# EpCAM-independent capture of circulating tumor cells with a 'universal CTC-chip'

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**Abstract.** Capture of circulating tumor cells (CTCs), which are shed from the primary tumor site and circulate in the blood, remains a technical challenge. CellSearch® is the only clinically approved CTC detection system, but has provided only modest sensitivity in detecting CTCs mainly because epithelial cell adhesion molecule (EpCAM)-negative tumor cells may not be captured. To achieve more sensitive CTC-capture, we have developed a novel microfluidic platform, a 'CTC-chip' comprised of light-curable resins that has a unique advantage in that any capture antibody is easily conjugated. In the present study, we showed that EpCAM-negative tumor cells as well as EpCAM-positive cells were captured with the novel 'universal CTC-chip' as follows: i) human lung cancer cells (PC-9), with strong EpCAM expression, were efficiently captured with the CTC-chip coated with an anti-EpCAM antibody (with an average capture efficiency of 101% when tumor cells were spiked in phosphate-buffered saline (PBS) and 88% when spiked in blood); ii) human mesothelioma cells (ACC-MESO-4), with no EpCAM expression but with podoplanin expression, were captured with the CTC-chip coated with an anti-podoplanin antibody (average capture efficiency of 78% when tumor cells were spiked in PBS and 38% when spiked in blood), whereas ACC-MESO-4 cells were not captured with the CTC-chip coated with the anti-EpCAM antibody. These results indicate that the novel 'CTC-chip' can be useful in sensitive EpCAM-independent detection of CTCs, which may provide new insights into personalized medicine.

## Introduction

Circulating tumor cells (CTCs) are tumor cells that are shed from the primary tumor and circulate in the peripheral blood. CTCs, as a surrogate of distant metastasis, can be potentially useful for the diagnosis and monitoring of therapeutic effects in malignant tumors (1). However, the isolation of rare CTCs contaminated in a large number of normal hematologic cells is a technical challenge. Among a variety of systems for the detection of CTCs that have been developed and tested, CellSearch® (Veridex, LLC, Raritan, NJ, USA) is the only system approved for clinical use (2). CellSearch is a semi-automated system for quantitative evaluation of CTCs; CTCs are isolated using ferrofluid nanoparticles coupled with an antibody against epithelial cell adhesion molecule (EpCAM), which is highly expressed in tumor cells of epithelial origin. The most important advantage of the CellSearch system is its high reproducibility and the CTC-testing peformed with CellSearch which has proven to be clinically useful in monitoring the blood from patients with metastatic breast, colorectal and prostate carcinoma (3-5). Conversely, the most critical issue with CellSearch is its low sensitivity in the detection of CTCs. In fact, our previous study evaluating the CTC-test in primary lung cancer revealed that CTCs were not detected in 29% of patients with clinically detectable distant metastases (6), suggesting a need for more sensitive detection systems. We also conducted a study with the CTC-test in malignant pleural mesothelioma (MPM), a highly aggressive malignant tumor associated with asbestos exposure (7), and it revealed a very low diagnostic capability with a sensitivity of 33%. Its low sensitivity may be largely caused by its incapability to capture EpCAM-negative tumor cells, such as MPM, which originates from the mesothelium and may not or only weakly express EpCAM (8), suggesting a need for novel systems of EpCAM-independent detection of CTCs.

Among a variety of EpCAM-independent CTC-capture systems including size-based or density-based separation systems (9), a microfluidic system called a 'CTC-chip' has an advantage with its capability of capturing specific cells with an antibody attached to microposts. Nagrath *et al* and Maheswaran *et al* first reported a higher sensitivity in the detection of CTCs with a CTC-chip coated with anti-EpCAM antibodies (10,11). Despite the promising results reported in a pilot study, no additional study to confirm or validate its high performance has been reported. A novel polymeric CTC-chip comprised of light-curable resins has been designed by the author (T.O.) (12). Among a variety of advantages of the novel CTC-chip over the 'original' CTC-chip including its lower

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*Key words:* circulating tumor cells, epithelial cell adhesion molecule, CTC-chip, malignant pleural mesothelioma, podoplanin

