Metastatic cell detection using a phage-peptide-modified light-addressable potentiometric sensor

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Detection of metastatic cells is clinically demanded to diagnose metastasis in the early stage and access the therapeutic response to anticancer drugs. We applied phage display technology to cultured cells with different metastasis potentials and obtained four metastasis-associated peptides. The association between peptides and metastatic cells was validated by ELISA as well as biosensor studies. The selected phage-peptides not only bound SW620, the metastatic cell against which the peptides were screened, but were also able to capture breast cancer cells of high metastasis. The phage-peptide-modified LAPS (light-addressable potentiometric sensor) was able to distinguish metastatic cells from non-metastatic cells and detect as few as 100 metastatic cells per ml of blood. Thus LAPS modified with specific phage-peptides may be developed to provide a new diagnostic approach that can aid the treatment of cancer.

Introduction

The malignancy of cancers is mainly attributed to tumour metastasis. Early detection of metastasis will translate into early treatment and increased curability. Two factors are critical to diagnose metastasis: the appropriate markers and the sensitive detection approach. Searching for metastasis markers has been attempted intensively in the last two decades. Many biomarker candidates, especially cell-surface proteins, were identified as diagnostic tools of metastasis. For example, HER-2 (human epidermal-growth-factor receptor 2) overexpression had been identified in various solid tumours [1] and evaluation status of HER-2 in the metastatic site led to more accurate treatment of metastatic patients [2]. Histochemical study of primary carcinoma and paired brain metastasis showed that high EGFR (epidermal growth factor receptor) expression was related to metastasis [3]. Higher levels of lectin, HGFR (hepatocyte growth factor receptor) and PDGFR (platelet-derived growth factor receptor) also suggested the progression towards metastasis [4–6]. With the known tumour markers, an antibody microarray had been developed towards clinical diagnosis. For example, immobilized antibodies corresponding to different antigens in leukaemia were designed to capture the tumorous lymphocytes [7,8]. However, the availability of antibodies with known markers is lagging behind clinical demand. As an alternative, phage display has been used to yield cell-specific peptides or antibodies without a prior knowledge of receptors on the target cell surface. A number of groups had identified many peptides targeting specific cells by differential screening [9–11].

On the other hand, detection of metastatic tumour cells with the patient-friendly approach becomes challenging. The current approach, biopsy, is invasive and less applicable if patients do not have a clearly defined pathological site [12]. The more promising approach would be based on the fact that tumour cells are loosened and released into the blood or lymph route, thus facilitating the migration. Obviously, the blood sample is a handy source for the detection.

Biosensors translated the biological process to the electronic signals and were applied in novel diagnostic and analytic devices, including DNA chips, electronic noses and so on. Compared with other types of microsensors, LAPS' (light-addressable potentiometric sensor) simple structure and great potential in multiplexed detection have made it deeply studied in immunoassay and cell metabolism [13–15]. The dependence of signal on the binding of charged species made it a good candidate to sense cell capture and protein interaction.

Key words: cell capture, differential phage display, light-addressable potentiometric sensor (LAPS), metastasis diagnosis, metastatic cell, phage-peptide.

Abbreviations used: HER-2, human epidermal-growth-factor receptor 2; HRP, horseradish peroxidase; LAPS, light-addressable potentiometric sensor; LSD, least significant difference; pfu, plaque-forming units; RT–PCR, reverse transcription–PCR; SPR, surface plasmon resonance.

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In the present study, we screened peptides from a 12-peptide phage library using the differential screening approach and obtained peptides that were selectively associated with metastatic cells. The cell selectivity was further validated with four other cancer cell lines. We further explored metastasis-specific phage-peptides in conjunction with LAPS. Application of phage-peptide-modified LAPS may propose a non-invasive approach for diagnosis of metastasis.

