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Analysis of Circulating Breast Cancer Cell Heterogeneity and Interactions with Peripheral Blood Mononuclear Cells.

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Abstract

For solid tumors, extravasation of cancer cells and their survival in circulation represents a critical stage of the metastatic process that lacks complete understanding. Gaining insight into interactions between circulating tumor cells (CTCs) and other peripheral blood mononuclear cells (PBMCs) may provide valuable prognostic information. The purpose of this study was to use single-cell RNA-sequencing (scRNA-seq) of liquid biopsies from breast cancer patients to begin defining intravascular interactions. We captured CTCs from the peripheral blood of breast cancer patients using size exclusion membranes followed by scRNA-seq of enriched CTCs and carry-over PBMCs. Transcriptome analysis identified two populations of CTCs: one enriched for transcripts indicative of estrogen responsiveness and increased proliferation and another enriched for transcripts characteristic of reduced proliferation and epithelial-mesenchymal transition (EMT). We applied interactome and pathway analysis to determine interactions between CTCs and other captured cells. Our analysis predicted for enhanced immune evasion in the CTC population with EMT characteristics. In addition, PD-1/PD-L1 pathway activation and T cell exhaustion were predicted in T cells isolated from breast cancer patients compared to normal T cells. We conclude that scRNA-seq of breast cancer CTCs generally stratifies them into two types based on their proliferative and epithelial state and differential potential to interact with PBMCs. Better understanding of CTC subtypes and their intravascular interactions may help design treatments directed against CTCs with high metastatic and immune evasive competence.

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DATA AVAILABILITY

The raw data is available in GEO - GSE139495.

Keywords

breast cancer; metastasis; tumor microenvironment; circulating tumor cells

INTRODUCTION

Metastatic breast cancer remains a major source of morbidity and mortality in the US ¹. Development of metastases involve a cascade of events, where primary tumor cells detach and invade, enter and survive in circulation, attach to a distant tissue and form a secondary site of disease. Each step within the cascade represents an opportunity for targeted therapy. While the vast majority of circulating tumor cells (CTCs) fail to survive the rigors of circulation, the few that endure represent a dangerous population of cells capable of propelling disease progression ². In an effort to better understand the mechanisms associated with CTC survival, both antibody-based and size-exclusion methods have been developed to capture CTCs from liquid biopsies ³.

Results from the SUCCESS trial, reported in 2010 and corroborated in follow-up studies, demonstrated that disease free survival, distant disease-free survival, and overall survival negatively correlated with the presence and quantity of CTCs in patients prior to chemotherapy ⁴. Based on the FDA approved CELLSEARCH® CTC test, 5 or more CTCs in 7.5 mL of blood is indicative of worse outcomes in breast cancer patients. Many CTC capture methods have attempted to garner information from CTCs by using immunohistochemical methods, but this approach is limited by the number of proteins that can be simultaneously queried. The recent advent of single cell sequencing has allowed the generation of robust, discovery-rich whole genome and transcriptome data sets ⁵. Furthermore, recent advances in bioinformatics have made it possible to improve assignment of cell identity based on their transcriptome ⁶. Together these advances provide increased utility for capturing data from CTCs.

Experimental models estimate that an aggressive tumor will release up to one million CTCs into circulation over a 24-hour period. This accounts for less than 0.5% of the total number of peripheral blood mononuclear cells (PBMCs), owing to the rarity of CTCs ². Further estimates suggest less than 0.02% of disseminated tumor cells actually produce a viable metastatic lesion ². Development of distal metastatic sites requires interaction of tumor cells with surrounding normal tissue cells that leads to formation of a unique tumor microenvironment. Similarly, there is evidence for CTCs travelling not only in isolation, but also in clusters, forming a mobile microenvironment supporting their survival in circulation ⁷⁻¹⁰. Our ability to analyze and manipulate CTCs is limited by the rarity of this cell population. We believe bioinformatic analysis can prove to be a valuable tool for identifying signaling pathways that favor CTC survival and can narrow down clinically meaningful and targetable signaling pathways. We hypothesized that the interactions between CTCs, PBMCs, and platelets in the blood stream could be characterized using single cell RNA sequencing (scRNA-seq) analysis of their transcriptomes. Disruption of the CTC microenvironment has the potential to serve as treatment target.

