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An Integrated Microfluidic System for Screening of Peptides Specific to Colon Cancer Cells and Colon Cancer Stem Cells Using the Phage Display Technology

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Abstract— **Reagents binding specifically to target molecules are essential tools for clinical diagnosis and targeted therapy. Screening of target cell-surface specific affinity reagents with bench-top methods has some drawbacks, including time-consuming, labor-intensive and requirement of large-scale instrument. Microfluidic platforms may overcome these drawbacks because they could automate and complete the screening process within a shorter period of time. Phage display is a promising technology in selection of cell-surface specific peptides. In recent years, a subpopulation of tumor cells named cancer stem cells is believed to be the tumorigenic cells and closely associated with metastasis. A specific peptide that can recognize and differentiate cancer stem cells from the rest of cancer cell population is therefore useful for early diagnosis and targeted therapy. In this study, selection of M13 phage displayed peptides that bind with colon cancer cells and colon cancer stem cells using an integrated microfluidic system was successfully demonstrated. Compared with the traditional methods, the total selection process was shortened to 36 hours while traditional method needs almost a month. More importantly, the screening process can be automated and performed on a single microfluidic chip. The developed technique may be promising for early diagnosis of cancer and target therapeutics.**

Keywords—microfluidics, colon cancer, cancer stem cell, phage display library, screening, biomarker

I. INTRODUCTION

Affinity reagents having high specificity are essential tools for diagnosis and targeted therapy. Generation of specific affinity reagents is therefore drawing significant attention nowadays. Several selection systems have been used to develop target-specific affinity reagents. Among them, phage display is one of the promising tools used for identification of target-specific peptides. Phage display technology, first developed in 1985, is based on the ability to express polypeptides as fusions to coat proteins on surface of phage [1]. Surface display of peptides has been achieved by inserting a random peptide encoding DNA sequence into the gene for coat proteins of phages. The technology was first successfully demonstrated using filamentous M13 phage [1]. The displayed peptides with a high affinity with the targeted biologically relevant molecules can be recovered together with the peptide encoding phage. Under a process called bio-panning, a high-affinity and specific ligand can be developed [2].

Traditionally, a selection experiment utilizing the phage display library is a relatively time-consuming and labor-intensive process. Furthermore, well-trained personnel and lab-scale instruments are needed for screening of specific peptides. Recently, microfluidic technology has been adapted for automating the entire screening process since it can be performed within a shorter period of time and it could control the stringency of each screening round [3]. Moreover, the traditional screening method suffers from a limitation that the amount of cell needed for the experiment is relatively large (10^5-10^7 cells). When the target is harvested from organs or tissues, it is almost impossible to get such a large amount of cells [4]. Screening of biomarkers on a microfluidic system may alleviate this issue [5].

Colorectal cancer (CRC) is one of the most common cancers worldwide and is one of the leading causes of morbidity. In 2008, more than 1.2 million new cancer cases and 600,000 deaths have occurred [6]. The research for more effective therapeutics for CRC is of great need to increase patients' survival rates [7]. Cancer stem cells (CSCs) are a rare subpopulation of cancer cells that can reconstitute a new tumor with the similar composition and phenotypes to the tumor of origin.

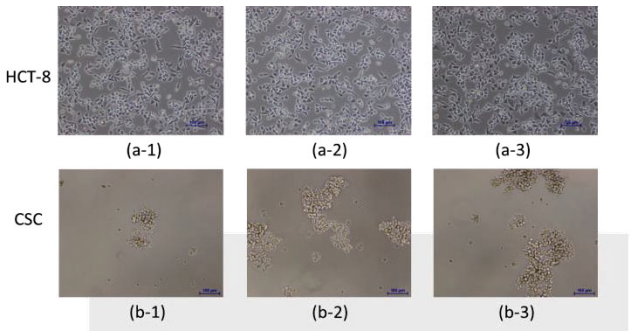

Figure 1. Photographs of colorectal cancer cells (HCT-8) and colon cancer stem cells.