Materials and methods

Cell culture and phage library

Cell lines SW480, SW620, MDA-MB-231, MDA-MB-435, T47D and MCF7 were obtained from A.T.C.C. Cells were adherently cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS (foetal bovine serum), 100 units/ml of penicillin and 100 mg/ml of streptomycin. The whole peripheral blood was obtained from Tianjin Medical University Cancer Institute. PhD-12 phage library (New England Biolabs) displayed 12 amino acids on the N-terminus of pIII coat protein and the complexity was 2.7×10^9 transformants.

Differential screening by phage display

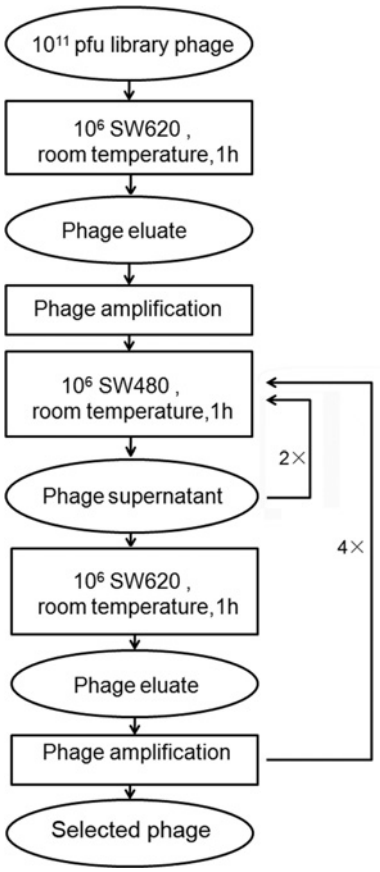
The selection protocol is outlined in Scheme 1. A total of 10^6 SW620 cells plated on to a 6-well plate were blocked with PBS and 1% (w/v) BSA for 1 h and then incubated with 10^{11} pfu (plaque-forming units) phages for 2 h. After washes, cell-bound phages were eluted with 100 mM glycine/HCl (pH 2.2), neutralized with 1 M Tris/HCl (pH 9.0) and then amplified using *Escherichia coli* ER2738 host strain. 10^{10} pfu amplified phages were incubated with 2 wells of SW480, each for 1 h. The depleted supernatant was applied to 10^6 SW620 cells and incubated for 2 h. The eluted phages were titred, amplified and three more subtraction/panning rounds were performed. The eluted phage pool or the individual phage clones were referred to as selected phages thereafter. Individual clones were identified by DNA sequencing using the primers recommended by the manufacturer.

Whole cell ELISA assay of cell-peptide binding

For binding analysis, cells were seeded at a density of 5×10^4 per well in a 96-well microtitration plate. After blocking with 5% BSA, cells were incubated with 10^{10} pfu selected phages or unselected library phages as control for 2 h. The wells were washed five times with PBST (0.1% Tween 20 in PBS). Anti-M13 phage antibody conjugated with HRP (horseradish peroxidase; Pharmacia Biotech) was then added to detect the binding of phages on cells and the association of phage with cells was presented by A_{450}.

Phage-peptide-modified LAPS

The setup of the phage-peptide-modified LAPS system has been previously described [16]. Briefly, after surface chemistry modification, the LAPS chip was mounted in a microchamber where the upper surface of the LAPS chip was in contact with the analyte solution. The backside was illuminated by modulated laser. The Lock-in Amplifier SR830 (Stanford Research Systems) was used to offer the bias voltage, amplify the weak photocurrent and transform current to voltage.



Scheme 1 Flow chart of differential phage display

Selected phage was added into the microchamber and allowed to react with aldehyde-terminated chip surface for 1 h. Unchanged aldehyde groups were blocked with BSA for 30 min. After washing with PBS, the output voltage was recorded as 'reference'. Signals were generated when cells were immobilized on the sensor chip (by phage-peptides) and presented by changes of output voltage.

