Research Article

Comprehensive isolation, identification, and nucleic acid analysis of single breast cancer cells: CTC-isoTECH

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Citation: Lori Millner, et al. Comprehensive isolation, identification, and nucleic acid analysis of single breast cancer cells: CTC-isoTECH. *Cancer Research Frontiers*. 2016 May; 2(2): 286-299. doi: 10.17980/2016.286

Competing Interests: The authors declare no competing financial interests.
Received Jan 8, 2016; Revised Mar 18, 2016; Accepted Mar 31, 2016. Published May 27, 2016

Abstract

The ability to isolate, phenotypically characterize, and profile the gene signature of single circulating tumor cells (CTCs) will provide deeper insight into cancer metastasis and will lead to improved diagnosis and treatment of cancer patients. Using current methods based on positive selection of epithelial markers, up to 40% of patients with highly aggressive metastatic breast cancer have no CTCs detected. This may be due to CTCs undergoing the process of epithelial to mesenchymal transition (EMT). This process causes epithelial characteristics to be down regulated as more mesenchymal features develop allowing the cells to have higher motility and increased ability to evade immune detection. Methods to enable capture and characterization of heterogeneous CTCs including those with non-epithelial phenotypes are needed. Additionally, having the ability to isolate pure, single CTCs allows for individual cell analysis providing information that is masked by bulk analysis. Here we provide details on an integrated method for isolating and characterizing heterogeneous single breast cancer cells based on a multiantigen, negative depletion strategy followed by sorting with dielectrophoresis (DEPArray). This negative depletion method removes unwanted leukocytes and allows heterogeneous, epithelial and non-epithelial cells to be retained. We were able to visually verify the isolation of single tumor cells with 100% purity. Following sorting, next generation sequencing and real-time PCR of single cells is described. This method allows single, heterogeneous CTCs to be analyzed which will have implications for prognosis and treatment options.

Keywords: Circulating tumor cells (CTCs), breast cancer, negative enrichment, dielectrophoresis, next generation sequencing

Introduction:

A circulating tumor cell (CTC) is a tumor cell that has been shed from a tumor and is found in systemic circulation. It is thought that these cells are shed from either primary or metastatic tumors, intravasate into the systemic circulation and have the potential to form metastatic lesions. The clinical significance of CTCs is very high because the major cause of morbidity in breast cancer patients is metastatic cancer (1). These cells must have the ability to withstand the shear forces encountered in the systemic circulation and the ability to evade immune detection (2). In order to create a secondary metastatic lesion, CTCs must extravasate into microvasculature and initiate neoangiogenesis. Because of these unique and demanding requirements, CTCs likely possess traits that enable them to carry out these tasks resulting in exploitable markers for identification and characterization.

Various method to enrich, isolate, and detect CTCs have been reported and are reviewed elsewhere (3). The only Food and Drug Administration (FDA) cleared method (CellSearch by Janssen) for identifying and enumerating epithelial CTCs uses peripheral blood mononuclear cells (PBMCs) enriched for cells expressing epithelial-cell adhesion molecule (EpCAM) and positively identifies CTCs based on expression of cytokeratins (CK) 8, 18, or 19. Since these markers are expressed in epithelial cells, this method identifies and enumerates only CTCs with an epithelial phenotype. Several studies have reported that overall and progression free survival is well correlated with the number of epithelial CTCs in metastatic breast, prostate, and colon cancers (4, 5) However, CTCs are not detectable in close to 40% of metastatic breast cancer patients (6-8). Additionally, CTC heterogeneity has been reported from a single blood draw of a single patient (9, 10). The process of epithelial to mesenchymal transition (EMT) is thought to be partly responsible for CTCs that are not detectable using epithelial markers. EMT is a biologic process that allows an epithelial cell to assume a mesenchymal phenotype including enhanced migratory capacity, invasiveness, elevated resistance to apoptosis and greatly increased production of ECM components (11, 12). EMT is known to occur early in development, wound healing, as well as in metastasis and has been reviewed elsewhere (13-17). A definition inclusive of CTCs that have experienced EMT needs to be established.

