Clinical Validation of an Ultra High-Throughput Spiral Microfluidics for the Detection and Enrichment of Viable Circulating Tumor Cells

The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.

Citation
Khoo, Bee Luan, Majid Ebrahimi Warkiani, Daniel Shao-Weng Tan, Ali Asgar S. Bhagat, Darryl Irwin, Dawn Pingxi Lau, Alvin S. T. Lim, et al. “Clinical Validation of an Ultra High-Throughput Spiral Microfluidics for the Detection and Enrichment of Viable Circulating Tumor Cells.” Edited by Hong Wanjin. PLoS ONE 9, no. 7 (July 7, 2014): e99409.

As Published
http://dx.doi.org/10.1371/journal.pone.0099409

Publisher
Public Library of Science

Version
Final published version

Citable link
http://hdl.handle.net/1721.1/89447

Terms of Use
Creative Commons Attribution

Detailed Terms
http://creativecommons.org/licenses/by/4.0/
Clinical Validation of an Ultra High-Throughput Spiral Microfluidics for the Detection and Enrichment of Viable Circulating Tumor Cells

Bee Luan Khoo1, Majid Ebrahim Warkiani2, Daniel Shao-Weng Tan4,5, Ali Asgar S. Bhagat6, Darryl Irwin7, Dawn Pingxi Lau4,5, Alvin S. T. Lim8, Kiat Hon Lim8, Sai Sakettee Krisna4,5, Wan-Teck Lim1, Yoon Sim Yap1, Soo Chin Lee9, Ross A. Soo9, Jongyoon Han2,10, Chwee Teck Lim1,2,3,6

1 Mechanobiology Institute, National University of Singapore, Singapore, Singapore, 2 BioSystems and Micromechanics (BioSyM) IRG, Singapore-MIT Alliance for Research and Technology (SMART) Centre, Singapore, Singapore, 3 Department of Biomedical Engineering, National University of Singapore, Singapore, Singapore, 4 Department of Medical Oncology, National Cancer Centre Singapore, Singapore, Singapore, 5 Cancer Therapeutics Research Laboratory, National Cancer Centre Singapore, Singapore, Singapore, 6 Clearbridge BioMedics Pte Ltd, Singapore, Singapore, 7 Sequenom Inc, San Diego, California, United States of America, 8 Department of Pathology, Singapore General Hospital, Singapore, Singapore, 9 Department of Hematology-Oncology, National University Hospital, Singapore, Singapore, 10 Department of Electrical Engineering and Computer Science, Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts, United States of America

Abstract

Background: Circulating tumor cells (CTCs) are cancer cells that can be isolated via liquid biopsy from blood and can be phenotypically and genetically characterized to provide critical information for guiding cancer treatment. Current analysis of CTCs is hindered by the throughput, selectivity and specificity of devices or assays used in CTC detection and isolation.

Methodology/Principal Findings: Here, we enriched and characterized putative CTCs from blood samples of patients with both advanced stage metastatic breast and lung cancers using a novel multiplexed spiral microfluidic chip. This system detected putative CTCs under high sensitivity (100%, n = 56) (Breast cancer samples: 12–1275 CTCs/ml; Lung cancer samples: 10–1535 CTCs/ml) rapidly from clinically relevant blood volumes (7.5 ml under 5 min). Blood samples were completely separated into plasma, CTCs and PBMCs components and each fraction were characterized with immunophenotyping (Pan-cytokeratin/CD45, CD44/CD24, EpCAM), fluorescence in-situ hybridization (FISH) (EML4-ALK) or targeted somatic mutation analysis. We used an ultra-sensitive mass spectrometry based system to highlight the presence of an EGFR-activating mutation in both isolated CTCs and plasma cell-free DNA (cf-DNA), and demonstrate concordance with the original tumor-biopsy samples.

Conclusions/Significance: We have clinically validated our multiplexed microfluidic chip for the ultra high-throughput, low-cost and label-free enrichment of CTCs. Retrieved cells were unlabeled and viable, enabling potential propagation and real-time downstream analysis using next generation sequencing (NGS) or proteomic analysis.