METHODS

Blood collection and processing.

Collection of whole blood samples from patients at the University of Colorado Denver (Aurora, CO) was in accordance with an IRB-approved protocol. 7.5mL of blood were collected directly into a Vacutainer tube containing ACD Solution A (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Whole blood was filtered through the CellSieve™ filter (CREATV MicroTech, Inc.) according to the manufacturer protocol with the following modifications: Due to possible interference with single cell sequencing, we did not use any fixation method prior to drawing the samples through the CellSieve™ filter. Refer to the Supplemental Methods for a detailed account of the CTC capture by filter backwash method and for cell capture numbers from each patient.

Detailed experiment design.

In a series of four individual experiments, we captured single cells using the 10X Genomics Chromium Box (10X Genomics, Pleasanton, CA, USA) from a total of 14 patients: 13 patients with active metastatic breast cancer and 1 patient with local breast cancer. For the patient with local breast cancer, (Sample 1) we removed 200uL of blood prior to filtration, we then applied the filter capture method to the remainder of the sample (Sample 1A). From the unfiltered portion of Sample 1, we captured 1,707 cells with 96% passing quality control filters, but the filtered portion of Sample 1 failed to pass bioinformatics filters as described in the previous section. We collected 3 samples from patients with metastatic disease (Samples 2, 3 and 4). From these samples, we captured a range of cells (Sample 2 = 336, Sample 3 = 71, and Sample 4 = 142 cells). Of the captured cells 89%, 41%, and 14% passed quality control filters for downstream analysis in samples 2, 3, and 4 respectively. In our next set of samples, we captured cells from 6 more patients with metastatic breast cancer (Samples 5, 6, 7, 8, 9, and 10). In samples 5, 6, and 7, we captured 794, 410, and 163 cells with 94%, 2%, and 2% passing quality control filters, respectively. Within the first two sets of samples captured, 3 samples from the metastatic cohort failed to pass bioinformatic quality control filters (Samples 13, 14, and 15).

The variability in cell capture abundance from the first and second set of samples led us to capture a third set of samples. In the third capture, we combined filter-captured cells with 2,000 non-filtered peripheral blood mononuclear cells from 3 patients with metastatic disease (Samples 9, 10, and 11) and then pooled the three samples for sequencing. Importantly, each patient's non-filtered cells were mixed with their filtered cells so that the pooled samples had both filtered and unfiltered cell representation from all three patients. The non-filtered cells were added as "carrier cells" to increase the total number of cells for capture as it was determined that increased cell concentration results in significantly higher percentage of cells successfully making it into oil droplets as single cells. In this manner, we were able to sequence 1,294 cells with 86% passing filter as single cells. CTCs were captured in the metastatic samples from Patient 2 (11 CTC-1, 13 CTC-2), Patient 5 (13 CTC-1, 12 CTC-2), Patient 6 (0 CTC-1, 3 CTC-2), Patient 7 (1 CTC-1, 0 CTC-2), the combined patients samples 9, 10, and 11 (20 CTC-1, 10 CTC-2) and from the unfiltered local breast cancer Patient 1 (7 CTC-1, 3 CTC-2). Taken together, the size exclusion method

we used in combination with the 10X Genomics Chromium Box produced significant variability across patients, but did enrich for CTCs.

Using the filtration method, we captured CTCs from MBC patient samples 2, 5, 6, 7, and the combined samples 9, 10, and 11. We did not capture CTCs from the filtered samples 3, 4, and 8. This correlates to 5/8 (62.5%) of the captured samples having CTCs. In comparison to Adams et al.^{11, 12}, our capture efficiency was slightly lower than what they reported but within reasonable expectations. A review by Eroglu et al.¹³, summarizes 8 CTC studies from patients with metastatic breast cancer where approximately 60% of patients had detectable CTCs using CellSearch, which also aligns our data to the common CTC capture values in breast cancer patients with metastatic disease.

Single-cell RNA-sequencing data processing.