In this study, we describe a method independent of epithelial phenotype status to enrich and isolate 2 distinct breast cancer cell line subtypes. A heterogeneous model is important because currently, only CTCs that express EpCAM are captured. We have chosen to include a breast cancer cell line that has undetectable or very low levels of EpCAM expression (MDA-MB-231, basal B) and a cell line that has moderate and varying EpCAM expression SK-Br-3 (luminal/ HER2). We used a combination of EpCAM, HER2, and CD44 for positive identification. EpCAM was chosen because it is the most commonly used extracellular marker for epithelial CTC detection. To detect non-epithelial cell types, HER2 and CD44 were chosen because they are likely to be expressed on more aggressive or non-epithelial CTCs. HER2 expression has been detected on CTCs in early and metastatic cancer and is not dependent on the primary tumor HER2 status (18, 19). In several studies, HER2 expression was found more frequently on CTCs than the primary tumor, and expression on CTCs has been associated with poor clinical outcome of early breast cancer (19-21). CD44 is involved in tumorigenesis, and it's presence on CTCs has been associated with lower overall survival than those with no CD44 expression (22). CD44 has been described as a marker of tumor-initiating cells and high expression has been observed on a subset of metastasis-initiating CTCs (23, 24). Studies have been conducted to characterize patients in which

no CTCs are detected by examining epithelial markers including EpCAM and cytokeratin. In metastatic (7) and non-metastatic (25) cancer, patients with the most aggressive diseases were found to have no detectable CTCs using epithelial markers. In metastatic breast cancer, it was observed that a higher proportion of patients with poor prognosis (high grade, hormone receptor and HER-2 negative disease, IBC patients, and patients with brain metastasis) had no detectable CTCs (7). Patients younger than 50 years old with early breast cancer possessing HER2-amplified and G1-G2 tumors had a higher possibility of having no detectable CTCs (25). It is not known if these patients have no CTCs or if they have CTCs that are simply not detected by epithelial markers. However, in a recent study, 64% of clinical samples with negative or undetermined EpCAM positive CTCs revealed at least one putative CTC ($EpCAM^{neg}$ CK^{pos}/$CD45^{neg}$ event) (8). Because cancer patients with no detectable epithelial CTCs may be at increased risk for having more aggressive disease, it is critical to develop methods to isolate and characterize these potential CTCs.

The goal of this study was to demonstrate feasibility to identify, isolate, and characterize single cells taken from a heterogeneous cell mixture including epithelial and non-epithelial type cells. To accomplish this goal, we describe a method based on dielectrophoresis to identify, capture, and characterize 2 subtypes of single tumor cells. This method using the DEPArray (Silicon BioSystems, Italy) is based on a microchip containing microelectrodes capable of creating dielectrophoretic cages that can house and manipulate cells to allow sorting (26). Fluorescently labeled cells can be visualized during the entire process to ensure 100% purity and absolute assurance of cell capture. Because this method is gentle on the cell, single cells can then be further characterized by methods including NGS and RT-PCR.

Sequencing a single CTC allows unique genetic profiles to be revealed that are masked by bulk analysis. A few previous studies have been conducted using the DEPArray to isolate single cells in order to perform single cell analysis by sequencing (27-30). Only a single paper has been previously published describing NGS on single tumor cells isolated by DEPArray. Small cell lung cancer cells enriched by RosetteSep Human Circulating Epithelial Tumor Cell Cocktail and isolated by DEPArray were analyzed by NGS whole genome sequencing (31). Here we describe NGS sequencing of DEPArray isolated single cells of both epithelial and mesenchymal phenotype using a targeted cancer panel.

Methods & Materials

Cell Culture

Subtypes were chosen based on molecular classification, invasion potential using modified Boyden chamber assays, and expression of EMT markers (32, 33). The breast cancer cell lines SK-BR-3 and MDA-MB-231, purchased from ATCC were cultured in DMEM Medium (Lonza, Basal, Switzerland) containing 10 % fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA) and 1 % mycozap (Lonza). All cells were cultured at 37°C and maintained under 5 % CO_2 in polystyrene flasks until confluent (70-80%). Cells were released using a 0.25% trypsin/EDTA solution and collected via centrifugation.

Blood Processing

Blood samples from healthy donors (Biospecialty, USA) were collected into K_3EDTA (BD Biosciences, USA) tubes and shipped within 24 hours. Breast cancer cell lines, a surrogate for CTCs, were spiked in before performing a 1-step RBC lysis and fixation (eBiosciences) step on 5 mL whole blood for 30 minutes at room temperature (RT). Next, the sample was centrifuged at 500 RCF for 5 minutes and washed with 50 mL 1xPBS. Before

the second centrifugation, a count of the cells was obtained using a Milipore Sceptor (Becton Dickinson, Franklin Lakes, NJ).

