CIRCULATING TUMOR CELL (CTC) ENRICHMENT: ULTRA HIGH THROUGHPUT PROCESSING OF CLINICALLY RELEVANT BLOOD VOLUMES USING A MULTIPLEXED SPIRAL BIOCHIP

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ABSTRACT

Detection and characterization of circulating tumor cells (CTCs) from minimally invasive "liquid biopsy" provides critical insights into tumor biology and is critical for companion diagnostics and care. Here, we present a multiplexed spiral biochip for ultra-high throughput isolation of CTCs using inertial microfluidics to realize a single step label-free enrichment process. This device is capable of efficient cell separation of clinically relevant blood volumes in a short period of time (7.5 mL blood in 35 min). CTCs were successfully detected and isolated from 100% (50/50) blood samples collected from patients with advanced stage metastatic breast and lung cancer. They were identified under immunofluorescence assays (cytokeratin positive), as well as molecular probes (EGFR or HER2 positive). CTC recovery rate ranges from 3-1535 CTCs/mL and obtained under high purity (1 CTC for every 30-100 white blood cells detected). Retrieved cells are unlabelled and hence more viable for propagation, drug development and other downstream analysis.

KEYWORDS: Circulating tumor cells, Clinical diagnostics, Inertial microfluidics, Cancer, Molecular probes

INTRODUCTION

Circulating tumor cells (CTCs) have been defined as cancer cells of solid tumor origin that can shed into the blood stream from either primary or secondary tumors of patients, leading to haematogenous metastatic spread and subsequent growth of tumour cells at distant sites within the body [1]. Microfluidic cell sorting devices in various forms have recently been adopted for circulating tumor cell (CTC) based diagnostics in cancer research. However, the limited sensitivity and throughput of the current platforms (e.g., affinity-based methods), combined with the complexity and heterogeneity of the disease, has restricted their usability [2]. In addition, the immunomagnetic isolation approaches involve chemical and mechanical manipulation of the cells, rendering them non-viable or challenging for downstream culture and molecular analysis of collected CTCs. Herein, we report the development of a multiplexed spiral biochip for ultra-high throughput isolation of CTCs to address the challenges of the next generation CTCs enrichment platform such as high sensitivity (near 100% detection rate), simplicity, high throughput (7.5 mL blood in 35 min) and label-free enrichment, representing a significant increase in blood processing rate from our previous demonstration [3]. Taking advantage of the Dean vortex flows present in curvilinear microchannels, the CTCs can be focused near the microchannel inner wall while driving the smaller hematologic cells (Platelets, WBCs) toward the microchannel outer wall, allowing an efficient separation at the outlet.

WORKING PRINCIPLE

When particles of different size flow inside a curvilinear microchannel, neutrally buoyant particles under the influence of inertial lift forces travel across the streamlines to an equilibrium position towards the channel walls. Simultaneously, they also experience a drag force due to the presence of transverse Dean flows, driving them along the direction of flow within the vortices [3,4]. Since both forces are a function of particle size, particles of different size occupy distinct lateral positions inside the microchannel, allowing size-based separation. By selecting the appropriate channel dimensions and also optimum flow rates, larger CTCs undergo inertial focusing and remain near the inner wall while smaller hematologic cells migrate toward the outer wall under influence of the Dean drag force. This allows continuous collection of CTCs at the inner outlet and blood cells (Platelets, WBCs) are removed from the outer outlet as waste (see Fig. 1A).

EXPERIMENTAL PROCEDURES

The microfluidic biochips were fabricated using standard soft-lithography techniques in polydimethylsiloxane (PDMS) as described elsewhere [4]. After stacking three separate devices together using manual alignment and oxygen plasma bonding, the fluidic inlets and outlets were punched inside the assembly (1.5 mm holes) and final device obtained by bonding the whole assembly to a microscopic glass slide using an air plasma machine. In all the experiments, the multiplexed biochip were initially mounted on an inverted microscope (Olympus IX71) equipped with a high speed CCD cam-

era (Phantom v9, Vision Research Inc., USA). High speed videos were captured at the channel outlet using Phantom camera control software and then analyzed using ImageJ® software. Immunofluorescence staining using standard markers (CK, CD45 and DAPI) for cancer cells and white blood cells (WBCs) was used for differentiation and quantification.
Human whole blood samples were obtained from healthy donors and metastatic lung and breast cancer patients. This study was approved by our institutional review board and local ethics committee according to a protocol permitted by the Institutional Review Board (IRB). Fluorescence in situ hybridization (FISH) was performed on HER2/neu positive SKBR3 and negative MDA-MB-231 cells lines as well as isolated CTCs according to the manufacturer's protocol.