CSCs are defined as having 3 critical properties: the capacity of self-renew indefinitely, the ability to recreate the

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full repertoire of cancer cells of the parent tumor and the expression of a distinctive set of surface biomarkers [8]. Furthermore, CSCs exhibited multiple drug resistance. A specific peptide to differentiate CSCs from the rest of cancer cell population is useful in early diagnosis and treatment of patients with CRC metastasis. This study therefore reports a new integrated microfluidic system for continuous selection of cancer cell-specific peptides. It can automate the biopanning process using M13 phage display peptide library. With these biomarkers specifically targeting CRCs and colon CSCs, it is possible to further apply these peptides in early diagnosis and drug delivery.

II. Materials and Methods

A. Experimental procedure

The phage display peptide library combined with an integrated microfluidic system was demonstrated in this study for screening cell-surface specific peptides. Negative and positive selection processes were performed alternately to screen peptides with higher specificity for CRCs and CSCs, respectively. The colon cancer cell used in this work was cell line HCT-8 and the colon CSC used was enrichment cultured from the HCT-8 cell line. Control cells and target cells were HCT-8 or CSC. Figure 1 shows photographs of the cells used in this study. Figures (a-1) to (a-3) were HCT-8 CRCs and Figures (b-1) to (b-3) were CSCs derived from HCT-8 CRCs.

The experimental procedures were as follows. First, 10 µl of M13 phage display peptide library (10^{11} pfu/ml, prepared in Tris-buffer saline, TBS) was pre-mixed with 200-µl control cells (HCT-8 or CSC, 10^5 cells/ml in phosphate buffer saline, PBS) grown on Epithelial Enrich magnetic beads (4×10^8/ml) and incubated for 10 min. A magnet was used to collect captured control cells (HCT-8 or CSC)/peptide complexes as negative selection. The supernatant contained phage binders of interest were then transported to an incubation chamber to perform subsequent positive selection. At the same time, magnetic beads coated with target cells (CSC or HCT-8) were added for 10-min incubation. After positive selection, a magnet was used to collect captured target cells (CSC or HCT-8)/peptide complexes. Two hundred microliters 1× PBS was then used to wash away the unbound phage binders for 5 times. The washing stringency (shear force) increased in each round by applying the washing vacuum pressure from -100 mmHg, -200 mmHg, -300 mmHg, -400 mmHg, -500 mmHg, to -600 mmHg. Finally, the collected magnetic bead complexes were added in *E. coli* K12 ER2738 for phage amplification. After five hours of amplification, the chamber was heated up to 65℃ for 15 min to remove *E. coli* residue. The amplified phage binders were then collected to perform next round of selection.

A schematic illustration of the operating principle of screening cell-surface specific peptides was shown in Figure 2. Note that the whole process was repeated six times and each time the stringency was increased to obtain high-specificity and high-affinity peptides. Table 1 lists the detailed experimental procedure performed on the integrated microfluidic chip and the time of each step.

B. Design and fabrication of the microfluidic chip

An integrated microfluidic chip was designed to perform the selection experiment mentioned above. Figure 3(a) shows a schematic illustration of the integrated microfluidic chip. It was made of two polydimethylsiloxane (PDMS) substrates (a thick PDMS layer and a thin PDMS membrane) and one piece of glass. An exploded view of the integrated microfluidic chip was shown in Figure 3(b) and a photograph of the chip in Figure 3(c). It could be used for performing samples/reagents delivering and incubating in screening process. In addition, a temperature control module using a thermoelectric (TE) cooler for controlling the temperature of the phage amplification process and the removal of *E. coli* residue process was developed. The dimensions of the developed chip were measured to be 6.2 cm (length) and 6.3 cm (width). The microfluidic chip was consisted of 3 identical incubation chambers, 2 identical wash buffer chambers, target cell inlets, a phage library inlet, a control cell inlet and 6 identical phage amplification chambers. Note that the temperature controlling area is located right below 6 phage amplification chambers.

