Published in final edited form as:

Lab Chip. 2018 July 10; 18(14): 2055–2064. doi:10.1039/c8lc00310f.

Dynamic CTC Phenotypes in Prostate Cancer Metastatic Models Visualized Using Magnetic Ranking Cytometry

Leyla Kermanshah^a, Mahla Poudineh^b, Sharif Ahmed^b, L. N. Matthew Nguyen^b, Sanjana Srikant^c, Rhema Makonnen^b, Fernando Pena Cantu^b, Michael Corrigan^b, and Shana O. Kelley^{*,a,b,c}

^aInstitute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto, ON M5S 3G9, Canada.

^bLeslie Dan Faculty of Pharmacy, University of Toronto, Toronto, ON M5S 3M2, Canada.

^cDepartment of Biochemistry, University of Toronto, Toronto, ON M5S 1A8, Canada.

Abstract

Tumors can shed thousands of cells into the circulation daily. These circulating tumor cells (CTCs) are heterogeneous, and their phenotypes change dynamically. Real-time monitoring of CTC phenotypes is crucial to elucidate the role of CTCs in the metastatic cascade. Here, we monitor phenotypic changes in CTCs in mice xenografted with tumors with varying aggressiveness during cancer progression and a course of chemotherapy to study the metastatic potential of CTCs and changes in the properties of these cells in response to treatment. A new device that enables magnetic ranking cytometry (MagRC) is employed to profile the phenotypic properties of CTCs. Overall, CTCs from metastatic xenografts in mice display dynamic and heterogeneous profiles while non-metastatic models had static profiles. Decreased heterogeneity followed by a reduction in metastasis incidence was observed after a course of chemotherapy administered to highly metastatic xenografts. Phenotypic profiling of CTCs could be employed to monitor disease progression and predict therapeutic responses.

Graphical Abstract

*Correspondence should be addressed to Shana O. Kelley (shana.kelley@utoronto.ca).

Conflicts of interest

The authors declare no competing financial interest.

Magnetic ranking cytometry profiles dynamic phenotypes in circulating tumor cells that are linked to metastatic potential.

Introduction

Primary tumors can shed thousands of cells into the blood every day¹. These circulating tumor cells (CTCs) are considered a missing link between a tumor and the development of metastatic disease^{2,3}. In addition, CTCs carry molecular signatures of primary tumors into the bloodstream. Therefore, their analysis, regarded as a real-time, non-invasive and cost-effective “liquid biopsy”, has attracted significant attention in the last two decades⁴. However, the rarity and heterogeneity of these tumor surrogates have presented obstacles toward their integration into routine clinical care^{2,5}.

Extensive heterogeneity exists among CTCs as they can change their phenotypes throughout their journey from their origin in the primary tumor site, to the blood circulation, and then to the metastatic niche². Not all CTCs have the potential to initiate metastasis and only a small fraction of them with more aggressive phenotypes will reach the final stage of the metastatic cascade¹. The phenotypic properties of this metastatic subpopulation have not been specifically identified. To investigate the role of CTCs and their phenotypic changes in metastasis, appropriate models are needed to mimic the multi-step metastatic cascade^{6,7}. A variety of animal models have been generated to explore tumor progression, CTC dissemination and metastatic tumor formation^{8,9}. Among these, xenograft mouse models have been widely used to understand the biology of cancer and develop effective cancer treatments¹⁰. Here, we generate three prostate cancer mouse models with differing metastatic potential by implantation of human prostate cancer cells into the mouse prostate (Figure 1). These pre-clinical models allowed us to monitor phenotypic changes in CTCs during tumour growth and explore their role in metastasis¹¹.

Microfluidic devices are a class of new solutions for isolation and characterization of rare CTCs that offer unique features including high capture efficiency, high system throughput, and high selectivity while requiring only small sample volumes¹². A variety of cell separation mechanisms have been applied in microfluidic-based platforms. Examples include immunomagnetic capture, size/deformability-based isolation, and dielectrophoresis, to name a few^{13–15}. In addition to CTC isolation, several on-chip strategies have been developed for characterizing CTCs based on their biological and functional phenotypes, such as protein expression, adhesion, and migration potential^{16–18}.

Previously, our group has described a microfluidic approach for sorting phenotypically-distinct CTCs based on the expression of surface proteins¹⁹. CTCs are tagged with magnetic nanoparticles and trapped by applying an external magnetic field. An array of X-shaped structures within a fluidic channel is used to decrease linear velocity and trap cells²⁰. This platform has been integrated with functional assays to identify CTCs with more aggressive phenotypes according to their metabolic activity, ability to digest collagen matrix, and migration towards a chemokine^{17,18}. A similar strategy was pursued in a recent study that sorted magnetically labelled CTCs into three subgroups based on the expression level of epithelial cell adhesion molecule (EpCAM) by manipulation of magnetic force acting on cells²¹. Using this approach, CTCs were sorted in discrete subgroups based on thresholds of surface protein expression.

In all these studies, CTCs were sorted in discrete subgroups based on thresholds of the marker expression. However, more resolution is needed to deconvolute patterns of CTC heterogeneity and to connect them with metastatic potential. Magnetic ranking cytometry (MagRC) has been developed in our laboratory to address this issue²². This highly sensitive and versatile microfluidic device classifies rare CTCs based on expression levels of any surface marker of interest. Heterogeneous populations of CTCs are sorted by progressively increasing magnetic field gradients using nickel micromagnets. The size of nickel micromagnets increases steadily along the length of the channel and creates 100 capture zones. Distinct phenotypic profiles are obtained on the basis of the number of CTCs trapped in each capture zones.

