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## Intratumor heterogeneity in epithelial cancer: Assessment in cell line models and circulating tumor cells

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## Intratumor heterogeneity in epithelial cancer: Assessment in cell line models and circulating tumor cells

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### Abstract

**Background:** CTCs are present only in small numbers in patients' blood. This study aimed to establish a protocol for enumeration and phenotypic characterization of circulating tumor cells (CTCs) by ImageStreamX MK II (AMNIS) imaging flow cytometry and to characterize the expression of epithelial, mesenchymal, and stem cell markers in CTCs. **Methods:** The study used the FaDu cell line at different passages, cisplatin-resistant (FaDu CDDP-R), and irradiation-resistant (FaDu IR-R) subclones, as well as blood samples from head and neck squamous cell carcinoma (HNSCC) and colorectal cancer (CRC) patients for CTC detection (n = 5). Cells were fixed using 4% paraformaldehyde and permeabilized by incubation in 0.3% Triton X-100. The cell suspensions were stained with 1:100 EpCAM AF-488, 1:50 CD45 AF-647, 1:50 Vimentin AF-555, and 1:50 ALDH1A AF-594 antibodies. **Results:** There were significant differences in EpCAM expression levels between FaDu at late passage and FaDu CDDP-R subclones, as well as between FaDu at late passage compared with FaDu IR-R. Furthermore, CTCs were successfully detected in five patients' samples with various CTC subpopulations. **Conclusions:** Intratumor heterogeneity in CTC phenotypes existed in CRC and HNSCC. Furthermore, three main subpopulations of CTCs were detected. Our findings strongly support future phenotypic studies of CTCs.

**Keywords:** *cancer stem cell, circulating tumor cell, epithelial-mesenchymal transition, imaging flow cytometry, liquid biopsy, neoplastic cells*

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### Introduction

As one of the major causes of worldwide morbidity and mortality, cancer is undoubtedly a prominent global health issue. In 2012, there were roughly 14 million new cancer cases reported worldwide, with reports of mortality reaching about 8 million patients.<sup>1</sup> Metastases cause 90% of cancer-associated mortalities and refer to cancer cells' ability to migrate into adjacent tissue, spread across the body, and form secondary tumors at distant organs.<sup>2,3</sup> Metastatic dissemination occurs as a two-step process. In the first step, a cancer cell from the primary tumor starts losing its adhesion and locally invades the surrounding tissue. Next, cancer cells enter the blood and lymph microvessel system (intravasation). Afterwards, the cancer cells with high survival capacity translocate through the blood vessels to the microvasculature of distant tissues (extravasation). After adapting to the microenvironments of distant tissue, the cancer cells colonize these tissues by starting to proliferate again and form distant metastases.<sup>4</sup> During their journey, cancer cells have to cope with various harsh conditions, such as anoikis, shearing forces, and

immune surveillance in the bloodstream.<sup>5</sup> As a result, only an estimated of 0.01% of the cancer cells in systemic circulation are able to form macroscopic metastases in distant organs.

The metastatic process begins when individual cells or a small group of cancer cells, with the ability to travel and invade, separate themselves from the primary tumor. Cancer cells migrate by means of a tightly regulated process defined as epithelial to mesenchymal transition (EMT). EMT plays a crucial role during embryonic development and organogenesis, but in pathological conditions such as tumorigenesis, it provides epithelial cells with a collection of individual traits that increase their migration capacity.<sup>6</sup> The main characteristics of the EMT program are the loss of adherence junctions and apical–basal polarities, as well as the acquisition of motility and invasion.<sup>7</sup>

Tumor cells can display different phenotypical and morphological states, as reflected by differences in gene expression, motility, metabolism, proliferation, and metastatic potential. These differences can be perceived

by comparing different tumors, within the same patient (intratumor heterogeneity) or by comparing one patient to another (intertumor heterogeneity).<sup>8</sup> Intratumoral heterogeneity originated from genetic, epigenetic, and environmental effects and produced marked diversity within cancer cell populations. Cancer heterogeneity presents a significant challenge for developing effective cancer treatment strategies. On the other hand, characterizing heterogeneity opens us up to better understanding its role in tumor progression and therapy resistance.<sup>9</sup>

Unfortunately, characterizing intratumor heterogeneity for diagnostic purposes, as well as to monitor clonal dynamics over the course of therapy using multiple temporally and spatially independent biopsies, is highly risky. This high risk is due to the invasiveness of the method. Therefore, liquid biopsies have attracted considerable attention due to their minimally invasive nature. Liquid biopsies allow serial sampling to characterize molecular alterations with the primary tumor or metastasis. Cell-free circulating free DNA, circulating tumor-derived exosomes, and circulating tumor cells (CTCs) are the three current components of liquid biopsy. However, the spread of these serum biomarkers makes them difficult to detect. Moreover, technologies to identify, quantify, and characterize these biomarkers require further development.<sup>10</sup>

At the same time, the cancer cell acquires a mesenchymal phenotype together with the expression of vimentin and N-cadherin as mesenchymal markers. Thus, technologies that rely only on EpCAM and cytokeratin expression for CTC detection might be unsuccessful to capture all subpopulations of CTCs.<sup>11,12</sup> N-cadherin and vimentin are proteins, which are mainly expressed in mesenchymal cells, and are considered as EMT markers. A hallmark of mesenchymal character is the down-regulation of E-cadherin and the up-regulation of N-cadherin. Moreover, vimentin expression increases cell motility and induces mesenchymal shape of cells. Conversely, SNAIL and TWIST are the transcription factors that control mesenchymal cell differentiation. Kallergi *et al.* demonstrated a correlation between EMT and disease progression through CTCs with mesenchymal marker expression that was detected intermittently in an advanced-stage patient as compared to patients with localized disease.<sup>12,13</sup>