Detection of cells by a phage-peptide-modified LAPS

Cells were detached by 0.5 mM EDTA and washed with PBS twice. For the validation experiment, cells were used at a concentration of 10^5 cells/ml. For cells mixed with blood, 10^2–10^4 cells/ml MDA-MB-231 cells were prepared by series dilution in blood. Blood samples were treated with erythrocyte lysing solution (Jingmei Biotech), centrifuged

Table 1 Screening of SW620-specific phages

10^{11} pfu phages were applied to SW620 cells in the first round. The eluted phages were amplified and 10^{10} pfu amplified phages were subtracted with SW480 before selection on SW620 in the next four rounds. Eluted phages of each round were titred and the enrichment of specific phages from each round of panning was presented by the ratio of input to output.

Rounds	Input phages (pfu)	Eluted phages (pfu) (output)	Enrichment (input/output)
1	1×10^{11}	1.1×10^{5}	9.1×10^{5}
2	1×10^{10}	3.2×10^{4}	3.1×10^{5}
3	1×10^{10}	6.0×10^{5}	1.7×10^{4}
4	1×10^{10}	8.9×10^{5}	1.1×10^{4}
5	1×10^{10}	1.3×10^{7}	7.7×10^{2}

and resuspended in PBS to remove erythrocytes. The prepared cells were injected to a LAPS microchamber and incubated for 30 min; signals were recorded where a laser of 75 kHz light frequency and a bias voltage of +1 V were applied.

Detection of cell-peptide binding by microscopic imaging

The glass coverslip was modified with phage-peptides covalently by glutaraldehyde [14,17,18]. In brief, APTES (aminopropyltriethoxysilane) was used to form a self-assembled layer on the glass surface and was modified by surface reaction with glutaraldehyde. When phages encoding different peptides were spotted on the surface of the glass slide, amine groups in phage reacted with the free aldehyde groups after incubating at 4°C overnight. Cells of interest were added on the glass slides at a density of 10^5 cells/ml. The surface was washed five times and observed under a light microscope.

Statistic analysis

Data were analysed with the software SAS/STAT Version 6 (SAS Institute), using ANOVA followed by the LSD (least significant difference) Student's *t* test. The level of significance was set at $P < 0.05$.

Results

Differential phage display and selection of cell-specific phage-peptides

SW620 and SW480 cells, derived from the same patient with colon cancer at different stages, were used to perform subtractive screening based on their different characteristics in metastasis when xenografted into nude mice [19,20]. Phage libraries encoding randomized 12-amino-acid peptides were applied to SW620 in the initial round. From the second round, phages were incubated with SW480 cells prior to SW620. The enrichment of peptides that bind preferentially to SW620 was calculated by