Here, we report a new device for magnetic ranking cytometry that takes advantage of manipulating the magnetic and drag force acting on CTCs to achieve high efficiency cell profiling. Incorporating nickel micromagnets with different sizes into a microfluidic channel with geometric width variation creates areas suitable for capturing magnetized cells (Figure 1). Based on the size of nickel micromagnets and the width of the channel, 10 discrete capture zones are defined within the channel. Having 10 capture zones makes the device more practical than those previously reported and facilitates visualizing CTC phenotypes as a profile in samples with low numbers of CTCs. This new generation of MagRC devices for analyzing cancer cells gives us reproducible phenotypic profiles that are comparable to those obtained using flow cytometry. This highly efficient and sensitive technique is adopted to monitor the dynamic phenotypic profiles of CTCs shed from tumor xenografts during cancer progression and link them to their metastatic potential. Dynamic CTC profiles are obtained from analyzing blood samples collected from the xenografted mice at different time points. Additionally, the effect of a chemotherapeutic agent administered to a highly metastatic prostate cancer xenograft model on CTC phenotypes is effectively monitored. This study is the first report that characterizes phenotypic changes in CTCs during administration of a chemotherapeutic in a xenograft model and links these changes with the appearance of metastatic lesions.

Materials and methods

Magnetic ranking chip fabrication.

Microfluidic chips were fabricated using glass slides coated with 1.5 μm nickel layer (EMF-Corp, US). Circular micromagnets were patterned through contact lithography; a layer of positive photoresist (AZ1600) was coated on the glass slide and exposed to UV for 10 s. The photoresist was developed subsequently and the unwanted nickel surrounding the contact area was etched away along with the resist. Fabricating the X-structures co-centered with nickel micromagnets was carried out by spin-coating of 50 μm of SU-8 3050 (Microchem, US) on top of the substrate. A 30 min soft-baking was followed by 20 s UV-exposure and development of SU-8. The channel was topped with a layer of polydimethylsiloxane (PDMS, Dow Chemical, US) with two holes for inlet and outlet connected to tygon tubes. Prior to use, the chip was sandwiched by two arrays of N52 NdFeB magnets (K&J Magnetics, PA, 1.5 mm by 8 mm).

Cell culture.

Four prostate cancer cell lines (VCaP, LNCaP, PC-3 and PC-3M) and two breast cancer cell lines (SKBR3 and MDAMB-231) were used in our analysis. VCaP, SKBR3, and MDAMB231 were obtained from American Type Culture Collection (ATCC). VCaP cells were cultured in Dulbecco's Modified Eagles's Medium (DMEM, ATCC). SKBR3 cells were cultured in McCoy's 5a Medium Modified (ATCC) and MDA-MB-231 cells were cultured in Leibovitz's L-15 medium (ATCC). LNCaP, PC-3 and PC-3M cell lines were obtained from Dr. Alison Allan's research group at London Health Sciences, London, ON, Canada. LNCaP and PC-3M cells were cultured in RPMI-1640. PC-3 cells were maintained in F12K media (ATCC). All media were supplemented with 10% fetal bovine serum (FBS). Cells were cultured at 37 °C and 5% CO₂.

Flow cytometry.

Cells were harvested from tissue culture using 0.25% trypsin/EDTA (Sigma-Aldrich, US) and incubated with blocking buffer (PBS + 1% BSA) for 30 minutes. For each cell line, 5×10^5 cancer cells were fixed and permeabilized using 4% paraformaldehyde (Sigma-Aldrich, US) and 0.2% Triton X (Sigma-Aldrich, US) in PBS, respectively. Cells were then washed with PBS and suspended in PBS containing 1% BSA and 0.1% Tween 20, and incubated with anti-EpCAM-APC, anti-Pan-CK-APC, anti-E-Cadherin-APC, anti-N-Cadherin-Alexa 488, and anti-Vimentin-Alexa 488 (BioLegend, US) at 1:50 dilution and stained at room temperature for 30 minutes. Mouse IgG (Abcam, US) was used as a negative control at the corresponding assay-specific concentrations. Samples were washed with PBS and resuspended in PBS. Samples were injected into a BD FACSCanto flow cytometer (BD Biosciences, US) and measurements were plotted as histograms. A total of 5,000–10,000 cells were analysed per cell line.

EpCAM profiling of cancer cells.

Cancer cells were incubated with 10 μl EpCAM magnetic nanoparticles (Miltenyi Biotec, US) for 30 minutes prior to running in the chip. These superparamagnetic nanoparticles are

50 nm in diameter and composed of iron oxide and dextran. Magnetized cells were loaded into the device at a flow rate of 400 μ l/h and were captured by an external magnetic field applied in the chip. Cells were then fixed, permeabilized and stained with 4% paraformaldehyde (Sigma-Aldrich, US), 0.2% Triton X-100 (Sigma-Aldrich, US) and DAPI (DAPI Prolong Gold reagent, Invitrogen, US), respectively. Finally, cells were washed with PBS. Chips were stored at 4 °C before scanning with a fluorescent microscope (Nikon).

EpCAM profiling of spiked samples.

To validate the immunostaining of cancer cells and mouse blood cells, LNCaP, PC-3 and PC-3M cells were spiked in 50 μ l of mouse whole blood. The final sample was incubated with 10 μ l EpCAM magnetic nanoparticles (Miltenyi Biotec, US) for 30 minutes. After incubation, blood was loaded into the chip at 400 μ l/h. Prior work performed with the original magnetic ranking cytometry devices¹⁹ indicated that unbound nanoparticles do not interfere with the analysis and thus the samples can be directly analysed. Any non-targeted cells were washed away using 300 μ l PBS-EDTA. Subsequently, trapped cells were fixed and permeabilized with 4% paraformaldehyde (Sigma-Aldrich, US) and 0.2% Triton X-100 (Sigma-Aldrich, US), respectively. Following permeabilization, cancer cells were stained with antibodies specific to cytokeratins (pan CK-APC (GeneTex, US) + CK-19-APC (BioLegend, US)) and vimentin-Alexa 555 (BioLegend, US), and mouse cells were distinguished with mouse anti-CD45-FITC (eBiosciences, US). Antibodies were prepared in 100 μ l PBS with 1% BSA. Finally, the nucleated cells were stained with DAPI (DAPI Prolong Gold reagent, Invitrogen, US) and washed with PBS. Chips were stored at 4 °C before scanning with a fluorescent microscope (Nikon).