Additionally, another subset of cancer cells is predicted to have stem cell properties, such as renewal and multipotency. The discovery of cancer stem cells (CSCs) shows that similar to normal cells, the cells within a tumor are hierarchically structured and reflect an important source of intratumor heterogeneity.<sup>14</sup> A CTC subpopulation with stemness phenotype can be identified through aldehyde dehydrogenase-1 (ALDH1) and C-Met expression, which is associated with

metastasis.<sup>11</sup> ALDH1 was associated with breast CSC properties both in vivo and in-vitro. The cells with stemness characteristics had an ability to form differentiated solid tumors in NOD/SCID mice. Thus, CTCs expressing ALDH1 indeed has potentials to form metastasis.<sup>15</sup>

CTC analysis is mostly performed by enrichment and subsequent detections using several technologies. CTC enrichment is achieved using physical properties, such as size, density, deformability, and electric charges. The enrichment may also be gained using biological features, such as the expression of cell surface markers. Conversely, detection of CTCs can be achieved by PCR-based methods or immunofluorescence staining followed by microscopy.<sup>16</sup> Even so, it needs to be noted that the limitation of CTC analysis still is a serious challenge for any analytical system. Furthermore, optimal sensitivity and specificity of analytical methods are required for identification and characterization of CTCs.<sup>17</sup>

Therefore, the aim of this study was to characterize the expression of epithelial, mesenchymal, and stem cell markers in CTCs that could be instrumental in assessing tumor heterogeneity and could serve as prognostic factors for tumor progression. Further, the study aimed to establish a protocol for enumeration and phenotypic characterization of CTCs using the ImageStreamX MK II (AMNIS) imaging flow cytometry. We hypothesized that the prognostic impact of CTCs on tumor progression might depend on their EMT/stemness phenotype. Using cell lines and CTCs from patients with epithelial cancers, we developed a protocol to assess intratumoral heterogeneity in the expression of CTC epithelial, mesenchymal, and stem cell markers.

## Methods

**Cell cultures.** FaDu cell lines was used at different passages, as well as the cisplatin-resistant (FaDu CDDP-R) and irradiation-resistant (FaDu IR-R) subclones. FaDu C54 and FaDu C46 were used as sensitive clones, whereas FaDu C5 and FaDu C78 were used as resistant clones. All cell lines were grown in minimum essential medium (MEM) and incubated in a CO<sub>2</sub> incubator for several days until the cells proliferated and were optimally differentiated. Firstly, all liquid in the Petri dish was removed. We then washed the cells using 7 mL PBS, which was later discarded. We added 3 mL trypsin–EDTA (0.25%) to detach the cells into a single cell and incubated the cell in a CO<sub>2</sub> incubator for 5 min. Next, the Petri dish was shaken smoothly, and then the cell suspension was transferred into a 5 mL Falcon, filled with 9 mL MEM. The cells underwent 3 min centrifugation at 1400 rpm at room temperature (RT). Afterwards, the supernatant was discarded and 3 mL of MEM was added into a new

15 mL Falcon tube so the cells could be suspended for immunostaining. Additionally, 10 mL of MEM was added into the new Petri dish for culturing the new passage. mRNA expression of vimentin and ALDH1A in the FaDu cell line, as well as in the HeLa and A549 cell lines, were assessed using Light Cycler 480 II (Roche, Switzerland) to confirm specific antibody binding, correlated with the cell line's mRNA expression pattern. TUBA was used as a reference gene.

**Patients and blood samples.** five patients were recruited for this cross-sectional study, all of whom provided written informed consent for research participation. Their blood was drawn at Charité Universitätsmedizin Berlin. Study inclusion criteria were locally advanced head and neck squamous cell carcinoma (HNSCC) with lymph node metastases ( $\geq$ T2, N1, MX) or colorectal cancer (CRC) (T2) with metastases to increase the probability of detecting CTCs. Additionally, only patients with serum CTCs underwent further analysis of CTC subpopulations. We analyzed an 8 mL sample of whole EDTA blood from each patient. Every patient's clinical data were recorded following ethical approval, which was granted by the Ethical Committee of Charité Berlin (EA4/087/15).

**Patient samples processing.** Whole blood samples were obtained from patients with HNSCC or CRC at Charité Universitätsmedizin Berlin. We carefully overlaid 8 mL whole blood samples, from each patient, in 15 mL Ficoll in 50 mL Falcon tube. The blood vial was rinsed twice by RPMI media and transferred to the tube. The next step was 35 min of centrifugation at 400  $\times$ g at RT. After that, the interphase layer containing peripheral blood mononuclear cells and CTCs was transferred into a new 50 mL Falcon tube and cold PBS was added, up to 50 mL following a 15 min centrifugation at 500  $\times$ g at 4 °C. The supernatant was discarded prior to immunostaining.

The cell suspension was stained against different extracellular and intracellular protein markers subjected to measurement using ImageStreamX MK II (AMNIS) imaging flow cytometry (Merck, Germany). The absolute numbers of CTCs were estimated using the total number of positive tumor marker events in the analyzed sample. Separately, to test our methods for CTC detection specificity, we examined two patients' blood samples by applying negative selection to enrich CTCs. We used RosetteSep (STEMCELL Technologies, Canada), combining it with Ficoll and gradient centrifugation, and comparing it with Ficoll by itself.

**Immunostaining of cell suspension from cell lines and patient samples.** The 100  $\mu$ L cell suspensions, obtained from cell lines or patients' blood samples, were first stained as vital cells with extracellular markers.

