A model to test and understand cancer cells that are circulating in the body

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Abstract

Circulating cancer stem cells (cCSCs) are a subset of circulating tumor cells (CTCs) that are released from primary tumors into the bloodstream, allowing them to spread to other parts of the body and evade the body's immune system. CTCs and cCSCs have the potential to be used as a non-invasive liquid biopsy to monitor a patient's disease progression or response to treatment but isolating and characterizing this rare and heterogenous population within the blood is difficult.

In this study, we have created an in vitro model of breast cancer CTCs. We cultured breast cancer cell lines in a non-adherent suspension condition to mimic the circulation of blood and a monolayer to mimic the primary tumor. We then used flow cytometry and immunoblotting to identify cell lines with upregulated cancer stem cell surface markers following suspension condition. We analyzed gene expression in the suspension-induced CSC cell lines and found changes in cell metabolism, reduced adhesion-related gene expression, and stimulated hypoxia-regulated genes. We also found that the hypoxia-regulated gene carbonic anhydrase IX (CAIX) was upregulated in a panel of suspension cultured cell lines and correlated with cells that expressed CD44, a known CSC marker. When we inhibited CAIX activity, we noted a decrease in the population of CSCs within the suspension cultured cells. Since CAIX is only expressed in highly hypoxic conditions, it may be a potential target for isolating cCSCs and furthering their use as a surrogate biomarker for monitoring treatment responses.

Introduction

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 over 140 clinical trials. However, recent evidence shows that CTCs are vastly heterogeneous, with 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) constitute a small subpopulation within the primary tumor and CTCs, and they are characterized by high surface expression of CD44 and low surface expression of CD24. These cells are more aggressive and possess the ability to self-renew and repopulate a tumor of heterogenous cancer cells.

Our goal is to characterize the molecular signature of circulating CSCs (cCSCs) in different types of breast cancer (TNBC, HER2$^+$, ER$^+$/PR$^+$) to understand their metastatic potential and resistance to anticancer treatments. To achieve this, we have created an in vitro model of CTCs by culturing a panel of breast cancer cell lines in suspension to simulate the blood stream, as well as in monolayer to simulate the primary tumor. The breast cancer cell (BCC) lines were cultured for up to seven days in a non-adherent or monolayer conditions. By conducting a detailed molecular analysis of CTCs, we can provide valuable insights to stakeholders who are interested in developing innovative CTC isolation technologies that can be integrated into routine clinical practice, ultimately advancing precision cancer medicine.

Reference: Twomey, J.D.; Zhang, B. Exploring the Role of Hypoxia-Inducible Carbonic Anhydrase IX (CAIX) in Circulating Tumor Cells (CTCs) of Breast Cancer. *Biomedicines* **2023**, *11*, 934. https://doi.org/10.3390/biomedicines11030934

Materials and Methods

Human BCC lines (BT20, MB-231, T47D, MCF7, ZR75-1, AU565 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, analyzed using multispectral imaging flow cytometry (MIFC), or treated with 0, 50, or 100 μM of U-104 (SLC-0111) for 24 hours to inhibit CAIX activity. 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.

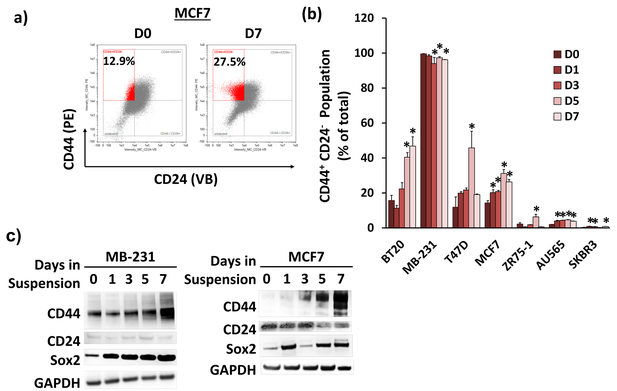

Figure 1: Suspension culture of breast cancer cell lines resulted in increased breast cancer stem cell (BCSC) subpopulations. Following monolayer (D0) or suspension culture (1, 3, 5, or 7 days), BCCs were dissociated and labeled with anti-CD44(PE) and anti-CD24(VB). MIFC was performed and the percentage 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