Citation: Khoo BL, Warkiani ME, Tan DS-W, Bhagat AAS, Irwin D, et al. (2014) Clinical Validation of an Ultra High-Throughput Spiral Microfluidics for the Detection and Enrichment of Viable Circulating Tumor Cells. PLoS ONE 9(7): e99409. doi:10.1371/journal.pone.0099409

Editor: Hong Wanjin, Institute of Molecular and Cell Biology, Biopolis, United States of America

Received December 17, 2013; Accepted May 14, 2014; Published July 7, 2014

This is an open-access article, free of all copyright, and may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose. The work is made available under the Creative Commons CC0 public domain dedication.

This is an open-access article, free of all copyright, and may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose. The work is made available under the Creative Commons CC0 public domain dedication.

Funding: Financial support by the Mechanobiology Institute (MBI) and Singapore-MIT Alliance for Research and Technology (SMART) Centre (BioSyM IRG). Clearbridge BioMedics Pte Ltd provided support in the form of salary for author AASB but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the ‘author contributions’ section.

Competing Interests: AASB is an employee of Clearbridge BioMedics Pte Ltd which commercialized the technology described here. AASB, JH and CTL, along with others, also have filed a patent application on the technology. Pending patent: Microfluidics Sorter for Cell Detection and Isolation and number PCT/US2011/027276. A product based on this technology is currently being developed by Clearbridge BioMedics Pte Ltd. This does not alter the authors’ adherence to all the PLOS ONE policies on sharing data and materials.

* Email: jyhan@mit.edu (JH); ctlim@nus.edu.sg (CTL)
† These authors contributed equally to this work.

Introduction

Circulating tumor cells (CTCs) is a collective term to describe cancer cells of solid tumor origin found in the blood of cancer patients. The heterogeneous nature of CTCs provides a comprehensive yet non-invasive means for characterizing tumor molecular subtypes, which can be utilized for stratifying patients to appropriate cancer therapy [1,2]. Current CTC capture platforms employ flow cytometry [5], fluorescence and magnetic-activated cell sorting methods [4], gradient centrifugation [5], and filtration [6,7,8]. These techniques are often limited by lengthy and complicated processing procedures, low purity and cell viability. An assay with high throughput, selectivity and specificity for CTC detection is pivotal for advancing CTC characterization and utility [9]. At present, Epithelial Cell Adhesion Molecule (EpCAM) is the most popular epithelial biomarker commonly used in the detection of CTCs [10]. However, EpCAM may not be expressed in all CTCs due to epithelial-mesenchymal transition (EMT) [11,12].
There is also growing interest in plasma cell-free DNA (cf-DNA) as an alternative for a non-invasive biomarker. Initial investigations suggest a degree of concordance between cf-DNA, CTCs [13], and disseminated tumor cells (DTCs) in metastatic breast cancer patients, highlighting the possibility that cf-DNA can be of prognostic value [11].

We previously developed a novel integrated spiral microfluidic device for CTC enrichment from whole blood [14]. Here, we adopted an enhanced and high-throughput multiplexed version that demonstrated high sensitivity by the consistent detection of viable putative CTCs (Breast cancer samples: 12–1,275 CTCs/ml; Lung cancer samples: 10–1,535 CTCs/ml) from 100% of patients’ blood samples (n = 56) of clinically relevant volumes (7.5 ml). Blood samples were completely fractionated to plasma, CTCs and PBMCs components for further downstream analysis such as immunostaining (Pan-cytokeratin+/CD45-), fluorescence in-situ hybridization (FISH) (EML4-ALK) or targeted somatic mutation analysis. We also demonstrated the rare presence of EGFR-activating mutation in isolated CTC-DNA and cf-DNA, as well as original tumor-biopsy samples via targeted somatic mutation. Retrieved cells were unlabeled and hence more viable for propagation and other informative downstream analysis such as next generation sequencing (NGS) and proteomic analysis.