Therefore, FcR Blocking was performed by adding 10  $\mu$ L blocking reagent and incubating it for 10 min at 4 °C. Afterwards, antibodies were added according to the established protocol 1:100 anti-human EpCAM AF488 (BioLegend, USA) and 1:50 anti-human CD45 AF647 (Exbio, Czech Republic) and incubated for 20 min at 4 °C. Following a washing step with PBS, cells were fixed using 4% paraformaldehyde in PBS and incubated for 15 min at RT. The cell suspension was then washed with 10% FBS in PBS. After that, cells were permeabilized by incubation in 0.3% Triton X-100 for 10 min at RT. Cells were then washed again in 1000  $\mu$ L of 10% FCS in PBS and centrifuged at 3500 rpm for 5 min at RT. The cell suspension was incubated with 1:50 anti-Vimentin AF555 (Cell Signaling, USA) and 1:50 anti-ALDH1A1 AF594 (Abcam, UK) antibodies for 20 min at RT. Cells were washed in 1000  $\mu$ L of 10% FBS in PBS and, after centrifugation, were stained with 1:250 Hoechst 33342 in PBS for 20 min at RT. The washed cell suspension was then transferred from a FACS tube into a 1.5 mL tube. The FACS tube was subsequently washed with PBS to remove the remaining cells then centrifuged to reduce its volume to the required 50–150  $\mu$ L. The sample can be analyzed immediately or stored at 4 °C until processing with ImageStreamX MK II (AMNIS) imaging flow cytometry (Merck, Germany).

**Cell line experiment and CTC detection from patient samples using imaging flow cytometry.** Imaging flow cytometry by the ImageStreamX MK II (AMNIS) acquires each cell in 12 different channels, enabling simultaneous display of qualitative and quantitative images of cells stained with different fluorochrome-labeled antibodies. Additionally, brightfield and side scatter images are also taken. In order to do this, fluorochromes are excited by 405, 488, 561, 592, and 642 nm lasers. Like conventional flow cytometers, fluorescence intensities are acquired for each sample beside the microscopic cell images. Beads and samples are automatically injected into the flow cell to form a single core stream that is hydrodynamically focused in front of the imaging 40 $\times$  objective. A slow flow rate was chosen in order to optimize the sensitivity and resolution of cell images. A compensation matrix was composed according to the data from single color compensation controls for each fluorochrome to remove spectral overlap. The results were analyzed using IDEAS software.

**Statistical analysis.** To compare differences in mean fluorescence intensity (MFI) of epithelial, mesenchymal, and stem cell markers in FaDu clones and subclones, as well as for each individual patient, paired and unpaired t-tests were performed. Calculations were done using SPSS 15.0 for Windows (Chicago, IL, USA). Two-sided  $p < 0.05$  were considered statistically significant.

## Results

**Method optimization.** A method was developed for the detection, enumeration, and characterization of CTCs that would rely not only on EpCAM but also on other antigens. An immunostaining protocol was established with 4% paraformaldehyde for fixation. This protocol applied after extracellular staining and continued with 0.3% Triton X-100 for cell membrane permeabilization before intracellular staining. The appropriate centrifugation speed and volume of antibodies were optimized. The cell suspensions were measured using ImageStreamX MK II (AMNIS) imaging flow cytometry, which can analyze the expressions of multiple biomarkers in an individual tumor cell (Table 1).

To optimize the staining, we used cell lines with high expression of each antigen as positive controls. This was done to test the sensitivity of this method. The cell lines consisted of FaDu pharynx squamous carcinoma cells for EpCAM, HeLa cervix adenocarcinoma cells for vimentin, A549 lung carcinoma cells for ALDH1A, and leukocytes from a healthy donor as a positive control for CD45 staining. We added the DNA dye Hoechst 33342 to stain the cell nucleus in order to discriminate vital cells from necrotic cells or debris. We found that this method was sensitive for detecting cells with marker features (Figure 1).

Additionally, we assessed the mRNA expression of vimentin and ALDH1A in FaDu, HeLa (positive control for vimentin), and A549 (positive control for ALDH1A) cell lines. This measurement was used to confirm that the antibody binding was specific and correlated with the mRNA expression pattern of the cell line. The values of mRNA expression are in fold change compared to the values of TUBA as a reference gene. HeLa cells had a higher Mrna expression of vimentin, whereas ALDH1A was strongly expressed in A549 cells. We also found that there was no mRNA expression of vimentin in FaDu cells. Moreover, both

FaDu and HeLa cells had a very low ALDH1A mRNA expression (Figure 2).

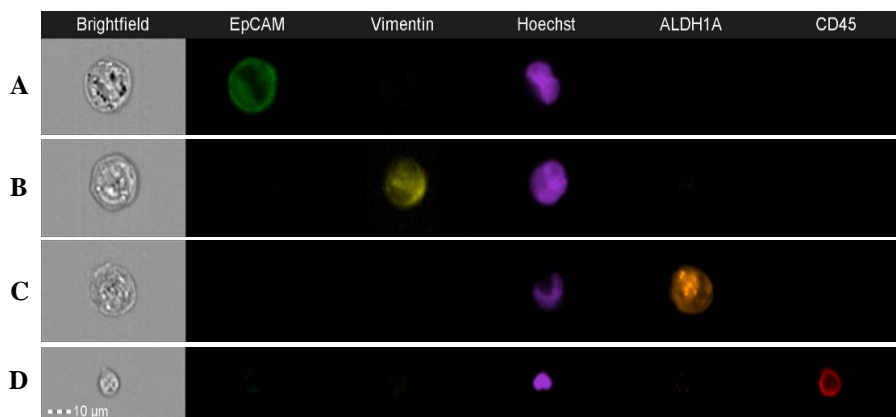
In addition, healthy whole blood was measured by applying Ficoll, which revealed no CTCs. To avoid bias, the experimenter was blinded as to the origin of the blood sample. Again, the result was negative for CTCs when a blood sample from a healthy donor was analyzed. Therefore, this method had an excellent specificity for determining CTCs as our target cells.

In general, the FaDu cells expressed EpCAM as an epithelial marker and ALDH1A as a stem cell marker; however, FaDu cells did not express vimentin as a mesenchymal marker. This finding was correlated with the absence of vimentin expression at the transcriptome level in FaDu cell subclones that was measured using real-time PCR (Figure 2A).