Single-cell RNA sequencing libraries were aligned to the human (GRCh38) and UMIs de-duplicated using Cell Ranger 2.1.1. All de-duplicated UMIs were processed using Seurat 3 for quality control, pre-processing, dimensionality reduction, and differential expression analysis. The normal patient cohort used for comparison of our patient samples was single-cell sequencing taken from open access data generated by 10X Genomics normal control PBMCs; <https://support.10xgenomics.com/single-cell-gene-expression/datasets/2.1.0/pbmc8k>. Refer to the Supplemental Methods for a detailed account of data processing and quality control. The raw data is available in GEO - GSE139495.

Marker determination and differential gene expression.

Marker genes for each cluster as well as differentially expressed genes for each relevant pairwise comparison were identified using a Wilcoxon rank-sum test (From Seurat 3). Restraints in the analysis also required: 1. the gene was detectable in 25% of cells for at least one of the compared groups and 2. the natural-log fold-change was greater than 0.25.

Interactome analysis.

The interactomes involved in the data were determined from the normalized expression data from the cells of 10x PBMC, filtered PBMCs, and combined along with the marker genes derived from the gene expression where positive and negative values are assigned to the marker genes and fit a gaussian mixture model to the expression values of each marker genes as described by Kumar et al. cell reports 2018¹⁴.

RESULTS

Unique subtypes of circulating tumor cells from patients identified using scRNA-seq analysis.

We collected whole blood samples from breast cancer patients treated at the University of Colorado in accordance with an IRB-approved protocol (Table 1). CTCs were enriched using a size exclusion filter with 7µm pores. After scRNA-seq, we analyzed the cohort by pooling the samples from patients with metastatic disease and compared them to scRNA-seq from a patient with early stage breast cancer and a publicly available scRNA-seq dataset of normal human control blood from 10X Genomics. See Materials and Methods and the

Supplemental Methods for detailed descriptions of the capture protocol, scRNA-seq quality control, and experimental design with capture efficiency for each sample.

We applied the standard uniform manifold approximation and projection (UMAP) analysis of combined scRNA-seq data from all filter-captured cell, which identified 10 distinct cell populations illustrated in Figure 1A. All three datasets (local breast cancer, metastatic breast cancer, and normal PBMCs) had representation across most cell populations (Figure 1B and Supplemental Figure 1). The presence of cell-specific makers expressed with high confidence ($FDR < 0.05$) constituted the basis for cell cluster identification. For PBMCs, these markers included: CD7, CD3D, IL7R, and CD8a in T-cells; CD19 and MS4A1 in B cells; CD207 in Dendritic cells; IL3RA in plasmacytoid dendritic cells (pDCs); CD33 and CD14 in CD14+ Monocytes; CD33 and FCGR3A (CD16) in CD16+ Monocytes; NCAM1 in Natural Killer cells; ITGA2B, ITGB3, SELP, and PF4 in Megakaryocytes/Platelets (Supplemental Figure 2). The frequency of CTC detection in the metastatic samples was an average of 5.6-fold (3.71% of total) higher compared to either the unfiltered sample from the patient with local recurrence (0.61% of total). Cells with epithelial characteristics in the normal PBMC sample (0.71% of total, Figure 1C) were identified. Component analysis places these cells in the CTC cluster. Compared to the local breast cancer and normal PBMC samples, the metastatic cohort had enrichment for dendritic cells (6.2-fold), plasmacytoid dendritic cells (11-fold), and megakaryocytes (45-fold). Two main sub-clusters of cells were identified within the CTC population, which we designated CTC-1 and CTC-2. Further breakdown of the CTC-1 cluster was possible into CTC1a and CTC1b (Supplemental Figure 3 and Supplemental Table 1). For final downstream analysis, we combined CTC1 clusters and compared CTC-1 and CTC-2 clusters.

The CTC-1 cells constituted 60% and the CTC-2 cells 40% of the total CTCs captured. Transcripts for standard epithelial cell markers EPCAM, KRT8, and KRT18 localized only to the CTC populations, although CTC-1 had significantly higher levels compared to CTC-2 cells (Figure 2A-C). Given the majority of our samples were from patients with ER+ disease, we were not surprised to discover enrichment of ESR1 expression in both clusters (Figure 2D). The two CTC populations shared 1,052 detected transcripts while CTC-1 uniquely expressed 6,825 and CTC-2 uniquely expressed 815 transcripts (Figure 3A). Consistent with an epithelial phenotype, the CTC clusters expressed nine keratin (KRT) proteins. Eight of these (KRT1, KRT7, KRT10, KRT19, KRT72, and KRT86) had increased expression in CTC-1 versus CTC-2 clusters, but KRT17 had increased expression in CTC-2 versus CTC-1 (Figure 3B). These data show the UMAP2 component analysis defined two distinct epithelial cell populations in our samples.

