Mesenchymal markers: the new avenue for circulating tumor cells detection

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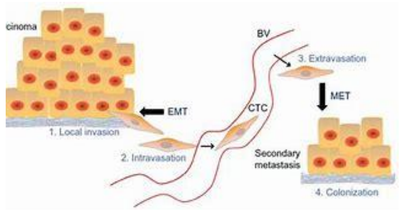
Introduction

Most CTC isolation systems are based on epitope-dependent CTC capture using epithelial markers. However, it is known that tumor cells can undergo epithelial-to-mesenchymal transition (EMT) when extravasating from the primary tumor to enter the bloodstream and eventually establish distant metastases (**Figure 1**). Epitope-dependent CTC detection platforms have limited sensitivity for detection of mesenchymal CTCs, leading to the inability to capture potentially clinically relevant CTCs, particularly in cancers with high mesenchymal phenotypes such as non-small cell lung cancer (NSCLC).

In this study, we evaluated the performance of a research use only assay developed for the identification of epithelial and mesenchymal CTCs enriched by the Parsortix® System, a label-independent microfluidic device that isolates cells based on their size and compressibility.

Figure 1. Epithelial to mesenchymal transition. Figure taken from Guttilla Reed I., *Cell Health and Cytoskeleton*. 2015



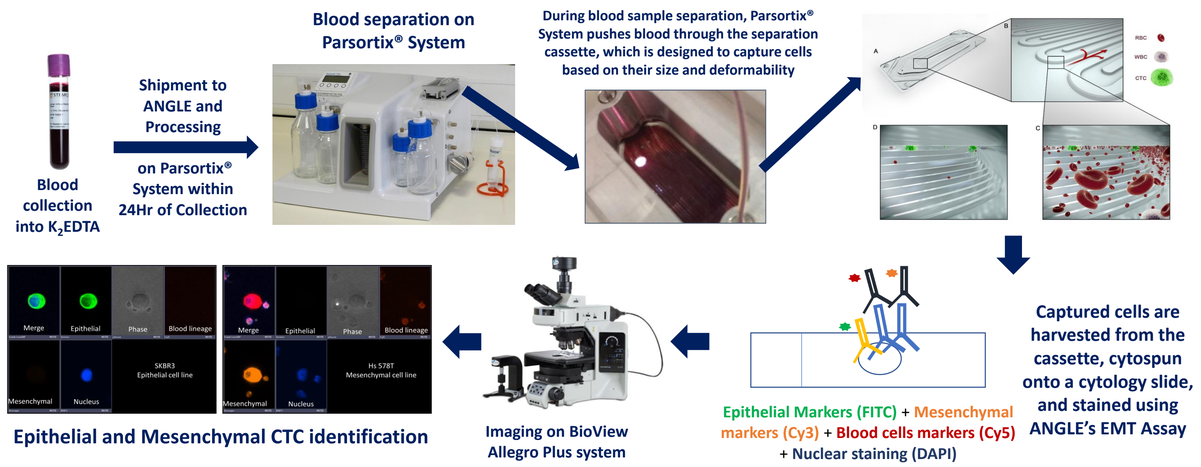
Workflow

Analytical performance of the assay was assessed by spiking known numbers of breast cancer cell lines into blood samples from 12 healthy volunteers. The contrived samples were processed as per the workflow in **Figure 2**. SKBR3 cells were used as a positive control for epithelial markers and as a negative control for mesenchymal markers. Hs 578T cells were used as a positive control for mesenchymal markers and as a negative control for epithelial markers. The sensitivity, specificity and consistency of the assay were defined as follow:

- Sensitivity = % of cancer cells stained for the positive expressed marker
- Specificity = 100 – (% cancer cells stained for the negative expressed marker)
- Consistency= Coefficient of variation (CV%) of fluorescent signal intensity across donors

Performance of the assay using clinical samples was assessed on 107 healthy volunteers (HV), 47 metastatic breast cancer (MBC) patients, and 48 non-small cell lung cancer patients (NSCLC) processed as depicted in **Figure 2**.

Figure 2. Schematic representation of assay workflow. Peripheral blood (8 – 10mL) was drawn into K_2EDTA tubes and processed on Parsortix® Systems within 24 hours from draw. Cells captured in the Parsortix® cassette due to their larger size and lower compressibility compared to other blood components were harvested, cytospun onto slides, and immunofluorescently stained for detection of epithelial and mesenchymal CTCs. Slides were imaged using a BioView AllegroPlus imaging system. Epithelial CTCs were defined as **FITC+**, **Cy3-**, **Cy5-**, **DAPI+** cells, mesenchymal CTCs were defined as **FITC-**, **Cy3+**, **Cy5-**, **DAPI+** cells, and EMT-transitioning CTCs were defined as **FITC+**, **Cy3+**, **Cy5-**, **DAPI+** cells. Identification was based on morphological evaluation and thresholding techniques using fluorescence intensity of the markers established on control cancer cell lines (SKBR3 as epithelial and Hs578T as mesenchymal-like breast cancer cell lines).