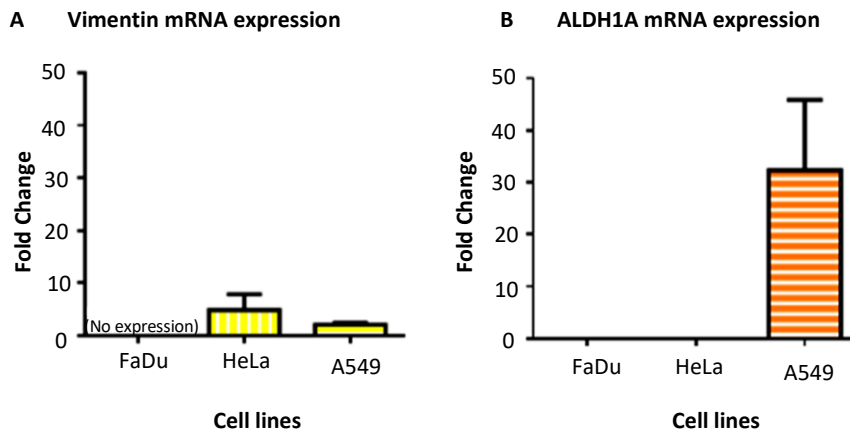
Statistically, the MFI of EpCAM expression was significantly higher in FaDu at late passage than that in FaDu CDDP-R subclones ( $410.9 \pm 39$  vs.  $270.4 \pm 15.7$  MFI,  $p = 0.029$ , respectively). On the other hand, FaDu at late passage was also significantly higher compared with FaDu IR-R ( $410.9 \pm 39$  vs.  $269.3 \pm 25.1$  MFI,  $P = 0.038$ , respectively) (Figure 3A), whereas the difference between FaDu at early passage and FaDu at late passage was not significant. Furthermore, there were no significant differences in expression of ALDH1A as a stem cell marker among all types of FaDu cells (Figure 3B).

**Table 1.** Inspire software settings for imaging flow cytometry

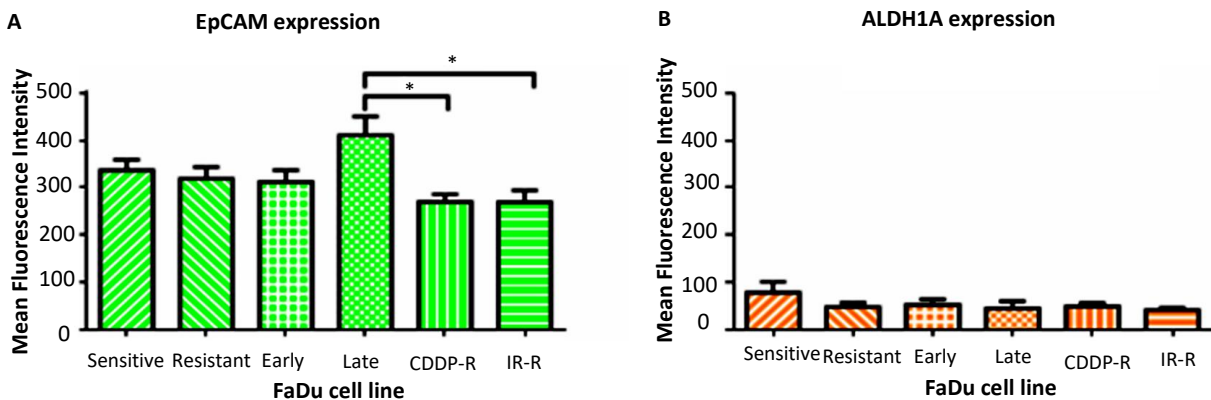
Marker	Channel	Wavelength	Power	Magnification
EpCAM	2	488 nm	25 mW	40X
Vimentin	3	561 nm	200 mW	40X
Hoechst	7	405 nm	40 mW	40X
ALDH1A	10	592 nm	250 mW	40X
CD45	11	642 nm	75 mW	40X



**Figure 1.** A. EpCAM expression in FaDu pharynx squamous carcinoma cells. B. Vimentin expression in HeLa cervix adenocarcinoma cells. C. ALDH1A expression in A549 lung carcinoma cells. D. CD45 expression in leukocytes



**Figure 2.** A. HeLa cells had a strong mRNA expression of vimentin, whereas FaDu did not express vimentin at the transcriptome level. B. A549 cells had a higher expression of ALDH1A compared with FaDu and HeLa cells.



**Figure 3.** A. FaDu at late passage was significantly higher than FaDu CDDP-R ( $p = 0.029$ ). Late passage was also significantly higher than FaDu IR-R for EpCAM expression ( $p = 0.004$ ). B. No significant differences were observed, in all types of FaDu cells, for ALDH1A expression ( $p = 0.039$ ). Values are expressed as mean fluorescence intensity  $\pm$  SEM. \* $p < 0.05$  ( $n = 3$ )

**Patients' characteristics.** Table 2 summarizes the patients' characteristics. Briefly, we recruited five patients for this research project, including two patients with HNSCC and three patients with CRC. All patients had documented metastases to either the liver or lungs. Three of the five patients were not being treated at the time of blood collection. Each patient underwent venipuncture to withdraw 8 mL whole blood at Charité Universitätsmedizin Berlin.

**CTC enumeration and characterization in patient samples.** CTCs were successfully detected in five patients' samples, with a mean CTC detection of  $69.8 \pm 33.4$  in 8 mL whole blood. The frequency of CTC subpopulations was expressed in various ways such as expressions of one marker, a combination of two, or even three markers for both interpatients and intrapatient (Table 3).

Patient #1 had the highest number of CTCs, which was more than double the amount found in patients #2 and #5. There were 9 CTCs found in patient #3 and 12 in patient #4 (Table 3 and Figure 4A).