The fabrication of the microfluidic chip adopted a typical PDMS soft lithography process [9]. Two molds was designed and made by computer-numerical-control (CNC) machining process on polymethylmethacrylate (PMMA) plates with a 0.5-mm drill bit. Then PDMS were casted on the PMMA molds to form two PDMS layers. Finally, the O_2 plasma treatment was used to bind the two layers and a glass substrate together to form a microfluidic chip.

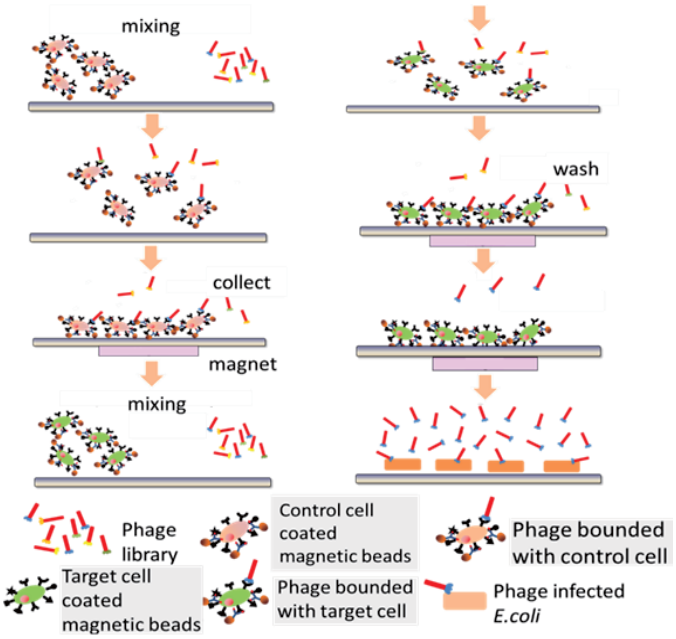

Figure 2. A schematic illustration of the experimental procedure implemented on the integrated microfluidic chip for screening of cancer cell specific oligopeptides. The steps include positive selection, negative selection, and phage amplification.

III. Results and Discussion

A. Characterization of the microfluidic chip

In order to screen peptides with higher affinity and specificity, we increased the screening stringency, in this case, shear stress of washing at each round of selection. The applied vacuum pressure was -100 mmHg, -200 mmHg, -300 mmHg, -400 mmHg, -500 mmHg and -600 mmHg, respectively. Figure 4 shows the relationship between the generated shear force and the applied vacuum pressure. It was found that the shear force increased when the applied vacuum pressure increased. By controlling the applied vacuum pressure, we could apply different shear forces to change the stringency during washing. The shear force of each panning round were characterized to be 12.8 μN, 54.4 μN, 80.8 μN, 116.9 μN, 153.1 μN and 189.3 μN, respectively.

Table 1. Details of the experimental protocol performed on the microfluidic system

Steps	Operating processes	Sample volume	On-chip operating conditions	Reaction time
I	Load control cell coated magnetic beads and phage library into the inlet chamber.	210 mL		
	Load control cell coated magnetic beads into chamber	200 mL		
	Load the washing buffer (1×PBS) into the washing buffer chamber.	1000 mL		
	Load E. coli into the phage amplification chamber	500 mL	37°C for E. coli incubation	
II	Incubate the control cell coated beads and phage library as negative selection.		-100mmHg, 0.2Hz	10 min
III	Attach the permanent magnet onto the bottom surface of the mixing chamber to attract the well-mixed magnetic complexes.			2 min
	Transport the supernatant to the next chamber for subsequent positive selection.	210 mL	-100mmHg	10 sec
IV	Incubate the collected phages and target cell coated beads as positive selection.		-100mmHg, 0.2Hz	10 min
	Attach the permanent magnet onto the bottom surface of the mixing chamber to attract the well-mixed magnetic complexes.			2 min
	Wash away the un-bonded phages using 1×PBS for 5 times.	1000 mL	Various (from -100 to -600 mmHg)	10 min
V	Suspend the bead/cell/phage complexes with 1×PBS	20 mL	-200mmHg	5 sec
	Pump the to the phage amplification chamber for amplification.	20 mL	-100mmHg	10 sec
VI	Amplification of phages		37°C	5 hr
	Heat to remove E. coli residue.		65°C	15 min
				Total ~6 hr