CTC imaging and analysis.

Immunostained cells were imaged by a fluorescent Nikon TiE eclipse microscope with an automated stage controller and CCD (Hamamatsu, Japan) under a 10 $\times$ objective. An automated program has been developed based on the geometry of the device. The scanning process for each chip takes 30 minutes. To improve the imaging resolution, CTC images were acquired using 50 $\times$ objective. NIS Elements AR software was used for image analysis. Finally, images were hand-checked to count CTCs captured in zones.

Orthotopic xenograft mouse models for prostate cancer.

All animal experiments were conducted according to the protocol approved by University of Toronto Animal Care Committee which follows the guidelines and standards of Canadian Council on Animal Care (CCAC). Mouse xenograft models were prepared by orthotopic injection of three prostate cancer cell lines, LNCaP, PC-3 and PC-3M. For each cell line, 1×10^6 cells per mouse were injected orthotopically via the right dorsolateral lobe of the prostate of 6–8 week old male athymic nude (nu/nu) mice. Mice were euthanized at each of designated time points after the injection (LNCaP xenografts at 2, 4, 8, 12, and 16 weeks, PC-3 xenografts at 2, 4, 8, and 12 weeks and PC-3M xenografts at 2 and 4 weeks, n=3 mice per cohort at each time point) and 500 μ l of blood was collected by cardiac puncture for CTC analysis. At each time point, tumors were dissected and their dimensions were measured by a caliper to assess tumor growth.

Histological analysis of xenografts.

After blood collection, tumors, lungs, and lymph nodes were extracted, formalin-fixed, paraffin-embedded, and sectioned to stained with hematoxylin and eosin (H&E). Histological examination of prepared tissues was done by a pathologist blinded to the difference of samples.

Profiling of mouse CTCs and immunostaining

Analysis of mouse blood was done in accordance with the analysis of spiked samples explained previously. 500 μ l of blood was incubated with 10 μ l of EpCAM magnetic beads (Miltenyi Biotec, US) for 30 minutes. After incubation, blood was loaded into the chip at 400 μ l/h. Samples were then washed 3–4 times with 100 μ l of PBS-EDTA to remove all non-specifically bound blood cells. Captured cells were fixed and permeabilized with 4% paraformaldehyde (Sigma-Aldrich, US) and 0.2% Triton X-100 (Sigma-Aldrich, US), respectively. Immunostaining of cells was conducted according to the protocol used for spiked samples mentioned in previous sections; cancer cells were stained for cytokeratins (pan CKAPC (GeneTex, US) + CK-19-APC (BioLegend, US)) and vimentin (BioLegend, US) and mouse blood cells were stained with mouse anti-CD45 (eBiosciences, US). Antibodies were prepared in 100 μ l PBS with 1% BSA. Cell nuclei were stained with DAPI (DAPI Prolong Gold reagent, Invitrogen, US). Immunostained cells were imaged by a fluorescent Nikon TiE eclipse microscope with an automated stage controller and CCD (Hamamatsu, Japan) under a 10 $\times$ objective. To improve the imaging resolution, CTC images were acquired using 50 $\times$ objective. NIS Elements AR software was used for image analysis. Finally, images were hand-checked to count CTCs captured in zones. It is noteworthy that the absolute concentrations of cells in samples does not affect the profiles.^{19, 22}

Docetaxel treatment of mice bearing PC-3M xenografts.

PC-3M xenografts were treated with two regimens of docetaxel. Two cohorts of mice were injected intravenously via the tail vein at a dose of 10 mg docetaxel/kg body weight for 2 and 3 doses every 10 days post-implantation. A docetaxel injectable solution was prepared by diluting the drug at a concentration of 10 mg/ml with saline to final concentration of 10 mg/kg per single injection. Control mice received pure saline. Docetaxel for research purposes only was obtained from Princess Margaret Cancer Centre (PMCC). All the PC-3M mice from both the control and treated groups were weighed twice weekly and sacrificed if body weight loss exceeded 20%. To measure the toxicity of the drug, tumor-free 6–8 week old male athymic nude (nu/nu) mice were treated with docetaxel via intravenous administration every 10 days. The weight of these mice was monitored and compared to healthy mice injected with saline.

Results and discussion

Recently, we developed a new technique, magnetic ranking cytometry, that allows simultaneous capture and sorting of CTCs as a function of their surface marker expression with high sensitivity and efficiency²². Using this approach, CTCs bound to immunomagnetic nanobeads were classified into 100 discrete zones by manipulating magnetic field gradients using nickel micromagnets with varying sizes along the length of the channel and applying

an external magnetic field. Here, we have designed a new MagRC device with ten capture zones (Figure 1, Figure S1). This device facilitates profiling CTC phenotypes with adequate resolution, particularly when the number of CTCs is very low. In the current design, we take advantage of manipulating magnetic force together with drag force acting on CTCs to achieve high efficiency of cell profiling.