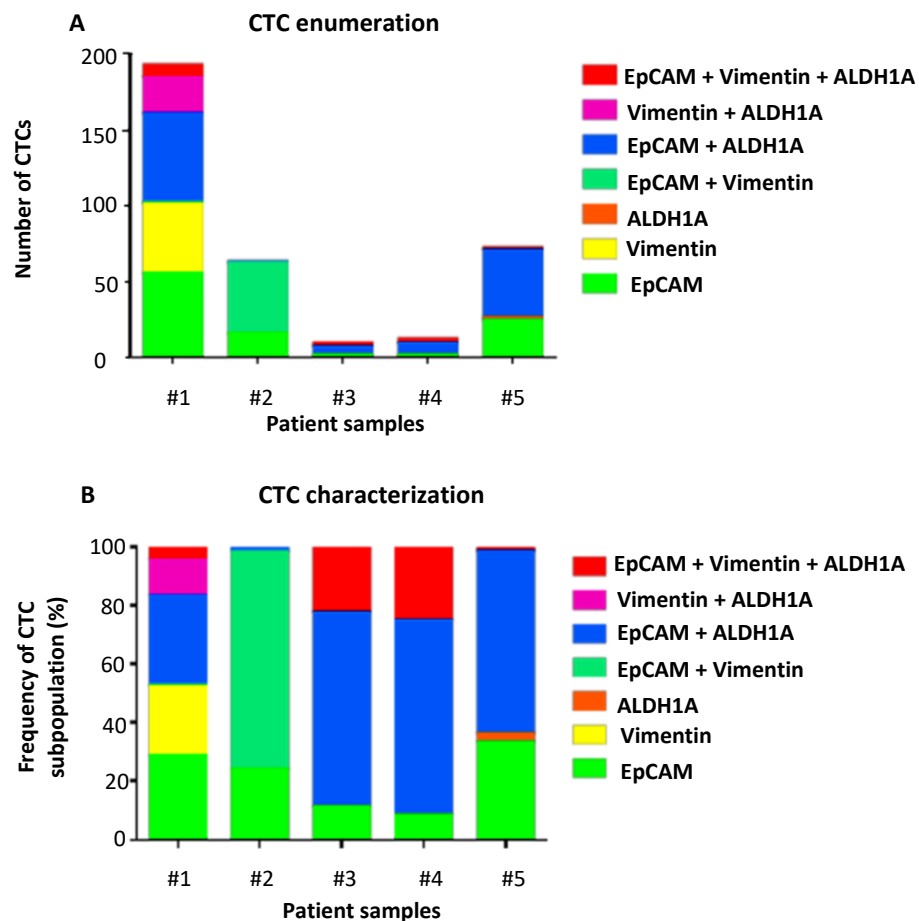
**Table 2.** Clinical characteristics of the study population

Patient	Age	Type of Cancer	Metastases	Therapy
1	52	Colorectal	Liver	Yes
2	36	Colorectal	Liver	No
3	49	Colorectal	Lung	No
4	50	Head and Neck	Lung	Yes
5	63	Head and Neck	Lung	No

Both EpCAM and a combination of EpCAM plus ALDH1A were expressed in all patients. However, CTCs expressing EpCAM plus ALDH1A comprised only 1.6% of all CTCs in patient #2. A combination of EpCAM plus vimentin expression was found in most CTCs (74.6%) in patient #2, whereas there was no expression of this combination found in other patients except in small numbers in patient #1 (0.5%). Interestingly, we detected a mixture of three CTC subpopulations in four patients (4.2%, 22.2%, 25%, and 1.4% in patients #1, #3, #4, and #5, respectively) (Table 3 and Figure 4B)

**Table 3.** Number and characterization of CTCs that were detected

Patient	Total CTCs	EpCAM only	Vimentin only	ALDH1A only	EpCAM + Vimentin	EpCAM + ALDH1A	Vimentin + ALDH1A	EpCAM + Vimentin + ALDH1A
#1	193	55 (28.5%)	46 (23.8%)	-	1 (0.5%)	59 (30.6%)	24 (12.4%)	8 (4.2%)
#2	63	15 (23.8%)	-	-	47 (74.6%)	1 (1.6%)	-	-
#3	9	1 (11.1%)	-	-	-	6 (66.7%)	-	2 (22.2%)
#4	12	1 (8.3%)	-	-	-	8 (66.7%)	-	3 (25%)
#5	72	24 (33.3%)	-	2 (2.8%)	-	45 (62.5%)	-	1 (1.4%)



**Figure 4.** A. Total number of CTCs that were detected in five patient samples. B. Various CTC subpopulations in every patient, expressed as percentages

The percentage of CTCs were calculated that would likely be missed if we analyzed the patients' samples using EpCAM as the only CTC detection marker. The result was 36% and 2.8% of potential CTC loss for patients #1 and #5, respectively. In contrast, none of the