B. Screening of cell specific peptides

Positive and negative selections were performed alternately to screen more specific and higher affinity peptides. The result of each round of selection was confirmed by polymer chain reaction and gel electrophoresis. Figure 5 shows the polyacrylamide gel electrophoresis analysis of screened cell specific peptides. Figure 5(a) shows the result using HCT-8 as target cells and CSC as control cells. Alternatively, Figure (b) shows the results using CSC as the target cells and HCT-8 as the control cells. Both results indicate that the selection process was successfully completed on the microfluidic chip. We then sequenced the nucleotide sequence encoding the peptide of the identified phages. Three candidates for HCT-8 specific peptides were determined while the candidates for CSC were still under investigation. More tests about the affinity and specificity of the screened peptides are ongoing.

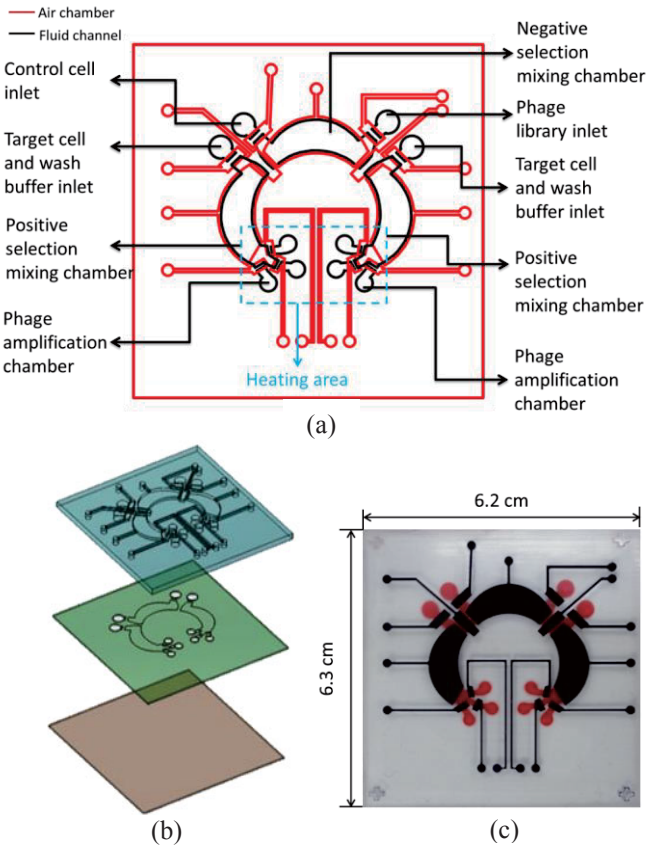

Figure 3. (a) A schematic (top view) of the microfluidic chip composed of normally-closed microvalves, micropumps, micromixers and a TE cooler; (b) An exploded view of the microfluidic chip; (c) A photograph of the microfluidic chip.

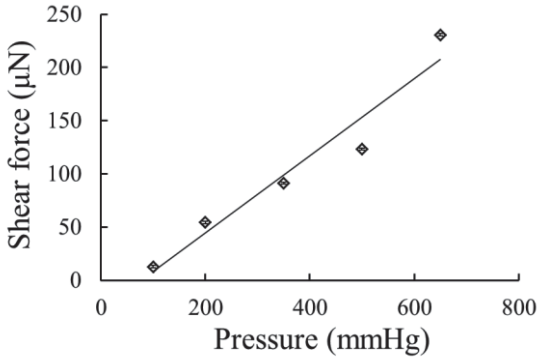

Figure 4. The relationship between the resultant shear force and the applied vacuum pressure. The shear force was increased in each panning experiment; the shear force of each round was calculated to be 12.8 μN, 54.4 μN, 80.8 μN, 116.9 μN, 153.1 μN and 189.3 μN, respectively.

