High-Throughput Single Cell Protease Analysis on Human Circulating Tumor Cells

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ABSTRACT
Detection of circulating tumor cells (CTCs) from cancer patients’ blood provides a convenient method for early-stage cancer diagnosis and to monitor disease progression. Many studies have confirmed that tumor consists of a heterogeneous cell composition. Only a small portion of CTCs can persist within the blood circulatory system. Residing CTCs may then extravasate to form a secondary tumor, mediated by the secretion of matrix metalloproteinases (MMP). Here, we present an integrated microfluidic approach for MMP activity measurement on sorted single CTCs. The assay results could contribute to identify CTC subpopulations of different metastatic potential and provide insights into cancer heterogeneity.

KEYWORDS: Single cell analysis, Matrix metalloproteinases functional assay, Circulating tumor cell

INTRODUCTION
Cancer initially arises as a local organ lesion, but can spread to distant organs as circulating tumor cells (CTCs) via the bloodstream. This process is known as metastasis and is responsible for approximately 90% of cancer related deaths [1]. Many studies have confirmed tumor cell population consists of a heterogeneous composition [2]. Such cell-cell variation renders any single-target treatment strategies ineffective. Microfluidic single-cell analysis technology has recently been adopted for cancer research to understand the inherent heterogeneity. However, the limited sensitivity and throughput of current platforms (e.g. micro-chamber methods) combined with system complexity, has restricted their clinical applications [3]. In addition, low CTC cell count also challenges the effectiveness of single cell study. Herein, we report the development of a microfluidic approach combining droplet-based single-cell encapsulation and high-throughput quantification of metalloproteinases (MMPs) expression to enable analysis on CTCs at the single-cell level. MMP activities are considered necessary for metastasis in order to degrade extracellular matrix (ECM) during invasation and extravasation. Sensitive detection of MMP activity in single CTCs may reveal its heterogeneity and be used as an indicator of the invasiveness of individual CTCs.

WORKING PRINCIPLE
CTCs are obtained with microwell-based cultures of red blood cell (RBC) lysed blood from cancer patients. Cultured CTCs are harvested after two weeks in culture for the single-cell MMP assay [4]. CTC suspension is pinched by fluorocarbon oil to form pico-liter droplets under the influence of viscous friction force and surface tension in a cross-junction microchannel [5]. By encapsulating various fluorescent reagents with cell solution, different cell assays can be performed inside droplets in a single run. This allows high-throughput single-cell analysis by monitoring the fluorescent signals from cell-containing droplets as shown in Fig. 1.

EXPERIMENTAL PROCEDURES
The microwell device for CTC culture was fabricated on uncoated petri dishes using a laser engraving/cutting system (Universal Laser System Inc) [4] and the droplet device for single CTC analysis...
was fabricated using standard soft-lithography in polydimethylsiloxane (PDMS) [5]. Nucleated cells extracted from cancer patients’ blood after RBC lysis were cultured in the microwells under hypoxia [4]. This study was approved by our institutional review board and local ethics committee (DSRB Reference 2012/00105, 2012/00979, 2010/00270, 2010/00691). All patients gave their informed consent for inclusion in this study. Cancer cell lines such as MDA-MB-231 were used for system characterization. They are cultured using normal culturing method described elsewhere [5]. In the assay experiment, the cell encapsulation device was mounted on an inverted microscope equipped with a high-speed camera (Phantom). The fluorescent detection was conducted on an epi-fluorescent microscope with an EMCCD camera (Andor). ImageJ software was used to analyze high-speed videos and fluorescent images. MMP activities were assayed using FRET-based substrate Pepdab010 (Biozyme). The MMP signals were calculated by subtracting the average intensity of empty droplets from the intensity of cell-containing droplets. CD45 antibodies (MACS) and cytokeratin antibodies (MACS) were used to differentiate residual WBCs from the cultured CTC population. Hoechst nuclei staining (Life technology) and live/dead staining (Life technology) were used to locate cells in the droplets and monitor cell viability.

**RESULTS AND DISCUSSION**

The amount of MMPs produced by each single viable cancer cell is small. To accumulate enough MMPs for detection, we encapsulated cancer cells into droplets with a volume of 100 pico-liter. MMPs released by cancer cells were stored inside individual droplets and accumulated to detectable concentrations over a period of time [5]. Under room temperature condition, above 80% of cancer cells were alive for two hours based on viability test on cultured cancer cell lines. We used MDA-MB-231 to verify the assay detection sensitivity. In order to suppress the fluorescent signals from empty droplets, cultured cancer cells were starved in serum-free medium overnight prior to MMP assay. The fluorescent signal of cell-containing and empty droplets were monitored over one hour. As shown in Fig. 2(a), cell-containing droplets demonstrated an average increase on fluorescent intensity of 150 a.u. and empty droplets demonstrated 20 a.u. increase over 1 hour. Therefore, the incubation time of subsequent single-cell MMP assay was set to 1 hour. During the experiments, we observed false signals from dead cells caused by the reaction between MMP substrate and intracellular proteases released from damaged cell membrane. The dead cells were identified with a live-dead staining and their signals were excluded from analysis.

Using the optimal assay parameters, two cancer cell lines (MDA-MB-231, MCF-7) and a breast epithelial cell line (MCF-10A) were studied at the single-cell level. Based on the results shown in Fig. 2(b), we can easily observe the inherent cell heterogeneity of cultured cell lines. Even though the cells origi-
nated from the same source and were cultured under the same condition, their phenotype in terms of MMP activity still differs from each other. Interestingly, we also found that the MMP activity of metastatic MDA-MB-231 cells is significantly higher than the less invasive MCF-7 cells and breast epithelial MCF-10A cells. This concurs with previous findings that high MMP expression could correlate with cancer invasiveness [2]. Next, we performed single-cell MMP assay on cultured CTCs from a breast cancer patient. CD45 antibodies was used to distinguish the residual WBCs in culture from CTCs, as WBCs may also secrete MMPs. Only MMP signals from CD45 negative cells were considered as CTC MMP results (Fig. 2(c)). Similarly to cancer cell lines, cultured CTCs also display a diverse MMP activity level, revealing the functional heterogeneity among the clinical CTC samples.

**CONCLUSIONS**

In this work, we demonstrated MMP functional assay on cultured CTC samples using a high-throughput droplet microfluidic device. The approach is capable of quantifying MMP activities of single viable CTCs suspended in water-in-oil droplet system rapidly and efficiently. The high sensitivity of the technique enables the detection of distinct MMP activities among various cancer cell lines and cultured CTCs. It has been observed that highly metastatic cancer cells have more intensive MMP activities comparing with less invasive cells. The single-cell MMP assay on cultured CTCs could shed insights on the functional heterogeneity among clinical CTC samples and guide development of personalized cancer treatment strategies.

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**REFERENCES**


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