The previous MagRC device consisted of a long channel that provides cells with residence times that enable efficient capture. However, the long length of the device impeded its mass production. To increase manufacturing yield while maintaining high capture efficiency, we steadily increased the width of the capture zones, which subsequently shortened the length of the device by half. The current design reduces fabrication time and cost more than three-fold. It also facilitates downstream analysis of CTCs by minimizing the time for scanning the chips using fluorescence microscopy and subsequent image processing. As the width of the channel increases, the linear velocity and therefore the drag force decreases. In addition, a local magnetic field gradient is engineered by using patterned nickel micromagnets that increase in size along the length of the channel. Based on the width of the channel and size of nickel micromagnets, the channel is divided into ten discrete zones. An array of X-shaped structures made of SU-8 are used to create areas with local slow flow velocity that enable cell capture. The final engineered design provides areas with favourable capture conditions (high magnetic force and low drag force). We defined a capture zone in the device as the region where the magnitudes of the magnetic and drag force are comparable, meaning that any cells that pass through a capture zone are expected to get captured. Magnetic and flow field simulations were performed in each zone and around the X-shaped structures using COMSOL Multiphysics in order to determine the size of a capture zone for cells with different levels of magnetic loading (Figure S2). The flow field simulation was performed at the optimized flow rate of 400 $\mu\text{l/h}$ which leads to the maximum linear velocity of 60 $\mu\text{m/s}$ in the first zone (Figure 1). More details on COMSOL simulation and modelling are provided in Supplementary Information.

With this unique device design, cancer cells are trapped in ten capture zones, according to the number of nanoparticles bound to the surface of cells, which reflects the expression level of the cell surface marker. Cells with higher marker expression (more magnetic beads) are mainly captured in initial zones where the nickel micromagnets are small and the linear velocity is high. Low-expressing cells (fewer magnetic beads) require the action of larger nickel micromagnets and lower velocity to become captured; therefore, they are found in the later zones.

Post-capture, cells within each zone are immunostained, counted and their number is plotted versus zone number to create an expression profile. The distribution of CTCs in the capture zones mirrors the expression levels of the surface marker that is used as the sorting agent, i.e. EpCAM. The position of the distribution reflects the expression levels, while the width of the distribution reports on the level of heterogeneity within the sample.

Three human prostate cancer cell lines were used to generate xenograft models of prostate cancer, LNCaP, PC-3 and PC-3M²³. The LNCaP cell line is androgen-responsive, highly epithelial and non-metastatic. A more aggressive cell line, PC-3, was originally derived from

bone metastases and is androgen-independent and highly metastatic⁹. Lastly, PC-3M cells are a metastasis-derived variant of PC-3 and are considered to have partially undergone the epithelial-to-mesenchymal transition (EMT)^{24,25}. First, these three cell lines were analyzed using the new device to benchmark performance. EpCAM was selected as the profiling marker, as it is well-established for CTC capture²⁶. Also, it is known to vary during EMT and its downregulation may contribute to the metastatic potential of cancer cells^{11,27}. In parallel, the EpCAM levels in all three cell lines were analyzed by flow cytometry capture efficiency of MDA-MB-231 cells, a triple negative breast cancer cell line with the lowest EpCAM expression levels (Figure S3). The number of EpCAM molecules displayed on the surface of MDA-MB-231 cells has been estimated around 1700 molecules²⁸. Given that the MDA-MB-231 cells are captured in late zones within our device, we can conclude that at least 1700 EpCAM molecules per cell are required for isolation of cells in our device.

One hundred target cells were spiked into 50 μ L of normal mouse blood and incubated with anti-EpCAM magnetic nanoparticles prior to processing of the sample. After a 30 minute incubation, samples were loaded into the device at a flow rate of 400 μ L/h. Non-target blood cells were then washed away using PBS-EDTA, whereas EpCAM-tagged CTCs were retained in the device under the influence of external magnetic field. Cancer cells were distributed in different capture zones based on the number of magnetic beads on their surface. Once CTCs were trapped in the device, they were stained with a cocktail of antibodies against cytokeratins 4,5,6,8,10,13,18 and 19 and vimentin¹¹. To distinguish cancer cells from mouse cells and eliminate false positives, mouse blood cells were stained with mouse anti-CD45. A cell with DAPI+/CK+/CD45- phenotype is considered a target cell. The number of cells in each zone was plotted against the zone number to obtain CTC phenotypic profiles (Figure 2A). High capture efficiencies were achieved for LNCaP, PC-3, and PC-3M cells (Figure 2B), and the profiles were highly reproducible. LNCaP and PC-3M cells with high levels of EpCAM expression were primarily captured in the earlier zones. PC-3 cells, which exhibit the lowest expression levels of EpCAM were mainly detected in later zones of the device. Flow cytometric analysis of EpCAM expression in these cells confirms their relative phenotypic profiles (inset to Figure 2A). It is noteworthy that 99.996% depletion of WBC from mouse blood is achieved with this device; this compares favourably to the 99.98% depletion achieved previously with human blood and the original magnetic ranking device.²²

The significant advantage of this technique over flow cytometry is that it is amenable to samples with low numbers of the target cells while flow cytometry needs at least 10^4 cells to provide reliable results. Also, cancer cells in unprocessed blood samples cannot be analyzed by flow cytometry due to the interference of blood cells, whereas whole blood can be easily analyzed with our device. The sensitivity of the device was challenged by processing samples with very low numbers of cells (Figure 2D). The number of recovered cells is very close to the actual number of cells measured by hemocytometer prior to loading into the device.

