Enhanced capture system for mesenchymal-type circulating tumor cells using a polymeric microfluidic device 'CTC-Chip' incorporating cell-surface vimentin

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Abstract. CellSearch, the only approved epithelial cell adhesion molecule (EpCAM)-dependent capture system approved for clinical use, overlooks circulating tumor cells (CTCs) undergoing epithelial-mesenchymal transition (EMT-CTCs), which is considered a crucial subtype responsible for metastasis. To address this limitation, a novel polymeric microfluidic device 'CTC-chip' designed for the easy introduction of any antibody was developed, enabling EpCAM-independent capture. In this study, antibodies against EpCAM and cell surface vimentin (CSV), identified as cancer-specific EMT markers, were conjugated onto the chip (EpCAM-chip and CSV-chip, respectively), and the capture efficiency was examined using lung cancer (PC9, H441 and A549) and colon cancer (DLD1) cell lines, classified into three types based on EMT markers: Epithelial (PC9), intermediate (H441 and DLD1) and mesenchymal (A549). PC9, H441 and DLD1 cells were effectively captured using the EpCAM-chip (average capture efficiencies: 99.4, 88.8 and 90.8%, respectively) when spiked into blood. However, A549 cells were scarcely captured (13.4%), indicating that EpCAM-dependent capture is not suitable for mesenchymal-type cells. The expression of CSV tended to be higher in cells exhibiting mesenchymal properties and A549 cells were effectively captured with the CSV-chip (72.4 and 88.4% at concentrations of 10 and 100 μg/ml, respectively) when spiked into PBS. When spiked into blood, the average capture efficiencies were 27.7 and 46.8% at concentrations of 10 and 100 μg/ml, respectively. These results suggest that the CSV-chip is useful for detecting mesenchymal-type cells and has potential applications in capturing EMT-CTCs.

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Abbreviations: EpCAM, epithelial cell adhesion molecule; CTCs, circulating tumor cells; EMT, epithelial-mesenchymal transition; CSV, cell surface vimentin

Key words: mesenchymal-type circulating tumor cells, polymeric microfluidic device, CTC chip

Introduction

Circulating tumor cells (CTCs) are shed from primary tumors and circulate in the peripheral blood. CTCs are considered the origin of metastases, and analyzing CTCs holds the potential for early metastasis detection and monitoring of therapeutic effects in malignant tumors (1,2). Among the available CTC detection devices, CellSearch (Veridex, LLC) is a semi-automatic system that utilizes antibodies against epithelial cell adhesion molecule (EpCAM), and is the only Food and Drug Administration (FDA)-approved device for clinical CTC detection (3). CellSearch consistently yields reproducible results and demonstrates its clinical relevance in epithelial cancers (4,5). However, epithelial tumor cells undergo epithelial-mesenchymal transition (EMT) to enhance their migration and invasiveness, resulting in the downregulation of epithelial markers such as EpCAM (6). Consequently, EpCAM-dependent systems, including CellSearch, may be limited in that they cannot capture CTCs undergoing EMT (EMT-CTCs), which is a pivotal subtype implicated in metastasis (7,8). To address this limitation, a novel polymeric microfluidic device 'CTC-chip' (9) was developed based on the conventional CTC-chip designed by Nagrath *et al* (10). The CTC-chip can capture diverse types of CTCs expressing targeted tumor cell antigens by easily attaching various antibodies to numerous microposts on the chip surface (11-13), and its clinical utility was previously reported by our group (14,15). In a previous study by our group, CTCs were undetectable in 40% of patients with lung cancer and metastases, indicating the need to develop an EpCAM-independent capture system using other antibodies (15). Recently, Satelli *et al* (16) identified cell-surface vimentin (CSV) as an EMT-CTC marker. The CSV monoclonal antibody clone 84-1 primarily associates with tumor cells and demonstrates clinical utility (17-19).

