic prostate cancer will be essential for therapeutic success, given the evident tumor cell heterogeneity that accompanies the emergence of resistance to initial androgen deprivation.

METHODS

Patients and Clinical Specimens

Patients with metastatic prostate cancer receiving treatment at the Massachusetts General Hospital (Boston, MA) were recruited according to an institutional review board (IRB)-approved protocol. Eligibility criteria included a histologic diagnosis of prostate adenocarcinoma and radiographic evidence of metastatic disease. For the CSPC cohort, recipients of prior hormonal therapy were excluded. Patients in the CRPC cohort required disease progression on ADT according to Prostate Cancer Working Group criteria (23), and may have received other therapies. A total of 25 patients donated 10 to 20 mL of blood on one or more occasions for CTC analysis. In addition, 21 male patients with no known diagnosis of cancer were recruited as controls using a separate IRB-approved protocol.

Cell Lines

LNCaP and VCaP cells were obtained from American Type Culture Collection after authentication by short tandem repeat profiling, and maintained as recommended. For generation of the AR signature, cells were cultured for 3 days in medium containing 10% charcoal-stripped serum (CSM) and maintained as recommended. For generation of the AR signature, cells were cultured for 3 days in medium containing 10% charcoal-stripped serum (CSM) and were used as controls using a separate IRB-approved protocol.

Immunofluorescence Staining and Automated Fluorescence Microscopy

Cells were captured on the infiniteCTC-chip as described (7), fixed and permeabilized as described (7), and stained with antibodies against PSA (rabbit polyclonal; DAKO), PSMA (J591 mouse monoclonal IgG1; N.H. Bander), and CD45 (mouse monoclonal IgG2a; Abcam), followed by appropriately matched secondary antibodies conjugated with DyLight 649 (Jackson ImmunoResearch), Alexa Fluor 555 (Invitrogen), and Alexa Fluor 488 (Invitrogen). Nuclei were stained with DAPI. An automated fluorescence microscopy scanning system (BioView)

In addition to altered AR signaling, AR-independent pathways, including PI3KCA-dependent signaling, have also been implicated in CRPC and may cooperate with partial AR reactivation in mediating disease progression in prostate cancer (20). Recent studies in mouse models of CRPC have suggested improved responses to combined AR and mTOR pathway inhibition (20). Given the potentially complex and heterogeneous mechanisms underlying CRPC, it is not surprising that treatment with the potent CYP17A1 inhibitor abiraterone acetate alone has a varied effect on the number and composition of CTCs. Some patients with CRPC who did have measureable "AR-on" CTCs showed more than 50% decline in the percentage of this CTC subset within 2 to 5 weeks of abiraterone acetate therapy (4 of 17 patients; 24%). Given the mechanism of drug action, these cases may be enriched for patients in whom intratumoral or adrenal gland synthesis of androgens plays a major role in the development of castration resistance. In contrast, tumors driven by ligand-independent AR gene activation would not be expected to show any suppression in "AR-on" CTC numbers. Indeed, a rising fraction of "AR-on" CTCs despite continued abiraterone acetate therapy was associated with a poor outcome, defined as decreased

Although AR reactivation is the dominant model to explain acquisition of resistance to androgen withdrawal, the limited human data available are consistent with our observations of an attenuated AR phenotype in CRPC. For instance, gene expression studies of bone metastases have shown increased AR mRNA levels in CRPC (12), and bone marrow biopsy studies (13) as well as CTC analyses (14) have shown nuclear AR localization in resistant disease. However, expression levels of androgen-activated genes seem to be reduced by 2- to 3-fold in CRPC, compared with primary untreated prostate cancer (12, 15). The most common acquired genetic alteration affecting AR, a median 1.6- to 5-fold gene amplification seen in approximately 30% of cases (16, 17), may not be sufficient to fully overcome the effects of ligand withdrawal and reestablish full AR-driven tumor cell proliferation. Indeed, a recent analysis of gene promoters targeted by AR in cells that are sensitive to androgen withdrawal versus cells with acquired resistance showed a qualitatively distinct subset of AR-activated genes (18, 19).

In our study, the complexity of AR signaling pathways in CRPC may be reflected by the presence of "AR-mixed" CTCs having simultaneous expression of androgen-induced and androgen-suppressed markers that was associated with decreased overall survival. Thus, expression analysis of AR target genes within CTCs may provide functionally relevant measures of aberrant AR activity.

To this end, we describe an exploratory study to evaluate the potential of an investigational AR inhibitor MDV 3100 (8, 22). If successful in human tumor imaging, radiisotope scanning for AR activity may complement single-cell CTC assays in providing ongoing monitoring for second-line hormonal agents in CRPC. Such individualization of second-line treatments in metastatic prostate cancer will be essential for therapeutic success, given the evident tumor cell heterogeneity that accompanies the emergence of resistance to initial androgen deprivation.

In summary, in this exploratory study, we show that PSA/PSMA-based AR signaling assay in CTCs may enable real-time quantitative monitoring of intratumoral AR signaling and its potential contribution to disease progression within an individual patient. Although this assay has potential as a promising biomarker, it requires validation in larger prospective studies of patients with prostate cancer undergoing second-line hormonal therapy. While this work was in progress, positron emission tomography imaging using radiolabeled antibodies against PSMA and PSA were reported as biomarkers of AR signaling in prostate cancer mouse xenografts treated with the investigational AR inhibitor MDV 3100 (8, 22). If successful in human tumor imaging, radiisotope scanning for AR activity may complement single-cell CTC assays in providing ongoing monitoring for second-line hormonal agents in CRPC. Such individualization of second-line treatments in metastatic prostate cancer will be essential for therapeutic success, given the evident tumor cell heterogeneity that accompanies the emergence of resistance to initial androgen deprivation.

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comprehensively imaged \textsuperscript{111m}CTC-chips under $\times 10$ magnification in seven z-planes in 4 colors at predetermined optimized exposure times, using modified Magnetron sputter-coated filter sets for the Cy3 and Cy5 spectra (Chroma; see Supplementary Methods for details). Potential CTCs were automatically classified using a previously described algorithm (6), followed by manual validation by a blinded human reviewer. High-resolution immunofluorescence images were obtained using an upright fluorescence microscope (Eclipse 90i, Nikon) under $\times 60$ magnification.