In this study, we endeavored to investigate the role of dynamic CTC phenotypes during cancer progression and their correlation to subsequent metastasis. Xenograft models have been used for real-time monitoring of CTCs during disease progression and elucidation of

their role in the metastatic cascade¹¹. To generate xenograft mouse models, human tumor cells can be implanted into immunodeficient animals intravenously, subcutaneously, or orthotopically²⁹. Intravenous injection of cancer cells via the tail vein is one model for metastasis, but the early steps of the metastatic cascade are neglected in this model⁹. In contrast to intravenous models, subcutaneous and orthotopic models include the first steps of metastasis which includes intravasation of CTCs into the bloodstream. A subcutaneous model is easy to establish, reproducible and cost-effective, however implantation of tumor cells under the skin does not mimic the native tumor environment⁸. Among all xenograft models, orthotopic xenografts are the most advantageous as they generate physiologically relevant primary tumors that shed CTCs and metastasize spontaneously³⁰. In orthotopic models, cells are implanted into the same organ from which the cancer originated. This gives us insight into the function of CTCs by resembling the actual tumor environment in which cells elect to disseminate³¹.

Various prostate cancer cell lines have been used for generating xenograft models. LNCaP is an androgen-sensitive prostate tumor³². Xenografts established from implantation of LNCaP cells exhibit slow tumor growth rates and low incidence of metastases²³. A more aggressive cell line, PC-3, is originally derived from bone metastasis and is androgen-independent³³. Xenografts with intravenous and orthotopic injection of PC-3 form lymph node metastases⁹. PC-3M cells are a metastasis-derived variant of PC-3 with much higher metastatic potential³⁴. PC-3M xenografts exhibit overt metastasis in the lymph nodes and in distant organs³⁵. These three xenograft mouse models have been commonly used for studying cancer biology and metastasis^{9,23,36}. Here, we implanted these three prostate cancer cell lines into the prostate of the immunodeficient mice to generate cancer models with different metastatic potential (Figure 3A). After inoculation, mice were sacrificed at different time points (2, 4, 8, 12, and 16 weeks) to assess tumor growth and metastasis. Since PC-3M xenografts are highly aggressive, the mice can only survive up to 4 weeks after implantation; therefore, they are analyzed at 2 weeks and 4 weeks post-injection. Mice implanted with PC-3 xenografts did not survive after week 12. At each time point 500 µl blood was collected via cardiac puncture for CTC capture and profiling. Upon sacrifice, tumors were collected and their dimensions were measured by a caliper. The tumor volume was calculated as reported in the literature: length × width × height × 0.52 (in millimeters)³⁷. All three cell lines started to form tumors shortly after cell implantation. Tumor growth rates in three xenografts are compared in Figure 3A. As expected, PC-3M xenografts had much faster tumor growth compared to LNCaP and PC-3 xenografts; 4 weeks post-injection, PC-3M xenograft mice were sacrificed due to the heavy burden of tumor volume and overt metastases. PC-3 and LNCaP xenografts exhibited similar tumor growth rates until week 4, however the growth rate in PC-3 xenografts accelerated by week 8. LNCaP xenografts exhibited a much slower growth rate were demonstrated to be the least aggressive model.

CTC profiling was performed on blood samples collected from the xenografted mice. The mice bearing different xenografted tumours were sacrificed at each time point and 500 µl of their blood was analyzed. For each time point, the number of CTCs isolated in capture zones was plotted against zone number to achieve a phenotypic profile based on EpCAM expression levels in CTCs. Representative images of an immunostained CTC and a mouse blood cell are shown in Figure 3B.

The raw data obtained from profiling of CTC phenotypes in three xenografts are shown in Figure S5. CTCs in LNCaP xenografts were mostly localized in the early zones (zone 1–4) where normally high EpCAM-expressing cells are captured. PC-3 CTCs, on the other hand, were isolated in later zones of the device (zone 8–10) indicating low EpCAM expression.

The metastasis-initiating potential of CTCs was investigated by comparing their phenotypic profiles before and after the detection of metastasis. The distribution of CTCs in capture zones at corresponding time points are shown in Figure 3C for all three xenografts (data from other time points are shown in Figure S5). At week 4, LNCaP and PC-3 models exhibited similar CTC profiles (Figure 3C). However, 4 weeks later (at week 8) a marked phenotypic shift was displayed in CTC profiles from PC-3 xenografts, whereas LNCaP profile remained unchanged. Despite the comparable number of bulk CTCs in LNCaP and PC-3 xenografts at week 4, PC-3 mice exhibited lung metastases at week 8, while LNCaP xenografts did exhibit detectable metastases. This difference in metastatic potential was reflected in the CTC phenotypic profiles obtained from these two xenografts highlighting the importance of CTC profiling.

For the PC-3M xenograft model, a large number of CTCs with heterogeneous phenotypes were shed from the xenografted tumor at early time points (Figure 3C). Overt lung and lymph node metastases were detected in these xenografted mice as early as 2 weeks post-injection (Figure 3E). The significant metastatic potential of this xenograft may be correlated to the heterogeneity of the CTCs and the existence of a large population of EMT-transformed CTCs. Overt lung and lymph node metastases were detected in these xenograft mice as early as 2 weeks post-injection (Figure 3E).

To investigate the effect of a chemotherapeutic agent on CTC profiles in vivo, mice bearing PC-3M xenografts were subjected to a course of docetaxel treatment. PC-3M xenografts were injected intravenously at a dose of 10 mg control cohort of mice received saline injections. At three time points (2 weeks, 4 weeks and 5 weeks after implantation), mice were sacrificed for blood collection and CTC analysis. Tumors and other organs were collected for histopathology analysis.

