An In Vitro Model to Identify the Molecular Signature of Circulating Cancer Stem Cells

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Abstract

Circulating cancer stem cells (cCSC) are a subset of circulating tumor cells (CTCs), which are cancer cells that are shed from primary tumors into the bloodstream, evading immunosurveillance to initiate distant metastases. CTCs and cCSCs are promising as a liquid biopsy for non-invasively monitoring a patient's disease progression or response to treatment. Exploring the use of CTCs, as a response biomarker for the early evaluation of drug product activity in clinical trials, is a matter of urgency. However, a big challenge in implementing CTCs as a predictive biomarker or surrogate endpoint is the difficulty in detecting and enumerating these cells within the blood, as the CTC population is rare and heterogeneous with distinct molecular and phenotypic features. Further evaluation of the subpopulations of CTCs to establish a molecular signature involved in metastatic progression of cancer, is important to advance the use of these cells. Using an in vitro model of breast cancer CTCs, in which breast cancer cell (BCC) lines are cultured in a non-adherent suspension condition to mimic the circulation within the blood and monolayer to mimic the primary tumor, we examined the molecular signature of BCCs using transcriptomic and proteomic approaches. Flow cytometry and immunoblotting was performed to identify the cell lines with upregulated cancer stem cell surface markers following suspension condition. Gene expression was then analyzed on the suspension-induced CSC cell lines, reporting alterations in cell metabolism, reduced adhesion-related gene expression, and stimulated hypoxia-regulated genes in the suspension cultured cells. Notably a hypoxia-regulated gene associated with cancer stem cells, carbonic anhydrase IX (CAIX), was upregulated in a panel of suspension cultured cell lines. As CAIX is expressed only in highly hypoxic conditions, this protein may have potential as a novel target for the isolation of CTCs to further their use as a surrogate biomarker for monitoring treatment responses.

Introduction

An accelerating number of targeted cancer therapies being approved by the Food and Drug Administration (FDA), accounting for about 35% of new molecular entities approved in 2017 alone, drives the need of predictive biomarkers that stratify individuals into sub-populations that are likely or unlikely to respond to a specific drug treatment. Circulating tumor cells (CTCs) have a great potential as a "liquid biopsy" for monitoring a patient's disease progression or treatment response; being evaluated as a surrogate endpoint in more than 140 clinical trials. However, recent evidence shows that CTCs are vastly heterogeneous, displaying distinct molecular and phenotypic populations, such as the circulating cancer stem cell (cCSC) population which is believed to contribute to metastatic spread. Cancer stem cells (CSCs) are a small subpopulation within the primary tumor and CTCs which are characterized by the high surface expression of CD44 and low surface expression of CD24. These cells are more aggressive, with the ability to self-renew and repopulate a tumor of heterogenous cancer cells. We aim to characterize the molecular signature of circulating CSCs (cCSCs) to understand their metastatic potential and anticancer treatment resistance. To achieve this, we have developed an in vitro model of CTCs, in which a panel of breast cancer cell lines are cultured in suspension to simulate the blood stream and monolayer to simulate the primary tumor. Breast cancer cell (BCC) lines were cultured in a non-adherent or monolayer condition for up to seven days. An in-depth molecular characterization of CTCs can inform stakeholders regarding the development of novel CTC isolation technology toward moving CTC utility into routine clinical practice for advancing precision cancer medicine.

Materials and Methods

Human BCC lines (BT20, MB-231, MCF7, T47D, ZR75-1, and SKBR3) were obtained from the American Type Culture Collection (ATCC) and cultured in either a monolayer adherent condition on tissue-culture polystyrene culture dishes or in a non-adherent suspension condition using Corning Ultra-Low Attachment plates for up to 7 days. At the indicated time points, cells were either harvested for immunoblotting, harvested for DNA microarray, or analyzed using multispectral imaging flow cytometry (MIFC). For MIFC, cells were dissociated using a non-enzymatic dissociation buffer (Cell Stripper), blocked using a 1.0% bovine serum albumin, 5.0% normal goat serum, PBS solution, and then labeled with a phycoerythrin (PE)-conjugated anti-CD44, Violet Blue (VB)- conjugated anti-CD24, and an AlexaFluor-488 conjugated anti-Carbonic Anhydrase IX (CAIX) in the dark on ice. MIFC was performed using an EMD Millipore FlowSight. Gene expression was analyzed using the Human OneArray® Plus gene expression profiling service (HOA version 6.2, Phalanx Biotech Group, Inc., San Diego, CA, USA). RNA was extracted from the MB-231 and the MCF7 cell lines, cultured in monolayer, or in suspension culture, for 7 days. Gene expression fold changes were calculated by the Rosetta Resolver 7.2, with an error model adjusted by the Amersham Pairwise Ration Builder. Differential expression of genes was determined through the selection criteria of log2|fold change| ≥ 0.585 and $p < 0.05$. Data shown are the log2 ratios (suspension compared to monolayer) of each cell-type, with the corresponding *p*-value.

