ApoStream™ Isolated Circulating Tumor Cells from Primary Breast Cancer Patients Reveals Heterogeneous Phenotypes Related to Epithelial-Mesenchymal Transition and Stem Cell Markers

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Abstract

Background: Detection of circulating tumor cells (CTCs) is an indicator of poor prognosis in patients with metastatic breast cancer and not in primary breast cancer (PBC). The classical phenotypic definition of a CTC is a nucleated (DAPI+) cell that is cytokeratin (CK) positive and CD45 negative. Several reports have shown that epithelial cell adhesion molecule (EpCAM) based capture methods detect only a fraction of CTCs and not the heterogeneous subpopulations of CTCs. Moreover, subsets of CTCs may acquire a more aggressive phenotype with features of invasiveness and motility by undergoing an epithelial to mesenchymal transition (EMT), and down regulating EpCAM. EMT is a hallmark of cellular invasion and metastasis and CTCs undergoing EMT (CTC-EMT) may express the putative cancer stem cell (CSC) like phenotype, $CD24^{low}CD44^{+}$. CTC-EMTs are not readily detected by current CTC detection technologies. Thus, in order to recover a heterogeneous CTC population for more extensive characterization, it is desirable to isolate CTCs using capture methods that are independent of EpCAM. In this report, we used ApoStream™, a novel antibody-free CTC isolation device, that does not rely on EpCAM to capture circulating rare cells, to evaluate the molecular heterogeneity of CTCs. **Objective:** Our aim is to determine whether the presence of CTC-EMT and CSCs in PBC patients receiving preoperative systemic therapy correlates with their ability to achieve a pathological clinical response (pCR). We hypothesize that patients with low EMT-CTC and cancer stem cells are more likely to have a higher pCR rate than patients with high CTC-EMT and CSC counts. **Methods:** Baseline blood samples (3 x 7.5 mL CPT tubes) were obtained from 14 newly diagnosed PBC patients prior to receiving preoperative systemic therapy in an IRB-approved clinical trial and processed using ApoStream™. Isolated cells were stained with anti-CK and anti-CD45 antibodies, and DAPI. In addition, a multiplexed immunofluorescence assay and laser scanning cytometry analysis were applied to identify multiple combinations of CTCs ($CK^{+}CD45^{-}$) for the expression and distribution of EpCAM, vimentin, CD44, CD24, β-catenin and E-cadherin. **Results:** ApoStream™ recovered both $EpCAM^{+}$ and $EpCAM^{-}$ cells. $CK^{+}CD45^{-}$ cells were detected in 10 out of 14 PBC patients. The expression of $EpCAM^{-}$ vimentin^{+} in the $CK^{+}CD45^{-}$ population was heterogeneous across the patient population. Among the $CK^{+}CD45^{-}$ population, E-cadherin and β-catenin were detected in 0-94% (Mean 52%) and 0-37% (Mean 8%), respectively. Patients with $CK^{+}CD45^{-}$ cells had a subset of cells with the putative cancer stem cell phenotype of $CD44^{+}CD24^{low}$. **Conclusions:** Heterogeneous CTC phenotypes with $CD44^{+}CD24^{low}$ in both $EpCAM^{+}$ and $EpCAM^{-}$ subsets were observed in baseline blood samples. This is an ongoing study to collect peripheral blood from patients post surgery to assess whether the detection of CTC-EMTs and cancer stem cells correlates with their ability to achieve a pathological complete remission.

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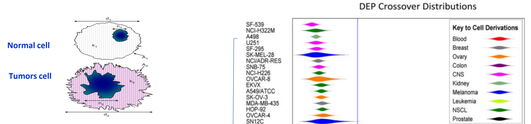
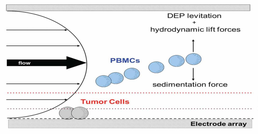
ApoStream™ Technology



(A) Dielectric properties polarizability of cells are dependent upon cell diameter, membrane morphology and conductivity. Inherent differences in morphology of CTCs and normal cells result in different dielectric polarization charges when exposed to an AC electric current.



