Microfluidic Analysis for Separating and Measuring the Deformability of Cancer Cell Subpopulations

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ABSTRACT: Increased deformability and softness endow tumor cells with highly invasive and metastatic capabilities. We exploited these characteristics to fabricate a high-throughput microfluidic device to measure cell deformability and separate cancer cells. Driven by hydrodynamic forces, the cells with better deformability passed through the chip faster, whereas stiffer cells passed through the device over a longer time period. The MDA-MB-231 and MCF-7 cell lines were used to evaluate the device because their metastatic potentials were known. We found that MDA-MB-231 cells, which were softer and exhibited stronger deformability, passed through the device more quickly. HeLa cells were also successfully separated into softer and stiffer subpopulations, whose distinct mechanical properties were confirmed by atomic force microscopy. We also measured the expression of metastasis-associated proteins (epidermal growth factor receptor and integrin β1) and found that subpopulations with varied deformabilities had different expression levels. Our results suggested that this high-throughput microfluidic device could be used to screen and evaluate the curative effects of drug and cancer progression by simultaneously testing cell deformability and expression levels of metastasis-associated proteins in separated cell subpopulations.

1. INTRODUCTION

Metastasis is the defining feature of cancer that involves the spread of tumor cells from a primary site to secondary tissues or organs and presents the main cause of cancer deaths.1,2 In addition to genetic and external environmental factors, metastasis is partially dependent on the mechanical properties of cancer cells.3 Successfully surviving and moving in the harsh and changing environments of the vasculature, lymphatic vessels, and stromal space requires that malignant tumor cells possess appropriately softer mechanical properties.4 These properties are determined by biophysical measurements, and cellular softness correlates with greater metastatic probability.5,6 It is suggested that mechanical properties can be used as diagnostic markers of the metastatic potential of cancer cells.6,7

The mechanical properties of cells can be estimated by evaluating their elasticity and viscosity using approaches such as micropipette aspiration,8,9 atomic force microscopy (AFM),10,11 optical tweezers,12,13 and so on. AFM and optical tweezer measurements are very accurate and provide quantitative data;11,12 however, they are extremely low throughput and require highly skilled operators. In micropipette aspiration, a cell is aspirated into a micropipette under negative pressure.8 This approach provides fairly accurate results with higher throughput than the two previously mentioned techniques, but it is still very slow, with a maximum throughput of a few cells/h.8,9 Microfluidic devices with microchannels are used to estimate cellular mechanical properties by measuring migration time and the entry velocity of single cells into a microchannel.14–21 This technology increases throughput but generally cannot distinguish and separate cell subpopulations with varied mechanical properties for further assays.

Some researchers have suggested that optimal mechanical properties for invasion and intravasation are inheritable cellular characteristics.17,22 This theory suggests that molecular targets could be used to track such malignant features. Mechanical signals act as mechanoreceptors and mediate mechanotransduction by transferring forces to specialized anchoring structures.23 For example, high integrin β1 levels activate epithelial-to-mesenchymal transition and increase invasive ability.23 High expression of epidermal growth factor receptor (EGFR) is also required for cell migration24 and survival.25 Greater EGFR expression on the surface of cancer cells is associated with the degree of tumor deterioration,26 but no studies have examined the association with EGFR expression.

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and mechanical properties due to cell sorting limitations. EGFR and integrin $\beta$1 expression levels are useful references to evaluate malignancy and cancer progression. In this study, we designed and developed a microfluidic device with microbarriers to separate and enrich cells with variable mechanical properties and used fluorescence signals to simultaneously detect EGFR and integrin expression in the separated subpopulations of cancer cells.

2. RESULTS AND DISCUSSION

2.1. Chip Design and Operation. The chip microstructures and fluidic parameters of the device are both essential to separate cancer cells. The device’s most notable feature is the increasingly sized placement of gaps to impede or allow the passage of cells with variable mechanical properties. Researchers suggested that the minimum passable blood capillary barrier for cancer cells in vivo is approximately 8 $\mu$m.27,28 We took this value into account when designing the chip to separate cells. Following the direction of fluid flow, the gaps decreased from 15 to 7 $\mu$m, allowing continuous analysis efficiency (Figure 1).

The chip was patterned and fabricated by mechanical microfabrication. A Tygon tube was connected to the chip to allow cells to be injected into the chip. A microinjection pump was used to control the flow rate at 1 mL/h. Chip-separated cells could be imaged by microscopy. The cells that flowed from the chip were collected and then subjected to protein expression analysis and immunofluorescence staining assays, and cells blocked in the chip were subjected to immunofluorescence staining assays.