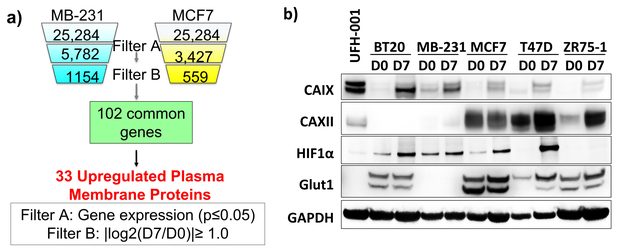

Figure 2: Gene expression analysis of suspension cultured BCCs identified a novel cCSC molecular profile. MB-231 and MCF7 cells were collected from monolayer and following 7 days of suspension culture and analyzed using the Human One Array (Phalanx Biotech). Significantly dysregulated genes were compared across the cell lines to identify a common molecular signature (a). Proteins which were significantly upregulated and predicted to localize to the plasma membrane were analyzed using immunoblotting (b) and MIFC, demonstrating suspension culture induced carbonic anhydrase IX (CAIX) expression across the selected cell lines. 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 HIF1α and the HIF1α-stimulated proteins of CAIX and Glut1 were also confirmed using immunoblot following suspension culture (b).

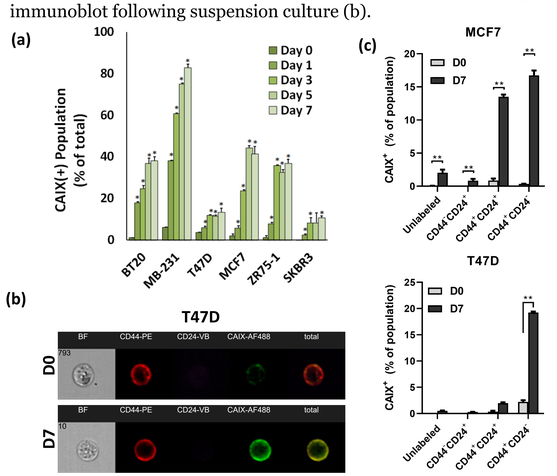

Fig.3: Surface expression of Carbonic Anhydrase IX (CAIX) increases following suspension condition predominantly in the CD44 positive population. BCCs were analyzed for CD44, CD24, and CAIX using MIFC. (a,b). BT20, MB-231, T47D, MCF7, and ZR75-1 cells saw a significant increase in the CAIX (+) populations at each time point (a). 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.

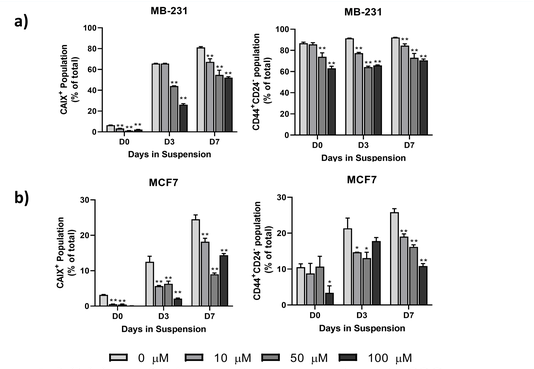

Figure 4: Inhibition of CAIX with U-104 reduced cCSC populations. MB-231 (a) and MCF7 (b) cells were cultured in a monolayer (D0) or suspension condition for 3 or 7 days and then treated with 0, 10, 50, 50, or 100 μM of U-104 for 24 h. Treated cells were then analyzed for CD44, CD24, and CAIX surface expression using MIFC. The CAIX$^+$ and the CD44$^+$CD24$^-$ decreased following U-104 treatment. (n = 3, * $p < 0.05$, ** $p < 0.01$).

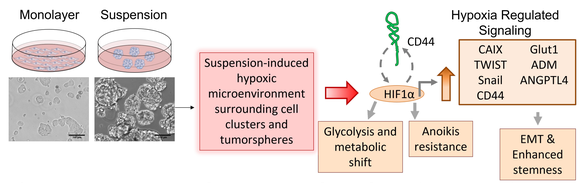

Figure 5: Suspension culture may result in a hypoxic microenvironment surrounding the "circulating" tumor spheres and clusters, resulting in activation of hypoxia-regulated signaling through HIF1α. Increased EMT factors, such as CAIX and Sox2 may result in enhanced stemness of the BCSC. CD44 and HIF1 act in a positive feedback loop through PI3K/AKT signaling, resulting in sustained CD44$^+$ cells through the 7 days of culture. CAIX is a potential marker that may be used to monitor or act as a therapeutic target for hypoxia-enriched cCSCs.

Conclusion

- Breast cancer cell lines cultured in suspension culture were able to undergo a shift in phenotype, which resulted in an increase in the cCSC like population characterized by the CD44$^+$/CD24$^-$ phenotype. Following suspension culture, CAIX was markedly upregulated, and the highest surface levels of CAIX were found in cells with high CD44$^+$ expression.
- Inhibition of CAIX activity following suspension culture resulted in a loss of CAIX$^+$ and CD44$^+$/CD24$^-$ populations.
- 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).
- 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.