**Quantitative Single-Cell Immunofluorescence Analysis**

Quantitative fluorescence intensity data for emission spectra (DAPI, FITC, Cy3, and Cy5) were obtained for each single cell as “Area pixels” measurements using image analysis software (Bioview). Data files were converted to CSV format and to FCS format (Text-

**Statistical Analysis**

AR activity and the proportion of CTC phenotypes between samples were compared using the Wilcoxon rank-sum test. Overall survival was defined as the interval between the start of therapy and the date of death or censorship. Serum PSA response was defined as a maximal decline of 50% or more in serum PSA (23). Survival curves were generated using the Kaplan–Meier method and compared using the log-rank test. Two-sided P values $< 0.05$ were considered statistically significant. Statistical analyses were conducted using R, version 2.12.0.

**Disclosure of Potential Conflicts of Interest**

N.H. Bander has ownership interest (including patents) and is a consultant/advisory board member in BZL Biologics, Inc. No potential conflicts of interest were disclosed by the other authors.

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In memory of Charles Evans and in recognition of philanthropic support for prostate cancer research from the Evans Foundation, we propose that this CTC-based test be called the Evans Assay.

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Androgen Receptor Signaling in CTCs

Editor's Summary

Positive and Negative Outcomes

Usually people want the good news first, to help cope with the bad news that inevitably follows. However, patients will soon desire both the positive and the negative outcomes together, according to the latest study by Ozkumur and colleagues. These authors have developed a multistage microfluidic device that is capable of sorting rare circulating tumor cells (CTCs) that are either positive or negative for the surface antigen epithelial cell adhesion molecule (EpCAM).

EpCAM+ cells found in the bloodstream have long defined the typical CTC. Many sorting technologies have been developed to enumerate EpCAM+ CTCs in cancer patient's blood; however, these cells are not always detectable in cancers with low EpCAM expression, like triple-negative breast cancer or melanoma. Ozkumur et al. engineered an automated platform, called the "CTC-iChip," that captured both EpCAM+ and EpCAM− cancer cells in clinical samples using a series of debulking, inertial focusing, and magnetic separation steps. The sorted CTCs could then be interrogated using standard clinical protocols, such as immunocytochemistry. The authors tested the "positive mode" of their device using whole blood from patients with prostate, lung, breast, pancreatic, and colorectal cancers. After successfully separating out the EpCAM+ CTCs, they confirmed that the cells were viable and had high-quality RNA for molecular analysis, in one example, detecting the EML4-ALK gene fusion in lung cancer. Using the "negative mode" of their device, the authors were able to capture EpCAM− CTCs from patients with metastatic breast cancer, pancreatic cancer, and melanoma. The isolated CTCs showed similar morphology when compared with primary tumor tissue from these patients, suggesting that the microfluidic device can be used for clinical diagnoses—delivering both positive and negative news at once.

Ozkumur et al. also demonstrated that CTCs isolated using the iChip could be analyzed on the single-cell level. One such demonstration with 15 CTCs from a prostate cancer patient reveals marked heterogeneity in the expression of mesenchymal and stem cell markers as well as typical prostate cancer–related antigens. The CTC-iChip can therefore process large volumes of patient blood to obtain not just EpCAM+ CTCs but also the EpCAM− ones, thus delivering both positive and negative news at once. With the ability to further analyze the molecular characteristics of CTCs, this CTC-iChip could be a promising addition to current diagnostic tools used in the clinic.
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Inertial Focusing for Tumor Antigen–Dependent and –Independent Sorting of Rare Circulating Tumor Cells

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Circulating tumor cells (CTCs) are shed into the bloodstream from primary and metastatic tumor deposits. Their isolation and analysis hold great promise for the early detection of invasive cancer and the management of advanced disease, but technological hurdles have limited their broad clinical utility. We describe an inertial focusing–enhanced microfluidic CTC capture platform, termed “CTC-iChip,” that is capable of sorting rare CTCs from whole blood at 107 cells/s. Most importantly, the iChip is capable of isolating CTCs using strategies that are either dependent or independent of tumor membrane epitopes, and thus applicable to virtually all cancers. We specifically demonstrate the use of the iChip in an expanded set of both epithelial and nonepithelial cancers including lung, prostate, pancreas, breast, and melanoma. The sorting of CTCs as unfixed cells in solution allows for the application of high-quality clinically standardized morphological and immunohistochemical analyses, as well as RNA-based single-cell molecular characterization. The combination of an unbiased, broadly applicable, high-throughput, and automatable rare cell sorting technology with generally accepted molecular assays and cytology standards will enable the integration of CTC-based diagnostics into the clinical management of cancer.

INTRODUCTION

The rarity of circulating tumor cells (CTCs) in the blood of cancer patients has required development of highly specialized technologies for their isolation (1, 2). Once detected, enumeration and molecular characterization of CTCs have been applied to prognostic classifications of breast, prostate, and colon cancers (3), and to predictive markers of targeted drug therapy in lung cancer (4). However, the limited sensitivity of commercially available approaches combined with the complexity and heterogeneity of the disease has restricted the broad acceptance and dissemination of CTC-based diagnostics (5).

Several strategies have been used to process blood for analysis of CTCs, including platforms for rapid scanning of unpurified cell populations (6–8). The most common enrichment approaches have used antibodies against the cell surface epithelial cell adhesion molecule (EpCAM). Labeling CTCs with anti-EpCAM–coated beads, followed by bulk magnetic enrichment methods (9–11), has been tested. The U.S. Food and Drug Administration (FDA)–approved Veridex system, CellSearch, immunomagnetically labels CTCs and then enriches the cells by bulk purification across a magnetic field. Conceptually, EpCAM-based CTC capture may have limited ability to identify tumor cells with reduced expression of this epithelial marker as a result of the epithelial–mesenchymal transition (EMT) (12). However, tumor antigen–independent CTC enrichment, such as bulk depletion of hematopoietic cells, suffers from poor yields and low purity (13, 14). Together, CTC isolation approaches have traditionally involved multiple batch processing steps, resulting in substantial loss of CTCs (14).