Positive Enrichment and Negative Depletion

For the negative depletion, each sample was resuspended in 80 µL autoMACS buffer (Miltenyi, Germany) for every 10^7 cells previously enumerated. Next, a cocktail of 20 µL of CD45 and 10 µL CD66 magnetic beads (Miltenyi) for every 10^7 cells was mixed with each sample. The sample was then incubated for 30 minutes at 4°C. Next, 3 mL of autoMACS buffer was added to the sample, which was centrifuged at 200 RCF for 10 minutes. The sample was resuspended in 1 mL autoMACS buffer and passed over a LS column (Miltenyi) primed with 3 mL autoMACS buffer contained in a high gradient magnetic field. Three wash steps with 3 mL of autoMACS buffer were performed to allow the CTCs to pass through the flow through representing the positive fraction. Finally, the column was removed from the high gradient magnetic field and 5 mL autoMACS buffer was added to plunge the remaining depleted leukocytes (negative fraction). For positive enrichment, the same method was performed, however 300 µL autoMACS buffer and 100 µL EpCAM magnetic beads (Miltenyi) were added to the sample. For the positive enrichment, the flow through containing leukocytes represents the negative fraction and the plunged fraction containing CTCs represents the positive fraction.

Cell Imaging

For quantification experiments, cells were incubated for fifteen minutes with 2 mM calcein AM (Life Technologies, USA) in 1xPBS at 37°C. The cells were centrifuged and washed once in 1xPBS, then counted using a hemocytometer. 20 µL of a 5,000 cells/mL dilution was added per 5 ml whole blood or to 80 µL 1xPBS in a 96 well plate, which served as a control to determine total cell count (n=8). Cells were captured and processed as described above. Positive and negative fractions were added to a 96 well plate and imaged using a Nikon Eclipse fluorescent microscope. Cells were enumerated using ImageJ and divided by the average total cell count to determine percent capture efficiency.

Cell Staining

For cell staining heterogeneity studies, cells were stained at a concentration of 5x10^5 cells/100 µL. Cells were stained with Hoechst 33342 (Life Technologies) at a concentration of 1 ug/uL, CD44 antibody conjugated with APC (1:100, Biolegend 103011), EpCAM antibody conjugated with PE (1:100, Biolegend 324305) and HER2 antibody conjugated with FITC (1:100, Biolegend 324404) for 15 minutes at 4°C. Cells were then fixed with 1% paraformaldehyde for 5 minutes at room temperature. To stain cells spiked into whole blood that have undergone negative depletion, 100 µL autoMACS buffer containing 1 % Tween-20 (Sigma) was added to the sample, which were then stained overnight at 4°C with Hoechst 33342 (Life Tech) at a concentration of 1 ug/uL, cytokeratin conjugated to FITC (1:25, Miltenyi), EpCAM conjugated to PE (1:25, Miltenyi) and CD45 conjugated to APC (1:25, Biolegend).

DEPArray single cell sorting

Cell sorting by the DEPArray was carried out by manufacturer's instructions. Briefly, 14 µL (13,000-40,000 cells) of sample was injected onto the DEPArray cartridge. Next, 830 µL of SB115 buffer (Silicon Biosystems, Bologna, Italy) was also injected onto the cartridge for later cell recovery. Following cartridge loading, 9.26 µL was automatically injected by the system onto the cartridge microchamber where non-uniform electric gradient was applied causing cells to become trapped in individual dielectrophoretic cages. Images were taken covering the entire surface area of the microchamber using 4 different fluorescent channels including PE, FITC, APC, and DAPI/Hoechst as well as brightfield images. Cells were automatically detected by the