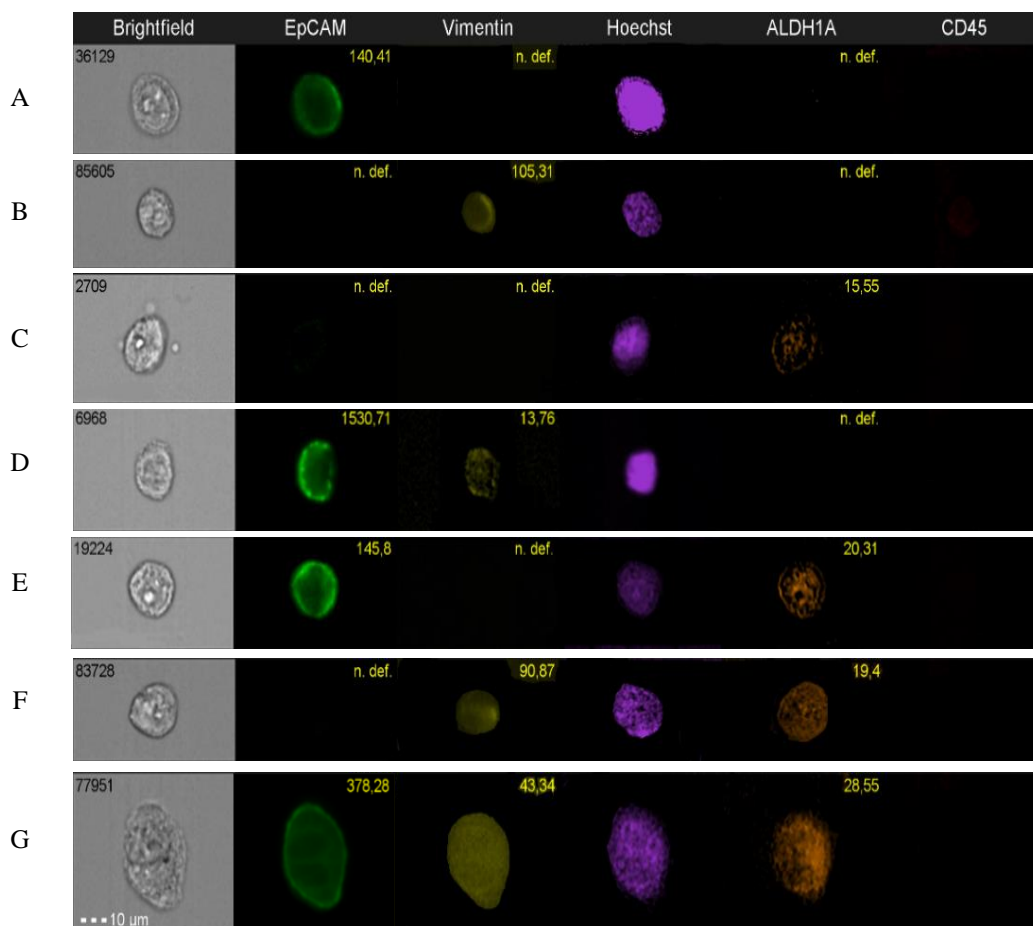
detected CTCs would have been missed in the remaining samples. On average, the potential CTC loss that was calculated from all patients was 7.8%. Figure 5 shows images of several CTCs that were detected by ImageStreamX MK II (AMNIS) imaging flow cytometry

and analyzed by IDEAS software. The number in the image from the brightfield channel is the object number of the cell, whereas the value in the images from the EpCAM, vimentin, or ALDH1A channels represents the corrected fluorescence intensity. This value was obtained by dividing the fluorescence intensity with the object area of the cell.

In general, EpCAM expression was significantly higher, compared with vimentin and ALDH1A, in CTCs from all patients. On the other hand, the expression of vimentin and ALDH1A was significantly different in all patients. This finding showed that CRC and HNSCC are associated with intratumor heterogeneity in CTC phenotypes. In patient #1, who had CRC with liver metastasis, we found 193 CTCs. The expression of EpCAM was significantly higher than ALDH1A ( $115.07 \pm 7.98$  vs.  $9.58 \pm 0.88$  MFI,  $p = 0.000$ ,

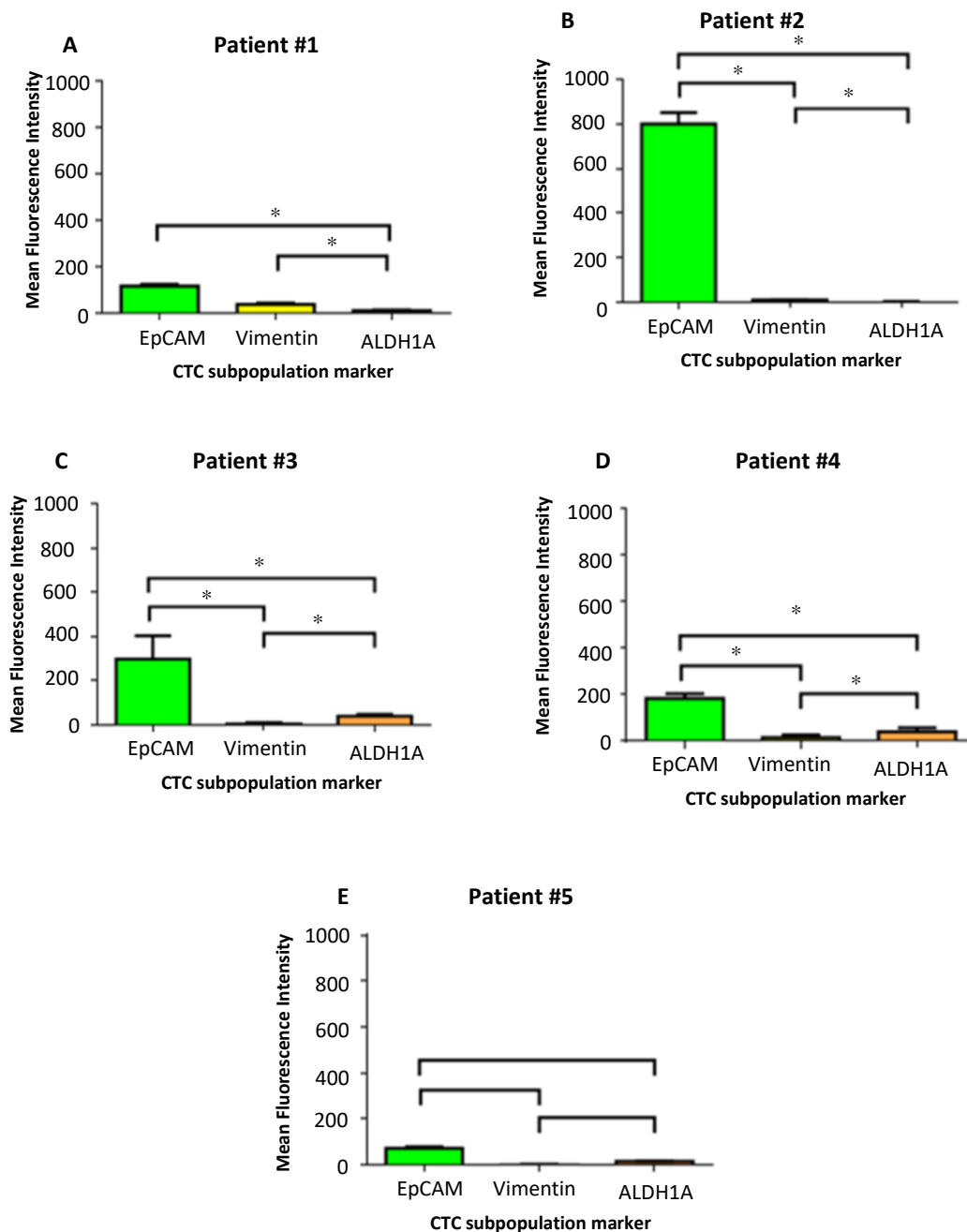
respectively), and vimentin had a significantly higher expression than ALDH1A ( $36.55 \pm 4.22$  vs.  $9.58 \pm 0.88$  MFI,  $p = 0.018$ , respectively) (Figure 6A).