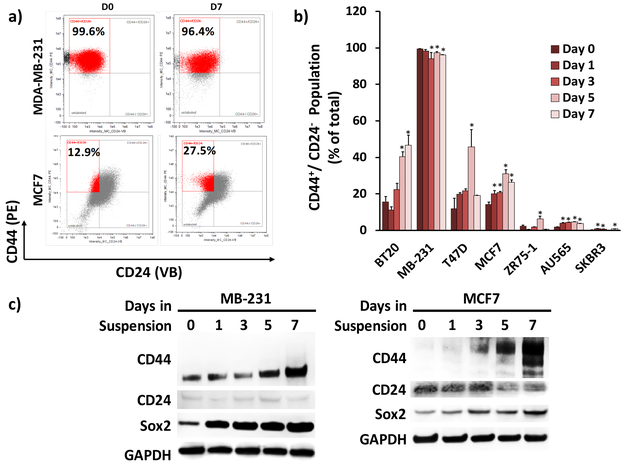
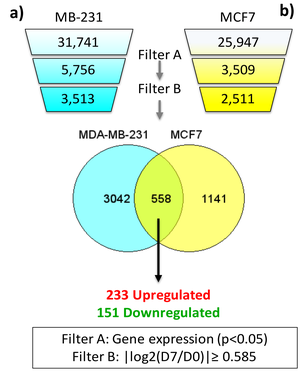


Figure 1: Suspension culture of breast cancer cell lines resulted in increased breast cancer stem cell (BCSC) subpopulations. Following monolayer or suspension culture (1, 3, 5, or 7 days), BCCs were dissociated and labeled with anti-CD44(PE) and anti-CD24(VB). MIFC was performed and the percentage of breast cancer stem cells within the total population was quantified by $CD44^+/CD24^-$ surface expression. Significant increases in the $CD44^+/CD24^-$ population were seen in BT20, MCF7, and T47D cell lines and MB-231 cells maintained a high basal level (a, b). (*p<0.05; n≥3). Expression of CD44 and Sox2, a stem cell transcription factor, increased over the seven days of suspension culture(c).

Results and Discussion



c)

Gene_symbol (protein)	M231S/ M231M	M7S/ M7M
CA9 (carbonic anhydrase IX)	1.380	5.581

b)

Gene Ontology Term: Biological Process	P-Value	Gene Count	Genes
Canonical Glycolysis	3.5E-04	6	ALDOA, GPI, PFKFB4, PFKFB3, ENO2, HK2
Response to Hypoxia	9.9E-04	13	MUC1, CAV1, CYP1A1, ARNT2, MECP2, ATM, DDIT4, PLOD1, ADM, CA9, VEGFA, ALKBH5, ANGPTL4
Positive Regulation of Gene Expression	1.9E-03	16	CAV1, VIM, GJA1, HSPA1B, PAWR, ACTG2, APOB, ID2, ID1, VEGFA, RGCC, MAPK8, WNT11, NFIL3, CTSH, FN1
Regulation of Apoptotic Process	2.1E-03	14	LGALS1, DAPK2, DAPK3, ATM, INHBB, INHBE, CASP8, NDRG1, CTSB, EIF2AK2, GDF15, IGFBP3, DEDD2, BMP8A
Cellular Response to Hypoxia	2.3E-03	9	EIF4EBP1, BCL2, RGCC, EDN1, VEGFA, STC1, FAM162A, NDRG1, MDM4