In the present study, CSV expression was validated in lung and colon cancer cell lines, and the capture efficiency of this polymeric CTC-chip coated with an anti-CSV antibody was assessed as a novel marker for mesenchymal CTCs, with the aim of establishing an EMT-CTC capture system.

Materials and methods

Cell lines. The human lung cancer cell lines PC9 (Riken BioResource Research Center) and H441 [American Type Culture Collection (ATCC)], and the human colorectal cancer cell line DLD1 (ATCC) were cultured in RPMI-1640 medium (Wako Pure Chemical Industries) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.). The human lung cancer cell line A549 (ATCC) was cultured in Eagle's minimal essential medium (Wako Pure Chemical Industries) supplemented with 10% FBS and 1% non-essential amino acids (Wako Pure Chemical Industries). All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37˚C.

Flow cytometry. Adhered cells were harvested using 1 mM EDTA (Wako Pure Chemical Industries) in PBS at 37˚C to maintain cell surface protein integrity, as it was found that CSV is degraded by trypsin treatment (20). Subsequently, cells were blocked with Protein Block (Dako) for 15 min and then sequentially incubated with primary antibodies, including an anti-EpCAM antibody (clone: HEA125; cat. no. sc-59906; Santa Cruz Biotechnology, Inc.), an anti-CSV antibody (clone: 84-1; cat. no. H00007431-M08; Abnova), an anti-E-cadherin antibody (clone: 36; cat. no. 610182; BD Biosciences), or an anti-Vimentin antibody (clone: RV202; cat. no. 562337; BD Biosciences), each diluted to 1:100, for 60 min at room temperature. Following primary antibody incubation, the cells were incubated with a goat anti-mouse IgG antibody conjugated with FITC (cat. no. 349031; BD Biosciences), diluted to 1:20, for 30 min at room temperature. Flow cytometry was performed using an EC800 cell analyzer (Sony Biotechnology) and FlowJo software version 10 (Becton-Dickinson and Co.) was used for data analysis. The mean fluorescence intensity was determined by comparison with the negative control.

Preparation of CTC-chip. The polymeric CTC-chip system was utilized following a two-step coating process with antibodies to capture CTCs, as previously described (11). Initially, the chip (cat. no. G4B442; Cytona, Inc.) was incubated overnight at 4˚C with a goat anti-mouse IgG antibody (cat. no. 1031-01; SouthernBiotech) in PBS at a concentration of 200 μg/ml. Subsequently, the chip was incubated for 60 min at room temperature with either an anti-EpCAM antibody (20 μg/ml; clone HEA125) or an anti-CSV antibody (10 and 100 μg/ml; clone 84-1) to capture the tumor cells. The chip coated with the respective antibody was denoted as the 'EpCAM-chip' and 'CSV-chip', respectively. After incubation, the chip surface was washed with PBS and kept moist. The chip is typically used following a 60-min antibody reaction.

Sample preparation and evaluation of cell-capture efficacy. Cells were labeled with the CellTrace™ carboxyfluorescein succinimidyl ester (CFSE) Cell Proliferation Kit (Thermo Fisher Scientific, Inc.) and suspended in 1 ml PBS containing 5% bovine serum albumin (Nacalai Tesque, Inc.) or blood sampled from a healthy volunteer (100 cells/ml). The cell suspension sample (1 ml) was applied to the CTC-chip and sent to the chip using a syringe pump at a constant flow rate of 1.0 ml/h. Images and videos of the cells on the chip were captured using a fluorescence microscope CKX41 (Olympus Corp.) and a digital video camera (Sony Biotechnology, Inc.), respectively. The actual number of cells (N-total) that were sent into the chip was determined by counting the number of cells that passed through the chip inlet and the number of captured cells (N-captured) was determined by counting the CFSE-labelled cells remaining on the chip. Cell capture efficiency was calculated as N-captured/N-total. The healthy volunteer (author MK) donated blood and provided formal written informed consent for its use in this study for experimental purposes. Data collection from healthy subjects was included in the Ethics Committee approval (approval no. H26-15).