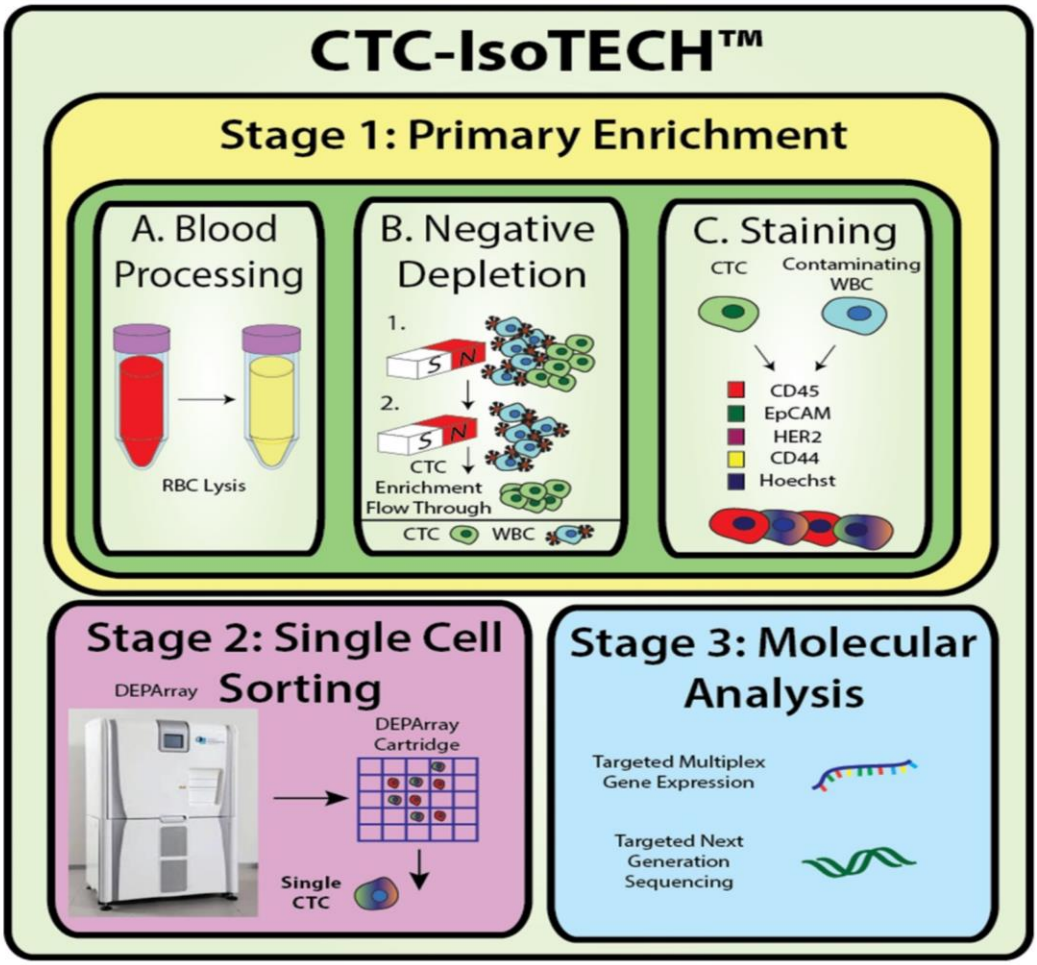

Figure 1. CTC-isoTECH platform description. Stage 1 includes whole blood processing, a negative immunomagnetic depletion of unwanted white blood cells followed by staining of the enriched cells. Stage 2 consists of single cell sorting by the DEPArray and, lastly, Stage 3 provides for molecular analysis of the single cells including gene expression analysis and next generation sequencing.

DEPArray based on Hoechst intensity. Captured images of the cells were visually confirmed by the user and cells of interest were moved to the parking chamber. Once in the parking chamber, cells were visually confirmed and then moved individually to the sorting chamber and sorted into individual 0.2 mL microfuge tubes. 100 µL of buffer was added and cells were centrifuged to remove supernatant.

Single Cell Transcriptional Analysis

Transcriptional analysis was performed using the Single Cell-to-Ct kit (Ambion) per the manufacturer's instructions on single cells isolated with the above described method. Briefly, 10 µl of cell lysis buffer was added to each single cell sorted by the DEPArray and allowed to incubate for 5 minutes at room temperature followed by the addition of 1 µl of stop solution. 4.5 µl of reverse transcription mix was added followed by reverse transcription reaction and 14

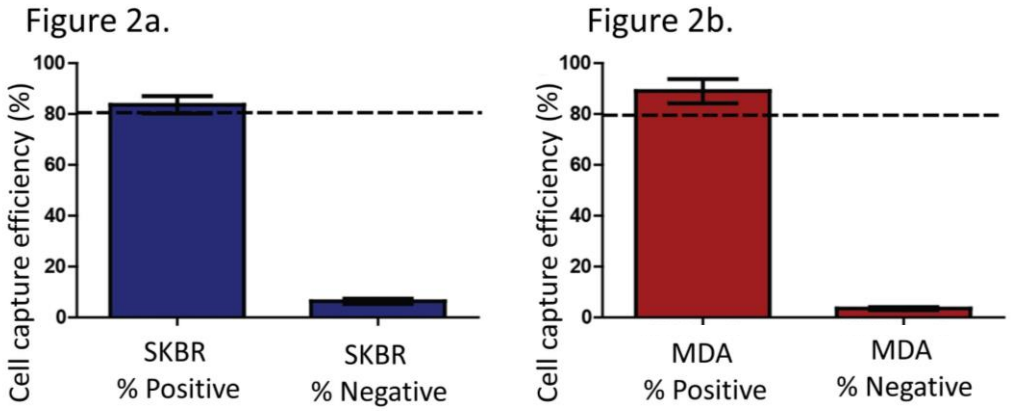

Figure 2. Negative depletion followed by positive identification (EpCAM, HER2, CD44) of breast cancer cell lines (a) SKBR (epithelial) and (b) MDA (mesenchymal) spiked into whole blood. % Positive and % Negative refer to the the positive (expected) fraction and the negative (unexpected) fraction.