Recently, we introduced microfluidic methods to improve the sensitivity of CTC isolation (15), a strategy that is particularly attractive because it can lead to efficient purification of viable CTCs from unprocessed whole blood (16–21). The micropost CTC-Chip (μCTC-Chip) relies on laminar flow of blood cells through anti-EpCAM antibody-coated microposts (15), whereas the herringbone CTC-Chip (HB- μCTC-Chip) uses microvortices generated by herringbone-shaped grooves to direct cells toward antibody-coated surfaces (16). Although promising, these methods require surface functionalization to bind to tumor antigens on CTCs and thus yield CTCs that are immobilized within a microfluidic chamber and are not readily subjected to either clinical cytopathological imaging or single-cell molecular characterization.

To address the shortcomings of the current approaches, we developed a strategy that combines the strengths of microfluidics for rare cell handling while incorporating the benefits of magnetic-based cell sorting. After the magnetic labeling of cells in whole blood, this capture platform integrates three sequential microfluidic technologies within a single automated system: (i) debulking by separation of nucleated cells, including CTCs and white blood cells (WBCs), from red blood cells (RBCs) and platelets using deterministic lateral displacement (22); (ii) alignment of nucleated cells within a microfluidic channel using inertial focusing (23); and (iii) deflection of magnetically tagged cells into a collection...
channel. In essence, these three integrated microfluidic functions replace bulk RBC lysis and/or centrifugation, hydrodynamic sheath flow in flow cytometry, and magnetic-activated cell sorting (MACS). We call this integrated microfluidic system the CTC-iChip, based on the inertial focusing strategy, which allows positioning of cells in a near-single file line, such that they can be precisely deflected using minimal magnetic force. This integrated microfluidic platform, with its ability to isolate CTCs in suspension using both tumor antigen–dependent and tumor antigen–independent modes, is compatible with high-definition imaging and single-cell molecular analyses, as well as standard clinical cytopathology. We demonstrate its capabilities for diverse cancer diagnostic applications in both epithelial and non epithelial cancers.

RESULTS

CTC-iChip design and function

The overall CTC-iChip isolation strategy is depicted in Fig. 1 and fig. S1. We explored two modes of immunomagnetic sorting to isolate CTCs: a positive selection mode (posCTC-iChip), whereby CTCs are identified and sorted on the basis of their expression of EpCAM, and a negative selection mode (negCTC-iChip), in which the blood sample is depleted of leukocytes by immunomagnetically targeting both the common leukocyte antigen CD45 and the granulocyte marker CD15.

Target cell labeling was developed and characterized for both operational modes (fig. S2). After labeling, the first stage within the CTC-iChip used hydrodynamic size–based sorting to achieve low shear microfluidic debulking of whole blood (22, 24). RBCs, platelets, plasma proteins, and free magnetic beads were discarded, whereas nucleated cells (WBCs and CTCs) were retained and presented to the second stage for inertial focusing. The efficient removal of free beads is critical because these may accumulate in the magnetophoresis channel and significantly reduce the sensitivity and specificity of the approach. The operational principle of microfluidic debulking is based on hydrodynamic size–dependent deterministic lateral displacement (22, 24), in which coincident flow of cell-containing and cell-free solutions through an array of microposts leads to rapid size-based separation (Fig. 1C and fig. S3). We tested two different array configurations with gaps between

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**Fig. 1.** The CTC-iChip system. (A) Three microfluidic components of the CTC-iChip are shown schematically. Whole blood premixed with immunomagnetic beads and buffer comprises the inputs. The figure demonstrates the positive isolation method; however, the system can be operated in negative depletion mode. (B) Integrated microfluidic system. The debulking array sits in a custom polycarbonate manifold that enables fluidic connections to the inputs, waste line, and second-stage microfluidic channels. The inertial focusing and magnetophoresis chip is placed in an aluminum manifold that houses the quadrupole magnetic circuit. Magnetically deflected cells are collected in a vial. (C) Hydrodynamic size–based sorting. A mixture of 2-µm (red) and 10-µm (green) beads enters the channel (i). Whereas the 2-µm beads remain in laminar flow and follow the fluid streamlines, the 10-µm spheres interact with the post-array (ii and iii) as shown in the scanning electron microscope (SEM) image (right panel). Larger beads are fully deflected into the coincident running buffer stream by the end of the array (iv). Scale bars, 100 µm. (D) Cell focusing and magnetophoretic sorting. Magnetically labeled SKBR3 (red) and unlabeled PC3-9 (green) cell populations are mixed and enter the channel in random distribution (i). After passing through 60 asymmetric focusing units (pictured in the SEM, right panel), the cells align in a single central stream (ii). Magnetically tagged cells are then deflected (iii) using an external magnetic field, and separation is achieved by the end of the channel (iv). Scale bars, 100 µm.
microposts of 20 or 32 μm. An array with 20-μm gaps retains virtually all nucleated cells with minimal contaminating RBCs but has a cutoff for cells larger than 21 μm and may therefore lose large CTCs or CTC clusters. In contrast, an array with 32-μm gaps has an extended operating range for cells between 8 and 30 μm but retains only 60% of WBCs. Because the cells lost in the 32-μm gap array are granulocytes and lymphocytes that are smaller than the reported CTC sizes (16), we selected this array for the CTC-iChip.

The second CTC-iChip component orders nucleated cells within the microfluidic channel, both laterally and longitudinally, so they can be precisely deflected into a collection channel with minimal magnetic moment. The rationale underlying the inertial focusing of cells in microchannels is based on the principles of pipe flow (23,25); essentially, a cellular fluid entering asymmetric, curved channels emerges as a tight row of individual cells traveling within a defined streamline position (Fig. 1D).

