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Multi-biomarker subtyping of circulating tumor cells using sequential fluorescence quenching

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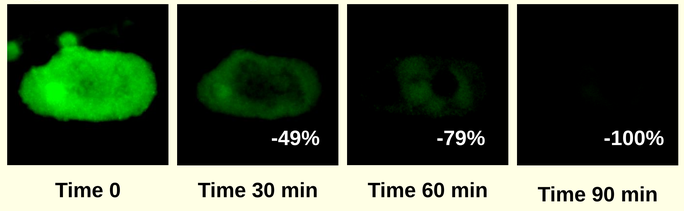
ABSTRACT

Circulating tumor cells (CTCs) are rare but clinically prognostic indicators of cancer status. However their clinical utility has been limited to 2-3 positive markers and 1 negative marker when using traditional fluorescent dyes. In contrast, tissue biopsies allow for numerous subtyping markers, yielding information about the tumor's biology and in predicting treatment response. If CTC analysis is to be useful as a "blood based biopsy", it must move beyond 2-3 identification markers. We describe a straightforward and inexpensive method to capture and identify CTCs using classical fluorescence biomarkers, followed by repeated quenching and restating of 9 unrelated fluorescent antibodies. Specifically, we sought to subtype CTCs with the epithelial to mesenchymal-like phenotype (EMTCTCs) identified using a CTC marker panel (Cytokeratin (CK), EpCAM, CD45) from 12 pancreatic patient samples. We sequentially subtyped specific EMTCTCs with an immunosuppression therapy panel (PD-L1, CXCR4, PD-1) and a mesenchymal marker panel (CD14, CD34, Vimentin), to better interrogate CTCs with the EMT phenotype. Our data demonstrate the ability to sequentially analyze, subtype and track 9 distinct cancer markers on every single isolated EMTCTC.

INTRODUCTION

The presence of circulating tumor cells (CTCs) is an indicator of metastatic disease, used to monitor therapy response and predict outcomes in late stage patients.[1-4] However, CTCs are not equally found in all tumor types, and the low frequency makes the tracking of therapeutic response difficult. EMT-like cells are found in almost all patients with solid malignancies and are theorized to be the aggressive CTC subtype responsible for tumor metastases. Typically, EMT-like cells are identified by their down regulation of EpCAM, and/or Cytokeratin, and up regulation of Vimentin, and/or N-Cadherin.[1-4] We previously reported that CellSieve™ microfilters rapidly and efficiently isolate both CTCs and EMT CTCs from peripheral blood, making it possible to study all CTC subtypes irrespective of proteomic expression.[3-4]

Figure 1. Decrease of signal over time.



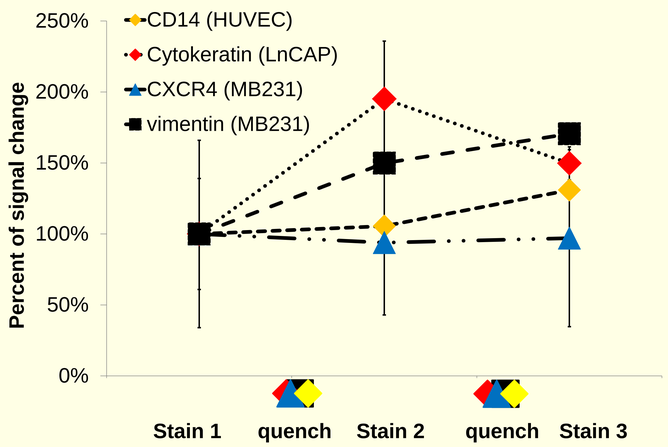


Figure 2. Representative examples of the percent change of signal during multiple rounds of the QUAS-R technique

RESULTS

- CTCs could be identified, marked, fluorescence quenched, and sequential restaining performed.
- No degradation was observed in cell surface/intracellular markers for 3 rounds of QUAS-R restaining.
- 764 EMTCTCs were identified as CK+/CD45-
- EpCAM was positive in only 2% of EMTCTCs.
- EMTCTCs had mesenchymal phenotypes (vimentin/CD34)
- PD-L1 and CXCR4 expressions were highly heterogeneous
- None of the CTCs were PD-1+ or CD14+.

CONCLUSIONS

- Sequential screening, analysis and tracking druggable targets is possible from a blood based biopsy.
- We performed sequential staining of CTCs, quantifying numerous actionable drug targets (e.g. EpCAM, CD31, CD34, CXCR4, Vimentin, PD1, and PD-L1).
- This approach can be used in research, patient selection for clinical trials, ~~and~~ companion diagnostics, and/or monitoring of response for immunotherapy.
- Using QUAS-R, patient samples can now be screened for multiple prognostic and predictive biomarkers from a single sample.