cycles of targeted pre-amplification was performed by adding Pre-Amp mix and diluted TaqMan Gene Expression Assays. Finally, pre-amplified cDNA products were subjected to real-time PCR for 4 marker genes and 4 housekeeping genes using an ABI 7300. The genes included were the epithelial marker EpCAM, the stem cell marker CD44, the marker for proliferation HER2, and the adhesion marker CDH1 as well as the reference genes ACTB, GAPDH, RPL13A, and RPL37A. Florigenic PCR reactions were performed at 40 cycles using TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix. A minus RT sample was included for each gene as a negative control. Because only single cells were used, all Ct values less than or equal to 35 were considered positive, while those that were greater than 35 were considered negative (34, 35). Due to the large cell-to-cell variation of any one gene when examining single cells, no normalization was used when comparing Ct values as a qualitative comparison (36).

Single Cell Whole Genome Amplification

Prior to sequencing, whole genome amplification of single cells was conducted by cell lysis, *Mse* I restriction digest, adaptor ligation and PCR amplification using the Ampli1 whole genome amplification (WGA) kit (Silicon Biosystems) per the manufacturer's instructions. Briefly, 2 µl of lysis mix was added to single cells sorted by the DEPArray and incubated for 15 hours. The DNA is then digested by adding 2 µl of digestion mix and incubated for 3 hours followed by addition of 5 µl of a ligation mix and an overnight ligation reaction. The primary PCR was carried out by adding 40 µl of the primary PCR mix and a 44 cycle PCR reaction (Eppendorf). A quality control end point PCR was carried out (Ampli1 QC kit, Silicon Biosystems) for 4 amplicons of 91, 108-166, 299, and 614 base pairs. Samples that had at least 3 out of 4 amplicons present were considered successfully amplified and were used for sequencing.

Single Cell Sequencing

Genomic DNA was extracted from SKBR and MDA cells using the DNeasy blood and tissue kit (Qiagen) according to the manufacturer's directions. Bulk genomic and single cell WGA DNA from SKBR and MDA cells were then quantified using the GeneRead DNA QuantiMIZE kit (Qiagen) according to manufacturer's directions. Whole exonic regions of 24 genes were amplified from 40