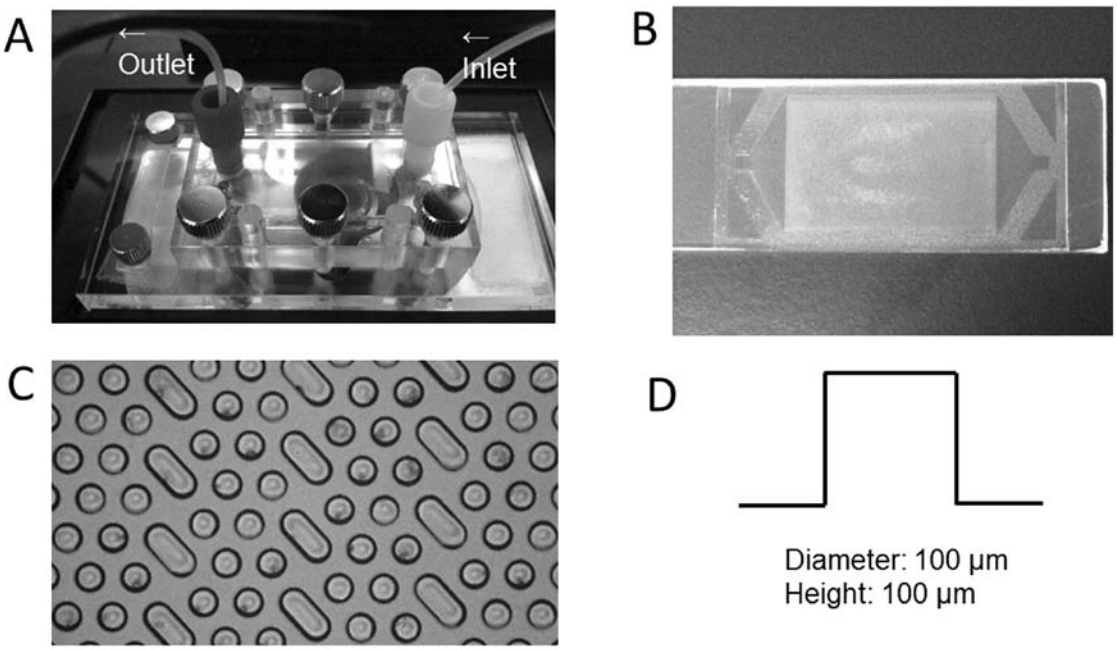


Figure 1. CTC-chip and sample flow system. (A) Whole image of the polymeric CTC-chip set in the holder. (B) Appearance of a CTC-chip. (C) The microstructure of the chip. (D) Schematic view of the micropost.

cost, higher durability and improved transparency, the most important and unique advantage is that any antibody which captures CTCs is easily conjugated to the chip, as the chip surface is made reactive with any antibody by the incorporation of monomers having an epoxy group in the resin (Fig. 1). Accordingly, EpCAM-negative CTCs can be potentially captured by the 'novel' CTC-chip coated with an antibody against a specific antigen which is expressed in tumor cells and which may be referred to as a 'universal CTC-chip'. In the present study, we first showed that EpCAM-negative MPM cells were effectively captured by the 'universal CTC-chip' coated with an antibody against podoplanin that is expressed in MPM cells.

## Materials and methods

*Cell lines*. Human lung cancer cell line, PC-9 and human mesothelioma cell line, ACC-MESO-4 established in Aichi Cancer Research Center (Nagoya, Japan) (13) were purchased from Riken BioResource Center (Tsukuba, Japan). These cells were cultured in RPMI-1640 medium (Wako Pure Chemical Industries, Osaka, Japan) and supplemented with 10% fetal bovine serum (Invitrogen, San Diego, CA, USA) at 37˚C in 5% $CO_2$.

*Immunocytochemistry*. Cells ($1x10^4$) were plated on a 24-well dish and cultured for three days. Cells were fixed with 4% paraformaldehyde followed by permeabilization with 0.25% Triton X-100 and blocking with Protein Block (Dako, Glostrup, Denmark). Then, cells were incubated with a primary antibody, a mouse anti-human EpCAM monoclonal antibody (clone HEA125) or a mouse anti-human podoplanin monoclonal antibody (clone E1; both from Santa Cruz Biotechnology, Dallas, TX, USA) diluted to 1:100 and incubated for 60 min at room temperature. After 30 min of incubation at room temperature with a secondary antibody (goat anti-mouse IgG) conjugated with Alexa Fluor 594 (Life Technologies, Carlsbad, CA, USA) and diluted to 1:100 containing 1 $\mu$g/ml Hoechst 33342 (Cell Signaling Technology, Danvers, MA, USA), images were acquired with the CKX41 inverted fluorescence microscope (Olympus, Tokyo, Japan) equipped with a DP73 digital camera (Olympus).