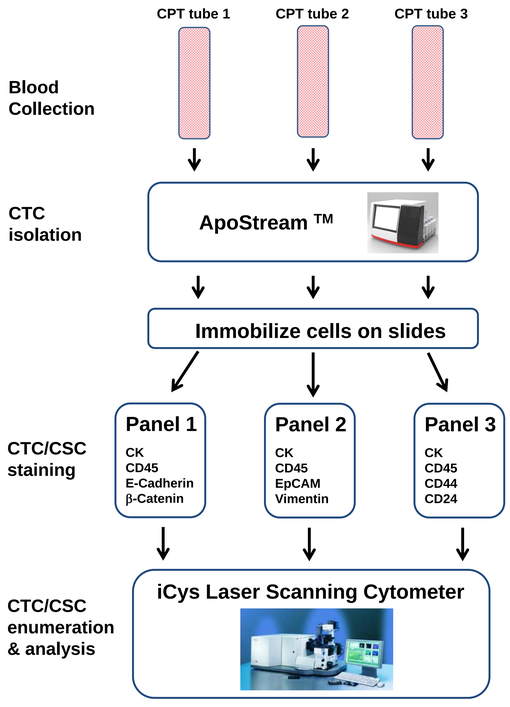
(B) Dielectrophoretic, hydrodynamic and sedimentation forces are utilized to attract CTCs and repel normal cells from the chamber floor. CTCs are collected through a port located in the chamber floor while normal cells flow into a waste port.

(C) Cross-over frequencies from different tumor cell types including breast, colon, ovarian, lung and melanoma cell lines and from peripheral blood mononuclear cells (PBMCs) were determined.[2] The differences in cross-over frequencies between cancer and normal cells enable ApoStream™ to separate CTCs from normal cells

ApoStream™ Prototype Device

Current prototype design shipped to National Cancer Institute & Massey Cancer Center, VA, in Dec 2012

Study Design



$CK^{+}CD45^{-}DAPI^{+}$ Enumeration

Patient ID	# of $CK^{+}CD45^{-}$ cells (per 7.5 mL blood)			Average	SD
	Tube 1	Tube 2	Tube 3		
MDACC-002	81	12	28	40	36
MDACC-003	0	0	0	0	-
MDACC-004	0	0	0	0	-
MDACC-005	0	0	4	1	2
MDACC-006	165	41	67	91	65
MDACC-007	17	8	22	16	7
MDACC-008	43	1	4	16	23
MDACC-009	2	7	3	4	3
MDACC-010	6	23	10	13	9
MDACC-011	21	3	77	34	39
MDACC-012	0	0	0	0	-
MDACC-013	38	38	60	45	13
MDACC-014	48	26	16	30	16
MDACC-016	0	0	0	0	-

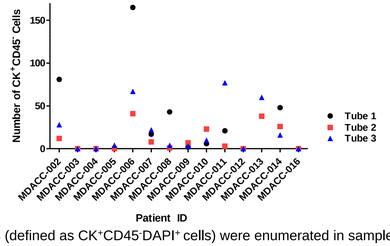


Figure 1. CTCs (defined as $CK^{+}CD45^{-}DAPI^{+}$ cells) were enumerated in samples collected from 14 PBC patients. CTCs were detected in 9 of 14 patients. The number of CTCs ranged from 0 to 165 with the average count per patient ranging from 0 to 91.

Biomarker Expression in CTCs

Patient ID	E-Cadherin		Beta-Catenin		EpCAM		Vimentin		CD44		CD24	
	% Positive CTCs	MFI	% Positive CTCs	MFI	% Positive CTCs	MFI	% Positive CTCs	MFI	% Positive CTCs	MFI	% Positive CTCs	MFI
MDACC-002	94	1,654	0	0	75	2,327	0	0	82	4,550	18	2,314
MDACC-003	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
MDACC-004	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
MDACC-005	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0	0	0	0
MDACC-006	2	1,735	0	0	2	3,106	5	1,030	16	5,013	0	0
MDACC-007	6	1,142	6	1,250	13	1,250	13	487	0	0	5	2,210
MDACC-008	19	1,626	7	2,400	100	3,316	100	484	67	4,827	67	2,119
MDACC-009	0	0	0	0	29	1,452	0	0	100	8,999	0	0
MDACC-010	50	2,419	0	0	22	1,532	0	0	100	2,937	0	0
MDACC-011	19	2,619	0	0	0	0	0	0	10	5,864	0	0
MDACC-012	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
MDACC-013	24	436	37	1,802	5	1,342	3	428	8	4,556	0	0
MDACC-014	0	0	0	0	15	1,866	0	0	25	8,265	0	0
MDACC-016	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