CTC-2 cells have EMT characteristics.

Classically, epithelial cells undergoing EMT are identified as having decreased CDH1 (E-cadherin) expression accompanied by increased CDH2 (N-cadherin). CTC-2 cells contained 1.2-fold less transcripts for CDH1 ($p = 3.35 \times 10^{-7}$, $FDR = 0.01$, data not shown) and 2.8-fold more transcripts for CDH2 compared to CTC-1 cells (Figure 4A). CTC-2 cells expressed a trend toward increased levels of the classical EMT marker ZEB1 and both populations expressed VIM. Furthermore, the breast cancer specific EMT marker BIRC3

was significantly increased in CTC-2 cells and trended toward increased expression for BCAR1 and EPSTI1 (Figure 4A; ^{15–17}). These data suggest the CTC-2 population demonstrated a more pronounced EMT phenotype compared to the CTC-1 cell cluster. Examination of apoptosis markers (BCL2, BAX, BID, CASP3, CASP6, CASP7, CASP8, CASP9, and CASP10) indicated all cell populations express apoptosis relevant genes. Interestingly, compared to CTC-1 cells, the CTC-2 profile for apoptosis markers was indicative of decreased propensity for apoptosis. CTC-2 had increased BCL2 expression and decreased expression of CASP3, CASP8, CASP9, and CASP10 compared to the CTC1 population (Supplemental Figure 4).

We used Ingenuity Pathway Analysis (IPA) to determine which signaling pathways were predicted to be active in the two CTC clusters. Activation scores in IPA are represented by a z-score, which is a measure of how well the actual gene pattern in a given dataset models the expected gene pattern in a curated pathway ¹⁸. We compared activation of pathways in CTC-1 to CTC-2 clusters for the IPA curated pathways common to breast cancer cell signaling, including: Nuclear Receptor Signaling, Growth Factor Signaling, Intracellular and Second Messenger Signaling, Cell Cycle Regulation, and Apoptosis Signaling. Within the broad family of curated pathways, we identified individual signaling pathways based on having significant z-scores (decreased activation based on z-score ≤ -2 , and increased activation based on z-score ≥ 2).

For Nuclear Receptor Signaling, three pathways were identified as significantly activated in CTC-1 cells (Aldosterone Signaling in Epithelial Cells, z-score = 5.10; Aryl Hydrocarbon Receptor Signaling, z-score = 3.12; Estrogen-mediated S-Phase Entry, z-score = 3.21; Figure 4B). Aldosterone Signaling in Epithelial Cells was also predicted to be activated in the CTC-2 cluster (z-score = 2.45). In the Growth Factor Signaling family, IGF-1 Signaling was activated in CTC-1 cells (z-score = 4.60) and NGF Signaling was predicted to be activated in both clusters (CTC-1 z-score = 6.40; CTC-2 z-score = 3.00; Figure 4C). Finally, AMPK Signaling (z-score = 2.10), PI3K/AKT Signaling (z-score = 2.29), and Regulation of eIF-4 and p70S6K Signaling (z-score = 2.69) pathways in the Intracellular and Second Messenger family had significant activation scores in the CTC-1 cluster (Figure 4D). Additionally, the CTC-1 cluster had significant activation scores in five Cell Cycle Signaling pathways, two of which overlapped with the Nuclear Receptor Signaling pathways (Figure 4E). Decreased activation was predicted for Cell Cycle: G1/S Checkpoint Regulation (z-score = -2.12). Increased activation was predicted for Cell Cycle Regulation by BTG Family Proteins (z-score = 2.33) and Mitotic Roles of Polo-Like Kinase (z-score = 3.80). In CTC-2 cells, the Role of CHK Proteins in Cell Cycle Checkpoint Control signaling was activated (z-score = 1.34).