The CTC phenotypic profiles for docetaxel-treated mice are shown in Figure 4. The total number of CTCs decreased at both week 2 and week 4 time points compared to that in control mice. The EpCAM profile at week 2 (4 days after first dose of docetaxel) is heterogeneous with two subpopulations similar to that of the control mice (Figure 4A). However, at week 4 (8 days after second injection), docetaxel-treated mice exhibited a lower number of CTCs with a different phenotypic profile (Figure 4B). Compared to the heterogeneous profile in PC-3M xenografts at week 4, only one population of CTCs remained in docetaxel-treated mice. This population is positioned in the later zones of the device where cells with low EpCAM expression are captured. This observation can be interpreted in two ways: 1) mesenchymal cells with a low expression of EpCAM are more resistant to chemotherapy²⁵, or 2) high EpCAM expressing cells change their phenotype after exposure to the drug and lose their EpCAM expression and therefore are captured in later zones. The second theory is backed by a body of research showing that EpCAM is downregulated in prostate cancer cells under docetaxel treatment³⁸. Following the decreased

cell heterogeneity, treated mice exhibited a decline in metastasis incidence and survived longer which correlates with literature studies³⁹. After the third dose of docetaxel, a much lower number of CTCs were remained which had mesenchymal phenotypes (Figure 4C). Overall, PC-3M mice treated with docetaxel shed lower number of CTCs with reduced heterogeneity and mesenchymal phenotypes.

To further study the effect of the docetaxel treatment on the properties of CTCs shed into the bloodstream, two regimens of docetaxel were administered to PC-3M mice. In first cohort, mice were treated with 10 mg docetaxel/kg body weight every 10 days for 3 doses. Mice from the second cohort received only 2 doses of docetaxel. The control cohort received saline injections. Figure 5A shows the comparison between the tumor growth rates in these three cohorts. Up to week 4, two groups had similar growth rates as they were treated the same. A marked decrease in the size of the tumor was observed in treated mice compared to control mice, confirming the effectiveness of treatment. However, at week 5 treated mice in cohort 1 and cohort 2 displayed different growth rates. The size of tumors in the mice treated with 3 doses of docetaxel decreased significantly while tumors receiving 2 doses of the drug continues to progress. At week 6, 12 days after completion of the treatment in cohort 1, tumor size was observed to increase.

To investigate the effect of docetaxel treatment on CTC phenotypes, CTC analysis was done on both groups of mice at week 6 (Figure 5B and Figure 5C, data from week 5 are shown in Figure S6 as well). The mice receiving two docetaxel injections displayed mesenchymal CTCs and also exhibited extensive lung metastasis (Figure 5D and Figure 5E). However, in the mice from the first cohort which are treated with 3 doses, a new population of CTCs with epithelial phenotypes emerged 12 days after the cessation of treatment (a similar profile is observed in the mice treated with 2 doses of docetaxel 15 days after the drug removal). This population of CTCs appears to be shed by the growing tumor after the removal of the treatment. In contrast to the mice with fewer treatment cycles, the mice with more dose of docetaxel formed much lower numbers of metastatic lesions.

Conclusions

We demonstrated a strategy for sorting rare CTCs built on a recently developed technology called magnetic ranking cytometry (MagRC). The new generation of MagRC allows sorting CTCs based on their surface marker expression with high sensitivity and efficiency. As few as ten cells could be recovered from the device. Specific immunostaining was utilized to ensure cells that are captured in early/late zones are CTCs. It is noteworthy that use of the relatively low flow rate (400 μ L/hr) can be a challenge for the processing of larger samples; however, device fabrication can be optimized in order to increase the chip aspect ratio and therefore, higher flow rates can be used for sample processing.

Reproducible phenotypic profiles were obtained from the analysis of cultured cells and xenograft CTCs. Real-time monitoring of CTC phenotypes provides a wealth of information that can be taken advantage of in cancer prognosis and treatment. Xenograft mouse models with different metastatic potential exhibited unique CTC profiles over the course of cancer progression. Non-invasive LNCaP-derived tumors shed a low number of CTCs with static

EpCAM profiles over 16 weeks, while aggressive xenografted tumors (PC-3 and PC-3M) exhibited dynamic EpCAM profiles. Extreme heterogeneity was observed in CTCs from highly metastatic PC-3M xenografts. Both aggressive models had a large population of EMT-transformed cells, indicating their role in forming metastasis. Furthermore, the effect of a chemotherapy drug was effectively traced by comparing dynamic CTC phenotypic profiles obtained from treated xenograft mice with CTC profiles from the untreated cohort of mice. A decrease in CTC counts and heterogeneity was observed after treatment with docetaxel and was followed by a reduction in the incidence of metastases. Capturing dynamic changes in CTC phenotypes during a course of chemotherapy treatment demonstrates the potential use of this technique for drug development and diagnostics. The high sensitivity, practicality and versatility of this microfluidic technique allow us to profile CTCs according to a surface marker expression. Its advantage over flow cytometry in dealing with a low number of CTCs and unprocessed blood samples makes it an appropriate platform for CTC phenotypic profiling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Research reported in this publication was supported by the Canadian Institutes of Health Research (grant no. CPG-146464), the Natural Sciences and Engineering Research Council of Canada (grant no. CHRPJ 493632-16), and the National Cancer Institute of the National Institutes of Health (grant no. R33CA204574). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the other funding agencies.