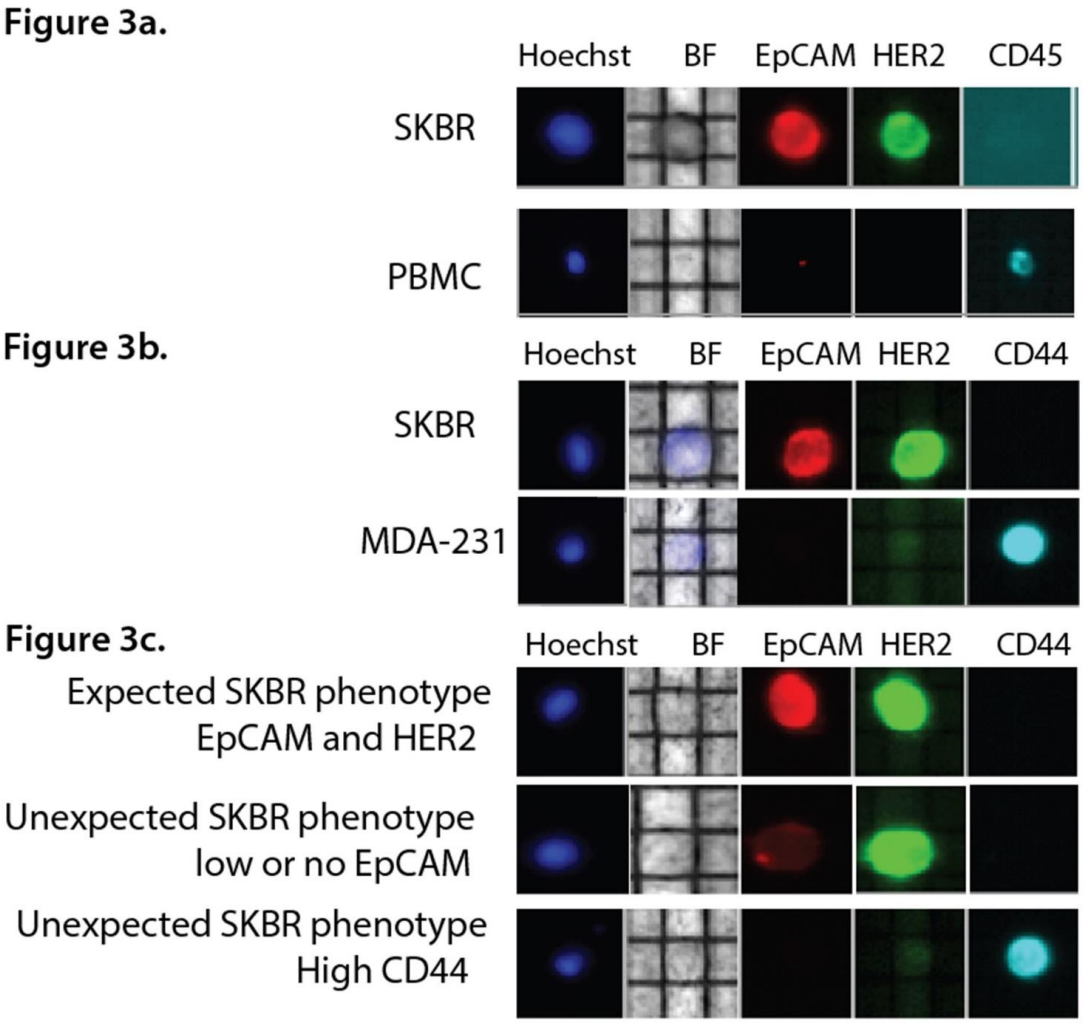

Figure 3. DEPArray images of single breast cancer cells. (a) DEPArray images of SKBR-3 cells that were spiked into whole blood followed by negative depletion. The remaining cells were stained with EpCAM, HER2, CD45, and Hoechst combined and analyzed by DEPArray. (b) SKBR and MDA-231 cells were combined, stained with EpCAM, HER2, CD44, and Hoechst in a single tube, and analyzed by DEPArray. (c) A single breast cell line, SKBR-3 was stained with EpCAM, HER2, CD44, and Hoechst and analyzed by DEPArray. SKBR-3 cells typically express high levels of EpCAM and HER2 and low or no expression of CD44 (stem cell marker).

ng of DNA per sample that was split into 4 pools, which then underwent multiplex PCR using the GeneRead DNAseq Clinically Relevant Tumor Panel (Qiagen, Version 2). Next, PCR amplicons of each sample were pooled and purified using the Agencourt AMPure XP Beads (Beckman Coulter, Brea, CA, USA). To construct a barcoded Illumina DNA library, the GeneRead DNA Library I Core Kit (Qiagen) and GeneRead Adapter I Set A 12-plex (Qiagen) kits were used according to manufacturer's directions. A size selection of the fragments was then performed using the Agencourt AMPure XP Beads. A final PCR was performed to amplify adapter-carrying fragments

Table 1. Breast cancer subtypes used to model patient CTC heterogeneity

Breast Cancer Cell Line	Molecular classification	HER2	EpCAM	CD44
SK-BR-3 (epithelial)	Luminal/HER2	**+**	**+/-**	
MDA-MB-231 (non-epithelial)	Basal B			**+**

and amplicons, which was again purified using the Agencourt AMPure XP Beads. Finally, the concentration of the amplicon library was determined by the GeneRead DNA seq Library Quant Assay (Qiagen) according to the manufacturer's protocol by qPCR. To ensure quality of the correct amplicon size it was analyzed using the QIAxcel DNA high resolution cartridge in a QIAxcel, a gel capillary electrophoresis instrument. Afterwards, samples were diluted to a final concentration of 2 nM and pooled according to manufacturer's instructions (Illumina) before being added to a MiSeq flow cell v2 (Illumina) to undergo sequencing on a MiSeq instrument, which generated fastq files.

NGS Analysis

Paired de-multiplexed fastq files from a MiSeq Illumina sequencer using Qiagen's Clinically Relevant tumor panel were imported into the CLC Biomedical Workbench (CLC Bio, version 2.5.1). Adapter sequences and bases with low quality scores (less than 20) were trimmed and reads were mapped to the reference genome, HG19. Variants were detected with the CLC variant caller using a threshold of 10% allele frequency and 450 minimum coverage.

Results:

This paper describes the CTC-isoTECH platform, which is an integrated method that starts with the enrichment of heterogeneous CTCs from whole blood followed by their identification, isolation, and downstream characterization (Figure 1). To enrich CTCs from whole blood, this method employs a negative depletion technique of CTCs from whole blood by removing CD45 and CD66b expressing cells from whole blood. When performing this negative depletion method with 50 SKBR-3 cells spiked into 5 mL of whole blood, we achieved an average of 84.2±8.2% recovery of our SKBR-3 cells in the positive (expected) fraction (Figure 2a). For the more mesenchymal cell line MDA-MB-231, we achieved an average recovery of 88.9±5.4% in the positive (expected) fraction (Figure 2b). This method resulted in a 402 fold depletion of leukocytes by reducing an average of approximately 13 million leukocytes prior to depletion down to 32,000 cells after depletion. This stringent depletion allows the cells to be sorted into single cell populations by the DEPArray which has a cartridge limitation of less than 80,000 cells.