Materials and Methods

Ethics statement and clinical sample preparation

This study was approved by respective institutional review boards (IRB) and local ethics committee (National Healthcare Group [NHG]) (DSR Reference 2012/00105, 2012/00979, 2010/00270, 2010/00691). Informed and written consent was obtained from all patients. IRB and ethics committee approval was also granted for NSCLC samples where retrospective archival specimens were retrieved (Singhealth 2010/516/B). Ten blood samples from healthy donors and 58 (56+2) blood samples from patients with metastatic lung or breast cancer were acquired. Blood samples were stored in EDTA-coated vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ, USA). Plasma was fractionated from whole blood for the lung samples by centrifugation (1500 g, 10 min). Blood samples were then lysed using red blood cell (RBC) lysis buffer (gBioscience, USA) according to manufacturer’s recommendations. The nucleated cell fraction was resuspended with phosphate buffered saline (PBS) to desired concentration (Fig. 1A).

Device fabrication

The microfluidic chips were fabricated using standard soft-lithography techniques in polydimethylsiloxane (PDMS) described elsewhere [14,15]. After fabrication of individual chips, the multiplexed device was obtained by stacking three separate devices together using manual alignment and oxygen plasma bonding. The fluidic inlets and outlets were punched into the assembly and final device obtained by bonding the whole assembly to a pre-cleaned microscopic glass slide using plasma machine.

Sample processing

Blood samples at 2X concentration (i.e., 7.5 ml of whole blood was lysed and resuspended in 3.75 ml of PBS) was placed into a 10 ml BD Luer-Lok syringe (Becton, Dickinson and Company) and pumped into the multiplexed chip using a syringe pump (NE-1000, New Era Pump Systems Inc., USA). Sheath consisted of 0.5% BSA in PBS supplemented with 2 mM EDTA and was similarly introduced into the biochip via a separate syringe pump (PHD 2000, Harvard Apparatus, USA). Sample and sheath were introduced into the device at a fixed flow ratio of 1:9 for optimal separation. Device was connected to syringes and collection tubes (falcon tubes; Becton, Dickinson and Company) by Tygon tubings (Spectra-tek-nika, USA). The enrichment process was visualised with an inverted microscope (Olympus IX71) linked to a high-speed CCD camera (Phantom v9, Vision Research Inc., USA) and operated using the Phantom Camera Control software.

Immunofluorescence staining and enumeration

Enriched cell fractions were fixed with 4% paraformaldehyde (PFA, Sigma, USA) for 10 min at room temperature, permeabilized with 0.1% Triton X-100 (Sigma Aldrich, USA), mixed and incubated with fluorescein isothiocyanate (FITC) conjugated pan-cytokeratin antibodies, allophtocyanin (APC) conjugated CD45 antibodies (1:100, Miltenyi Biotec Asia Pacific, Singapore) and Hoechst 33342 dye (1M, Sigma) in PBS buffer supplemented with 0.5% BSA on ice for 30 min. Other antibodies used included EpCAM (APC), CD44 (FITC) and CD24 (APC) (all 1:100, from Miltenyi Biotec Asia Pacific, Singapore). Stained cells were concentrated and imaged with an Olympus inverted microscope (Tokyo, Japan) (Emission filters ET460/50m, ET535/50m and ET 605/70; Olympus, Tokyo, Japan) equipped with an automated stage. Enriched cells were placed within the well of a 96-well plate (Thermo Scientific, USA) and the well was automatically scanned in a 1 mm x 1 mm grid format using a programmable stage and Metamorph software (California, United States). Corresponding image sets (at 40X magnification) were compared to determine presence of putative CTCs. Hoechst-positive/pan-cytokeratin-positive (CK+/CD45-negative (CD45-)) enriched cells generally (but not exclusively) with round nucleus and high nuclear to cytoplasmic ratio were considered putative CTCs. Enriched cells of some samples were also seeded onto 2D Gelretx (Invitrogen) coated substrates and incubated at 37°C and 5% CO2 to allow adherence of viable cells. Non-adherent cells were washed and removed gently with 1X PBS after 72 hr. Adherent cells were then stained with FITC conjugated pan-cytokeratin antibodies and APC conjugated CD45 antibodies. Some samples were also stained for 15 min on ice with potassium iodide (PI) after microfluidic processing to determine viable proportion of enriched cells.

