Development of a multi-parameter immunofluorescence assay for identification of circulating tumor cells with epithelial-mesenchymal phenotype

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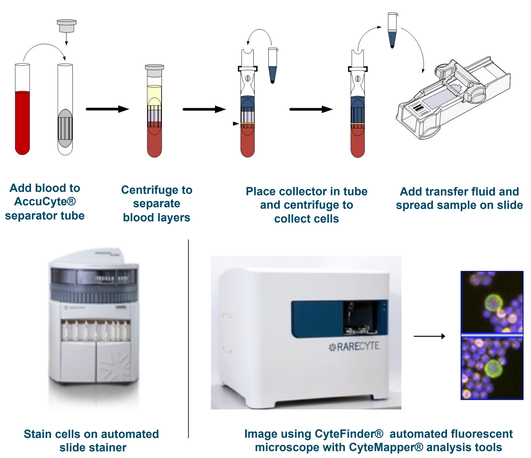
Background

The epithelial-mesenchymal transition (EMT) is understood to be an important step in invasion and metastasis of cancer. It is of increasing investigational interest to identify circulating tumor cells (CTCs) that express mesenchymal markers that indicate entrance into EMT. Such cells may not express surface epithelial markers (such as EpCAM) which are often used to capture CTCs. RareCyte has developed a platform for automated visual identification and retrieval of rare cells in blood by immunofluorescence (IF) that does not rely on surface marker capture. We developed a 5-parameter assay to identify epithelial CTCs with or without mesenchymal differentiation.

Methods

Buffy coats isolated from blood were spread onto slides using the AccuCyte® sample preparation system. A 5-parameter IF assay was developed with the following markers: Sytox orange (nuclear dye) / cytokeratin (CK) and EpCAM (epithelial cocktail) / vimentin (mesenchymal) / CD45, CD11b, and CD105 (exclusion cocktail) / EGFR (investigational biomarker). Slides were stained with the assay on an auto-stainer and scanned with the CyteFinder® imaging system. Specificity of the assay was validated on positive and negative control cell lines spiked into healthy donor blood. The assay was also applied to a pilot set of blood samples from 5 prostate and 3 breast cancer patients that were collected under an IRB-approved protocol. Nucleated cells that were epithelial marker-positive and exclusion marker-negative were identified as CTCs. CTCs were categorized as epithelial (epiCTC) if they were vimentin-negative and epithelial-mesenchymal (E-M CTC) if they were vimentin-positive. In a sample from a patient with a known mutation in TP53 (G244S) from tissue tumor analysis, individual epiCTCs, E-M CTCs and WBC control cells were mechanically retrieved using the CytePicker® module and whole genome amplification was performed. The resulting DNA was PCR amplified at the TP53 mutation site and sequenced.

Sample preparation and analysis workflow



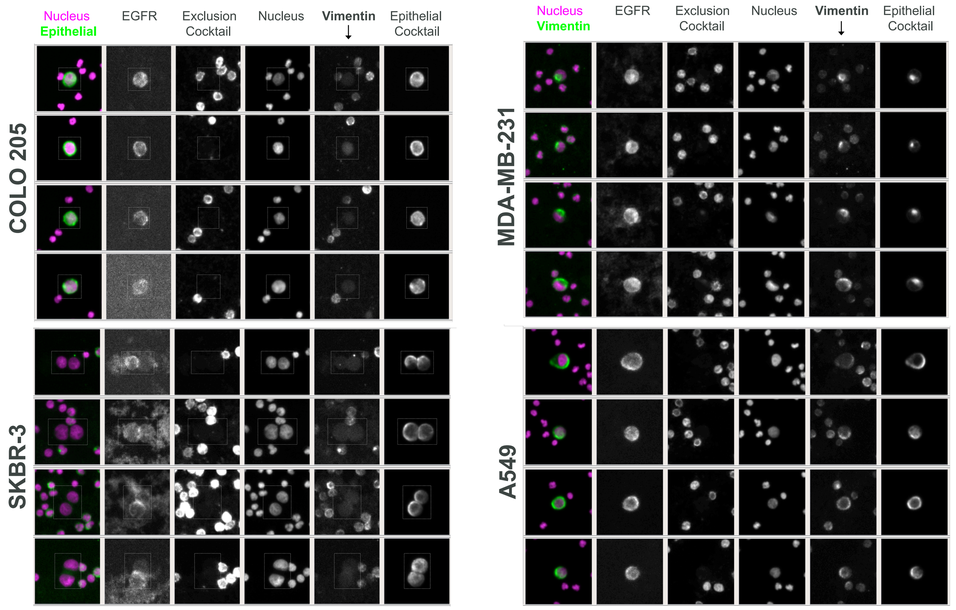


Figure 1. Mesenchymal differentiation assay applied to spike-in samples. Panels show individual channel images of cells detected after spiking into blood, processing to slides using the AccuCyte system, and imaging with the CyteFinder system. COLO 205 and SKBR-3 are epithelial cell lines without mesenchymal differentiation since vimentin is not expressed. MDA-MB-231 and A549 are epithelial cell lines with mesenchymal differentiation since vimentin is expressed.