To demonstrate our ability to distinguish cancer cells from peripheral blood monocyte cells (PBMCs), we performed an integrated experiment by spiking SKBR cells into whole blood, followed by negative depletion of CD45 and CD66b expressing cells and DEPArray analysis. We were able to distinguish SKBR-3 (EpCAM+, HER+, CD45-) cells from PBMCs (CD45+) (Figure 3a). To demonstrate our ability to identify and sort single heterogeneous cancer cells, a mixture of SKBR-3

Table 2. Single cell gene expression.

	CD44	CDH1	EPCAM	ERBB2	GAPDH	RPL13A	RPL37A	ACTB
SKBR 1					+			+
SKBR 2	+						+	+
SKBR 3	+	+		+	+	+	+	+
SKBR 4								+
SKBR 5	+		+		+	+	+	+
MDA 1	+				+	+	+	+
MDA 2	+			+	+			+
MDA 3	+				+			+
MDA 4	+				+	+		+
MDA 5	+			+	+			+
Pos Con	+	+	+	+	+	+	+	+
Neg Con								

RT-PCR was conducted on single cells sorted by the DEPArray. The top section examines single SKBR cells while the lower section examines single MDA cells. Expression is given as positive (+) or negative, with positive expression being any Ct value less than 35.

(epithelial phenotype) and MDA-MB-231 (basal/mesenchymal phenotype) cells were stained with EpCAM, HER2, CD44, and Hoechst and analyzed by DEPArray (Figure 3b). Two distinct phenotypes were observed. The putative SKBR-3 cells were found to be EpCAM and HER2 positive while the putative MDA-MB-231 cells were found to be singly positive for the stem cell marker, CD44. In an experiment to examine heterogeneity on the single cell level within a single cell line, we stained SKBR-3 cells and analyzed single cell expression by DEPArray (Figure 3c). The majority of cells were found to express the expected phenotype of $EpCAM^{+}$, $HER2^{+}$, and $CD44^{-}$. Two subsets of cells with unexpected phenotypes were identified; 27% of cells expressed low or no EpCAM with high HER2 expression and 0.5% of cells expressed only the stem cell marker, CD44. This demonstrates the ability of the DEPArray to detect heterogeneous and rare cell populations.

Following DEPArray sorting, SKBR-3 (epithelial phenotype) and MDA-MB-231 (mesenchymal phenotype) cells were further characterized by single cell real-time quantitative PCR (RT-PCR). Four target genes including EpCAM, ERBB2, CD44, and CDH1 and four housekeeping genes including GAPDH, ACTB, RPL13A and RPL37A were interrogated in 5 single SKBR cells (Table 2). Using a CT of 35 to discriminate between positivity and negativity, 3/5 cells were positive for at least one target gene and one housekeeping gene. Two cells (SKBR 1 and 4) were found to express no target genes and one housekeeping genes, ACTB or GAPDH. Expression varied in all target genes, including EpCAM, ERBB2, CD44, and CDH1. Less

Table 3. Single cell NGS examining a single (a) SKBR-3 cell replicates or single (b) MDA replicates. Column 1 refers to the chromosome, column 2 is the position on the reference genome, column 3 refers to the type of variant, column 4 is the variant allele, column 5 is the gene name, and column 6 is the dbSNP ID, and columns 7-9 refer to the presence (X) or absence (blank) of the variants in each of 3 triplicates.

Table 3a. SKBR-3 single cell replicates of NGS

Chromosome	Region	Type	Variant	gene	dbSNP ID	SKBR Rep 1	SKBR Rep 2	SKBRRep 3
4	55141055	SNV	G	PGDFRA	1873778	X	X	X
4	55151958^	Insertion	A	PGDFRA		X	X	X
7	140481417	SNV	A	BRAF	121913348	X	X	X
17	7578645	SNV	T	TP53	2909430	X	X	X
17	7579801	SNV	C	TP53	1642785	X	X	X
1	160786670	SNV	G	LY9	560681	X	X	X
1	162740327	SNV	C	DDR2	1780003	X	X	X
1	162748588	SNV	A	DDR2	907074	X	X	X
3	178922274	SNV	A	PIK3CA	2699896	X	X	X
6	12059954	SNV	A	HIVEP1		X	X	X
17	37868715	SNV	T	ERBB2	4252627	X	X	X