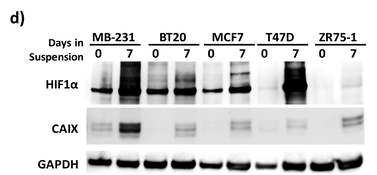


Figure 2: Gene expression analysis of suspension cultured BCCs identified a novel cCSC molecular profile. MB-231 and MCF7 cells were collected from monolayer and following 7 days of suspension culture and analyzed using the Human One Array (Phalanx Biotech). Significantly dysregulated genes were compared across the cell lines to identify a common molecular signature (a). Signaling pathway analysis performed using DAVID determined that *Response to Hypoxia* and *Cellular Response to Hypoxia* were 2 of the top biological processes activated following suspension culture for both MB-231 and MCF7 cells (b). The Human Protein Atlas, Uniprot and Reactome were used to identify the predicted novel cell surface markers. 33 dysregulated genes were identified with predicted plasma membrane localization. Carbonic Anhydrase IX (CAIX) was significantly upregulated in both cell lines (c). CAIX expression is known to be exclusive to tumors with low or non-existent expression in normal tissues, making this a potential biomarker or target for cCSCs. Upregulation of CAIX and HIF1α was confirmed using immunoblot following suspension culture (d).

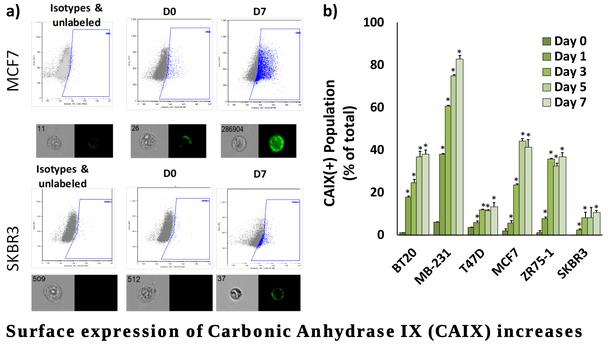
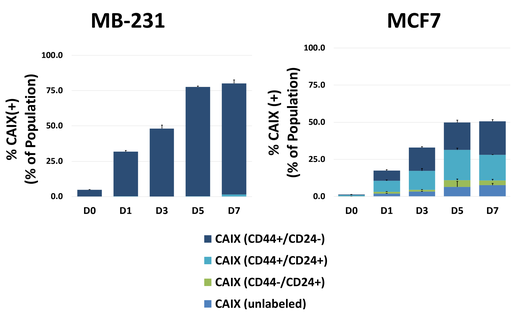


Fig.3: Surface expression of Carbonic Anhydrase IX (CAIX) increases following suspension condition. BCCs were analyzed for CAIX surface expression using anti-CAIX (AF488) during monolayer or suspension culture (a). BT20, MB-231, T47D, MCF7, and ZR75-1 cells saw a significant increase in the CAIX (+) populations at each time point (b).



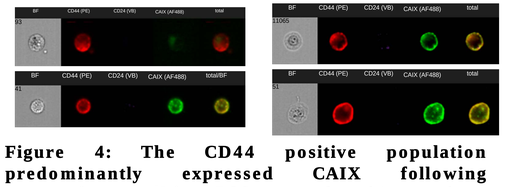


Figure 4: The CD44 positive population predominantly expressed CAIX following suspension condition. BCCs were cultured in monolayer or suspension condition and then analyzed for CD44, CD24, and CAIX using MIFC. CSC populations were gated using isotype controls and the contribution of CAIX(+) cells from each subpopulations of CSC markers (CD44+/CD24-; CD44+/CD24+; CD44-/CD24+; unlabeled) was quantified. CD44(+) cell populations expressed CAIX following suspension, regardless of CD24 expression. n=3.

Conclusion

- Breast cancer cell lines cultured in suspension culture were able to undergo a shift in phenotype, increasing the cCSC like population characterized by $CD44^+/CD24^-$.
- Carbonic Anhydrase IX was markedly upregulated following suspension, with highest surface levels found in cells with high $CD44^+$ expression.
- Ongoing work aims to verify our molecular signature using stable CTC lines generated from a metastatic cancer patient provided by the Laboratory of Rare Circulating Human Cells (LCCRH, Montpellier, Fr) and primary CTCs provided through a collaboration with Johns Hopkins University.
- Further characterization of cCSC like cancer cells within the CTCs and their resistance to anticancer therapies is necessary to fully develop the use of circulating tumor cells as predictive biomarkers.