DNA extraction and sequencing

DNA extraction was carried out on pooled cells (QIAamp DNA Blood Minikit (Qiagen, Hilden, Germany)), plasma DNA (QIAamp circulating nucleic acid kit (Qiagen, Hilden, Germany)) and formalin-fixed paraffin embedded tumour specimens (QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany)). Absolute number of intact copies of extracted genomic DNA was determined by Sequenom Sample ID panel (Sequenom Inc, CA, USA). Targeted somatic mutation analysis was performed by PCR amplification followed by Single Allele Base Extension Reaction (SABER) [16] and standard iPLEX chemistry [17,18]. Resultant mutant allele products were detected by mass spectrometry (Sequenom, CA, USA). PCR amplification was performed with Sequenom PCR Reagents Set (Sequenom) (95°C, 2 minutes; 45 cycles -95°C, 30 sec; 56°C for 30 sec; 72°C, 60 sec; 72°C, 5 min). Residual dNTPs were dephosphorylated (0.5 units SAP enzyme (Sequenom)), incubated (37°C, 40 min) and enzyme deactivated (5 min, 85°C) followed by single base extension (Sequenom iPLEX Pro Kit (Sequenom) (94°C, 30 sec; 40 cycles -95°C, 5 sec; 5 internal cycles -52°C, 5 sec; 80°C, 5 sec; 72°C, 5 min)). PCR products were de-salted using 6 mg of ion exchange resin (Sequenom) in 16 µL HPLC water. Cleaned PCR product was spotted onto MassArray SpectroCHIPS II (Sequenom) using
the MassARRAY RS1000 Nanodispenser. MALDI-TOF MS analysis was performed using the MassArray MA4.

DNA Fluorescence in-situ hybridization (FISH)

Cell spots were prepared with Cytospin centrifuge (Thermo Scientific, USA) (600 rpm, 6 min), fixed (acetic acid/methanol) and dehydrated via ethanol series (80%, 90%, and 100%). Slides were treated with RNase (4 mg/ml) (Sigma, USA) (40 min, 37°C), washed (1X PBS/0.2% Tween 20 (Sigma, USA)), denatured (70% formamide/2X SSC; 10 min, 80°C) and quenched dehydrated via ice-cold ethanol series. EML4-ALK probe (Vysis LSI ALK breakapart, Abbott, USA) hybridization was carried out under dark and humid conditions (42°C, overnight). Hybridized slides were washed with 50% formamide/2X SSC and 2X SSC at 45°C, counterstained with 4’, 6-diamidino-2-phenylindole (DAPI) and sealed.

Statistical methods

As CTC levels in patients were not normally distributed, results were presented as counts and medians with the corresponding percentages and ranges.

Results

Enhanced spiral microfluidic device

The microfluidic device consisted of three stacked spiral microfluidic chips with two inlets and two outlets (Fig. 1B). Suspended cells under flow within a curvilinear microchannel experience inertial lift forces coupled with the rotational Dean drag force in the fluid regime. The combination of these forces focus the cells at certain equilibrium positions of the channel cross-section [19]. Since these forces are a function of cell size, cells of different sizes (larger CTCs and smaller hematologic cells) occupy distinct lateral positions away from the microchannel walls, and this allows for size-based separation at the outlets [14,20].

Enrichment of putative CTCs from patients with metastatic breast or lung cancer

Blood samples (7.5 ml) from 10 healthy donors (Table S1 in File S1) and 58 patients (Table S2 in File S1) with metastatic breast or non-small cell lung cancer (NSCLC) were processed using the multiplexed spiral microfluidic chip. Two samples included in the table were not enumerated for CTCs and their enriched samples were directly processed for SABER molecular analysis (see section below). Hoechst positive/pan-keratin-positive (CK+)/CD45-
negative (CD45-) enriched cells were considered putative CTCs (Fig. 2A, 2B). These cells generally (but not exclusively) exhibit round nucleus and high nuclear to cytoplasmic ratio. CTCs were detected in 100% (n = 56) of all samples, with a varied range of CTCs isolated for breast cancer samples (12-1,275 CTCs/ml) (Median: 55 CTCs/ml) and NSCLC samples (10–1,535 CTCs/ml) (Median: 82 CTCs/ml) respectively (Fig. 2B). CK+<sup>+</sup>/CD45- cells were detected at significantly lower counts in healthy samples (2–7 CK+ cells/ml). These could be attributed to epithelial cells present at trace amounts in blood. However, due to their small number in comparison with that of cancer patients, a detection threshold at < sup>< sup>7 CK+/CD45- cells was thus determined for a