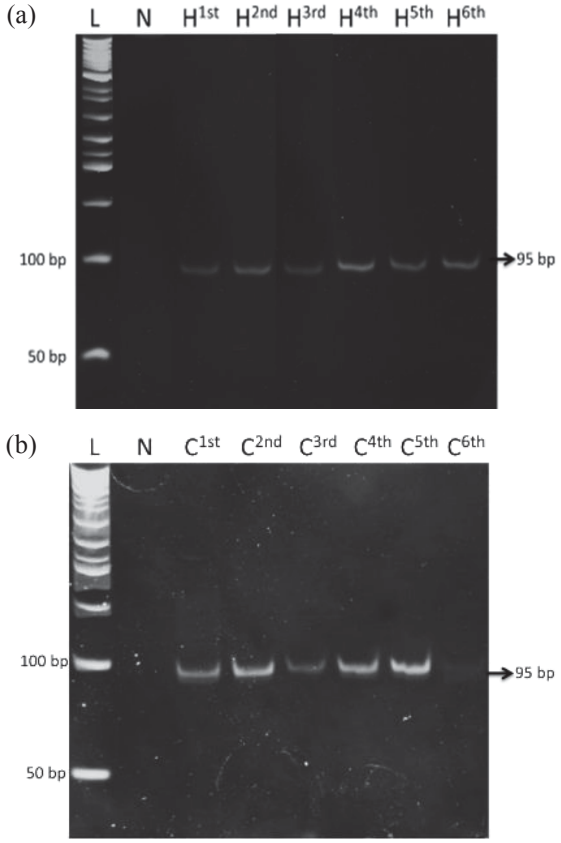

Figure 5. Polyacrylamide gel analysis of (a) HCT-8 as the target cells and (b) CSC as the target cells for screening of cell specific peptides. N: negative control using ddH_2O; L, 50-bp ladders. Lanes H^{1st} to H^{6th} and C^{1st} to C^{6th} represented the first to the sixth round of the selection, respectively.

C. Comparison of the traditional method and the microfluidic chip

Specific peptides screened from the developed microfluidic chip have several advantages over the traditional phage display screening method. First, the entire method was automated that errors caused by manual operation would be reduced to minimum. Second, microfluidic platforms may produce precise control of shear force such that the stringency of each round can be increased by adjusting the applied vacuum pressure. Third, the time of the proposed microfluidic chip for each selection round is only 6 h while the traditional method needs 5-6 days. For each selection process, at least 3 rounds of bio-panning are needed. For this reason, to screen a specific peptide traditionally takes about a month while less than 3 days are needed using the microfluidic chip. Finally, the microsystem also has better selectivity as demonstrated that only three candidates were found after completing the whole screening experiment yet the traditional methods typically have hundreds of candidates and may require lengthy sequencing and verification processes.

IV. Conclusion

In summary, we have successfully demonstrated a new integrated microfluidic system based on magnetic beads and microfluidic techniques for automating the entire process of cell specific peptides screening using M13 phage display peptide library. Compared to the traditional phage display method, this new integrated microfluidic system is smaller in size and has the ability to perform the whole screening experiment in an automatic format. The total time took for a single round of the screening process on the microfluidic system is approximately 6 h, which is much faster than the time required for the traditional phage display process. This integrated microfluidic system screened a cell specific peptide to recognize HCT-8. It may provide a useful platform for rapid screening of specific ligands for clinical diagnosis. Furthermore, the identified peptides may be applied in target therapy for colon cancer treatment.

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