Statistical analysis. The average and standard error of the capture efficiency were calculated from 3 independent experimental repeats. All analyses were performed using SPSS (version 27.0; IBM Corp.).

Results

Classification of cell lines based on E-cadherin and vimentin expression. The classification of the cell lines based on flow cytometric analysis of E-cadherin and vimentin expression is summarized in Fig. 1. According to these results, the cell lines were classified into three types: Epithelial (PC9; high E-cadherin expression and low vimentin expression), mesenchymal (A549; low E-cadherin expression and high vimentin expression) and intermediate (H441 and DLD1).

EpCAM expression and capture efficiency. EpCAM was strongly expressed in the epithelial and intermediate types but weakly expressed in the mesenchymal type (Fig. 2). The capture efficiency of the EpCAM-chip was in accordance with the expression intensity of EpCAM. High capture rates were observed in the epithelial and intermediate types (average capture efficiencies: PC9, 99.4%; H441, 88.8%; and DLD1, 90.8%), and a low capture rate was observed in the mesenchymal type (average capture efficiency: A549, 13.4%) (Fig. 3).

CSV expression and capture efficiency. The expression of CSV was inversely in accordance with that of EpCAM and increased in the following order: Mesenchymal, intermediate and epithelial (Fig. 3). The results obtained by examining the capture efficiency of the CSV-chip when tumor cells were spiked in PBS or blood at two concentrations (anti-CSV antibody: 10 and 100 μg/ml) are provided in Figs. 4 and 5. When spiked in PBS, the average capture efficiencies of PC9, H441, DLD1 and A549 were 37.1, 68.2, 60.4 and 72.4%, at a concentration of 10 μg/ml, and 48.7, 82.1, 75.5 and 88.4%, at a concentration of 100 μg/ml, respectively (Fig. 4). When spiked in blood, the average capture efficiencies were 13.7, 33.0, 21.2 and 27.7% at a concentration of 10 μg/ml, and 19.5, 38.2, 33.0 and 46.8% at a concentration of 100 μg/ml (Fig. 5),

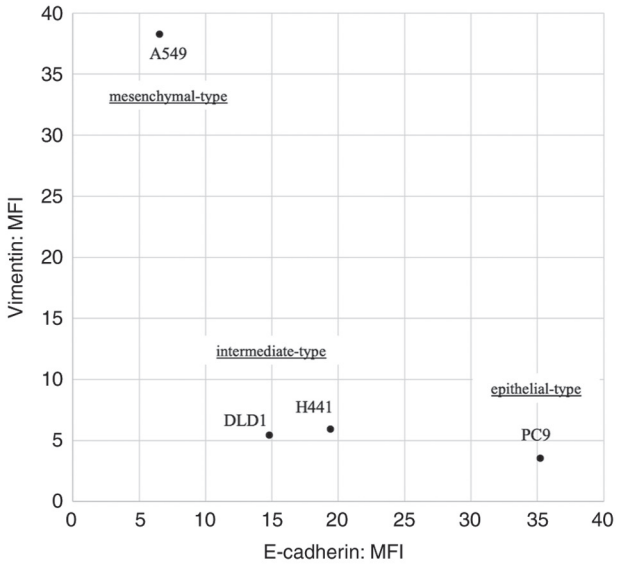

Figure 1. Classification of cell lines based on the flow cytometric analysis of E-cadherin and Vimentin expression. MFI, mean fluorescence intensity.

respectively. The capture efficiency of the CSV-chip was also in accordance with the expression intensity of CSV.

Discussion

The present study focused on CSV, a novel marker for EMT-CTCs, as an alternative to traditional EpCAM-based capture methods. The findings demonstrated that the CSV-chip effectively captured mesenchymal-type cells, which are often overlooked by EpCAM-dependent systems. This advancement represents a significant improvement in cancer diagnostics and monitoring, offering a more comprehensive tool for detecting metastatic cancer and studying EMT-CTCs, which are pivotal subtypes involved in invasive growth and metastatic spread.