Figure 2. Enumeration of CTC from cancer patients. (A) Immunofluorescence staining of isolated CTCs. CTCs (marked by white arrow) were identified by the following criteria: Hoechst+, pan-CK+ and CD45-. Scale bar: 20 μm (B) Box plot summary indicating the range of CK+ cells/ml recovered from the sample outlet for blood samples extracted from healthy volunteers, as well as breast and lung cancer patients. The box plot presents the median, lower and upper quartiles (25<sup>th</sup>, 75<sup>th</sup> percentiles). Data points that lie outside the 10<sup>th</sup> and 90<sup>th</sup> percentiles are shown as outliers (Anova, p<0.001). Encapsulated image of PAP stained isolated cells shows a large CTC with high nucleus to cytoplasmic ratio (N/C) ratio (labeled with white arrow). (C) Staining of CTC for pan-CK and CD45. Upper panel depicts a representative image of cells which were double positive (CK+/CD45+); while lower panel shows a double negative (CK-/CD45-) cell. Scale bar: 20 μm (D) Staining of CTC for pan-cytokeratin and EpCAM. Scale bar: 20 μm. doi:10.1371/journal.pone.0099409.g002
### Table

| Sample no | Sample type | CellSearch® | Multiplexed Spiral chip |
|-----------|-------------|-------------|-------------------------|
| 1         | Lung        | 3           | 2580                    |
| 2         | Lung        | 0           | 3442                    |
| 3         | Lung        | 5           | 1110                    |
| 4         | Lung        | 2           | 37                      |
| 5         | Lung        | 2           | 270                     |
| 6         | Breast      | 0           | 1275                    |
| 7         | Breast      | 5           | 870                     |
| 8         | Breast      | 4           | 322                     |
| 9         | Breast      | 1           | 247                     |
| 10        | Breast      | 0           | 300                     |

### Figure

- **A**: Images of Hoechst, CD44, CD45, and Bright Field.
- **B**: Table showing sample type, CTC count, CellSearch® count, and Multiplexed Spiral chip count.
- **C**: Images with arrows indicating specific areas.
- **D**: Mass spectra with peaks labeled (i) 6324 Da (Mutant) 33%, (ii) 6404 Da (Wild-Type) 32%, (iii) 6404 Da (Wild-Type) 1.4%.
sample to be considered significantly positive for CTCs. Also, a negligible amount of double positive CK+/CD45+ cells (<5%, data not reported) were detected in our enriched samples, as was similarly reported elsewhere [21]. However, since the nature of these cells is yet to be established, they are not considered for enumeration in our study. We also observed many Hoechst+/CK-/CD45- cells among the captured putative CTCs (Fig. 2C). This population varied in distribution across all samples, and was present at an average proportion of 51.5±17.3% of the total nucleated cells (Table S2 in File S1). Several hypothesis generated to explain their presence include the theory of cancer cell intermediates due to EMT [12,22]. Five enriched samples were also immunostained for EpCAM, and EpCAM-/CK+ and EpCAM+/CK+ cells were detected in the isolated CTCs (Fig 2D). These EPCCAM- cells constituted more than half of the enriched cell population (~89.1±6.0%) (Fig. S1 in File S1) and are generally CD45+. A portion of CK+ putative CTCs from these 5 enriched samples were also positive for EMT markers such as E-cadherin and Vimentin (Fig. S2 in File S1). Enriched samples generally retained viability, as determined by potassium iodide staining (~87.5%, Fig. S3 in File S1). Furthermore, a fraction of these enriched viable CTCs maintained on 2D substrates expressed CD44 (Fig. 3A), and some CD44+ cells also co-expressed CD24 (~24.7±14%) (Fig. S4 in File S1). The expression of CD44 is associated with cancer stem cell-like traits [23].