We tested variable cell suspensions for focusing performance; WBCs as well as cancer cell lines were well focused within the operational parameters (hematocrit less than 0.4%; flow rate between 50 and 150 μl/min; nucleated cell concentration less than 3 × 10⁶/ml) (fig. S4). Inertial focusing operational parameters were matched to output of the preceding debulking array, and the in-line integration of these complex microfluidic structures within the CTC-iChip thus avoided cell losses associated with commonly used batch processing strategies.

In the final CTC-iChip component, magnetically labeled cells are separated from unlabeled cells within a deflection channel. The precise control over cell position provided by inertial focusing prevents cellular collisions during magnetophoresis; therefore, cell displacement occurs as a predictable function of magnetic load. We modeled the forces exerted on cells labeled with 1-μm beads using a quadrupole magnetic circuit (fig. S5) and predicted deflection patterns under different flow and magnetic load conditions (Fig. 2A). This model was tested using magnetically labeled PC3-9 human prostate cancer cells. The measured deflection distance, plotted as a function of magnetic load, matched the prediction (Fig. 2B).

To demonstrate the dependence of sensitivity on flow speed, we processed labeled cells at various flow rates and quantified the number of beads per cell for deflected and nondeflected outputs (Fig. 2C and fig. S6). The improvement in sensitivity with increasing magnetic residence time (by reducing flow speed) correlated with the predictive model (Fig. 2D), indicating high magnetic sensitivity for the overall system (5 to 20 beads per cell, depending on cell size). The process parameters characterized for the PposCTC-iChip applied similarly to the negCTC-iChip.

**Evaluating the CTC-iChip using cells spiked into whole blood**

To evaluate the efficiency of the CTC-iChip, we spiked five cell lines spanning a broad range of EpCAM expression into healthy whole blood and isolated using PposCTC-iChip or negCTC-iChip modes. The EpCAM expression of each cell line was quantified by comparing the anti-EpCAM signal to that of a matched irrelevant antibody (Fig. 3A).

Recovery of SKBR3 human breast cancer cells [24-fold EpCAM signal over control immunoglobulin G (IgG)] was 98.6 ± 4.3% (mean ± SD), and capture of human prostate PC3-9 cancer cells (3.7-fold EpCAM signal) was 89.7 ± 4.5% (Fig. 3B). Even cells with minimal EpCAM expression, such as MDA-MB-231 (26), a “triple-negative” mesenchymal breast
cancer cell line (only 2.5-fold EpCAM signal over control), were recovered with 77.8 ± 7.8% capture efficiency by posCTC-iChip. Virtually complete abrogation of EpCAM expression was achieved by ectopic expression of the EMT master regulator LBX1 in MCF10A human breast cancer cells (MCF10A-LBX1) (27), resulting in 10.9 ± 3.0% capture efficiency.

Switching to the negCTC-iChip, both the epithelial parental MCF10A cells and their highly mesenchymal MCF10A-LBX1 derivatives were captured at equal efficiency (96.7 ± 1.9% for MCF10As and 97.0 ± 1.7% for the MCF10A-LBX1 derivatives) (Fig. 3B). Together, these two modes demonstrate the flexibility of the CTC-iChip to isolate a broad spectrum of rare cells with high efficiency in both tumor antigen–dependent and tumor antigen–independent modes.

Sample purity was analyzed for both operating modes. Using EpCAM-based positive selection, we achieved an average >3.5-log purification (mean, 1500 WBCs/ml of whole blood; range, 67 to 2537 WBCs/ml). In the leukocyte depletion mode, purification was 2.5 log (mean, 32,000 WBCs/ml; range, 17,264 to 39,172 WBCs/ml) (Fig. 3C). In the posCTC-iChip, the vast majority of contaminating WBCs carried magnetic beads, suggesting that nonspecific interactions between WBCs and either the anti-EpCAM antibody or the beads themselves caused the contamination. In the negCTC-iChip, contaminating WBCs were free of beads, suggesting that they comprise a population of leukocytes with reduced CD45 or CD15 expression, as confirmed by flow cytometry (table S1).

posCTC-iChip isolation of CTCs
We tested the posCTC-iChip in patients with prostate cancer, a disease in which metastatic lesions primarily affect bone, and hence, CTC analysis is key to analyzing recurrences after resection of the primary tumor. On average, 10 ml (range, 6 to 12 ml) of whole blood was analyzed from these patients. Using triple staining for cytokeratins (CKs) (epithelial marker), CD45 (leukocytes), and 4′,6-diamidino-2-phenylindole (DAPI) (nuclear marker), we identified ≥0.5 CTC/ml in 37 of 41 (90%) prostate patients with recurrent (castration-resistant) disease (mean, 50.3/ml; range, 0.5 to 610/ml; median, 3.2/ml) (Fig. 4A). The detection cutoff of 0.5 CTC/ml was more than 2 SDs above the mean number of CK+ cells detected in 13 healthy donors (excluding an outlier with 0.7/ml; mean ± SD, 0.17 ± 0.12/ml; median, 0.19/ml; range, 0 to 0.33/ml). WBC contamination in the posCTC-iChip product was low (mean, 1188/ml; median, 352/ml; range, 58 to 9249/ml), resulting in high sample purities (mean, 7.8%; median, 0.8%; range for samples with ≥0.5 CTC/ml, 0.02 to 43%) (fig. S7).

We performed a detailed comparison of the posCTC-iChip with the FDA-approved CellSearch system (Fig. 4B). To minimize reagent variability between platforms, we used anti-EpCAM capture as well as CK and CD45 staining antibodies from the same source, and consistent criteria were used to evaluate putative CTCs. CTCs were defined as DAPI+/CD45−/CK+, and WBCs were defined as DAPI+ or DAPI+/CD45+ events. Specimens from prostate (n = 19) and other cancers (breast, n = 12; pancreas, n = 6; colorectal, n = 2; lung, n = 2) were compared. Although both assays performed well with high CTC loads (>30 CTCs per 7.5 ml), at lower CTC numbers, there was a marked differential in capture efficiency. Among the 86% (36 of 42) of metastatic cancer patients with fewer than 30 CTCs/7.5 ml, the number of CK+ CTCs isolated with the posCTC-iChip was significantly higher in 22 cases (P < 0.001, paired t test analysis). The remaining 14 cases had CTCs below detection limits for both systems (Fig. 4B and table S2). Thus, the sensitivity of the CTC-iChip is particularly critical in patients with a lower CTC burden.