RESULTS AND DISCUSSION

To reduce the cellular components flowing in the spiral biochip, we employed a conventional RBC lysis technique (i.e., using ammonium chloride) in order to process larger volume of clinical samples. The lysis process did not compromise the recovery and isolation of cancer cells significantly (data not shown). While white blood cells (WBCs) constitute just 1% of total blood volume fraction, it is still challenging to separate minute quantities of CTCs from them efficiently. Extensive characterization of the proposed methodology was carried out to study the depletion capability of WBCs in the multiplexed spiral biochip. To demonstrate the impact of input sample cell concentration on the device performance and final purity, we carried out the processing of blood under different nucleated cell concentrations. Initial 7.5 mL whole blood collected from healthy donors had the RBC lysed and the nucleated cell fraction was then spun down and resuspended back to 7.5 ml (1x concentration), 3.75 mL (2x concentration) and 2.5 mL (3x concentration) respectively. Figure 1B shows the total cells count collected from the CTC outlet at different sample concentrations in 5 min. Since the total number of WBCs varies from one patient to another and with cancer type/stage [5], we decided to use 2x concentration (~ 14×10^6 WBCs) as optimal for future tests and clinical validation. This translates to a total processing time of 35 min for 7.5 mL blood sample using a single biochip. To test the performance of the spiral biochip for CTC isolation and recovery, we characterized the biochip with commercially available cancer cell lines. Using the spiral biochip, we demonstrate high recovery of breast (MCF-7) and bladder (T24) cancer cells spiked into healthy blood samples. Following enrichment, cancer cells were identified by immunofluorescence staining either by enumerating under epi-fluorescence microscope or by flow cytometry analysis with common surface markers (CK+/CD45-). For both cell lines spiked at clinically relevant concentrations of 500/7.5 mL of whole blood, a recovery of 87.6% for MCF-7 and 76.4% for T24 cells was achieved (Fig. 1C).

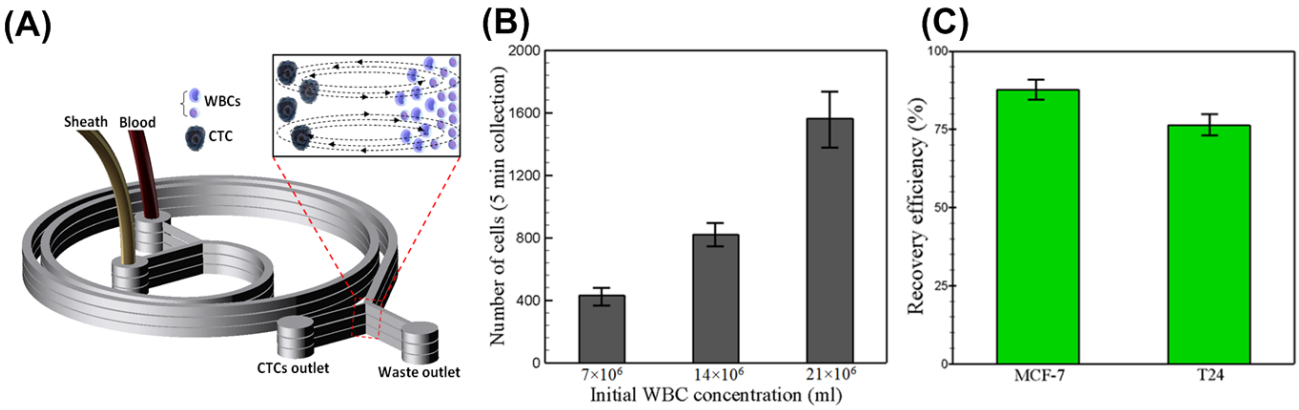

*Figure 1: **(A)** Schematic representation of the configuration and operational mechanism of a multiplexed spiral microfluidic chip for capturing circulating tumor cells with two inlets and two outlets, **(B)** different WBC concentration affects the total number of nucleated cells collected for the first 5 min of processing time (Anova (single factor), $P<0.05$) and **(C)** recovery of spiked cancer cells introduced at clinically relevant concentration (500 cells per 7.5 mL whole blood) (Anova (single factor), $P<0.05$).*