Although CTC-1 cells had increased activation of cellular signaling and cell cycle signaling compared to CTC-2 cells, the CTC-1 cluster also expressed consistently higher activation of apoptosis pathways. CTC-1 cells had increased activation for Death Receptor Signaling (z-score = 5.21), Telomerase Signaling (z-score = 2.61), and Aryl Hydrocarbon Receptor Signaling (overlap with Nuclear Receptor Signaling; Figure 4F). Both CTC-1 and CTC-2 clusters were predicted to have decreased activation in PTEN Signaling (CTC-1 z-score =

-5.05; CTC-2 z-score = -3.13). Taken together, IPA analysis suggests CTC-2 cells are more quiescent and less prone to apoptotic cell death compared to CTC-1 cells.

CTC-2 cells interact with immune cells through receptor/ligand pairs that decrease immune surveillance.

Survival of CTCs in the blood stream is critical to the development of distant metastases. Recently published reports describe CTCs traveling in clusters potentially in tandem with cells from the tumor microenvironment⁷, which suggests cell-cell interactions in clusters may play a critical role in CTC survival. We applied a cell-cell communication tool developed by Kumar et al., which utilizes scRNA-seq data and compared the interactome of the captured immune cells from the metastatic samples with the CTC-1 and CTC-2 cell populations¹⁴. The tool predicts interactions between two cell types based on the gene expression of ligand-receptor pairs present in the cell populations defined by highly-expressed and cell-specific markers (Supplemental Table 2). The predicted interactions between CTC-2 cells and PBMCs included megakaryocytes (MK), CD14+ and CD16+ monocytes, dendritic cells (DC), plasmacytoid dendritic cells (pDC), B cells, T cells, CTC-1 cells, and with CTC-2 cells themselves (Figure 5). These interactions were bi-directional for MKs, CD16+ monocytes, and pDCs. In addition to interacting with CTC-2 cells, CTC-1 cells were predicted to interact with MKs, CD16+ monocytes, DCs, and pDC cells. CTC-1 interactions were bi-directional only for MK cells. For interactions with CD16+ monocytes, pDC, and CTC-2 cells, the predicted direction was ligand expression on CTC-1 and receptor expression on the PBMC or CTC-2 cell. Since MKs are responsible for platelet production, it is not surprising that several MK interactions included proteins involved in platelet activation (i.e. B2M, CXCR1, CXCR2, PDL2, PF4, PPBP, TGFB1, THBD, THBS1, and VWF). These data suggest both CTC-1 and CTC-2 cells participate in interactions with CTCs and PBMCs and generally, the CTC-2 cells have a more diverse pattern of interaction compared to CTC-1 cells.

While size exclusion capture of cells enriches for larger cells including CTCs, a significant number of PBMCs were retained in the backwash. This allowed us to compare lymphocyte activity between the local breast cancer sample, the metastatic breast cancer samples, and the 10x Genomics reference data generated from normal controls. The comparison revealed significant z-scores for 21 pathways that broadly grouped hierarchically according to T-cell activation/differentiation (tumor cell surveillance) and immune checkpoint inhibition pathways (tumor cell immune evasion; Figure 6). Compared to lymphocytes from the normal control group, patient-derived lymphocytes had a general trend of decreased activation scores for pathways associated with T-cell activation/differentiation suggestive of a T-cell suppression phenotype (z-scores presented in Supplemental Table. 3). Pathways associated with tumor cell immune evasion were activated in the patient samples. Peripheral T-cells from the normal control set were predicted to have decreased activation ($z = -4.27$) of the PD-1, PD-L1 cancer immunotherapy pathway immune evasion compared to T-cells from patient samples (metastatic disease, $z = 4.60$; local breast cancer, $z = 2.65$). This is in line with data that activation of the PD-1/PD-L1 immune checkpoint is commonly up-regulated in the cancer setting. In tumor immune evasion, T-cell exhaustion represents an exacerbated loss of immune reactivity toward the cancer cells. IPA analysis predicted the T

Cell Exhaustion Signaling Pathway to be significantly activated in the filtered samples from patients with metastatic disease (z-score = 2.45). Taken together these data suggest that peripheral lymphocytes from patients with metastatic disease are programmed for CTC tolerance.