Table 3b. MDA-MB-231 single cell replicates of NGS

Chromosome	Region	Type	Variant	gene	dbSNP ID	MDA Rep 1	MDA Rep 2	MDA Rep 3
3	178917762	Insertion	T	PIK3CA		X	X	X
3	178917762	Insertion	TT	PIK3CA		X	X	X
3	178917763	Deletion	-	PIK3CA		X	X	X
3	178917763	Deletion	-	PIK3CA		X	X	X
4	55152040	SNV	T	FIP1L1	2228230	X	X	X
17	7572980	SNV	G	TP53		X	X	X
17	7579644	Deletion	-	TP53	146534833	X	X	X
17	37865713	SNV	C	ERBB2		X	X	X
17	37881451	SNV	T	ERBB2		X	X	X
17	37881451	MNV	TA	ERBB2		X	X	X

heterogeneity was observed in the 5 single MDA-MB-231 cells that were examined (Table 2). All MDA-231 cells expressed CD44 and the housekeeping gene, ACTB. The only target gene with heterogeneity in expression was ERBB2. These experiments demonstrate our ability to characterize single cells by gene expression following DEPArray sorting to the single cell level.

In addition to single cell RT-PCR, cells sorted by DEPArray were also further characterized using next generation sequencing (NGS) by Illumina's massively parallel sequencing technology. Following DEPArray sorting, single SKBR-3 and MDA-231 cells were subjected to whole genome amplification and library preparation for NGS. A targeted 24 gene panel was analyzed for each single cell and single nucleotide variants (SNVs) were reported for 3 replicates of each single cell. We found 100% concordance in both the single SKBR-3 cell and in the single MDA-231 cell (Table 3). This experiment demonstrates feasibility to perform NGS on single cells sorted by the DEPArray.

Discussion:

The majority of CTC capture methods are based on enrichment and/or identification of epithelial markers. Studies have shown that through the process of epithelial to mesenchymal transition (EMT) known to occur in cancer, epithelial markers are down-regulated in favor of mesenchymal markers that allow for increased motility and ability to evade immune detection (2) . Therefore, using epithelial markers as the basis for enrichment and identification will not detect those cells that express only mesenchymal markers. To combat this limitation, we describe here a method of negative depletion following identification using both epithelial and mesenchymal targets.

Circulating tumor cells have demonstrated the potential to be key players in metastasis and therefore in cancer morbidity (37). CTCs have been well associated with overall survival and progression free survival (4) and have great potential to serve as biomarkers and therapeutic monitors. Because they can be obtained serially in a non-invasive manner, the use of CTCs has been touted as a non invasive liquid biopsy. CTCs may possess potential druggable targets and studies have been conducted to enumerate CTCs before, during, and after treatment. Although there is great interest in studying these cells, technical challenges have not been fully addressed to allow for robust and reproducible methods to study these cells. The identification, capture, and characterization of single CTCs remains a challenge and much work is underway to address these limitations (38).

Single cell analysis is growing in popularity for studying disease and has been recently reviewed (39). Research is growing on single cell sequencing (40-42), single cell transcriptomics, and single cell metabolomics. Because circulating tumor cells have been shown to be heterogeneous even from the same blood draw (43), it is crucial to be able to capture and characterize single CTCs. This will ultimately allow researchers to develop methods that will eradicate those cells from cancer patients and prevent metastasis.

Our study demonstrates the ability to examine single, heterogeneous CTCs regardless of EMT status. Two heterogeneous subtypes that have varying expression of epithelial markers were sorted from a mixed population. We were able to identify extracellular protein phenotypes using mean fluorescent intensity and then sort the cells into single cell populations. These single cells were further characterized by gene expression and NGS. The ability to perform genetic analysis on single CTCs from patient samples will provide physicians with the most comprehensive information to determine the best course of treatment for each patient. The ability to study single, heterogeneous CTCs will also allow researchers to discover new drug targets and methods of preventing metastasis from occurring. Because it is likely that only a small subset of a patient's CTCs are capable of causing metastases, it is crucial to characterize a comprehensive collection of a patient's CTCs including epithelial and non-epithelial subtypes as we have demonstrated here.

Abbreviations:

CK, cytokeratins;
CTCs, circulating tumor cells;
EpCAM, epithelial cell adhesion molecule;
EMT, epithelial to mesenchymal transition;
FDA, Food and Drug Administration;
NGS, next-generation sequencing;
PBMC, peripheral blood mononuclear cells;
SNVs, single nucleotide variants

Funding Sources:

NCI Contract #HHSN261201300034C

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