*Flow cytometry*. Cells were collected and incubated with a primary antibody, an anti-EpCAM antibody (clone HEA125) or an anti-podoplanin antibody (clone E1), diluted to 1:100 and incubated for 20 min at room temperature. Then, the cells were incubated with a goat anti-mouse IgG antibody conjugated with FITC (BD Biosciences, San Jose, CA, USA) diluted to 1:20. Flow cytometric analysis was performed using the EC800 Cell Analyzer (Sony Biotechnology, Inc., Tokyo, Japan) and FlowJo software (Tree Star, Inc., Ashland, OR, USA).

*Preparation of CTC-chip*. The polymeric CTC-chip system (Fig. 1) (12) was used after a two-step coating with an antibody to capture CTCs (Fig. 2). In the first step, the chip surface was incubated with a goat anti-mouse IgG antibody (SouthernBiotech, Birmingham, AL, USA) in phosphate-buffered saline (PBS) at a concentration of 20 $\mu$g/ml overnight at 4˚C and then the chip surface was washed with PBS. In the next step, the chip surface was incubated with an antibody to capture CTCs, an anti-human EpCAM antibody (clone HEA125) or a mouse anti-human podoplanin antibody (clone E1), diluted with PBS at a concentration of 20 $\mu$g/ml and incubated for 1 h at 4˚C in order to react with the surface anti-mouse IgG antibody. After being washed with PBS, the chip surface was kept wet. The antibody-coated chip was

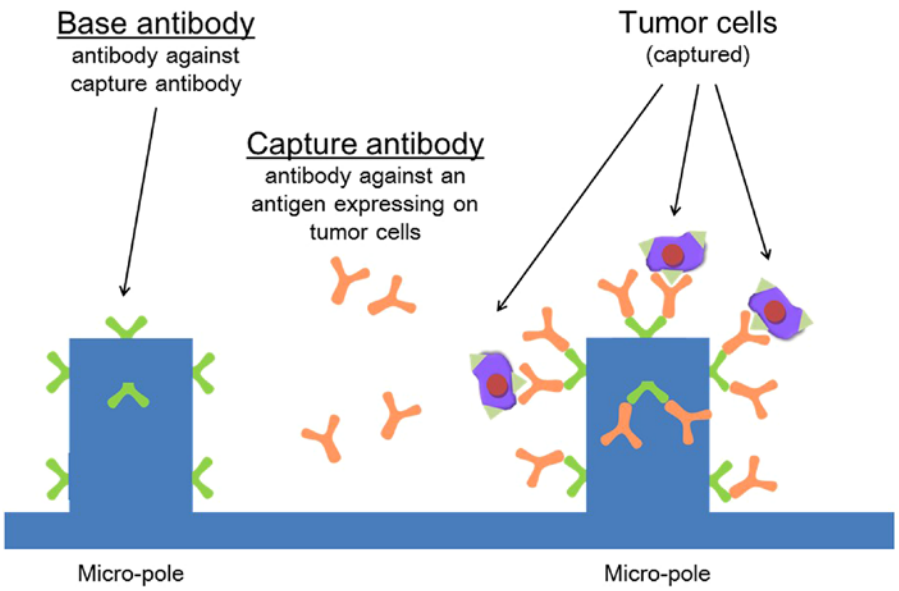


Figure 2. Capture of tumor cells with the CTC-chip after a 'two-step' coating with a capture antibody.