In addition to capturing more CTCs in patients with lower CTC burdens, the iChip isolates these cells in suspension, which in turn enables their immobilization on a standard glass slide for high-resolution imaging and standard clinical cytopathological examination (fig. S8), as well as simultaneous staining for multiple biomarkers (Fig. 4, C and D). Beyond imaging, molecular genetic tools are increasingly applied to the characterization of CTCs. Nowhere is this more evident than in non–small cell lung cancer (NSCLC), where targeted therapies can provide marked clinical benefit (28). Among the most challenging assays is detection of the EML4-ALK translocation in about 3% of cases, which marks those responsive to the selective

**Fig. 3.** Evaluation of overall system performance using cancer cell lines spiked into whole blood. (A) Quantification of variable EpCAM expression in five cell lines using flow cytometry. (B) Capture yield of positive selection and negative depletion modes using various cell lines expressing different levels of EpCAM. (C) Background in posCTC-iChip product is measured, achieving >3.5-log depletion of WBCs. In contrast, posCTC-iChip has an order of magnitude lower purification. In both (B) and (C), each data point is an experimental result. Upper and lower bounds of the boxes signify the 75th and 25th quantiles, respectively. Perpendicular line in the box represents median value, and data points left above or below the error bars are outliers.
targeted inhibitor crizotinib. Detection of this intrachromosomal trans-
location by fluorescence in situ hybridization (FISH) is difficult, and, at
the molecular level, the variability of chromosomal breakpoints neces-
sitates RNA-based detection of the fusion transcript, which cannot be
readily achieved using either fixed CTCs or free plasma nucleic acids.

We established a reverse transcription polymerase chain reaction
(RT-PCR) assay capable of detecting the \textit{EML4-ALK} translocation
in H3122 lung cancer cells spiked into WBCs at a purity of 0.1% or
introduced into whole blood (10 cells/10 ml) and processed through
the \textsuperscript{pos} CTC-iChip (Fig. 4E). In patient specimens, the \textit{EML4-ALK} tran-
script was detected in CTCs from four cases known to have this chro-
mosome rearrangement by FISH analysis of the primary tumor. It was
absent in CTCs from two NSCLC patients and one patient with pro-
state cancer whose tumors were all known to lack this abnormality. In
cases where CTC-based RNA analysis identified the expected product,
nucleotide sequencing confirmed the breakpoint in the fusion tran-
script (Fig. 4F). Thus, the \textsuperscript{pos} CTC-iChip allowed purification of CTCs
for RNA-based molecular genotyping.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{CTC isolation by \textsuperscript{pos} CTC-iChip in cancer patients. (A) CTCs isolated
from castrate-resistant prostate cancer (CRPC) patients were enumerated
and compared with blood specimens processed from healthy donors. (B)
EpCAM-based isolation using \textsuperscript{pos} CTC-iChip was compared with the Cell-
Search system. Clinical samples were metastatic cancer patients of prostate
(\textit{n} = 19), breast (\textit{n} = 12), pancreas (\textit{n} = 6), colorectal (\textit{n} = 2), and lung (\textit{n} = 2).
All counts were normalized to 7.5 ml. (C) For enumeration of CTCs from
CRPC patients, CK8/18/19 staining was used (green). CD45 antigen (red)
was used to identify contaminating leukocytes. Scale bars, 10 \textmu m. (D) A
CTC from a CRPC patient was stained for prostate-specific antigen (PSA)
(red), prostate-specific membrane antigen (PSMA) (yellow), and DAPI (blue)
to demonstrate dual immunofluorescence staining for PSAs. (E) Validation
of \textit{EML4-ALK} RT-PCR assay was completed with cell lines. \textsuperscript{pos} CTC-iChip
products of whole blood from a healthy donor (HD) spiked with 0, 10,
and 100 H3122 cells (expressing \textit{EML4-ALK} variant 1) per 10 ml were
subjected to RT-PCR for detection of the \textit{EML4-ALK} fusion. Product isolated
from healthy donor blood spiked with 500 VCaP cells/ml was processed as
a negative control. (F) \textsuperscript{pos} CTC-iChip products from patient samples known
to harbor the \textit{EML4-ALK} translocation by FISH were similarly processed as
in (E), and the bands were sequenced to confirm the presence of the
fusion transcript. A representative sequence trace from patient 3 shows
the translocation breakpoint between exon 13 of \textit{EML4} and exon 20 of
\textit{ALK}. CTC analysis of three patients whose cancer lacks the translocation
was used to establish specificity: a prostate cancer patient (lane 1), an
EGFR mutant lung cancer patient (lane 2), and a HER2-amplified lung
cancer patient (lane 6).}
\end{figure}
**negCTC-iChip to isolate CTCs**

Given the heterogeneity of circulating cancer cells, including the subset thought to undergo EMT, depletion of normal blood cells from clinical specimens should allow characterization of unlabeled nonhematopoietic cells. We analyzed CTCs from 10 patients with metastatic breast cancer, including luminal (ER+/PR+, n = 6), triple-negative (ER-/PR-/HER2-, n = 2), and HER2+ (n = 2) subtypes. Triple-negative breast cancers are noteworthy in that they express primarily mesenchymal markers and are unlikely to be captured efficiently using positive selection for EpCAM+ cells (20).

We stained the enriched CTC specimens using the Papanicolaou (Pap) stain, which is used for cytopathology analysis in clinical laboratories. In selected cases, the hematoxylin and eosin (H&E)–stained primary tumor tissue was compared with Pap-stained fine needle aspirates (FNAs) of the tumor or pleural effusions from the same patient. A remarkably similar morphological appearance was evident between cancer cells in the primary breast tumors and the isolated CTCs, as shown for three different patients in Fig. 5A. An ER+ breast cancer patient revealed small and regularly shaped cells in H&E, cytology, and CTC samples. Similarly, larger and more irregular tumor cells were found in a HER2+ primary breast cancer by H&E cytology and CTC analysis. In another example from a triple-negative high-grade breast cancer patient, pleomorphic CTCs similar to the patient’s previously sampled cytology specimen were seen.