Histological evaluation remains a cornerstone of oncology diagnostics, providing essential insight into prognosis and treatment planning for various cancer types. Traditionally, this method relies on invasive tissue biopsies, which pose several challenges, including patient discomfort, potential complications and the risk of sampling errors due to tumor heterogeneity. By contrast, liquid biopsies offer a less invasive alternative by enabling real-time monitoring, comprehensive profiling and early detection of relapse (21,22). Although CTCs are promising biomarkers for liquid biopsies, their clinical utility has been limited by low detection rates. The CellSearch system, which is the only FDA-approved device for clinical CTC detection, relies on an EpCAM-based capture method (3) that can overlook tumor cells that have undergone EMT. This limitation underscores the need for alternative detection methods and further refinements (7,8).

EMT is a critical process in tumor invasion and metastasis (6), which highlights the necessity for EpCAM-independent capture systems. Vimentin, a key marker of EMT, has been associated with tumor metastasis (23-25). Traditionally, the expression of vimentin in blood cells has previously restricted its use in CTC capture (26). However, recent research has identified a subset of aggressive CTCs that express vimentin on their surface (CSV) and exhibit an EMT phenotype. This finding suggests a higher likelihood of tumor recurrence and a poorer prognosis for patients with CSV-positive CTCs (19).

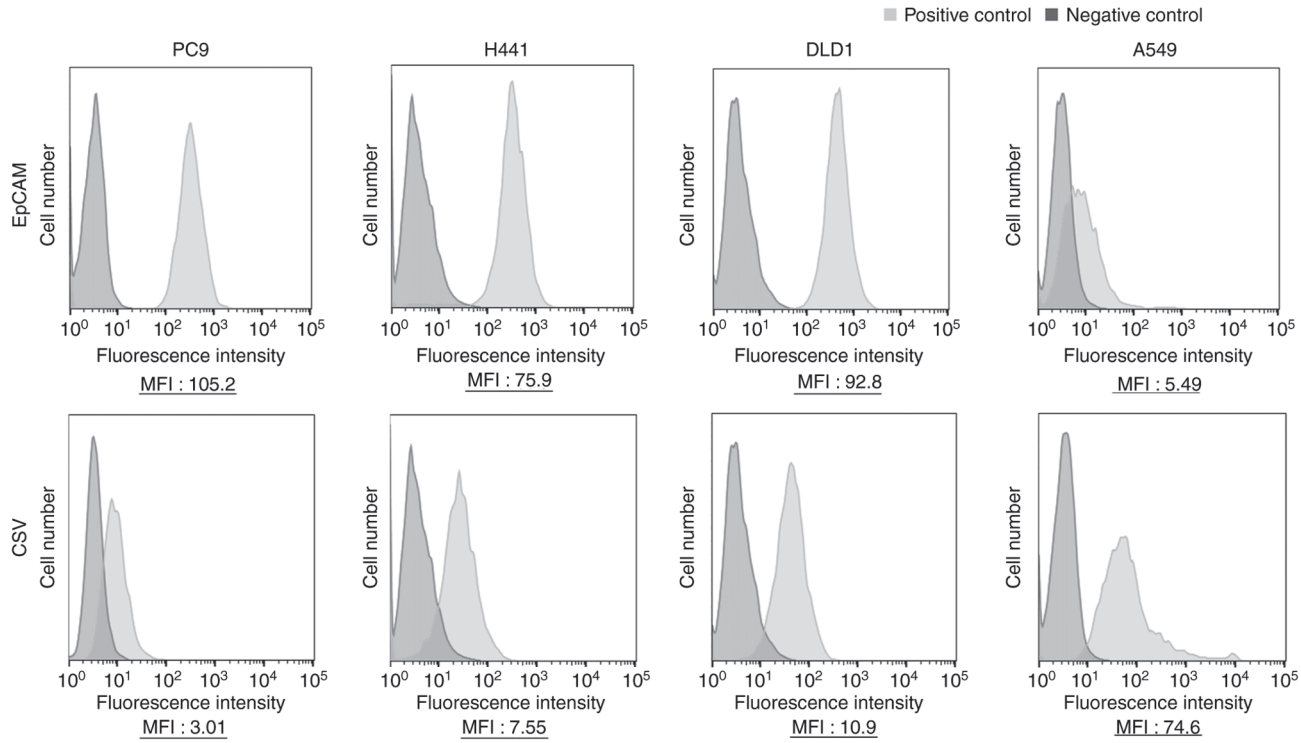

Figure 2. Flow cytometric analysis. EpCAM and CSV expression on cell lines. The left population represents the unstained control group used to establish baseline fluorescence levels. EpCAM, epithelial cell adhesion molecule; CSV, cell surface vimentin; MFI, mean fluorescence intensity.

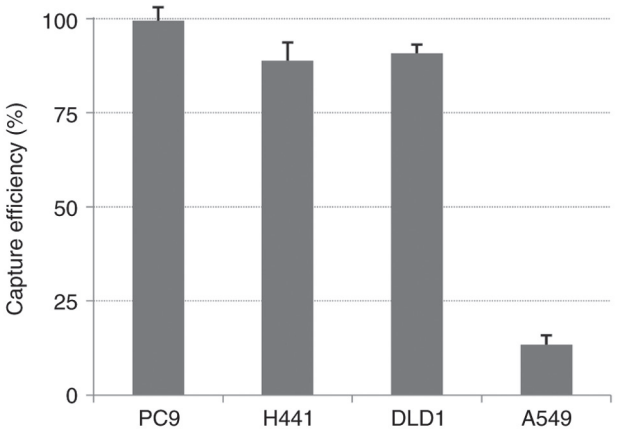

Figure 3. Capture efficiency of EpCAM-chip when tumor cells were spiked into blood. EpCAM, epithelial cell adhesion molecule.

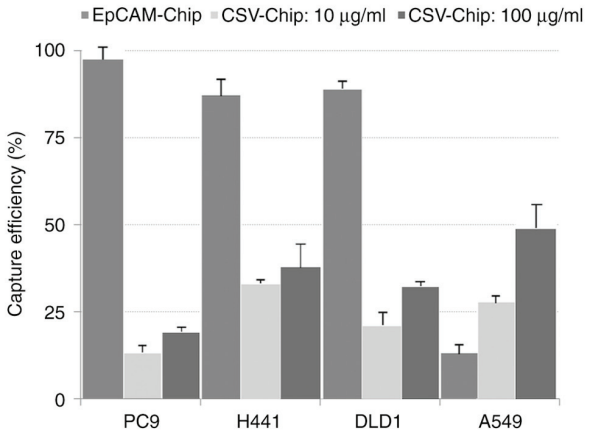

Figure 5. Capture efficiency of CSV-chip when tumor cells were spiked into blood; comparison with EpCAM-chip. CSV, cell surface vimentin; EpCAM, epithelial cell adhesion molecule.

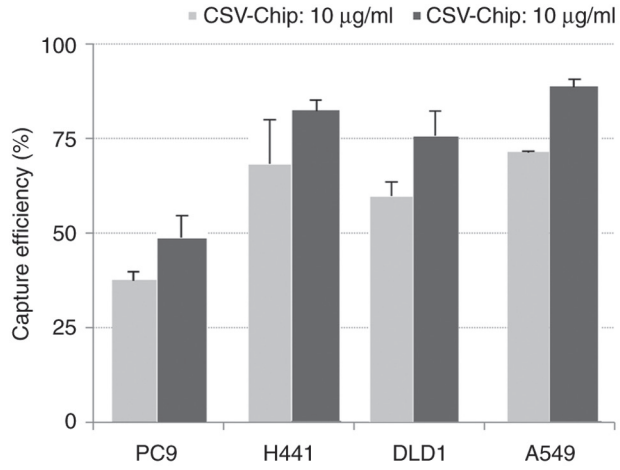

Figure 4. Capture efficiency of CSV-chip when tumor cells were spiked into PBS. CSV, cell surface vimentin.