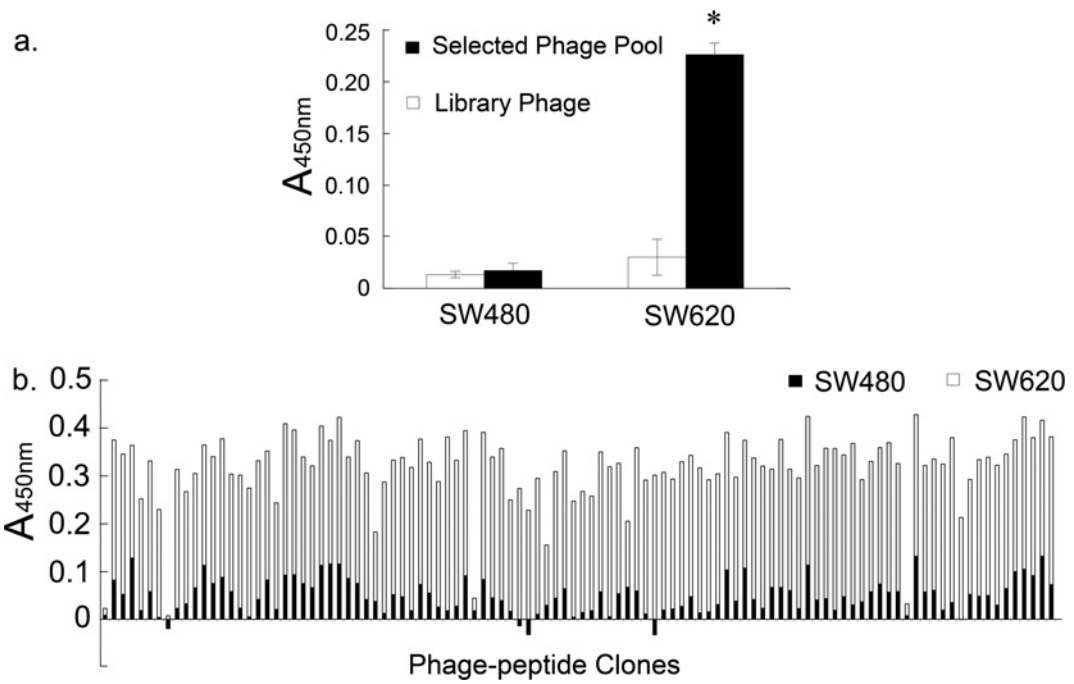


Figure 1 Evaluation of cell specificity of selected phages by ELISA

Selected phages from the fourth round panning (**a**) and individual phage clones (**b**) were incubated with SW620 or SW480 cells respectively. Bound phages were detected by anti-phage antibody conjugated with HRP and determined by A_{450}. The library phage (the first column in **b**) was used as a non-selective control. For (**a**), results were obtained from three independent experiments. *$P < 0.05$ compared with the SW480 group calculated by the two-sided independent sample *t* test.

titration phages eluted from each panning cycle, and presented by input-to-output ratio stepwise. As shown in Table 1, the first round of panning resulted in 10^6-fold enrichment. Greater than 10^4-fold enrichment was obtained from each of the subsequent three rounds, indicating an effective screening against SW620 cells. The last round only produced 770-fold enrichment from the input, suggesting a plateau of enrichment reached at the fifth round.

To validate the selectivity of peptides towards SW620, binding with SW480 and SW620 was evaluated by ELISA. As shown in Figure 1(a), the selected phage pool bound SW620 cells 7-fold more than SW480 (as measured by A_{450}), indicating specific binding between the selected pool of phage-peptides and SW620 cells.

Characterization of individual clones

Individual clones were isolated and amplified. The binding specificity of 105 individual clones was evaluated by ELISA. Out of 105 phage clones, 102 showed at least a 2-fold higher attenuance for SW620 than for SW480 (Figure 1b). In contrast, the original phage library displayed a basal reading to both cells. Thus the 102 clones were specific for SW620 but not for SW480 cell lines. Among the 102 positive clones, 26 with the highest SW620-to-SW480 ratio were submitted for sequencing analysis. Four peptide sequences were concluded and listed in Figure 2(a). The former three peptides shared a common motif of 'P-W-x-E-P-x-Y', implying a similar binding target on the cell surface. The last peptide appears to be more hydrophilic compared with the others.

We next tested the binding of these phage-peptides with several breast cancer cell lines, including highly metastatic cell lines, MDA-MB-231 and MDA-MB-435 [21–23], and non-metastatic cell lines, T47D and MCF7 [24–26]. The specific binding between the peptides and cell lines was verified by using ELISA, with the peptide library as non-specific binding control. As illustrated in Figure 2(b), all four selected phage-peptides preferentially bound to cells known to be highly metastatic (MDA-MB-231 and MDA-MB-435), whereas they only minimally bound to non-metastatic cells (T47D and MCF7). Thus these selected phage-peptides were specific to antigens shared by different metastatic cell lines, regardless of the background of targeting cells.

Capture of metastatic cells by phage-peptides

We performed proof-of-principle studies to capture cells by phage-peptide immobilized on appropriate surfaces such as coverslips as well as biosensors.