Using the optimal test parameters, 7.5 mL of blood samples from 10 healthy volunteers (control) and 25 patients with metastatic breast cancer and 25 patients with non-small cell lung cancer (NSCLC) were processed. CTCs were successfully detected and isolated from 100% (50/50) of blood samples under high purity (1 CTC for every 30 WBCs detected). They were identified with immunofluorescence staining using standard probes (pan-cytokeratin+/CD45-), molecular probes such as HER2 (Fig. 2A, 2B), as well as sequencing for known mutations such as EGFR. We could successfully identify EGFR activating mutation in CTCs from 5 patients and also in matched free plasma DNA as well as tumor biopsy of the same patients (Fig. 2C). The fast processing time, ability to collect more CTCs from larger patient blood volume, and label-free nature of the spiral chip lends itself to a broad range of potential genomic and transcriptomic applications. The incessant collection of enriched CTCs and short residence time in the microchannels (<10 msec) eliminates the long-shear exposure to the CTCs, therefore minimizing any undesirable shear-induced phenotype changes.

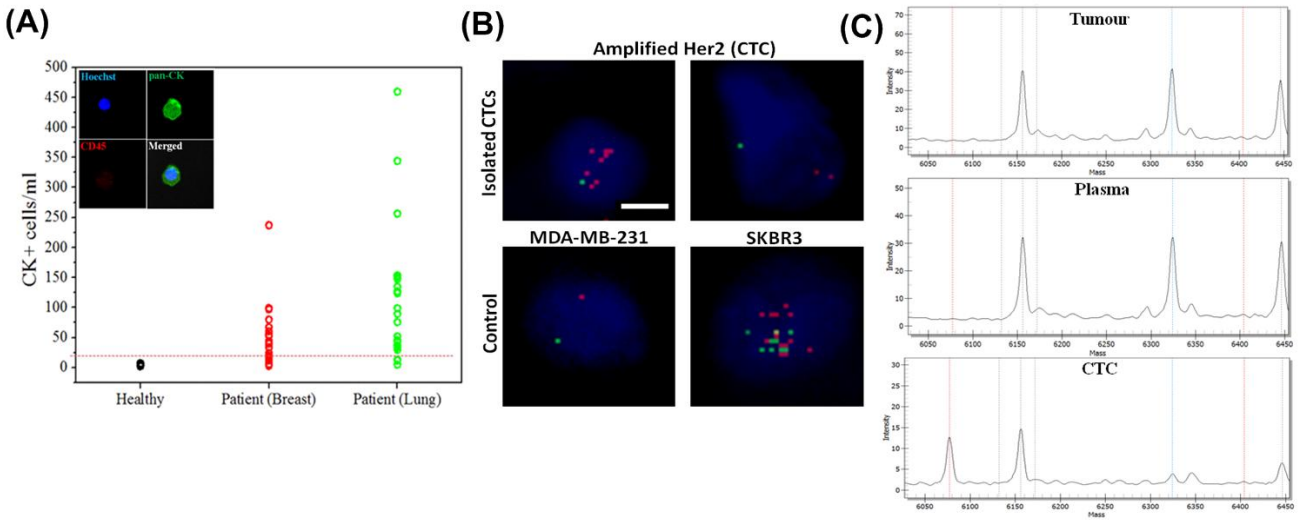

Figure 2: **(A)** *CTCs enumeration plot for healthy donors (black), breast cancer patients (bred) and lung cancer patients (green). (Inset) Representative image (60X magnification) of isolated CTCs,* ***(B)*** *FISH analysis for detection of HER2 gene on the enriched CTC from patient's blood. Two breast cancer cell lines (SKBR3 and MDA-MB-231) were used as a control, and* ***(C)*** *tracings of EGFR nucleotide sequencing from the tumor, DNA free plasma and circulating tumor cells.*

CONCLUSIONS

In this work, we demonstrated label-free size-based inertial separation of cancer cells from lysed blood using an ultra high-throughput multiplexed spiral microfluidic device. This approach utilizes the combined effect of inertial and Dean drag force to separate rare cells from large volume of blood samples rapidly and efficiently. The fast processing time of the technique critically enables even the time-sensitive molecular assays (e.g., RNA analysis), and allows the collection of more CTCs (by processing larger blood volume), as well as plasma and WBCs, from the same patient's blood. Given the current scientific uncertainty of CTC's role in cancer pathophysiology, the flexibility enabled by our CTC enrichment technique would be critical in advancing the CTC-based cancer diagnostics. We will report on the various downstream cellular and molecular assays from the primary CTCs enabled by this technique.

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