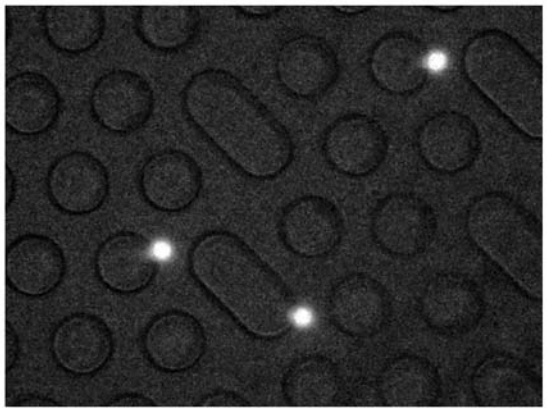

Figure 3. Captured tumor cells.

referred to as the 'EpCAM-chip' when coated with the anti-EpCAM antibody or as the 'podoplanin-chip' when coated with the anti-podoplanin antibody.

*Sample preparation and flow test*. Cells were labeled using the CellTrace™ CFSE Cell Proliferation kit (Life Technologies) according to the manufacturer's protocol, and then 1,500 cells were suspended in 3 ml of PBS containing 5% BSA or in 3 ml of the blood sampled from a healthy volunteer (the author, C.Y.). A cell suspension sample of 1 ml (500 cells/ml) was applied to the CTC-chip system.

Each sample was sent to the chip using a syringe pump at a constant flow rate (1.5 ml/h when suspended in PBS or 1.0 ml/h when suspended in the blood). Meanwhile, each sample tube was shaken to ensure that the cell suspension was homogeneous. Images and videos of the cells in the chip were monitored and recorded with a fluorescence microscope CKX41 (Olympus) and a digital video camera (Sony Biotechnology, Inc.) (Fig. 3). Each experiment, sample preparation and flow test was performed in triplicate.

*Evaluation of cell capture efficiency*. We determined the actual number of cells that were sent into the chip (N-total) by counting the number of cells that passed through the inlet of the chip. We also determined the number of captured cells (N-captured) by counting CFSE-labeled cells remaining on the chip after completion of the flow test. The cell capture efficiency was evaluated as N-captured/N-total. The average value of capture efficiency was calculated from the results obtained in the triplicate experiments.

## Results

*Expression of EpCAM and podoplanin*. PC-9, a human lung adenocarcinoma cell line, strongly expressed EpCAM in immunocytochemical staining (Fig. 4) and flow cytometry (Fig. 5). In contrast, ACC-MESO-4, a human MPM cell line, did not express EpCAM (Figs. 4 and 5).

Conversely, ACC-MESO-4 strongly expressed podoplanin, an MPM-specific antigen, in immunocytochemical staining (Fig. 4) and flow cytometry (Fig. 5), while PC-9 did not express podoplanin (Figs. 4 and 5).

*Cell capture efficiency*

*Capture from the cell suspension spiked in PBS (Fig. 6 and Table I)*. When PC-9 cells were suspended in PBS, the average capture efficiency for the EpCAM-chip was 101.1%. However, when ACC-MESO-4 cells were spiked in PBS, the average capture efficiency for the EpCAM-chip was only 3.0%.

In contrast, when the podoplanin-chip was used to capture ACC-MESO-4 cells, the average capture efficiency was markedly increased (78.3%). In contrast, when PC-9 cells were spiked, the average capture efficiency for the podoplanin-chip was only 2.3%.

*Capture from cell suspension spiked in the blood (Fig. 7 and Table II)*. When ACC-MESO-4 cells were suspended in the blood, the average capture efficiency for the EpCAM-chip

Table I. Capture from the cell suspension spiked in PBS.

| | EpCAM (HEA125) | | | | Podoplanin (E1) | | | |
|---|---|---|---|---|---|---|---|---|
| | No. of cells captured (N-captured) | No. of total cells (N-total) | Cell capture efficiency (%) | | No. of cells captured (N-captured) | No. of total cells (N-total) | Cell capture efficiency (%) | |
| | | | Values | Average | | | Values | Average |
| PC-9 | 511 | 519 | 98.5 | 101.1 | 11 | 847 | 1.3 | 2.3 |
| | 584 | 571 | 102.2 | | 19 | 614 | 3.1 | |
| | 611 | 596 | 102.5 | | 15 | 626 | 2.4 | |
| ACC-MESO-4 | 11 | 669 | 1.6 | 3.0 | 340 | 499 | 68.1 | 78.3 |
| | 17 | 535 | 3.2 | | 534 | 682 | 78.3 | |
| | 27 | 663 | 4.1 | | 643 | 727 | 88.4 | |

EpCAM, epithelial cell adhesion molecule.