The device employs hydrodynamic force to define cancer cell mechanical properties (Figure 1). The gap size distribution from first to ninth arrays ranges from 15 to 7 $\mu$m, respectively (Figure 1A). The diameter of the middle blocking column is 15 $\mu$m (Figure 1B). Each array region consists of 100 rows of blocking columns. Cell flow through these gaps requires continuous movement and deformation (Figure 1C).

2.2. On-Chip Cell Analysis. The chip was used to separate and analyze the mechanical properties of two breast cancer cell lines: MDA-MB-231 and MCF-7. The mechanical properties of these lines have been quantified and confirmed by optical deformability assays in previous studies.10,25 MDA-MB-231 is a mesenchymal-like breast cancer cell line with greater softness, whereas the epithelium-like MCF-7 line is stiffer. Cell suspensions at the density of $1 \times 10^5$ cells/mL were prepared and injected into the chip, and the numbers of retained cells in each array were counted. From the first (15 $\mu$m gap) to the fifth array (11 $\mu$m gap), more MCF-7 cells were blocked than MDA-MB-231 cells. From the sixth (10 $\mu$m gap) to the ninth array (7 $\mu$m gap), MDA-MB-231 cells were trapped but in lower numbers compared with MCF-7 cells (Table 1). The statistical data for each gap array revealed a greater blocking rate as the gap diameter decreased, confirming that each array trapped cellular subpopulations with different mechanical properties.

Trapped cells were imaged by fluorescence microscopy (Figure 2). In the 15 $\mu$m array (7 $\mu$m gap) of the chip, more MCF-7 cells were trapped than MDA-MB-231 cells. Immunofluorescent MCF-7 and MDA-MB-231 cells present diverse morphologies, which implies that the device successfully separates cell populations with different mechanical properties.

2.3. Young’s Modulus Measurement by AFM. More MCF-7 cells were blocked in the chip, whereas more MDA-MB-231 cells flowed through (Table 2). This result indicated that MDA-MB-231 cells were softer, with higher deformability.

Stiffness is quantitatively evaluated using Young’s modulus or the elastic modulus. AFM was used to measure Young’s modulus of MCF-7 and MDA-MB-231 cells. Considering the

![Figure 1](image1.png) Pattern of the designed separation chip. (A) Overall scheme of the separation chip with the gaps ranging in size from 15 to 7 $\mu$m. (B) Detailed scheme of the separation chip. (C) Pattern of cell flow in a chip, resulting in continuous cell movement and deformation in this device.

![Figure 2](image2.png) Retained cells in different arrays in the chip. The left line is the image of MCF-7 cells in 12 and 7 $\mu$m arrays, and the right image is the MDA-MB-231 cells. The scale bar is 100 $\mu$m.

| Table 1. Retained Rates ± Standard Error (SE, %) of Two Cells in Each Gap Array |
|-----------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| gaps ($\mu$m)   | 15    | 14    | 13    | 12    | 11    | 10    | 9     | 8     | 7     |
| MCF-7           |       |       |       |       |       |       |       |       |       |
| Retained Rate   | 0     | 0     | 0.1 ± 0.04 | 0.1 ± 0.01 | 0.2 ± 0.01 | 0.1 ± 0.01 | 0.3 ± 0.03 | 24 ± 2.31 | 42 ± 2.19 |
| MDA-MB-231      |       |       | 0     | 0     | 0.1 ± 0.04 | 0     | 0.2 ± 0.04 | 0.1 ± 0.05 | 16 ± 2.65 | 33 ± 2.08 |
thickness of the attached cells, we calculated Young’s modulus at a 500 nm indentation depth to decrease disturbance at the bottom of the dish (adherent cell height is \( \sim 1.5 \mu m \)). The average value of Young’s modulus for MDA-MB-231 cells was significantly lower than that of MCF-7 cells \( ^{10} \) (Figure 3).

**2.4. Separated Cell Subpopulation Analyses.** The HeLa cell line was used to explore device separation of cancer cell subpopulations based on mechanical properties. Five minutes after injection, HeLa cells trapped in the ninth array were imaged (Figure 4). The red arrow indicates a stiffer cell with limited deformability, whereas the green arrow points to a more flexible dumbbell-shaped cell with relatively higher deformability allowing it to pass through the gap. The results show that cell subpopulations with variable mechanical properties can be separated in the different chip arrays.