MATERIALS & METHODS

CellSieve™ microfilters were used to isolate CTCs and EMTs cells from 7.5 mL of peripheral blood from patients with breast, lung or pancreatic cancer. Collected cells were fixed, permeabilized, and stained with DAPI and antibodies against cytokeratin 8, 18 and 19, EpCAM and CD45. Cancer derived cells were identified and imaged under a fluorescent microscope. After cell identification, enumeration and subtyping, cells on the filter were marked and samples archived. Archived samples can be reanalyzed for additional markers. The fluorescence is removed using the QUAS-R technique in which each cell is quenched of fluorescence and restained for additional markers of interest. Each cell can be sequentially stained, quenched and reanalyzed for 2 additional sets of relevant markers. The same cells are reevaluated, allowing IHC based scoring of each cell for multiple biomarkers.

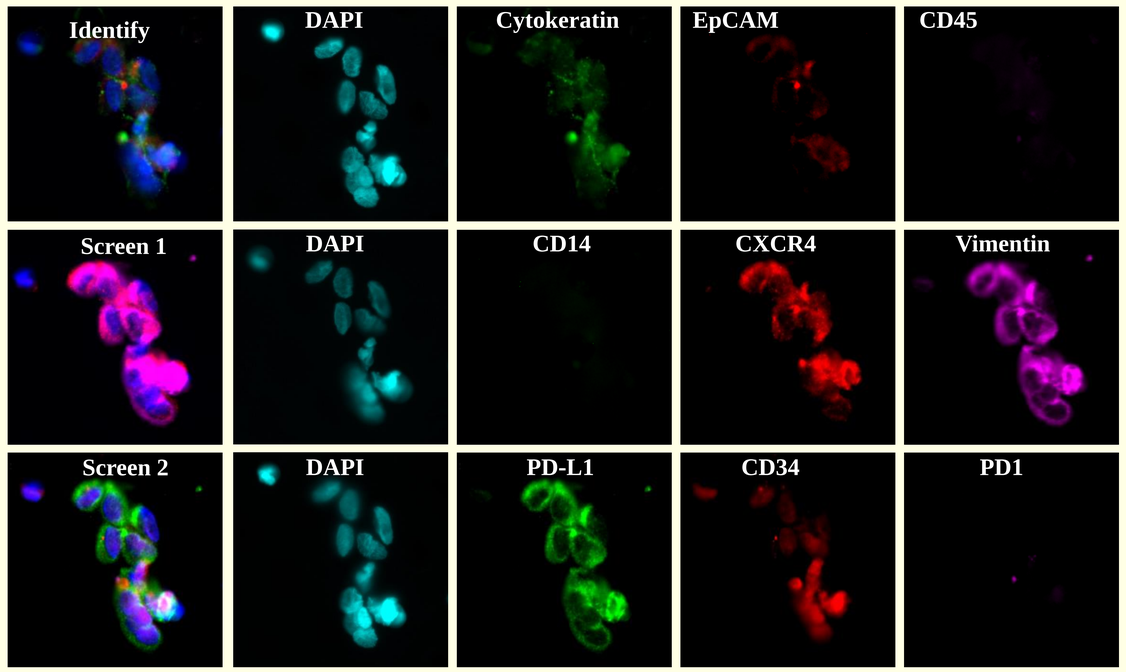


Figure 3. Sequential staining, qualification and quantification with an array of biomarker panels against the same cell cluster. An EMT-like CTC cluster, identified with a "classical" CTC stain (top), a panel of motility markers (middle), and a panel of immune activation markers (bottom).

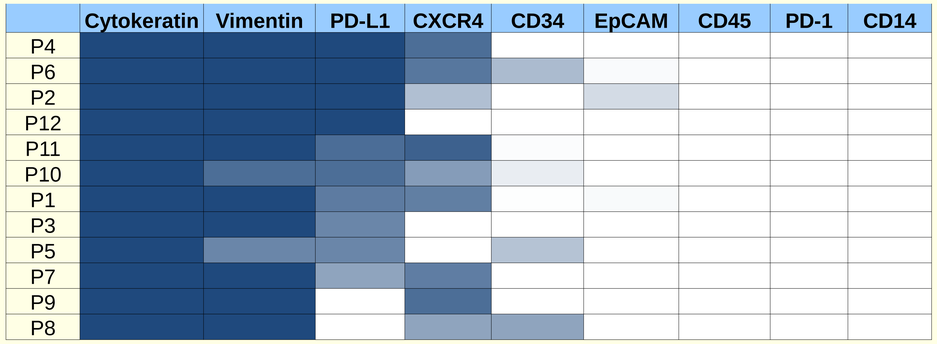


Figure 4. Heat map of the percent of EMT-CTCs positive for the 9 markers (n= 12 pancreatic cancer patients). Dark blue is 100% cell positivity and white is no cell positivity.

Funding Sources

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