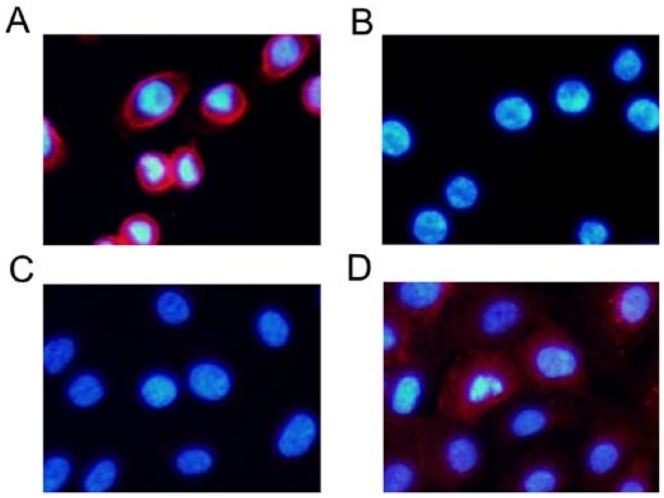


Figure 4. Immunostaining of (A and B) PC-9 and (C and D) ACC-MESO-4 cells for (A and C) EpCAM or (B and D) podoplanin expression. EpCAM, epithelial cell adhesion molecule.

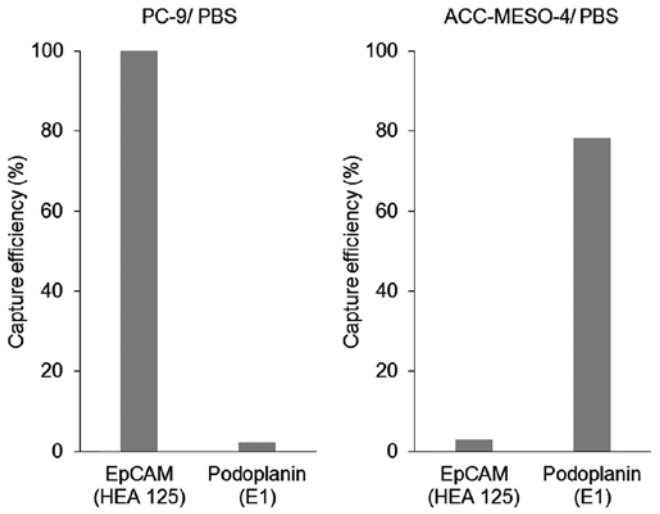


Figure 6. Capture efficiency of the CTC-chip when tumor cells were spiked in phosphate-buffered saline (PBS). EpCAM, epithelial cell adhesion molecule.

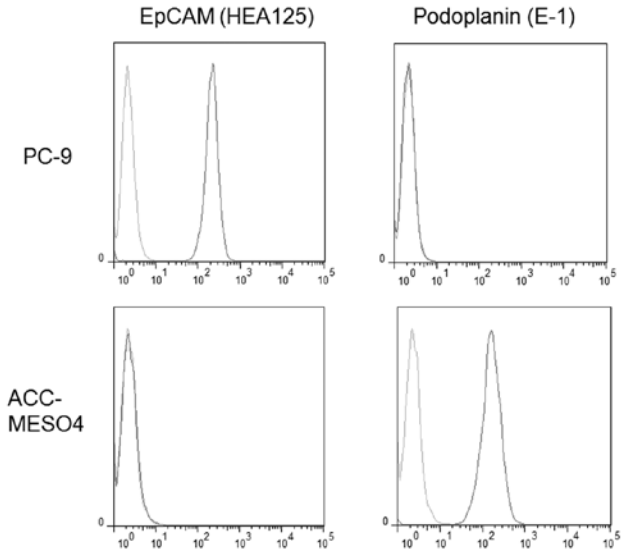


Figure 5. Flow cytometry of PC-9 cells and ACC-MESO-4 cells for EpCAM or podoplanin expression. EpCAM, epithelial cell adhesion molecule.

was only 2.2%, but that for the podoplanin-chip was much higher (38.4%). When PC-9 cells were suspended in the blood, the average capture efficiency of the EpCAM-chip and the podoplanin-chip were 88.0 and 6.9%, respectively.