Cells of interest were added on to coverslips modified with different phage-peptides. Unbound cells were washed off and cell binding was monitored by a phase-contrast microscope.

A patch of SW620 cells deposited on the spots of phage-peptides 1, 2 and 3 (representative of phage-peptide 1 in Figure 3a), consistent with substantial sequence similarities among them. Very few cells bound to library phage (Figure 3b). Fewer SW480 cells were captured by all these spots (Figures 3c and 3d). Phage-peptide 1 was the most frequently appearing sequence obtained from phage display, and is thus more representative.

a.

No.	Phage displayed peptide sequence	Frequency
1	LPWKEPYYLMPP	12/26
2	SPWSEPAYTLAP	8/26
3	VPWMEPAYQRFL	5/26
4	SVSVGMKPSPRP	1/26

b.

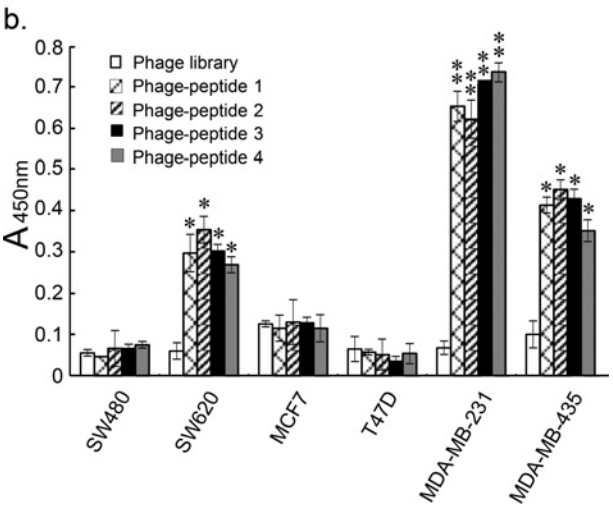


Figure 2 Binding of selected phage-peptides to cells of metastatic tumours

(**a**) Four phage-peptide sequences were obtained and listed with designated numbers. (**b**) Selected phage-peptides and the library phage were incubated with a panel of cells as indicated. The binding between phage-peptide and each cell line was measured with ELISA by reading the A_{450}. Results were obtained from three independent experiments. $^*P < 0.05$ for SW620 and MDA-MB-435, and $^{**}P < 0.01$ for MDA-MB-231 compared with non-metastatic cells (SW480, MCF7 and T47D) computed by one-way ANOVA followed by the LSD Student's *t* test.

Fewer cells bound to the peptide 4 spot, which has the least consensus sequence among the peptides; thus no further characterization was carried out.

This method, although primitive, supported the fact that phage-peptides could be used in a cell separation device to efficiently and selectively capture metastatic cells.

We validated metastatic cell detection with phage-peptides using more defined methodology, namely LAPS. The apparatus is sketched in Figure 4. The selected phage-peptide (using peptide 1 in this case) was covalently immobilized on a LAPS surface. SW480, SW620 or MDA-MB-231 cells were injected into the chamber. Cells captured by the immobilized phage-peptide probes on the surface were sensed by a Lock-in amplifier and translated into output voltages. SW480 output voltage changed