Notes and references

1. Pantel K and Speicher MR, *Oncogene*, 2015, 1–9.
2. C. A. Klein, *Nature*, 2013, 501, 365–372. [PubMed: 24048069]
3. Siravegna G, Marsoni S, Siena S and Bardelli A, *Nat. Rev. Clin. Oncol*, 2017, 1–18.
4. Perakis S and Speicher MR, *BMC Med*, 2017, 15, 75. [PubMed: 28381299]
5. Hajba L and Guttman A, *TrAC Trends Anal. Chem*, 2014, 59, 9–16.
6. Valkenburg KC and Williams BO, *Prostate Cancer*, 2011, 2011, 1–22.
7. Rea D, del Vecchio V, Palma G, Barbieri A, Falco M, Luciano A, De Biase D, Perdonà S, Facchini G and Arra C, *Biomed Res. Int*, 2016, 2016, 1–11.
8. van Marion DMS, Domanska UM, Timmer-Bosscha H and Walenkamp AME, *Crit. Rev. Oncol. Hematol*, 2016, 97, 107–117. [PubMed: 26321372]
9. Havens AM, Pedersen EA, Shiozawa Y, Ying C, Jung Y, Sun Y, Neeley C, Wang J, Mehra R, Keller ET, McCauley LK, Loberg RD, Pienta KJ and Taichman RS, *Neoplasia*, 2008, 10, 371–379. [PubMed: 18392141]
10. van Weerden WM, Bangma C and de Wit R, *Br. J. Cancer*, 2009, 100, 13–18. [PubMed: 19088719]
11. Ruscetti M, Quach B, Dadashian EL, Mulholland DJ and Wu H, *Cancer Res*, 2015, 75, 2749–2759. [PubMed: 25948589]
12. Poudineh M, Sargent EH, Pantel K, Kelley SO, *Nature Biomed. Eng*, 2018, 2, 72–84.
13. Sarioglu a F., Aceto N, Kojic N, Donaldson MC, Zeinali M, Hamza B, Engstrom A, Zhu H, Sundaresan TK, Miyamoto DT, Luo X, Bardia A, Wittner BS, Ramaswamy S, Shioda T, Ting DT, Stott SL, Kapur R, Maheswaran S, Haber D. a and Toner M, *Nat. Methods*, 2015, 12, 685–691. [PubMed: 25984697]
14. Gascoyne PRC and Shim S, *Cancers (Basel)*, 2014, 6, 545–579. [PubMed: 24662940]

15. Li P, Stratton ZS, Dao M, Ritz J and Huang TJ, *Lab Chip*, 2013, 13, 602. [PubMed: 23306378]
16. Issadore D, Chung J, Shao H, Liong M, Ghazani AA, Castro CM, Weissleder R and Lee H, *Sci. Transl. Med*, 2012, 4, 1–10.
17. Green BJ, Kermanshah L, Labib M, Ahmed SU, Silva PN, Mahmoudian L, Chang I-H, Mohamadi RM, Rocheleau JV and Kelley SO, *ACS Appl. Mater. Interfaces*, 2017, 9, 20435–20443. [PubMed: 28548481]
18. Poudineh M, Labib M, Ahmed S, Nguyen LNM, Kermanshah L, Mohamadi RM, Sargent EH and Kelley SO, *Angew. Chemie - Int. Ed*, 2017, 56, 163–168.
19. Mohamadi RM, Besant JD, Mephram A, Green B, Mahmoudian L, Gibbs T, Ivanov I, Malvea A, Stojcic J, Allan AL, Lowes LE, Sargent EH, Nam RK and Kelley SO, *Angew. Chemie Int. Ed*, 2015, 54, 139–143.
20. Besant JD, Mohamadi RM, Aldridge PM, Li Y, Sargent EH and Kelley SO, *Nanoscale*, 2015, 7, 6278–6285. [PubMed: 25784586]
21. Jack R, Hussain K, Rodrigues D, Zeinali M, Azizi E, Wicha M, Simeone DM and Nagrath S, *Lab Chip*, 2017, 17, 1349–1358. [PubMed: 28294230]
22. Poudineh M, Aldridge PM, Ahmed S, Green BJ, Kermanshah L, Nguyen V, Tu C, Mohamadi RM, Nam RK, Hansen A, Sridhar SS, Finelli A, Fleshner NE, Joshua AM, Sargent EH and Kelley SO, *Nat. Nanotechnol*, 2017, 12, 274–281. [PubMed: 27870841]
23. Wu X, Gong S, Roy-Burman P, Lee P and Culig Z, *Endocr. Relat. Cancer*, 2013, 20, 155–170.
24. Verdone JE, Parsana P, Veltri RW and Pienta KJ, *Prostate*, 2015, 75, 218–224. [PubMed: 25327565]
25. Pavese JM and Bergan RC, *Cancer Lett*, 2014, 352, 179–186. [PubMed: 25016063]
26. de Wit S, van Dalum G, Lenferink ATM, Tibbe AGJ, Hiltermann TJN, Groen HJM, van Rijn CJM and Terstappen LWMM, *Sci. Rep*, 2015, 5, 12270. [PubMed: 26184843]
27. Wu S, Liu S, Liu Z, Huang J, Pu X, Li J, Yang D, Deng H, Yang N and Xu J, *PLoS One*, 2015, 10, 1–14.
28. Prang N, Preithner S, Brischwein K, Göster P, Wöppel A, Müller J, Steiger C, Peters M, Baeuerle PA and Da Silva AJ, *Br. J. Cancer*, 2005, 92, 342–349. [PubMed: 15655555]
29. Khanna C and Hunter K, *Carcinogenesis*, 2005, 26, 513–23. [PubMed: 15358632]
30. Hoffman RM, *Nat. Rev. Cancer*, 2015, 15, 451–452. [PubMed: 26422835]
31. Kolostova K, Pinterova D, Hoffman RM and Bobek V, *Anticancer Res*, 2011, 31, 1535–1539. [PubMed: 21617207]
32. Hudson TS, Perkins SN, Hursting SD, Young HA, Kim YS, Wang TC and Wang TTY, *Int. J. Oncol*, 2012, 40, 1113–1121. [PubMed: 22266918]
33. Sánchez AM, Sánchez MG, Malagarie-Cazenave S, Olea N and Díaz-Laviada I, *Apoptosis*, 2006, 11, 89–99. [PubMed: 16374544]
34. Lakshman M, Xu L, Ananthanarayanan V, Cooper J, Takimoto CH, Helenowski I, Pelling JC and Bergan RC, *Cancer Res*, 2008, 68, 2024–2032. [PubMed: 18339885]
35. Lowes LE, Goodale D, Xia Y, Postenka C, Piaseczny MM, Paczkowski F and Allan AL, *Oncotarget*, 2016, 7, 76125–76139. [PubMed: 27764810]
36. Yang F, Song L, Wang H, Wang J, Xu Z and Xing N, *PLoS One*, 2015, 10, 1–15.
37. Ni J, Cozzi P, Hung T, Hao J, Graham P and Li Y, *Transl. Oncol*, 2016, 9, 41–45. [PubMed: 26947880]
38. Massoner P, Thomm T, Mack B, Untergasser G, Martowicz A, Bobowski K, Klocker H, Gires O and Puhr M, *Br. J. Cancer*, 2014, 111, 955–964. [PubMed: 24992580]
39. Denslow A, Switalska M, Nowak M, Maciejewska M, Chlopicki S, Marcinek A, Gebicki J and Wietrzyk J, *BMC Cancer*, 2017, 17, 177. [PubMed: 28270133]