We extended these morphological analyses to pancreatic cancer and melanoma with similar findings (Fig. 5A). For these, pancreatic adenocarcinoma showed CTCs of comparable size to the primary tumor by both histology and Pap cytology. Conversely, melanoma consisted of dyshesive tumor cells. The spindled cytoplasm in melanoma was also

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**Fig. 5.** Classification of CTCs with cytopathology and ICC. (A) Specimens from H&E-stained primary and metastatic tumors (upper row) are compared with matched Pap-stained cytology samples from FNAs or pleural effusions (FNA/E) (middle row) and Pap-stained CTCs enriched from blood samples of the same patient using **negCTC-iChip** (lower row). Marked morphological similarity is seen between isolated CTCs and main tumors or cytology samples. (B) ICC profiles of primary and metastatic tumors (upper panel) matched to CTCs from the same patient (lower panel). All images: ×1000 original magnification. Scale bar is 30 μm and valid for all images.
seen on the cytology preparation, but the CTCs appeared round. As a neural crest–derived malignancy, melanoma cells do not express EpCAM, and hence, their detection requires the negCTC-iChip isolation mode. Nevertheless, on the basis of established cellular and nuclear morphology criteria, our CTC analyses were considered to be of sufficient quality to enable a clinical diagnosis of suspicious for malignancy.

Pap-stained CTC slides were destained and then subjected to immunocytochemistry (ICC), which was first validated through cell lines (fig. S9). ICC of CTCs identified estrogen receptor (ER) protein in luminal breast cancer cells, keratin in triple-negative cells, and strong HER2 staining in cells from HER2+ breast cancers (Fig. 5B and fig. S10). Similarly, CTCs from patients with pancreatic cancer stained positive by ICC for CK, and CTCs from melanoma patients stained positive for the melanocytic marker Melan-A (Fig. 5B and fig. S10). The combination of Pap staining followed by ICC enabled enumeration of CTCs isolated by negCTC-iChip despite the presence of surrounding leukocytes.

Not all cytologically suspicious cells (for example, large cells with large, irregular nuclei as identified on the Pap-stained CTC slide) could be confirmed as tumor cells by ICC staining. Conversely, cells that were not scored as CTCs on initial cytological evaluation were subsequently identified as tumor cells by ICC, reflecting substantial heterogeneity in CTC size and morphology (fig. S11). Thus, by not relying exclusively on immunofluorescence-based scoring of CK+ cells, we were able to apply to CTCs the same rigorous morphological and immunohistochemical criteria used by clinical cytopathologists in the diagnosis of malignancy.

We observed large variation in CTC size among different cancer types. Although some CTCs were larger than leukocytes, there was considerable overlap between the two cell populations (Fig. 6). The variation in CTC size was not restricted to different cancer histologies. In one patient with ER+/PR+ breast cancer whose CTCs were isolated using the negCTC-iChip and analyzed using a combination of Pap stain and ICC, we identified CTCs ranging from 9 to 19 μm in diameter.

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**Fig. 6.** Variation of CTC sizes and morphologies. (A) CTCs from breast cancer and melanoma patients consecutively stained with Pap and either anti-CK (breast) or anti-Melan-A (melanoma) antibodies. (B) Quantitative analysis of the effective diameter (maximum feret diameter) for individual cells isolated in three cases. The top two panels are from different melanoma patients (M1 and M2). The bottom panel is from a breast cancer patient (B3). (C) Occasional very large cells with ample cytoplasm and multilobed nuclei were initially considered suspicious but were CK−. The same cells were subsequently restained for the platelet marker CD61, which supports their identification as circulating megakaryocytes. (D) CTCs were occasionally observed as clusters and confirmed by positive CK staining. All images: ×1000 original magnification. Scale bar, 30 μm.
Although most melanoma CTCs were large in size (>12 μm), one patient with metastatic melanoma had numerous CTCs less than 10 μm in diameter, detected using Pap and ICC for Melan-A (Fig. 6, A and B). In breast cancer and melanoma patients, some very large atypical cells (>30 μm) identified by Pap staining as having multilobed nuclei were at first assumed to be CTCs. However, ICC staining for the platelet marker CD61 confirmed their identity as megakaryocytes (Fig. 6C). Finally, application of the negCTC-iChip platform identified clusters of two to six CK+ CTCs in breast and pancreatic cancers, consistent with our previous detection of CTC clusters using the HbCTC-Chip (isolated fixed cells. EpCAM+ CTCs were distinguished from contaminating CD45+ leukocytes. Arrowhead denotes an erythrocyte. Dashed line outlines the microchannel. (Fig. 6D). The negative selection mode of the CTC-iChip thus provided a comprehensive and unbiased view of nonhematological cells in the bloodstream of cancer patients.

### Single-cell RNA expression in CTCs

Global CTC expression analyses may identify major pathways involved in metastasis (20), but the inherent heterogeneity of CTCs necessitates the identification of expression patterns and signaling pathways within individual cells. We therefore applied a series of single-cell micromanipulation approaches to interrogate individual CTCs isolated from a patient with prostate cancer using the negCTC-iChip. Although micromanipulation approaches require expertise and can be time-consuming, the fact that the CTCs are unadulterated allows for more accurate RNA-based expression profiling than isolated fixed cells. EpCAM+ CTCs were distinguished from contaminating CD45+ leukocytes within the negCTC-iChip product by immunostaining (Fig. 7, A and B). CTCs identified as EpCAM+/CD45− were individually isolated and subjected to RNA analysis by multigene microfluidic quantitative RT-PCR (qRT-PCR), profiling for a panel of transcripts implicated in androgen receptor (AR) signaling, cellular proliferation, stem cell, epithelial and mesenchymal cell fates, and leukocyte-specific lineage (Fig. 7C). Single cells from the human prostate cancer cell line LNCaP were used to optimize assay conditions (fig. S12).