***N/A denotes no CTCs detected in sample; MFI = Mean Fluorescence Intensity.**

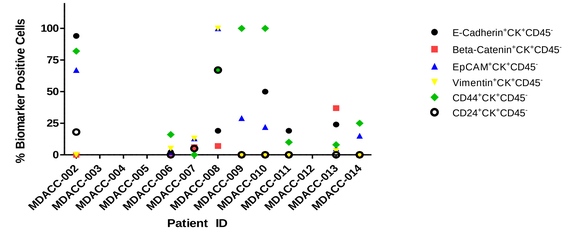
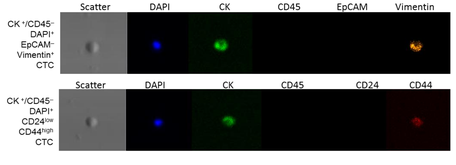


Figure 2. The CTC population was analyzed for expression of EMT (Vimentin) and CSC ($CD44^{high}$ $CD24^{low}$) markers. Expression levels of each biomarker was calculated as: (Number of cells expressing marker positive cells/CTC number) *100. Representative images shown below.



EMT Subpopulation of CTCs
$EpCAM^{-}/Vimentin^{+}$

Patient ID	% $EpCAM^{-}/Vimentin^{+}$ cells among $CK^{+}/CD45^{-}$ population
MDACC-002	0
MDACC-003	0
MDACC-004	0
MDACC-005	0
MDACC-006	2
MDACC-007	0
MDACC-008	0
MDACC-009	0
MDACC-010	0
MDACC-011	0
MDACC-012	0
MDACC-013	3
MDACC-014	0
MDACC-016	0

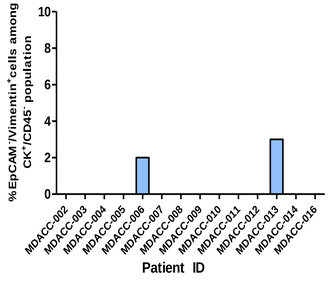
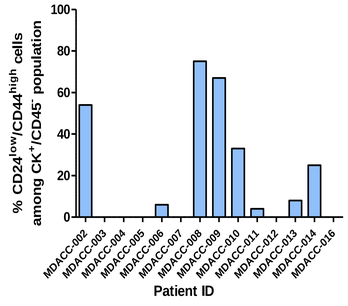


Figure 3. The CTC population was analyzed for $EpCAM^{-}Vimentin^{+}$ expression, which has been shown to signify the EMT state. Two of 14 (14%) patients were shown to express $EpCAM^{-}$ $Vimentin^{+}$ cells, ranging from 2-3%.

CSC Subpopulation of CTCs
$CD24^{low}CD44^{high}$



Patient ID	% $CD24^{low}/CD44^{high}$ cells among $CK^{+}/CD45^{-}$ population
MDACC-002	54
MDACC-003	0
MDACC-004	0
MDACC-005	0
MDACC-006	6
MDACC-007	0
MDACC-008	75
MDACC-009	67
MDACC-010	33
MDACC-011	4
MDACC-012	0
MDACC-013	8
MDACC-014	25
MDACC-016	0

Figure 4. CTCs were analyzed for the expression of $CD24^{low}CD44^{high}$. Eight of 14 (57%) patients had $CD24^{low}CD44^{high}$ CSCs, ranging from 8-75%.

Summary

- CTCs ($CK^{+}CD45^{-}DAPI^{+}$ cells) were detected in 71% (10/14) primary breast cancer patients prior to receiving preoperative therapy.
- EMT and stem cell markers range of expression and frequency of detection in PBC patients:
  - E-Cadherin^{+} range 2-94% in 78% (7/9) patients
  - β-Catenin^{+} range 6-37% in 21% (3/14) patients
  - $EpCAM^{-}Vimentin^{+}$ was 3% in 14% (2/14) patients
  - $CD24^{low}CD44^{high}$ range (8-75%) in 57% (8/14) patients
- In this ongoing clinical trial, we will test the hypothesis that low EMT-CTC and CSCs in baseline blood samples is correlated with a higher pCR rate compared to PBC patients with high EMT-CTC and CSC counts.

References

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