Results

- ANGLE's EMT assay showed a high degree of analytical sensitivity (97-98%) and specificity (96-98%), as well as good consistency (CV=22-25%), which is illustrated in **Figure 3A.** Results from clinical samples confirmed this finding, showing high specificity (96%). CTC-like cells were observed in only 4% (4/107) of the healthy volunteers, of which 2 declared suspected endometriosis at the time of blood donation, indicating the possible presence of Circulating Endometrial cells (Chen Y, *et al.,* Evaluation of Circulating Endometrial Cells as a Biomarker for Endometriosis, Chin Med J (Engl), 2017). The assay identified ≥1 CTC in 31% of the NSCLC patients and 40% of the MBC patients (**Figures 3B** and **3C**). CTC clusters were observed in 63% of the CTC-positive MBC patients and 33% of the CTC-positive NSCLC patients. Cluster size ranged from 2-22 CTCs (**Figures 3B** and **3C**).
- Phenotypically, more CTCs expressing only mesenchymal markers were harvested from NSCLC patients compared to MBC patients, 38% vs 25% respectively (**Figures 3B** and **3C**). A larger proportion of CTCs expressing both epithelial and mesenchymal markers were detected in both cancer types (59% in NSCLC and 74% in MBC) and a small percentage of the CTCs harvested expressed just the epithelial markers.
- In MBC, the positivity rate for CTC presence was significantly higher in Stage IV compared to Stage III patients, and a significantly higher number of CTCs was captured in T3-4 patients compared to T1-2. In NSCLC patients, a significantly higher number of EMT CTCs was captured in patients diagnosed with squamous cell carcinoma compared to those diagnosed with adenocarcinoma.

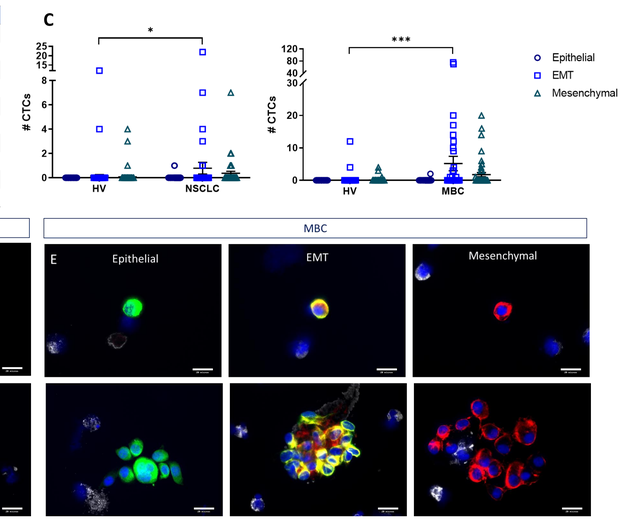
Figure 3. CTCs identification and phenotyping in MBC and NSCLC patients. (A) Table show analytical study results; (B) Table shows number of donors included in each cohort (N), % donors with at least 1 CTC across all donor or across only metastatic patients, range and mean of CTCs captured, % donors with at least 1 CTC cluster, range of the number of CTCs found in clusters, % of CTC by EMT phenotype; (C) Dot plots show mean ± SEM of the number of CTCs captured across the three cohorts and divided by staining phenotype (*P≤0.05, ***P≤0.001, Two-way ANOVA followed by Sidak's multiple comparison test); (D) Representative images of NSCLC and (E) MBC single CTC (top) or CTC clusters (bottom), including epithelial (left), EMT (center) and mesenchymal cells (right). Merge colors: FITC (epithelial markers) in green, Cy3 (PD-L1) in red, Cy5 (blood lineage markers) in white, DAPI (nuclear staining) in blue.

A

	Specificity	Sensitivity	Consistency (CV%)
Epithelial Markers	97.1%	98%	22%
Mesenchymal markers	97.9%	96%	25%

B

	HV	MBC	NSCLC
Number of donors	107	47	48
Donor with ≥1 CTC	4% (4/107)	40% (19/47)	31% (15/48)
Metastatic donors with ≥1 CTC	-	52% (11/21)	25% (11/44)
CTC range	1-12	1-96	1-22
CTCs mean across CTC+ (≥1) donors	6	17	4
Donors with ≥1 CTCs' cluster / CTC+ donors	50% (2/4)	63% (12/19)	33% (5/15)
CTCs per cluster (range)	2-12	2-17	2-22
Epithelial CTCs	0%	1%	3%
EMT CTCs	75%	74%	59%
Mesenchymal CTCs	25%	25%	38%



Conclusions

- ANGLE's EMT assay successfully allowed for the identification of epithelial AND mesenchymal CTCs in the population of cells harvested by the Parsortix® System from blood samples of MBC and NSCLC patients.
- This study highlights the importance of the inclusion of mesenchymal markers into CTC characterization, as a large proportion of the CTCs harvested by the Parsortix® System would have been missed using an epithelial-only based approach, particularly in NSCLC patients.