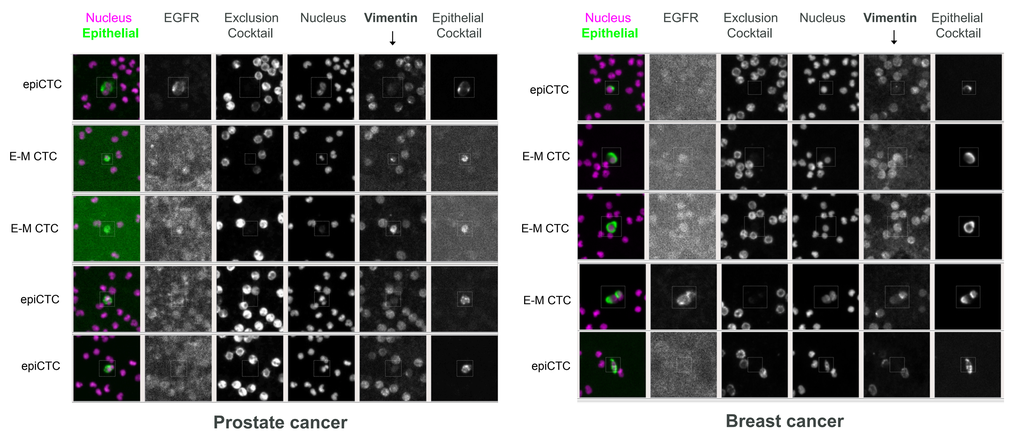


Figure 2. Mesenchymal differentiation assay applied to patient samples. Panels show individual channel images cells of CTCs identified from prostate and breast cancer patients. Expression of vimentin is present in three of the prostate CTCs and two of the breast CTCs, indicating mesenchymal differentiation.

Table 1. Quantitation of epiCTCs and E-M CTCs in patient samples

Patient	Breast cancer			Prostate Cancer				
	1*	2	3	1	2	3	4	5
CTCs counted	84	15	15	164	56	86	1	1
epiCTCs	55	5	13	140	56	34	1	0
E-M CTCs	29	10	2	24	0	52	0	1
% epiCTC	**65.5**	**33.3**	**86.7**	**85.4**	**100.0**	**39.5**	**100.0**	**0.0**
% E-M CTC	**34.5**	**66.7**	**13.3**	**14.6**	**0.0**	**60.5**	**0.0**	**100.0**

*male

Results

The mesenchymal differentiation CTC assay identified epiCTCs in samples spiked with COLO 205 or SKBR-3. In contrast, the assay identified E-M CTCs in samples spiked with MDA-MB-231 or A549; this is consistent with reported mesenchymal differentiation of these lines.

Between 1 and 164 CTCs were counted in the patient samples (Table 1). The average percentage of epiCTCs was 64% (range 0-100%) and of E-M CTCs was 36% (range 0-100%). EGFR-positive epiCTCs and E-M CTCs were identified.

In a sample from a prostate cancer patient with the tumor-associated TP53 G244S mutation, individual CTCs were picked for sequencing. 2 of 2 epiCTCs and 6 of 7 E-M CTC contained the mutation, demonstrating that the E-M cells identified were malignant. 0 of 6 control WBCs contained the mutation.

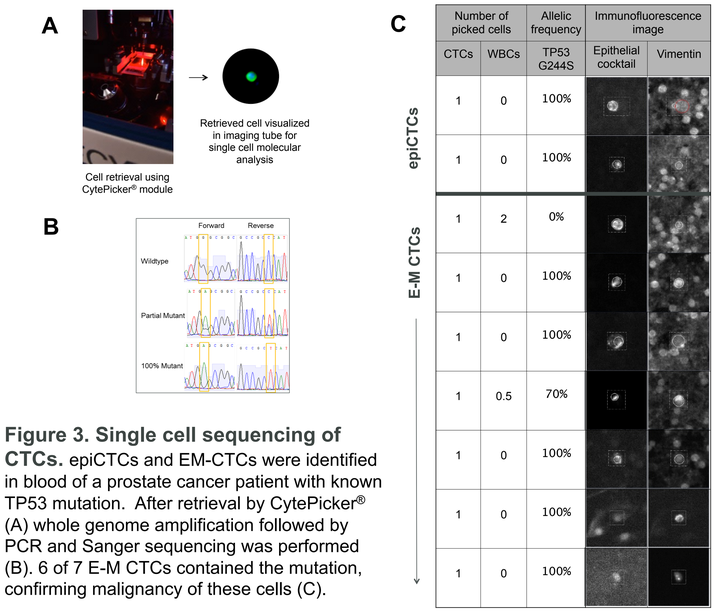


Figure 3. Single cell sequencing of CTCs. epiCTCs and EM-CTCs were identified in blood of a prostate cancer patient with known TP53 mutation. After retrieval by CytePicker® (A) whole genome amplification followed by PCR and Sanger sequencing was performed (B). 6 of 7 E-M CTCs contained the mutation, confirming malignancy of these cells (C).

Conclusions

We have developed a multi-parameter immunofluorescence assay for identification of circulating tumor cells with epithelial-mesenchymal phenotype and applied it to cancer patient samples. Confirmation of malignancy of epiCTCs and E-M CTCs by single cell mutational analysis was demonstrated. The expression of EGFR on CTCs in prostate and breast cancer confirms literature reports.