Head to head comparison with CellSearch assay

Comparisons on the CTC enumeration values between the FDA approved CellSearch assay and our multiplexed spiral biochip were conducted with 10 blood samples from patients with breast or lung cancer. CTCs were detected in 80% (8/10) samples using CellSearch, and 100% of the samples (10/10) by the multiplexed spiral microfluidic chip. A significantly lower range of CTC count was obtained from CellSearch as compared to the multiplexed spiral device (Fig. 3B), implying loss of EPcam- CTCs using CellSearch. Data illustrating similar limitations in detecting lung CTCs and contrast between CTC counts obtained has been previously highlighted in comparison study between CellSearch and ISET [24].

Identifying therapeutically tractable alterations in CTCs and plasma

Given the challenge with low tissue yield from lung biopsies, we sought to determine therapeutically tractable alterations in enriched NSCLC CTCs. EML4-ALK gene translocation is found in approximately 1 to 6.7% of NSCLC patients [25,26]. In an index ALK positive NSCLC sample no 18, Table S2 in File S1), we demonstrated ALK rearrangement in CTCs (Fig. 3C) using the ALK Vysis breakapart probe (Abbott Molecular, USA) after enumeration and fixation of spotted cells. Out of 177 enumerated cells, 25.4% were found to have positive signals for ALK rearrangement, with the same fusion signal identified in 54% of 200 cells in the original FFPE sample. We also performed targeted mutation profiling of both plasma and CTCs in three NSCLC patients using SABER [16], previously shown to detect rare alleles down to <0.5% frequency [18] in a single reaction. Technical replicates were performed where the amount of input template, as determined by Sample ID panel, was <150 intact template copies, such that a single mutant strand would be observed by the SABER method. Up to 128 technical replicates were performed, depending on the amount of intact extracted DNA template isolated from each sample. In all three samples, EGFR mutations were detected in diagnostic tumour specimens using the Sequenom massarray using standard iPLEX chemistry. One baseline sample (sample no 32, Table S2 in File S1) demonstrated concordance across formalin-fixed paraffin-embedded tumor (FFPE) tumor block, plasma and CTCs, although at differing mutant allele frequency (33%, 32% and 1.5% respectively, Fig. 3D). Interestingly, one patient was sampled serially and showed no mutations in plasma and CTCs after treatment with gefitinib (sample no 33, Table S2 in File S1), an EGFR TKI inhibitor. In the last sample (sample no 11, Table S2 in File S1), while no mutation was detected in the circulating plasma DNA, it was detected at very low concentrations in pooled CTCs (0.05%) (TIL).

Discussion

Progression in CTC characterization critically hinges on the development of techniques to enrich CTCs under high concentrations and purity [27]. The development of label-free and high throughput assays to obtain reliable ‘real-time’ analysis of the disease status is necessary to facilitate personalized treatment strategies [28]. Previously, we demonstrated a novel spiral microfluidics technique for the detection and enrichment of CTCs. The multiplexed version presented here had been further enhanced to provide a device of high throughput (20 times faster) (7.5 ml in less than 5 min), high sensitivity (100% detection) (3–1,535 CTCs) and selectivity [Mean: 750 WBCs/ml]. Isolated CTCs remained viable and can be potentially propagated in culture.

Blood samples can be completely fractionated to plasma, CTCs and PBMCs components, which provides the opportunity to interrogate each component with genomic and transcriptomic tools. We obtained high definition images of immunostained putative CTCs (Hoechst+/pan-cytokeratin+/CD45-) and further identified therapeutically tractable genomic alterations (EMLA-ALK translocation) in CTCs, using gold standard FISH assays as well as a mass spectrometry based method for mutational profiling. In a patient with paired CTCs and plasma, we demonstrated concordance in EGFR mutation in both cf-DNA and CTCs. Despite the low WBC count through the use of the spiral microfluidic biochips, somatic mutations were found in pooled CTCs at very low frequencies (1.5% and 0.05%). Possible reasons...
include the presence of heterogeneous cell populations in circulation whose molecular profiles are discordant from the primary. Given the amount of DNA required for this high sensitivity assay, multiplexed mutational analysis may be feasible to reliably obtain genetic patterns of CTCs. The fast processing time and nature of the spiral microfluidic biochip lends itself to a broad range of potential genomic and transcriptomic applications. There are currently ongoing studies to apply RNA-based single-cell molecular analysis and next generation sequencing (NGS) on captured CTCs. The improvisation of such upcoming techniques for CTC enrichment and characterisation will hopefully shed new light on the CTC biology (origin, progression) and utilisation for therapeutics and treatment.