## Discussion

In the present study, we showed that EpCAM-negative tumor cells were effectively captured with the novel CTC-chip. This is the first study to report the isolation of EpCAM-negative tumor cells using the 'universal' CTC-chip, suggesting that the system is a promising modality to detect a variety of CTCs without EpCAM expression due to non-epithelial origin or undergoing epithelial-mesenchymal transition (EMT).

As an EpCAM-negative tumor, we selected an MPM cell line, ACC-MESO-4, and tested the capture efficiency of the CTC-chip coated with anti-podoplanin antibody. MPM is a rare malignant tumor associated with asbestos exposure, but its incidence is increasingly prevalent worldwide.

Table II. Capture fromthe cell suspension spiked in the blood.

| | EpCAM (HEA125) | | | | Podoplanin (E1) | | | |
|---|---|---|---|---|---|---|---|---|
| | No. of cells captured (N-captured) | No. of total cells (N-total) | Cell capture efficiency (%) | | No. of cells captured (N-captured) | No. of total cells (N-total) | Cell capture efficiency (%) | |
| | | | Values | Average | | | Values | Average |
| PC-9 | 118 | 116 | 101.7 | 88.0 | 38 | 538 | 7.1 | 6.9 |
| | 350 | 409 | 85.6 | | 22 | 435 | 5.1 | |
| | 304 | 397 | 76.6 | | 39 | 464 | 6.9 | |
| ACC-MESO-4 | 17 | 404 | 4.2 | 2.2 | 287 | 483 | 59.4 | 38.4 |
| | 6 | 447 | 1.3 | | 123 | 487 | 25.2 | |
| | 6 | 534 | 1.1 | | 159 | 518 | 30.7 | |

EpCAM, epithelial cell adhesion molecule.

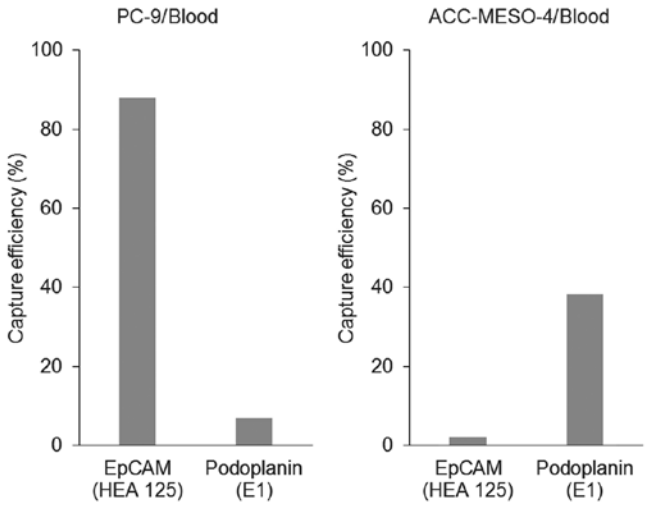


Figure 7. Capture efficiency of the CTC-chip when tumor cells were spiked in blood. EpCAM, epithelial cell adhesion molecule.

MPM is a highly aggressive tumor with a median survival of 4-12 months due to lack of effective diagnostic and/or treatment modalities (7). The diagnosis of MPM is principally established with histologic examination, which usually requires invasive procedures such as a core-needle biopsy or a video-assisted thoracoscopic biopsy. These invasive procedures may not be feasible for mass-screening or for patients with poor performance status, and the development of less invasive diagnostic procedures is clinically important. A number of noninvasive markers including serum soluble mesothelin-related protein (SMRP) and serum osteopontin have been evaluated, but there has been no established marker for the diagnosis of MPM. In a previous study (8), we evaluated the diagnostic performance of the CTC-test using CellSearch, and revealed a significant, but modest diagnostic performance of MPM with a sensitivity of 33%. We also examined EpCAM expression in MPM originating from the pleural mesothelium that did not essentially express EpCAM, and revealed that only 11 of the 21 MPM tumors were EpCAM-positive. In the present study as well, the MPM cell line, ACC-MESO-4, did not express EpCAM (Fig. 2A and B). These results clearly indicate that MPM tumor cells without robust expression of EpCAM could not effectively be captured with an EpCAM-dependent CTC-capture system such as CellSearch, which led us to develop an EpCAM-independent CTC-capture system such as the 'universal' CTC-chip.