EpCAM was highly expressed in patient #2 compared with the other patients and was also significantly higher compared with vimentin and ALDH1A ( $p = 0.000$ ,  $p = 0.000$ , respectively). Vimentin expression was significantly different from ALDH1A ( $p = 0.000$ ) (Figure 6B). Furthermore, in patients #3, #4, and #5, EpCAM expression was significantly higher compared with vimentin and ALDH1A (Patient #3;  $p = 0.024$ ,  $p = 0.025$ , Patient #4;  $p = 0.000$ ,  $p = 0.000$ , Patient #5;  $p = 0.000$ ,  $p = 0.000$ , respectively). In addition, ALDH1A was significantly higher compared with vimentin in patients #3, #4, and #5 ( $p = 0.001$ ,  $p = 0.002$ ,  $p = 0.000$ , respectively) (Figure 6C, 6D, 6E).



**Figure 5.** A representative example of intrapatient heterogeneity in CTC expression profiles. Cell suspensions from patient samples were stained with fluorescence-labeled EpCAM, vimentin, ALDH1A, and CD45 antibodies and were measured with ImageStreamX MK II (AMNIS) imaging flow cytometry with the laser excitation at 405, 488, 561, 592, and 642 nm. A. A CTC with EpCAM expression. B. A CTC with vimentin expression. C. A CTC with ALDH1A expression. D. A CTC with combined EpCAM and vimentin expression. E. A CTC with combined EpCAM and ALDH1A expression. F. A CTC with combined vimentin and ALDH1A expression. G. A CTC with mixed EpCAM, vimentin, and ALDH1A expression





**Figure 6.** CTC subpopulation marker expressions in individual patients #1, #2, #3, #5, and #6. EpCAM was significantly higher in all patients, compared with vimentin and ALDH1A. Values are expressed as mean ± SEM.

**Discussion**

Using ImageStreamX MK II (AMNIS) imaging flow cytometry, which has optimal speed and sensitivity, combined with phenotyping abilities of flow cytometry plus the detailed imagery of microscopy, we successfully established a method for detecting EMT and stem cell markers in individual tumor cells. In our study, FaDu were used as a model for HNSCC, since two previous studies failed to detect HNSCC CTCs using this technology.<sup>18,19</sup> In addition, we investigated intratumor heterogeneity using the EMT program. We found that our cell line models significantly differed in their EpCAM

expression levels. However, there was no intratumor heterogeneity with respect to the presence of EpCAM-negative subpopulations within EpCAM-positive bulk cells. This may be because we selected cells with homogenous phenotypes when establishing cell lines. Furthermore, intratumor heterogeneity was reflected through seven CTC subpopulations that were detected and observed in all patients. Herein we discuss the appearance of CTC subpopulations in order to better understand CTCs and their correlation with the EMT program, potentially producing metastases and intratumor heterogeneity.

EpCAM is a specific marker for epithelial cells and is highly expressed in many carcinomas. Due to this, the only CTC detection assay that is approved by the U.S. Food and Drug Administration, the CellSearch system, relies on EpCAM+ expression. Unfortunately, CTCs can escape EpCAM-dependent capturing due to EMT reprogramming where EpCAM is down-regulated on cancer cells. Consequently, epithelial cells lose cell-cell adhesion and their cell polarity. Simultaneously, cancer cells gain migratory and invasive properties to become mesenchymal stem cells. Some studies observed that EMT is critical for metastasis by stimulating motility, invasion, and dissemination of epithelial cancer cells.<sup>6,20,21</sup>

A recent study established vimentin as a marker for detection of CTCs that had undergone EMT in a patient with metastatic colon cancer. The same study also demonstrated an association between the number of vimentin+ CTCs and treatment outcomes.<sup>22</sup> Furthermore, as a cancer stem cell marker, ALDH1A is important for the differentiation, expansion, tumor cell self-protection, and development of therapy resistance, although less is known about its role in mediating metastasis. ALDH1A expression in CTCs has been correlated with metastatic progression, poor clinical outcomes, and therapy responses in patients with metastatic breast cancer.<sup>23,24</sup>

FaDu is one of the human cancer-derived cell lines that are widely used to study the biology of squamous cell carcinoma. This study demonstrated that there was a significantly different expression of EpCAM in FaDu cell line model, particularly between FaDu at late passage and FaDu cisplatin-resistant as well as FaDu irradiation-resistant subclones. One of the reasons for the different expression could be epigenetic regulation. The down-regulation of the EpCAM gene expression via hypermethylation of its promoter is the cause of EpCAM expression loss in human embryonic stem cells and breast cancer cells.<sup>25,26</sup> Previous study reported the increasing invasiveness of lung adenocarcinoma cell lines as a result of hypermethylation of the EpCAM promoter and decreased expression of EpCAM.<sup>27</sup> In this study, the reduction of EpCAM expression in both FaDu cisplatin-resistant and FaDu irradiation-resistant subclones may correlate with the invasiveness traits, which could be tested in future studies.