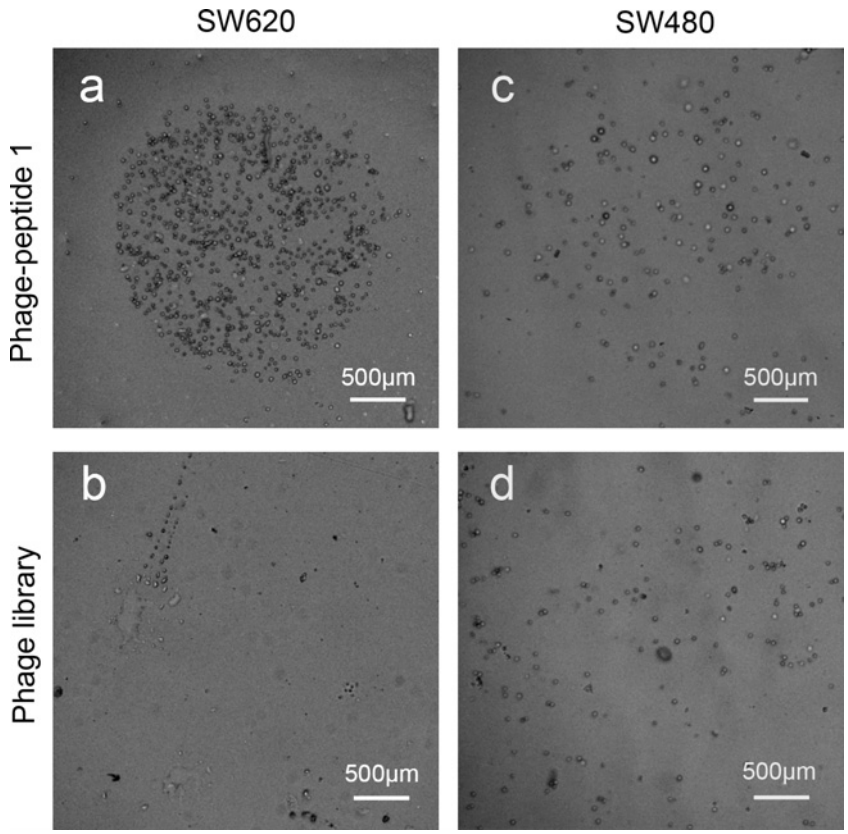


Figure 3 Capture of cells by modified glass surface

The surface of glass was modified with phage-peptide 1 (**a**, **c**) or library phage (**b**, **d**) by spotting phage on a glutaraldehyde-treated coverslip. Modified glass was incubated with SW620 (**a**, **b**) or SW480 (**c**, **d**) respectively. After washing, captured cells were observed under a light microscope.

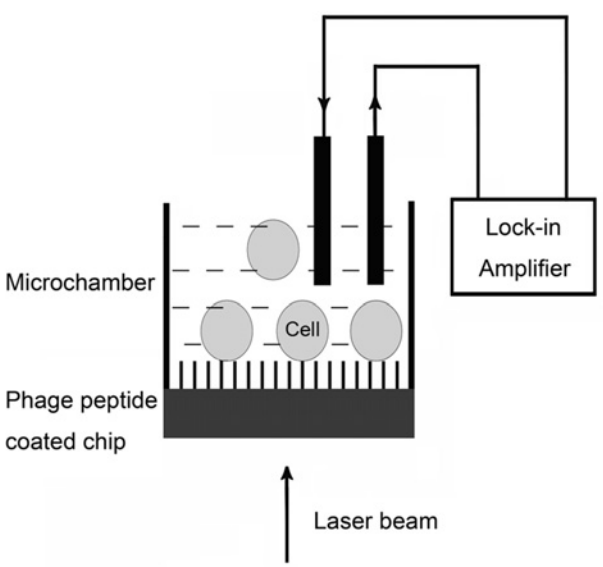


Figure 4 Schematic drawing of phage-peptide-modified LAPS

by 3 μV from the baseline, while SW620 and MDA-MB-231 showed significant increases of 40 and 60 μV respectively (Figure 5a).

To elucidate further the specificity of the detection, we performed a competition binding by incubating MDA-MB-231 or SW620 cells with soluble phage-peptide 1 or library phage prior to subjecting to LAPS surface. As shown in Figure 5(b), pre-incubation of cells with specific peptides (open bars), but not library peptides (solid bars), completely eliminated binding signals detected by LAPS, indicating that signals were attributed to the specific immobilization of cells by the surface peptide molecules.