The mechanical properties of HeLa cells that pass through the chip at different times can be estimated by AFM with spherical probes. Cells that passed through the chip after 5 and 30 min were collected, whereas those not separated by the chip were used as a control (labeled as N). Compared with the control group, we observed statistically significant reductions of Young’s modulus (N group: 0.257 ± 0.05 kPa, \(^{30} \) 30 min group: 0.227 ± 0.01 kPa, 5 min group: 0.209 ± 0.05 kPa, \( p < 0.01 \); Figure 5). The results confirmed that cells that passed through the chip after 5 min were the softest among the three groups. The cells in the 30 min group were obviously softer than cells that were not separated by the chip.

**2.5. Protein Expression of Cell Subpopulations.** To further explore whether EGFR and integrin expression levels correlated with deformability, we performed flow cytometry and western blotting to detect metastatic-associated proteins in separated subpopulations (Figure 6). Although flow cytometry testing requires a large supply of cells, we still collected the cells that passed through the chip during different time points (normal culture and passed through the chip for 5 and 30 min) to test and verify the accuracy of this microfluidic chip for the detection and separation of cell subpopulations of varied deformability. EGFR and integrin expression levels measured by flow cytometry are shown in Figure 6AB. From the results, we found that HeLa cells that passed through the chip within 5 min had the highest integrin \( \beta 1 \) levels among the three groups (Figure 6A). HeLa cells that passed through the chip within 5 min had the highest EGFR levels among the three groups; however, numerical comparison revealed that there are no significant differences. This result may be related to finding the small cell numbers in every sample passing through the device (Figure 6B). To further verify the accuracy of the results, we collected subgroups of cells through repeated experiments to accumulate enough cells for western blotting. The results showed that the protein expression of each group exhibited significant differences. Western blotting confirmed the flow cytometry results for integrin (Figure 6C), and EGFR (Figure 6D) showed significantly higher expression in the 5 min group. Notably, integrin \( \beta 1 \) and EGFR expressions are highly relevant

**Table 2. Proportions of MCF-7 and MDA-MB-231 Cells Passing through the Chip**

| Cells          | Passing Rate (%) | SE  |
|----------------|------------------|-----|
| MCF-7          | 4.31             | 0.32|
| MDA-MB-231     | 8.83             | 1.47|

Figure 3. Young’s modulus of MCF-7 and MDA-MB-231 cells that passed through the chips. * means \( p < 0.05 \).

Figure 4. Magnified field showing the deformed cells in the gap of 8 \( \mu m \). The red arrow points to a stiff cell with limited deformability trapped in the chip. The green arrow points to a malleable cell with good deformability.

Figure 5. Young’s modulus of HeLa cells. One hundred curves were acquired, including 50 cells randomly for each group. The statistical significance values are from comparison with the control group. ** means \( p < 0.01 \) and * \( p < 0.05 \).

Figure 6. Expression of cell-migration-related and overexpressed proteins. (A, B) Flow cytometry and (C, D) western blotting assays to measure the expression of integrin \( \beta 1 \) and EGFR in cells in normal culture and passed through the chip for 5 and 30 min.
to mechanical properties. Cell subpopulations with relatively higher EGFR and integrin β1 expression were softer and more deformable, enabling them to pass through the chip more quickly.

### 2.6. Cell Subpopulation Analysis in One Chip.

Gold cluster probe-based assays have been applied for protein quantitative analyses. The peptide YHWYGTPQNYI is a specific target sequence for EGFR. Compared with fluorescent dye analysis, the fluorescence of the gold cluster was less likely to quench; thus, the analysis accuracy using fluorescence intensity is improved. In the present work, a gold cluster probe was used to target EGFR expressed on trapped HeLa cells in different arrays on the chip. Cells blocked in the chips were labeled with the probe and then fixed. Forty images in each array (from the sixth to the ninth) were collected by inverted fluorescence microscopy for statistical analyses. Figure 7A shows the merged bright-field and fluorescence images, and Figure 7B depicts the corresponding fluorescence intensity histogram. Greater EGFR expression corresponded with enhanced fluorescence intensity (normalized to the substrate), and the signal change was imaged and analyzed. The images are displayed by MATLAB software as data cube histograms (Figure 7B). The highest fluorescence intensity value for cells in the ninth array was higher than that in the other arrays. The statistical analysis also revealed that the cell subpopulation in the ninth array had higher EGFR expression than those in the sixth to eighth arrays. The relatively high EGFR expression was confirmed to correspond with cell deformability. This finding demonstrates that we successfully separated subpopulations of HeLa cells with varied mechanical properties and confirmed that EGFR expression correlated with cell deformability.