CTCs detected in all five patients' samples with metastasis. The purpose of recruiting patients with metastasis was to increase the probability of CTC detection, considering this was our first study for CTC detection and characterization using ImageStreamX MK II (AMNIS) imaging flow cytometry. In three patients with CRC, we detected at least 9 CTCs/8 mL whole blood. Previous clinical studies showed that the cut-off value for CTC numbers at baseline, which identified patients with metastasized colorectal cancer with

significantly worse prognosis, was  $\geq 3$  CTCs/7.5 mL.<sup>28</sup> Moreover, in the samples of two patients with HNSCC, we detected 12 and 72 CTCs, respectively, and the detection of  $\geq 2$  CTCs/7.5 mL correlated with a poor prognosis in HNSCC using CellSearch system.<sup>29</sup> Based on these two cut-off values of CTC for CRC and HNSCC, all patients in this study may have a poor prognosis for their diseases.

Interestingly, observing of seven CTC subpopulations with distinct marker expression, the result found that these subpopulations consisted of CTCs that only expressed EpCAM, vimentin, or ALDH1A, a combination of two markers, such as EpCAM plus vimentin, EpCAM plus ALDH1A, vimentin plus ALDH1A, and a combination of three markers. The subpopulation of EpCAM+/vimentin-/ALDH1A- CTCs might represent cancer cells, which lost their apical-basal polarization and cell-cell contacts in primary tumor, but are yet to acquire a partial or complete mesenchymal state at least at the proteomic level. Conversely, EpCAM-/vimentin+/ALDH1A- CTCs might stand for cancer cells that have changed completely from epithelial to mesenchymal phenotype through the EMT program. Based on the previous studies, in which the expression of mesenchymal markers in tumor tissue was associated with poor prognosis,<sup>30-32</sup> it could be speculated that the detection of EpCAM-/vimentin+/ALDH1A- CTCs may identify the patient with worse prognosis.

In a model hamster oral keratinocytes model, a previous study demonstrated the cooperation between epithelial and mesenchymal cells during the formation of metastasis.<sup>33</sup> While both populations of pure epithelial and mesenchymal cells developed tumors at the subcutaneous injection site in mice, interestingly, only mesenchymal cells intravasated into the bloodstream and none of the cell types developed lung metastasis on their own. In contrast, when pure epithelial and mesenchymal cells were injected intravenously, only the epithelial cells formed lung metastasis. Additionally, the mixed epithelial and mesenchymal cells were administered subcutaneously resulting in both cell types entering the blood stream and creating lung metastasis from the epithelial cells. These experiments suggest that the process of completion of metastasis might require cellular cooperation between epithelial and mesenchymal cells via mesenchymal invasion, which enables "passenger" epithelial cells to follow.<sup>33</sup> The "passenger" epithelial cells may be another explanation of EpCAM+/vimentin-/ALDH1A- CTCs. More importantly, EpCAM-/vimentin+/ALDH1A-CTCs may have a role as the "leader" mesenchymal cells in terms of cell cooperation that was observed in this study.

Two further possible models for establishing metastases have been proposed. The first model is the "plasticity

type I” metastasis model where cancer cells respond to signals from the microenvironment and in which a dynamic EMT/MET program drives metastasis formation.<sup>34</sup> Based on this model, EMT/MET plasticity is increased, and as a result, a partial EMT or a hybrid epithelial/mesenchymal phenotype appears on a single CTC.<sup>35</sup> In this study, we detected CTCs in patient samples with combined EpCAM plus vimentin (EpCAM+/vimentin+/ALDH1A-) expression, supporting the occurrence of such hybrid epithelial/mesenchymal phenotype in patients and not only in experimental models. In many cancer types like breast cancer and melanoma, cells that collectively express epithelial and mesenchymal genes appear dominant. In this manner, a hybrid epithelial/mesenchymal phenotype of CTCs may mean a worse prognosis for patients compared with patients with CTCs of exclusively a mesenchymal phenotype.<sup>35,36</sup>

The second model of metastasis formation is the “genetic type II,” which suggests that cancer cells go through a permanent and irreversible EMT. Here, cancer cells display stem-like properties, and their phenotypic plasticity is lost upon genetic alterations that endow them with the capacity to colonize distant organs and outgrow without the MET process.<sup>37</sup> This model is supported by our observation of EpCAM-/vimentin+/ALDH1A+ and EpCAM-/vimentin-/ALDH1A+ CTCs. Interestingly, in intermediary cells, mixed epithelial, mesenchymal, and stemness features (EpCAM+/vimentin+/ALDH1A+) were expressed in our observations of four patients.

An interesting previous study demonstrated a correlation between mesenchymal CTCs and disease progression via sequential CTC monitoring in 11 patients. In a longitudinal monitoring of EMT phenotypes in CTCs from an index patient using RNA *in situ* hybridization, reversible shifts between epithelial and mesenchymal cell fates accompanied each cycle in response to therapy and disease progression. Every time an index patient had a good treatment response, most CTCs showed epithelial features. Conversely, when the patient had disease progression, most CTCs were in a mesenchymal state.<sup>35</sup> Therefore, characterization of CTCs using EMT and stem cell markers could assist prognostication of tumor progression, and applying ImageStreamX MK II (AMNIS) imaging flow cytometry, which has high sensitivity and specificity, would be beneficial for managing patients who are undergoing cancer treatment.

## Conclusions

By using samples from patients with cancer as the positive control and samples from healthy donors as the negative control, we demonstrated that this platform was very specific for CTC detection. This platform was also reproducible and very sensitive, considering we

managed to identify CTCs in 100% of our patients. The CTC subpopulations present per patient reflect the heterogeneity of biomarker expression in CTCs. Future studies may investigate the clinical utility of CTCs by correlating frequencies and phenotypic changes in CTCs with clinical outcomes that reflect cancer prognosis and investigate CTC subpopulation expression over the course of therapy in a large cohort of patients with HNSCC or CRC.

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## Conflict of Interest Statement

We confirm that none of the authors have any competing interests in the manuscript.

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