**Supporting Information**

File S1 Contains the following Supporting Information files:

**Table S1**: List of healthy samples as controls. **Table S2**: List of patient samples for clinical validation. Clinico-pathological characteristics are provided for patients with metastatic lung or breast cancer who provided samples for CTC enumeration. Samples may be serially obtained from a single patient and these are indicated by the patient number. C: Cycle, D: Day. Post-surgery pre AC samples are stated to be <3 weeks post-treatment. **Figure S1**: EpCAM staining of enriched cell populations. (A) Immunostaining with EpCAM-FITC and CD45-APC antibodies. (B) Flow cytometry analysis of EpCAM/CD45 cell populations. Scale bar: 20 μm. **Figure S2**: CTC images displaying variation in EMT biomarker expression. (A) CK+ cells can either be E-cadherin+ or E-cadherin- on breast CTCs. (B) CK+ cells can either be Vimentin+ or Vimentin- on breast CTCs. Scale bar: 20 μm. **Figure S3**: Scattered plot obtained with flow cytometry analysis. Potassium iodide staining of enriched samples to determine viability. **Figure S4**: Flow cytometry analysis of CD44+FITC/CD45-APC cell populations. (DOC)

**Acknowledgments**

We would like to express our sincere gratitude to all healthy and patient donors who participated in this trial. Technical support by the Mechanobiology Institute (MBI) and Singapore-MIT Alliance for Research and Technology (SMART) Centre (BioSyM IRG) are gratefully acknowledged. We also appreciate the help provided by NTU’s Micro-Machine Center (MMC) facilities for wafer fabrication and the lab facilities at the Nano Biomechanics Laboratory, National University of Singapore (NUS). Clinical samples and data collection were supported by the Singapore National Medical Research Council grant NMRC 1225/2009.

**Author Contributions**

Conceived and designed the experiments: BLK MEW DSWT JH CTL. Performed the experiments: BLK MEW DI. Analyzed the data: BLK MEW DSWT CTL. Contributed reagents/materials/analysis tools: DSWT CTL. Wrote the paper: BLK MEW DSWT JH CTL.