DISCUSSION

The metastatic process is extremely inefficient and only a small proportion of CTCs have the capacity to generate metastatic lesions. Although distinguishing “dangerous” from inert CTCs has proven challenging, having the ability to identify CTCs competent of metastasis formation and their interaction will help improve treatments. Our data support other studies showing CTCs are a heterogeneous cell population and a size exclusion filtration method successfully captures this diversity^{10, 11, 19–22}. Based on gene expression analysis, we identified two CTC populations. Consistent with increased gene expression, IPA analysis predicted CTC-1 cells were more transcriptionally active and dependent on estrogen signaling than CTC-2 cells. Validating and monitoring distinctions such as these may provide insight into a therapeutic vulnerability for eradicating the CTC-1 population using anti-endocrine therapy and/or cell cycle inhibitors. In contrast, analysis predicted CTC-2 cells to be more quiescent than CTC-1 cells and showed CTC-2 cells expressed increased markers associated with EMT.

The existence of an EMT CTC phenotype is in agreement with other studies reporting CTCs with increased EMT markers in breast cancer patients^{23–26}. This EMT phenotype may impart CTCs with increased ability to survive in circulation and enhance their metastatic competence. Previously, Yu et al.²⁷ demonstrated CTCs with an EMT phenotype were associated with response to breast cancer therapy with increased numbers of EMT CTCs indicating loss of therapeutic efficacy and disease relapse. Consistent with this observation, we found the CTC-2 population had decreased expression of estrogen responsive pathways that are commonly associated with disease progression. Gene expression pathway activation analysis also suggested the CTC-2 population was less prone to activation of apoptotic pathways, which could indicate an increased potential for resistance to chemotherapy.

One of the challenges CTCs face in circulation is the fact that they do not live in isolation and need to escape immune surveillance by PBMCs. One proposed mechanism for survival is through association with stromal cells that leave from the primary tumor with the CTCs. Duda et al. demonstrated in pre-clinical models that CTCs traveling in clumps contain stromal cells from the tumor microenvironment and these stromal cells help CTCs establish a metastatic niche²⁸. Ao et al., using a size exclusion method to capture CTCs, reported increased presence of cancer-associated fibroblasts in the blood of patients with metastatic breast cancer²⁹. We were unable to identify any fibroblasts or fibrocytes, which could be due to our cohort having few of these cells present or to a limitation in our ability to identify these populations using single-cell sequencing.

Another proposed mechanism used by CTCs to survive circulation and evade immune detection is through interaction with platelets^{27, 30–33}. Megakaryocytes are responsible for producing platelets and so it is not surprising that we found expression of several platelet

markers in the MK cluster. Considering MK interactions as a surrogate for platelet interactions, our data suggested CTC-1 and CTC-2 cells likely do interact with platelets, consistent with previous reports. Activated platelets produce abundant TGF β , which has been shown to be important for inducing and maintaining an EMT phenotype in CTCs^{27, 30}. Utilizing the Interactome analysis, the CTC-1 population reported for 29 interactions with MKs whereas CTC-2 cells reported 33 MK interactions. One unique MK : CTC-2 interaction included TGF β -SDC2, 2 interactions included PF4 and stronger MK interactions with CTC-2 compared to CTC-1 cells included ARF1 and B2M. Syndecan 2 (SDC2) has been shown to regulate TGF β signaling³⁴. Platelet activation factor 4 (PF4, alias CXCL4) is chemokine released during platelet activation and has recently been suggested to be associated with poor outcomes in breast cancer³⁵. Phospholipase D2 (PLD2) is involved in platelet activation³⁶ and ARF1 has been reported to be highly expressed in breast cancer metastasis³⁷. Taken together, these data merit further validation as they may indicate a survival mechanism for CTC-2 cells via platelet interactions.