Detection of metastatic cells in blood by phage-peptide-modified LAPS

Patient-friendly diagnosis of tumour malignancy from clinically relevant samples, such as blood, requires phage-peptides to distinguish metastatic cells from highly abundant circulating leucocytes. Therefore we investigated the

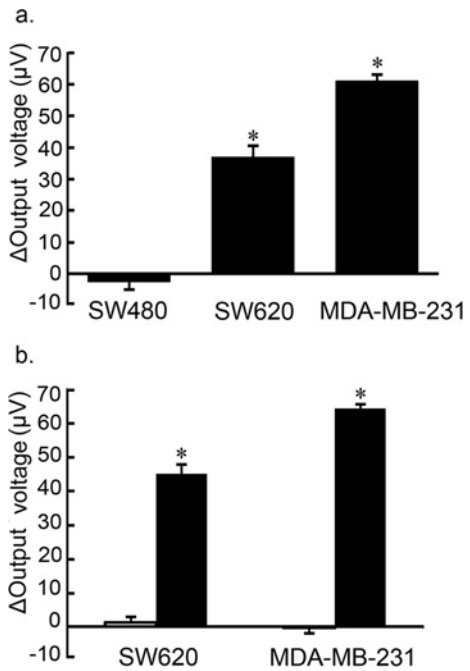


Figure 5 Detection of metastatic cells with phage-peptide-modified LAPS

(**a**) SW480, SW620 or MDA-MB-231 was incubated with phage-peptide-modified LAPS and signals were recorded. Results were obtained from three independent experiments. *$P < 0.05$ compared with the SW480 group calculated by the *t* test. (**b**) Cells were incubated with soluble phage-peptide 1 (open bars) or library phage (solid bars) and then added to LAPS modified with phage-peptide 1. Signals of cell binding were detected. Results were obtained from three independent experiments. *$P < 0.05$ compared with library phage-treated cells calculated by the *t* test.

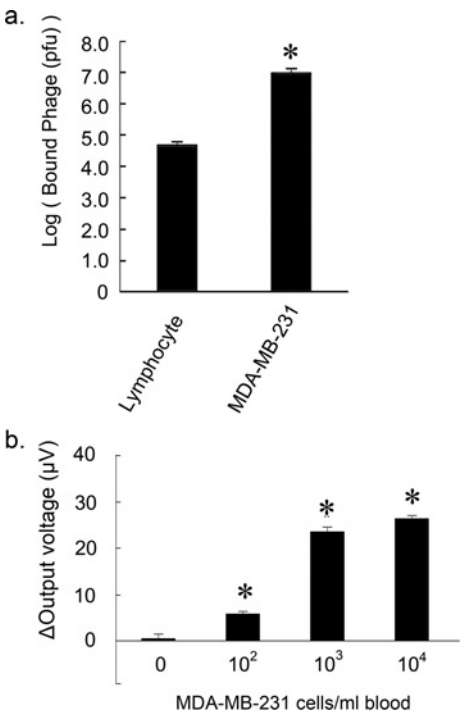


Figure 6 Detection of metastatic cells containing blood

Phage-peptide 1 was incubated with MDA-MB-231 or lymphocyte, washed and eluted. The recovered phage was titred. Results were obtained from three experiments. $P < 0.05$ compared with lymphocyte calculated by the *t* test. (**b**) Increasing concentrations of MDA-MB-231 cells in blood were added on to a LAPS microchamber and signals were recorded after gentle washing. Results were obtained from three independent experiments. *$P < 0.05$ compared with blood containing no tumour cells calculated by the *t* test.

detection of target cells in a blood sample by using phage-peptide-modified LAPS.

Addition of a blood sample containing no cancer cells did not lead to appreciable voltage changes relative to PBS buffer, indicating that leucocytes in the blood sample did not bind (Figure 6b). This was also supported by the fact that selected phage bound with MDA-MB-231 100 times higher than it did with control lymphocyte Jurkat cells determined by titration (Figure 6a).