**References**

1. Pantel K, Brakenhoff RH, Brandt B (2008) Detection, clinical relevance and specific biological properties of disseminating tumour cells. Nature Reviews Cancer 8: 329–340.
2. Majid EW, Lim CT (2013) Microfluidic Platforms for Human Disease Cell Mechanics Studies. Materiomics: Multiscale Mechanics of Biological Materials and Structures. Springer, pp. 107–119.
3. He W, Wang H, Hartmann LC, Cheng JG, Low PS (2007) In vivo quantitation of rare circulating tumor cells by multiphoton intravalve flow cytometry. Proceedings of the National Academy of Sciences 104: 11760.
4. Allan AL, Vantyghem SA, Tuck AB, Chambers AF, Chin-Yee IH, et al. (2005) Detection and quantification of circulating tumor cells in mouse models of human breast cancer using immunomagnetic enrichment and multiparameter flow cytometry. Cytometry Part A 65: 4–14.
5. Gertler R, Rosenberg R, Fuehrer K, Dahm M, Nekarda H, et al. (2003) Detection of circulating tumor cells in blood using an optimized density gradient centrifugation. Recent Results in Cancer Research 162: 149–156.
6. Tan SJ, Lakshmi RL, Chen P, Lim WT-Y, Yobas L, et al. (2010) Versatile label free biochip for the detection of circulating tumor cells from peripheral blood in cancer patients. Biosensors and Bioelectronics 26: 1701–1705.
7. Warkiani ME, Bhart AAS, Khoo BL, Han J, Lim CT, et al. (2013) Isoporous micro/Nanoeengineered membranes. ACS nano 7: 1882–1904.
8. Zheng S, Lin HK, Lu B, Williams A, Datur R, et al. (2011) 3D microfluidic device for viable circulating tumor cell (CTC) enrichment from blood. Biomedical microdevices 13: 207–213.
9. Paris PL, Kobayashi Y, Zhao Q, Zeng W, Sritharan S, et al. (2009) Functional phenotyping and genotyping of circulating tumor cells from patients with castration resistant prostate cancer. Cancer letters 277: 164–173.
10. Nagrath S, Sequist LV, Maheswaran S, Bell DW, Irimia D, et al. (2007) Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature 450: 1235–1239.
11. Alix-Panabières C, Schwaarzbech H, Pantel K (2012) Circulating tumour cells and circulating tumor DNA. Annual review of medicine 63: 199–215.
12. Thiery JP (2002) Epithelial-mesenchymal transitions in tumour progression. Nature Reviews Cancer 2: 442–454.
13. De Matteo-Arreda L, Cortes J, Santapria L, Vivancos A, Tabernero J, et al. (2013) Circulating tumour cells and cell-free DNA as tools for managing breast cancer. Nature Reviews Clinical Oncology 10: 377–389.
14. Hou HW, Warkiani ME, Khoo BL, Li ZR, Soo RA, et al. (2013) Isolation and retrieval of circulating tumor cells using centrifugal forces. Scientific Reports 3:1259.
15. Guan G, Warkiani ME, Luan KB, Lim CT, Chen PC, et al. (2013) High throughput circulating tumor cell isolation using trapezoidal inertial microfluidics. Proceedings of 17th International Conference on Miniaturized Systems for Chemistry and Life Sciences, Freiberg, Germany: 23–25.
16. Ding C, Chau RW, Lau TK, Leung TN, Chan LC, et al. (2004) MS analysis of single-nucleotide differences in circulating nucleic acids: application to noninvasive prenatal diagnosis. Proceedings of the National Academy of Sciences of the United States of America 101: 10762–10767.
17. Parker WT, Lawrence RM, Ho M, Irwin DL, Scott HS, et al. (2011) Sensitive detection of BCR-ABL1 mutations in patients with chronic myeloid leukemia after imatinib resistance is predictive of outcome during subsequent therapy. Journal of Clinical Oncology 29: 4250–4259.
18. Yip PY, Yu B, Cooper WA, Selinger CI, Ng CC, et al. (2013) Patterns of DNA mutations and ALK rearrangement in resected node negative lung adenocarcinoma. Journal of Thoracic Oncology 8: 408–414.
19. Majid EW, Guan G, Khoo BL, Lee WC, Bhagat AAS, et al. (2013) Slanted spiral microfluidics for the ultra-fast, label-free isolation of circulating tumor cells. Lab Chip.
20. Khoo B, Warkiani M, Guan G, Tan DS-W, Lim AS, et al. (2014) Ultra-High Throughput Enrichment of Viable Circulating Tumor Cells. Springer, pp. 1–4.
21. Stott SL, Hsu C-H, Tsukrov DI, Yu M, Miyamoto DT, et al. (2010) Isolation of circulating tumor cells using a microvortex-generating hemoringe-chip. Proceedings of the National Academy of Sciences 107: 10392–10397.
22. Thiery JP, Lin CT (2013) Tumor dissemination: an EMT affair. Cancer cell 23: 272–273.
23. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. Proceedings of the National Academy of Sciences 100: 3983–3988.
24. Farace F, Massard C, Vinuol N, Drusch F, Jacques N, et al. (2011) A direct comparison of CellSearch and ISET for circulating-tumor-cell detection in patients with metastatic carcinomas. British journal of cancer 103: 475–473.
25. Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, et al. (2007) Identification of the transforming EML4–ALK fusion gene in non-small-cell lung cancer. Nature 448: 561–566.
26. Koivunen JP, Mermel C, Zejnullahu K, Murphy C, Lifshits E, et al. (2008) Sensitive and rapid detection of circulating tumor cells and cell-free DNA as tools for non-invasive prenatal diagnosis. Proceedings of the National Academy of Sciences 107: 18392–18397.
27. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, et al. (2004) Circulating tumor cells, disease progression, and survival in metastatic breast cancer. New England Journal of Medicine 351: 781–791.
28. Jin C, McEfail SM, Duffy SP, Dong X, Tavassol P, et al. (2013) Technologies for Label-free Separation of Circulating Tumor Cells: From Historical Foundations to Recent Developments. Lab Chip.