Immune checkpoint blockade recently emerged as a viable treatment for triple negative breast cancer, but has had minimal success in ER+ breast cancer. ER+ breast cancer is generally less immunogenic compared to triple negative and HER2+ breast cancer³⁸. We show that T-cells isolated in the peripheral blood from patients with breast cancer have increased activation of pathways associated with increased immune checkpoint inhibition and T-cell exhaustion. Furthermore, pDCs are a rare population of immune cells found in the peripheral blood that can regulate T-cell activation versus T-cell anergy (i.e. the lack of T cell response to appropriate antigen stimuli;³⁹). Recent evidence suggests pDCs that secrete granzyme B (GZMB) suppress T-cell expansion⁴⁰. Intriguingly, our analysis indicated GZMB and insulin-like growth factor 2 receptor (IGFR2) interaction was present between CTCs and pDCs. This result may indicate CTCs directly interact with this regulatory subset of dendritic cells in order to stimulate immunotolerance. Overall, this study demonstrates the potential of using a bioinformatic approach to instruct on signaling pathways between PBMCs and CTCs that may provide CTC with survival advantages and would be of interest to further validate.

There is clinical need for effective, easily tolerated, and real-time diagnostic tools. Decreased CTC numbers were shown to correlate with improved chemotherapy outcomes in breast cancer and neuroendocrine neoplasms^{41, 42} and changes in cytokines and lymphocytes have been tracked before and after treatment^{43, 44}. Findings such as these demonstrate the clinical value of evaluating both the CTC and PBMC fraction to follow disease progression. Our approach of using scRNA-seq followed by Interactome analysis has the advantage of simultaneously querying dozens of receptor/ligand interactions that may inform on CTC survival and could be tracked over time and throughout therapy. We acknowledge deeper validation of this approach is required for it to have clinical impact, but an important purpose of this manuscript is to demonstrate the potential application of a bioinformatic approach to evaluating critical signaling pathways in a very rare cell population. Overall, this study demonstrates that accounting for PBMC interactions and stratifying CTCs not only on their number, but also on their subtype may indicate their metastatic potential and guide monitoring and treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Cell types identified in breast cancer patients and 10X normal human sample after scRNA-seq.

(A) The uniform manifold approximation and projection (UMAP) for dimensionality reduction algorithm identified 10 unique cell type clusters from scRNA-seq analysis of the local breast cancer (BC) sample, the metastatic BC patient cohort, and the Normal 10X Genomics control dataset. (B) Single cells from each group - Normal, Local BC, and Metastatic BC - had representation in most cell populations. (C) Cell distribution frequency revealed that size-exclusion filtration enriched for CTCs, megakaryocytes, dendritic cells and plasmacytoid dendritic cells in filtered patient samples from the Metastatic BC cohort, compared to the unfiltered Local BC sample and Normal control.

Figure 2. The CTC population expresses common breast cancer epithelial markers.

(A) Expression of Epithelial Cell Adhesion Molecule (EPCAM) was highly localized to the CTC clusters and CTC-1 cluster expressed 1.4-fold more EPCAM than CTC-2 ($p = 3.81 \times 10^{-17}$, $FDR = 1.28 \times 10^{-12}$). (B-C) Breast cancer cells commonly express keratins 8 (KRT8) and 18 (KRT18) and these keratins were localized to the CTC populations. Compared to CTC-2, CTC-1 expressed significantly more KRT8 (1.6-fold, $p = 5.05 \times 10^{-14}$, $FDR = 1.69 \times 10^{-9}$) and KRT18 (4-fold, $p = 1.44 \times 10^{-20}$, $FDR = 4.82 \times 10^{-16}$). (D) Our patient cohort consisted of individuals diagnosed with estrogen receptor positive breast cancer, which is consistent with estrogen receptor alpha (ESR1) gene expression localized to the CTC cluster. ESR1 was expressed significantly more in CTC-1 vs. CTC-2 clusters (1.7-fold, $p = 2.57 \times 10^{-10}$, $FDR = 8.62 \times 10^{-6}$). Boxed insets shown for additional clarity of the expression profile in the CTC-1 and CTC-2 clusters. The purple scale bar represents normalized expression counts.

Figure 3. CTC-1 and CTC-2 cell types identified as different cell types based on differences in their overall gene and keratin expression profiles.