Figure 1: Monitoring dynamic CTC phenotypes in mouse xenografts using a 10 zone devices for Magnetic Ranking Cytometry (MagRC).

i) Three xenograft models with varying phenotypic properties and anticipated aggressiveness are generated by orthotopic implantation of prostate cancer cell lines into the prostate of immunodeficient mice. ii) At various time points post-injection, mice are sacrificed, and their blood is analyzed with the new generation of 10 zone MagRC devices for CTC profiling. The blood is mixed with antibody-functionalized magnetic nanoparticles that facilitate capture and profiling of CTCs. iii) The 10 zone devices feature progressive increases in channel width and the size of embedded nickel micromagnets, providing suitable areas for capture and sorting CTCs based on their phenotype. The length and width of the device is 5.4 cm and 4.3 cm, respectively. By increasing the width of the channel, the linear velocity and therefore, the drag force that cells experience decreases in capture zones. In addition, a local magnetic field gradient is engineered by using patterned nickel micromagnets with varying sizes along the channel. The radii of the nickel magnets increase by 10 μm sequentially from $r=145 \mu\text{m}$ to $r=235 \mu\text{m}$. Based on the width of the channel and the size of nickel micromagnets, the channel is divided into ten discrete zones. An array of X-shaped structures made of SU-8 are used to create areas with local slow flow velocity and thus facilitates cell capture. CTCs bound to a surface antibody (here anti-EpCAM) conjugated to magnetic beads are passed through the channel and get captured when the magnetic force exceeds the drag force acting on the cells. The distribution of CTCs in the capture zones mirrors the protein expression of cells. iv) Cells captured in each zone are immunostained, counted and plotted as a profile of captured cells vs. zone number. The position of the distribution reflects expression levels, while the width of the distribution reports on the level of heterogeneity within the sample. Finally, the correlation between CTC phenotypic profiles and their metastatic potential is investigated.

Figure 2: Profiling prostate cancer cell lines.

(A) Analysis of spiked samples: Capture profiles obtained from LNCaP, PC-3 and PC-3M cells spiked separately into mouse blood. Subsequent to on-chip cell capture, cells were stained with cytokeratins, vimentin specific antibodies and DAPI. Mouse blood cells were stained with mouse anti-CD45 to eliminate false positives. (B) Capture efficiencies for LNCaP, PC-3 and PC-3M cells. (C) Sensitivity of the 10-zone device where the sorting device was challenged with low numbers of cells. Error bars represent standard error from five trials. (D) Representative images of a PC-3M cell and a mouse blood cell stained with antibodies against cytokeratins, vimentin and mouse CD45.

Figure 3: Profiling CTCs from mice bearing human prostate cancer xenografts. (A) Tumor growth rate in three xenografted animals. (B) Representative images of a CTC from PC-3M xenograft and a mouse white blood cell. CTCs are stained for cytokeratins and vimentin and mouse cells for mouse anti-CD45. DAPI is used to stain nuclei. (C) CTC distribution based on EpCAM expression in prostate cancer xenograft mouse models at two time points: Week 4 and week 8 post-injection for LNCaP and PC-3 xenografts and week 2 and week 4 for PC-3M xenograft. (D) Representative immunohistochemistry (IHC) images of lung metastasis in PC-3M and PC-3 mice, at 4 and 12 weeks post injection, respectively. (E) Tabulation of metastatic lesions observed in the lung and lymph nodes of xenografted mice.

Figure 4: Docetaxel treatment of mice bearing PC-3M xenografts.

(A) CTC profiles in PC-3M mice from control and docetaxel-treated cohorts at week 2 post-injection. A decrease in CTC heterogeneity is observed after docetaxel treatment. (B) CTC profiles in PC-3M mice from control and docetaxel-treated cohorts at week 4 post-injection. One subpopulation of CTCs is eliminated after treatment with docetaxel. (C) CTC profile in PC-3M mice treated with 3 doses of docetaxel every 10 days at week 5 post-injection.

Figure 5: Effects of docetaxel treatment on PC-3M mouse xenografts and CTCs. (A) Comparison of tumor growth rate in control PC-3M mice with PC-3M xenografts treated with two regimens of docetaxel: i.v. injection of 10 mg/kg docetaxel every 10 days for 2 or 3 doses. (B) CTC profile in PC-3M mice treated with 2 doses of docetaxel 6 weeks post-injection. (C) CTC profile in PC-3M mice treated with 3 doses of docetaxel 6 weeks post-injection. (D) Representative immunohistochemistry (IHC) images of lung metastasis in PC-3M mice treated with two regimens of docetaxel 6 weeks post-injection. (E) Mice treated with 2 doses of docetaxel displayed more lung micrometastases compared to the mice treated with 3 doses of docetaxel.