Among a variety of EpCAM-independent CTC-capture systems including size-based or density-based separation systems (1), we adopted a microfluidic system called the 'CTC-chip' due to its capability to capture specific cells with an antibody attached to microposts. The original CTC-chip coated with an anti-EpCAM antibody could capture only EpCAM-positive tumor cells, because another antibody capturing EpCAM-negative tumor cells was not available in the chip system (10,11). The novel 'universal' CTC-chip, used in the current study, has overcome this critical issue, as any antibody for capture can be easily attached to the chip (12). In fact, EpCAM-positive cells of lung cancer (PC-9) spiked in PBS were perfectly captured with an average capture efficiency of 101.1% using the chip coated with an anti-EpCAM antibody ('EpCAM-chip'); while, when coated with an anti-podoplanin antibody ('podoplanin-chip'), podoplanin-positive cells of MPM (ACC-MESO-4) were effectively captured with an average capture efficiency of 78.3% (Table I and Fig. 6). Podoplanin is a mucin-type transmembrane glycoprotein. Podoplanin expression seen in limited normal tissues such as lymphatic vessels and in type I alveolar epithelium, is increased in some malignant tumors including MPM (14). In fact, ACC-MESO-4, an MPM cell line, strongly expressed podoplanin (Figs. 4 and 5) and its cells were captured using the podoplanin-chip. Some MPM cells do not express podoplanin, and failed to be captured with an anti-podoplanin antibody. The 'universal' chip can capture such tumor cells by attaching other antibodies against specific antigens such as mesothelin (14) and CD146 (15), which will be examined in future studies. In addition, the 'universal' chip may be useful for capturing a variety of tumor cells which originate from the epithelium but do not express EpCAM due to various reasons such as they may be undergoing EMT, and this topic will also be examined in a future study.

We examined the capture efficiency of the 'universal' chip for tumor cells spiked in the blood to simulate isolation of CTCs from the blood. PC-9 cells, spiked in the blood, were effectively captured using the EpCAM-chip with an average capture efficiency of 88.0%. However, ACC-MESO-4 cells could be captured using the podoplanin-chip, but the efficacy was only modest with an average capture efficiency of 38.4% (Table II and Fig. 7). These results indicate that some components included in the blood may weaken or inhibit antigen-antibody reaction, resulting in decreased capture efficiency documented when ACC-MESO-4 cells were spiked in the blood. In capturing PC-9 cells with the EpCAM-chip, the antigen-antibody reaction may be stronger, and may be strong enough for efficient capturing of tumor cells even if it is decreased in the blood. Considering its clinical application, the capture efficiency of the 'universal' chip in capturing tumor cells contaminated in the blood should be increased, even when the chip is coated with any capture antibody such as the anti-podoplanin antibody. In addition, the sensitivity in detecting CTCs from the blood sampled from cancer patients should be examined and may be compared with that using the CellSearch system.

For monitoring cancer genetics in the blood ('liquid biopsy'), cell-free methods detecting fragments of DNA derived from tumor cells may provide several advantages such as superior sensitivity, as compared with cell-based methods detecting CTCs. However, morphological visualization of tumor cells circulating in the blood can be achieved only by direct detection of CTCs using cell-based methods such as CellSearch and CTC-chips. More importantly, molecular characterization of tumor cells can be achieved at not only the genomic level (e.g. genomic alterations in tumor cells) but also the cellular level (e.g. expression of tumor-specific antigens on tumor cells) (16). In a future study, we will analyze the expression of tumor-specific antigens such as HER2, especially in correlation with the therapeutic effects of targeting agents, which may provide new insights into personalized medicine.

In conclusion, using the novel CTC-chip, we successfully captured EpCAM-positive tumor cells (PC-9) when the chip was coated with an anti-EpCAM antibody, and also captured EpCAM-negative tumor cells (ACC-MESO-4) when the chip was coated with an antibody against an MPM-specific antigen (podoplanin). The 'universal' CTC-chip may provide new insights for the detection of CTCs and personalized medicine.

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