(A) Venn diagram showing the total number of genes expressed in CTC-1 (7877) and CTC-2 (1867) cells, including the number of genes in common. **(B)** UMAP feature plots specific to keratin expression in the CTC-1 and CTC-2 clusters. Keratin expression for CTC-1 vs CTC-2 was KRT1 1.8-fold, $p = 1.16 \times 10^{-05}$, FDR = 0.390; KRT7 2.9-fold, $p = 4.60 \times 10^{-15}$, FDR = 1.54×10^{-10} ; KRT10 2.9-fold, $p = 4.11 \times 10^{-19}$, FDR = 1.38×10^{-14} ; KRT19 2.86-fold, $p = 5.77 \times 10^{-11}$, FDR = 1.94×10^{-06} ; KRT72 1.8-fold, $p = 9.65 \times 10^{-06}$, FDR = 0.324; KRT86 2.0-fold, $p = 5.85 \times 10^{-06}$, FDR = 0.196. KRT17 was expressed more in CTC-2 vs CTC-1 2048-fold in CTC-2 compared to CTC-1 ($p = 1.79 \times 10^{-05}$; FDR = 0.599).

Figure 4. CTC-1 and CTC-2 cells are a distinct set of epithelial breast cancer cells.

(A) Compared to CTC-1 cells, CTC-2 cells had significantly increased expression for the classical EMT marker CDH2, alias NCAD (1.7-fold, $p = 1.67 \times 10^{-07}$, FDR = 0.006) and breast cancer (BC) specific EMT marker BIRC3 (12.3-fold, $p = 1.48 \times 10^{-08}$, FDR = 5.00×10^{-04}). Expression of classical EMT marker ZEB1 (1.7-fold, $p = 0.001$, FDR = 1) and breast cancer specific EMT markers BCAR1 (883.5-fold, $p = 1.83 \times 10^{-05}$, FDR = 0.614) and EPSTI1 (18.7-fold, $p = 0.001$, FDR = 1) trended toward increased expression in CTC-2 vs. CTC-1 cells. Heat map representations of Ingenuity Pathway Analysis (IPA) predicted increased pathway activation scores for (B) Nuclear Receptor Signaling, (C) Growth Factor Signaling, (D) Intracellular and Second Messenger Signaling, (E) Cell Cycle Regulation, and (F) Apoptosis Signaling pathways in CTC-1 compared to CTC-2 cells. Boxes without grey dots indicate significant activation scores (considered $-2 > z > 2$). The heat map expression bar represents the activation z-score.

Figure 6. Signaling pathways for T-cells in peripheral circulation predicted increased activation of immune checkpoint inhibition and T-cell exhaustion.

Heat map representation of Ingenuity Pathway Analysis (IPA) predicted overall decreased activation in T-cell pathways of patient samples compared to normal PBMC controls, with the exception of increased activation for PD-1, PD-L1 Cancer Immunotherapy and T-Cell Exhaustion Signaling Pathways. Boxes without grey dots indicate significant activation scores (considered $-2 > z > 2$). The heat map expression bar represents the activation z-score.

Table 1.

Patient Data.

Status for estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor kinase 2 (HER2), and metastatic recurrences in patient samples. Only those samples that passed bioinformatic filters for downstream analysis are included in the table.

Sample ID	ER Status	PR Status	HER2 Status	Metastatic Sites
1	Positive (3+, 99%)	Negative (<1%)	Negative (FISH 1.1)	N/A - local recurrence
2	Positive (3+, 85%)	Positive (3+, 15%)	Positive (IHC 3+)	Skin, Liver, Bone, Brain
3	Positive (70%)	Positive (2%)	Positive (FISH 7.82)	Pleural effusion
4	Positive (3+ 85%)	Positive (3+ 15%)	Positive (IHC 3+)	Skin, Liver, Bone, Brain
5	Positive (86%)	Positive (87%)	Negative (IHC 1)	Bone
6	Positive (3+, 92%)	Positive (3+, 90%)	Negative (IHC 1+)	Bone
7	Positive (1+, 2+, 65%)	Positive (3+, 35%)	Equivocal (IHC 2+ , FISH 1.47)	Lymph nodes, Bone, Lung
8	Negative	Negative	Positive (IHC 3+)	Brain, Lung, Lymph node, Bone, Liver
9	Positive	Positive	Negative	Pleural effusion, Bone
10	Negative	Negative	Positive (++) FISH)	Bone, Liver, Lymph node
11	Negative	Negative	Negative	Skin, Lymph node