### 3. CONCLUSIONS

We designed and fabricated a novel microfluidic device to exploit unique mechanical properties to separate and analyze cancer cell subpopulations. The distinct mechanical properties and expression of metastasis-associated proteins in these cell subpopulations were verified by AFM analysis, flow cytometry, and western blotting assays. These results provide support for using the chip as a screening or diagnostic tool to explore connections between cancer progression and mechanical properties of cell subpopulations by testing cancer cell deformability. The device may be used as a supplement for clinical high-throughput detection and could be integrated with other analyses to identify cells associated with invasion, metastasis, and cancer progression.

### 4. MATERIALS AND METHODS

#### 4.1. Cell Culture.

A human cervical cancer cell line (HeLa cells) and breast cancer cell lines (MDA-MB-231 and MCF-7) were purchased from the Cell Bank of Xiehe. HeLa cells were cultured in Dulbecco’s modified Eagle’s medium with high glucose (HyClone Laboratories, Logan, UT) and 10% fetal bovine serum (FBS, HyClone Laboratories) and then incubated at 37 °C in a humidified incubator containing 5% wt/vol CO2. The cell density was determined using a hemocytometer prior to any experiments.

#### 4.2. Microfluidic Chip Design and Fabrication.

The microchip was designed on glass slides. The width of the chip channel was 2 mm. The size limitation in the channel is achieved by the compact arrangement of small columns (the diameter is 15 μm). The gaps decreased from 15 to 7 μm from the left to right of the chip (see the gap size distribution in Figure 1: array 1, 15 μm; array 2, 14 μm; array 3, 13 μm; array 4, 12 μm; array 5, 11 μm; array 6, 10 μm; array 7, 9 μm; array 8, 8 μm; and array 9, 7 μm).

Microfluidic channel patterns and the corresponding master were designed and fabricated by mechanical microfabrication. Sylgard 184 PDMS (Dow Corning) precursor was mixed thoroughly with its curing agent (Dow Corning) at 10:1 (w/w) and degassed by vacuum for 30 min. The precursor mixture was cast against the silicon mold and polymerized at 65 °C for 3 h in a vacuum drying oven (Yiheng Scientific Instrument Co., LTD., Shanghai, China). The cured poly(dimethylsiloxane) replica was then gently peeled from the master, and the columnar inlet and outlet were manually drilled for each channel and reversibly sealed against a clean microscope cover glass to form a microfluidic chip. The liquids were pumped into the microfluidic chip via syringe pumps (Longer Inc., China).

#### 4.3. Sample Preparation.

To evaluate the device’s enrichment efficiency, HeLa cells were incubated and resuspended at a specific concentration in 1 mL of phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) to reduce the cell aggregation.

#### 4.4. Experimental Setup.

A syringe pump was used to inject the prepared sample continuously into the microchannel. The flow rate was initiated at 1 mL/h to observe the enrichment efficiency and to count the number of collected cells in the chip in the microscope. Then, to identify the cancer cells, the cells were stained with antibodies (EGFR and integrin β1, Abcam) before capture.
4.5. Flow Cytometry Analysis. Cells were suspended in PBS containing 2% FBS with antibody staining. Antibodies against integrin β1 and EGFR were diluted 1:100. Isotype control antibodies were used as the gating controls.

4.6. Protein Expression Analysis. Cells separated at different time points were suspended in PBS by scraping and were lysed by sodium dodecyl sulfate lysis buffer (Beyotime Biotechnology, China). The protein concentration was determined using the bicinchoninic acid protein assay kit (Beyotime Biotechnology, China). The separated proteins were electroblotted onto nitrocellulose transfer membranes (0.2 μm, Whatman, U.K.). The membranes were then blocked with 5% BSA in TBST (Tris-buffered saline and Tween 20) for 1 h and incubated with primary antibodies (mouse anti-EGFR antibody, 1:100 dilution, and mouse anti-integrin β1 antibody, 1:200 dilution, Abcam) at 4 °C overnight. The membranes were rinsed three times with TBST for 10 min and then incubated with secondary antibody (HRP-labeled goat anti-mouse IgG, dilution 1:2000, Beyotime Biotechnology). After washing three times with the TBST for 10 min, protein bands were visualized with the BeyoECL Plus kit (Beyotime Biotechnology). GAPDH was used as a loading control.

4.7. Statistical Analysis. All experimental permutations were duplicated, and independent experiments were repeated at least in triplicate. The data are presented as the mean ± standard error of three independent experiments. Student’s t-test was used to calculate the statistical significance. The asterisks * denote p values of less than 0.05 compared to untreated cells.

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Notes
The authors declare no competing financial interest.

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