A cell-concentration-dependent voltage increase was observed at 100 metastatic cells per ml of blood (Figure 6b), which is 10^5 times lower than the concentration of leucocytes in blood, indicating that the detected signals were contributed merely by the specific binding of peptide and cancer cells. A plot of data showed that the output voltage change was directly proportional to metastatic cell concentration and a detection limit of 100 metastatic cells per ml of blood was obtained.

Discussion

Metastasis causes deterioration and death of cancer patients. The reliable diagnosis for metastasis is only possible by series biopsy, but it is not applicable in the early stage of metastasis because there is no clearly perceptible site for biopsy. Because release of tumour cells into blood vessels is the hallmark of metastasis, their detection represents a promising metastasis diagnosis approach but is still an unresolved issue.

One challenge is the lack of selective metastatic markers (antigens unique to tumours with high invasive potential that are not expressed by leucocytes). Enrichment of metastatic cells with epithelial or organ-specific antibodies can only identify cells from specific organs but do not demonstrate their tumorous nature, thus giving false positive results [27]. Here, we used cell lines that were from the same genetic background, yet were dramatically different in metastasis phenotype [17,18], and differential phage display techniques to isolate peptides that are specific to metastatic cells. We obtained four metastatic cell-specific peptides, among which peptide 3 was reported previously as the selected phage using neuroblastoma cell line WAC2 as target [28]. Notably, it was reported that this peptide bound to a range of highly metastatic cells such as MDA-MB-435, but not to lymphocyte, erythrocyte and monocyte in blood. The peptide shared a similar sequence motif

'P-W-x-E-P-x-Y' as we screened, supporting the existence of a common target on the metastatic cells. Identification of the binding target will provide new insight into the mechanism of tumour metastasis, explore the invasive potential of tumour cells and orient anticancer treatment.

In the early stage of metastasis, just a few tumour cells are mixed with 10 million leucocytes and 5 billion erythrocytes in 1 ml of blood, and thus a very small quantity of tumour cells can be isolated [12,27]. The only testing of the presence of enriched tumour cells is RT–PCR (reverse transcription–PCR) assays to detect tumour markers. So far, few studies have attempted direct quantification of isolated tumour cells with biosensor. To avoid multiple operations in RT–PCR, such as RNA extraction, RT–PCR of marker genes and PCR product analysis by gel electrophoresis, we developed a label-free and real-time method to detect live metastatic cells. We reported that phage-peptide-modified LAPS was able to detect a cell line, against which phage-peptides were selected, in a homogeneous solution [16]. Here, we show that the metastasis-associated phage-modified LAPS apparatus can specifically detect metastatic cells in blood with a detection limit of 100 metastatic cells. The improvement can be made to allow continuously the flow of a larger volume of blood through the chip and the detection limit can be further reduced to meet the clinical need. In addition, LAPS measures the signals generated inside the illuminated area; thus one can design the chip to contain multiple detection zones [13,29]. Hence multiplexed detection could be achieved by using various capture molecules or, in this case, phages bearing different peptides.

Other biosensor detection methods have been investigated using SPR (surface plasmon resonance) modified with synthesized peptides [12] or small ligands [30]. However, SPR modified with phage was not able to detect cell binding (results not shown) because phage's dimension of 800 nm is much larger than SPR's sensitive distance, which is approx. 100 nm [30,31]. Compared with SPR, LAPS has the advantage of using phage-peptides as probes, which are considered to be an unlimited and cost-effective resource compared with synthesized peptides.

In summary, we reported the screening of metastasis-specific peptides by using differential phage display technology. Four specific peptides were characterized, among which most peptides contained a common sequence motif. The selectivity of peptides binding to metastatic cells was validated with various cell lines as well as using different methods, such as ELISA or LAPS modified with phage-peptides. The selective capture of metastatic cells by fixed phage-peptides provides a proof-of-principle study for the development of diagnostic LAPS chips with a multiplex of phage-peptide probes.

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