A marked heterogeneity was apparent among 15 CTCs isolated from a single patient with metastatic CRPC who had progressed through multiple lines of therapy, including androgen deprivation therapy with leuprolide, the chemotherapeutic drug docetaxel, and the second-line androgen biosynthesis inhibitor abiraterone acetate. Consistent with EpCAM+ immunostaining, 13 of the 15 CTCs were positive for epithelial gene expression, of which 2 CTCs were dual positive for epithelial as well as mesenchymal markers vimentin and N-cadherin (Fig. 7D). Thus, a subset of CTCs appears to have undergone partial EMT. CTC heterogeneity was also evident with expression of stem cell markers [Nanog, Oct-4 (POUSF1), and c-Myc] in 10 of the 15 CTCs, which overlapped primarily with epithelial markers within individual CTCs (Fig. 7C). Proliferation markers cyclin B, cyclin D, Aurora A kinase, and MYBL2 were detected in another subset of seven CTCs.

AR activity, previously defined in CTCs as the ratio of androgen-driven PSA to androgen-repressed PSMA expression (21), was heterogeneous among CTCs. The “AR on” phenotype (PSA expression only) was only seen in 2 of the 15 CTCs, whereas the “AR off” state (PSMA only) was evident in 2 CTCs, and the “mixed AR” state (PSA+/PSMA−) in 10 CTCs (Fig. 7D). This distribution is concordant with single-cell immunofluorescence analysis of AR signaling status in CTCs from patients with CRPC (21).

### DISCUSSION

The CTC-iChip described here has the ability to process large volumes of whole blood (8 ml/hour), with high throughput (107 cells/s) and...
at high efficiency, in positive selection (tumor antigen–dependent) and negative depletion (tumor antigen–independent) modes, thus enabling cytopathological and molecular characterization of both epithelial and nonepithelial cancers. Traditional magnetophoresis requires the attachment of either hundreds of beads per cell or very large beads to provide sufficient magnetic moment for cell isolation (11, 29). In contrast, by virtue of its ability to precisely position cells within the channel using inertial focusing, the fluidic design of the CTC-iChip allows for efficient fractionation of cells with only a few 1-μm beads, resulting in high yields and purity of CTC isolation.

We have tested initial “proof-of-principle” clinical applications of both the positive and negative selection modes of the CTC-iChip. The \(^{30}\)CTC-iChip isolated CTCs at a purity of >0.1%, which is sufficient for molecular analyses, including detection of the EML4-ALK fusion transcript in NSCLC. Total CTC capture yield is critical to both genotyping and other applications, including enumeration for either prognostic or drug response measurements. The median number of CTCs detected by \(^{30}\)CTC staining of \(^{30}\)CTC-iChip product was 3.2 CTCs/ml, with 90% of clinical samples having CK* cells above the threshold set using healthy donors. In a similar cohort using the CellSearch system, a median of 1.7 CTCs/ml was detected, with 57% of samples above the threshold (30). In our direct comparison between the \(^{30}\)CTC-iChip and the CellSearch system, the microfluidic device was significantly more sensitive at low CTC numbers (<30 CTC/7.5 ml). These results suggest that a subpopulation of EpCAM\(^{low}\) cells was missed by the CellSearch bulk processing approach. Thus, whereas current commercially available approaches may be effective in patients with EpCAM\(^{hi}\) CTCs, the CTC-iChip displayed increased sensitivity for patients with low numbers of circulating cancer cells, which may also have EpCAM\(^{low}\) expression.

Previously, we demonstrated the efficacy of two microfluidic systems to isolate CTCs from whole blood. CK* CTCs were detected in 99% of patients with high purity (18 to 70%) in the first-generation micropost chip (15), and application of disease-specific markers for staining (PSA) and computer-assisted enumeration methods were later found to improve system reliability and specificity (19). Building on the improved heuristics and staining, CTCs were subsequently detected in 64% of prostate patients using the first-generation micropost chip (19), and in 93% of patients using the second-generation herringbone chip (16). Yet, these systems remain limited by low throughput (~1 to 2 ml of blood/hour), the inability to conduct single-cell or slide-based analyses, the requirement for three-dimensional image scanning platforms, and the availability of only a positive selection mode. The CTC-iChip system presented here thus encompasses major advances over our previous methods. Whole blood is now processed through a microscale system at speeds comparable to bulk systems (8 ml/hour) while preserving the high sensitivity afforded by microfluidic isolation techniques. Furthermore, rapid and gentle isolation of CTCs, as well as their collection in suspension, increases the integrity of these cells and their RNA quality, which are crucial for downstream analyses, such as cytopathology and single-cell expression profiling.

Moreover, the system can be run in either a positive selection or a negative depletion mode, thus broadening its potential application in the clinic and in basic research studies. The \(^{30}\)CTC-iChip allows for depletion of normal blood cells, uncovering an unselected population of nonhematopoietic cells for analysis. The robustness of this platform was demonstrated by staining CTCs per clinical pathology protocols, which yielded high-quality diagnostic images. The \(^{30}\)CTC-iChip allowed for isolation of CTCs from a nonepithelial cancer (melanoma) and from cancer that has undergone EMT and lost virtually all detectable EpCAM expression (triple-negative breast cancer). Hence, the \(^{30}\)CTC-iChip will be broadly applicable to all cancers that demonstrate vascular invasion, a major limitation of current technologies.

However, several additional optimizations should be considered before the CTC-iChip technology can be deployed for large-scale clinical applications. These include further improvements in CTC purity to facilitate routine molecular analyses of CTCs and in total blood volume processed to enable early cancer detection. From a manufacturing standpoint, we envision the CTC-iChip being integrated into a single monolithic device made of plastic and incorporating all three components of the CTC-iChip within a single footprint. Integration of such an economical chip into a fully automated device would potentially enable broad dissemination of this technology.