The development and validation of monoclonal antibody clone 84-1, specific to CSV, represents a significant advancement in targeting and capturing these cells (17-19).

In the present study, by conjugating anti-CSV antibodies to a universal CTC-chip designed to accommodate various antibodies, a system for capturing EMT-CTCs was developed. This system allowed for a more nuanced understanding of the role of CSV-positive CTCs in cancer progression and metastasis. The comprehensive classification of lung and colon cancer cell lines based on EMT markers revealed that EpCAM was strongly expressed in epithelial-type cells but was significantly reduced in mesenchymal-type cells. Conversely, CSV expression was more prevalent in cells with mesenchymal traits, highlighting its potential as a specific marker for mesenchymal tumors.

Despite these encouraging results, the CSV-chip demonstrated only moderate capture efficiency when cells were spiked into blood samples, likely due to the inhibitory effects of blood components on antigen-antibody interactions (12). This suggests that, while the CSV-chip shows considerable promise, optimizing its conditions and making additional refinements are essential for enhancing its clinical performance. The present study identified a CSV concentration of 100 μg/ml as optimal based on blood-based concentration studies; however, further research is required to validate this concentration for clinical applications. In addition, pretreatment processes such as hemolysis, CD45 depletion or peripheral blood mononuclear cell fractionation should be further explored to enhance the capture efficiency and ensure more accurate detection.

Recent studies have highlighted the increased counts of CTCs and the higher proportions of CSV-positive CTCs in patients with lymph nodes or distant metastases (27). Notably, patients with CSV-positive CTCs experienced significantly shorter disease-free survival compared to those with EpCAM-targeted CTCs, whose prognosis remained unaffected (18,28,29). These findings underscore the critical role of CSV-positive CTCs in patient prognosis and support ongoing investigations into antibody therapeutics targeting CSV to disrupt tumor-forming cells (30,31). Our previous reports examining the prognostic significance of EpCAM-CTCs have demonstrated the clinical impact of CTC counts (15), and the study of CSV-CTCs provides an opportunity to gain deeper insights into the complexities of CTCs and their role in cancer progression.

Despite these promising results, one limitation of the present study was the absence of clinical sample data. To address this limitation, work is in progress to condition and collect additional clinical samples for analysis to validate the efficacy and clinical relevance of the CSV-chip in real-world settings. This validation is essential for the broader application and acceptance of the CSV-chip in clinical practice. The ongoing investigation into CSV-CTCs is expected to refine our understanding of CTCs in clinical contexts and potentially enhance the prognostic and therapeutic strategies in cancer care.

In conclusion, the development of the CSV-chip represents a significant advancement in CTC detection technology. By enabling the capture of a broader range of CTCs, including those undergoing EMT, the CSV-chip enhances our ability to monitor and understand metastasis more effectively. Future research should focus on validating these findings in patient samples and integrating this technology with downstream molecular analyses. This approach will provide deeper insights into the biology of metastasis and guide personalized cancer

treatment strategies, ultimately contributing to more effective management of metastatic cancer.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MK, KY and FT conceived and designed the study. TK, TM, RO, HM, MT and KK assisted with the study setup and provided critical insights. MK, TK and MM performed cell experiments. TO provided the CTC-chip and technical support. MK drafted the manuscript. FT was involved in the project management and supervised the study. MK and FT confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was reviewed and approved by the Institutional Review Board of the University of Occupational and Environmental Health, Japan (Approval No: 10-127). The healthy volunteer (author MK) donated blood and provided formal written informed consent for its use in this study for experimental purposes. Data collection from healthy subjects was included in the Ethics Committee approval (approval no. H26-15).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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