The emerging field of CTC biology brings with it unprecedented insight into the mechanisms underlying the blood-borne metastasis of cancer, as well as powerful new clinical applications to help diagnose and manage disease. As the technology matures, these are likely to include the initial genotyping and molecular characterization of cancer, as well as repeated noninvasive sampling of tumors during treatment. Because targeted therapies increasingly shape the clinical paradigm of cancer therapeutics, such serial “real-time” monitoring of cancer for indicators of drug response and emerging resistance is likely to become a mainstay of clinical oncology. The integrated microfluidic technology platform presented here provides a major step in this direction by enabling processing of large blood volumes with high throughput and efficiency, isolating CTCs regardless of tumor surface epitopes, and providing an end product that is compatible with both standardized clinical diagnostics and advanced molecular analyses. Because rare cell detection technologies continue to improve in sensitivity, they may ultimately provide novel approaches for early detection of invasive cancer before the establishment of metastatic disease.

**MATERIALS AND METHODS**

**Samples**

MDA-MB-231, SKBR3, and MCF10A cell lines were obtained from the American Type Culture Collection. PC3-9 cells were obtained from Veridex, LLC, and LBX1-expressing MCF10A cells were derived from a stable cell line previously published by our laboratory (27). Device performance was evaluated by prelabeling the cell lines with a fluorescent marker and spiking them into whole blood at ~200 to 1000/ml of whole blood (Supplementary Materials and Methods).

Fresh whole blood was collected from healthy volunteers under an Institutional Review Board (IRB)–approved protocol or commercially sourced from Research Blood Components. Samples from metastatic breast, colorectal, pancreas, lung, melanoma, and prostate cancer patients were collected under a separate IRB-approved protocol.

**Chip design and fabrication**

Hydrodynamic sorting chips were designed at Massachusetts General Hospital (MGH) and fabricated by Silex with deep reactive ion etching to form the microfluidic chamber. A custom polycarbonate manifold was used to form the fluidic connections to the microchip (fig. S3). The
inertial focusing and magnetophoresis chips were designed and fabricated at MGH with soft lithography and polydimethylsiloxane (fig. S4). The chip was placed within a custom stainless steel manifold that held four magnets in a quadrupole configuration to create a magnetic circuit enabling cell deflection (fig. S5) (Supplementary Materials and Methods).

**Magnetic bead labeling of target cells in whole blood**

Before processing the whole blood, samples were incubated with functionalized magnetic beads 1 μm in diameter (Dynal MyOne 656-01, Life Technologies) (fig. S2). For posCTC-iChip, beads were functionalized with a biotinylated anti-EpCAM antibody, and active magnetic mixing was applied to achieve good labeling of EpCAMlow cell lines. For negative depletion, anti-CD45 and anti-CD15 functionalized beads were used (Supplementary Materials and Methods).

**Immunofluorescence staining of CTCs**

For enumeration analysis, isolated cells were incubated with saponin, DAO, and anti-CK [phycocerythrin (PE)] and anti-CD45 [allophycocyanin (APC)] antibodies, acquired from Veridex, still in suspension. Cells were plated on a poly-l-lysine–functionalized glass slide with a closed chamber (fig. S8), and glass slide was scanned with the BioView imaging system while the chamber was still intact. Cells that were CK+/DAPI+/CD45− were scored as CTCs. Samples evaluated for PSA/PSMA expression were stained with a primary/secondary approach. All antibodies are catalogued in table S3.

**Comparison to CellSearch**

For the CellSearch and posCTC-iChip comparison, two blood tubes were drawn: one CellSave tube for CellSearch run and one EDTA tube for posCTC-iChip run. Samples in CellSave tubes were processed within 3 days after the draw as optimized and recommended for CellSearch approach, and EDTA samples were processed with the posCTC-iChip within 4 hours of draw. CellSearch product was scanned in MagneCyt (N. Hartford, MGH) and then formally reviewed by an expert cytologist (E.B.). Slides were then destained and exposed to ICC process (N. Hartford, MGH) and then formally reviewed by an expert cytologist (E.B.).

**Single-cell micromanipulation and qRT-PCR**

Blood samples from a patient with metastatic prostate cancer were processed through the posCTC-iChip, and unfixed CTCs and contaminating leucocytes were stained in solution with fluorophore-conjugated antibodies against EpCAM and CD45. Single CTCs were identified based on an EpCAM+/CD45− phenotype and transferred under direct microscopic visualization to individual PCR tubes with a TransferMan NK2 micromanipulator (Eppendorf AG). Single-cell cDNA was prepared and amplified for single-cell transcriptome analysis, followed by specific target preamplification (Fluidigm Corp.). Microfluidic qRT-PCR was performed with the BioMark Real-Time PCR system (Fluidigm Corp.). The normalized gene expression in each cell (−ΔCt) was calculated as the negative of the difference between the Ct value for each gene and the GAPDH Ct value for the cell. Heat maps of normalized gene expression (−ΔCt) were generated with the Heat Map image module of GenePattern, with global color normalization.

**RT-PCR analysis**

RNA isolation was done with RNeasy Micro Kit (Qiagen). After RNA isolation, reverse transcription of RNA to complementary DNA (cDNA) using oliog(1T) was performed with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). For detection of EML4–ALK fusion cDNAs, partial nested PCR analysis was done with FidelityTaq PCR Master Mix (Affymetrix). PCR amplification was performed in a thermocycler (Peltier Thermal Cycler, MJ Research). Gel electrophoresis was done with an aliquot of RT-PCR products. The amplified EML4–ALK products were sequenced, and results were analyzed with the ABI PRISM DNA sequence analysis software (Applied Biosystems) (Supplementary Materials and Methods).

**Cytology and ICC**

CTCs were enriched via posCTC-iChip from the whole blood of cancer patients and plated on a poly-l-lysine surface (fig. S8). Pating chamber was removed after cell adhesion to facilitate standard cytopathology processing. Pap stain was done with hematoxylin, eosin–azure, and orange G and initially reviewed for suspicious cells by a certified cytopathologist (N. Hartford, MGH) and then formally reviewed by a staff cytopathologist (E.B.). Slides were then destained and exposed to ICC process (Supplementary Materials and Methods).

**RESEARCH ARTICLE**


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