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Cytometric Characterization of Circulating Tumor Cells Captured by Microfiltration and Their Correlation to the CellSearch® CTC Test

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• Abstract

Recent studies reporting hundreds, to thousands, of circulating tumor cells (CTCs) in the blood of cancer patients have raised questions regarding the prevalence of CTCs, as enumerated by the CellSearch® CTC Test. Although CellSearch has been shown to consistently detect clinically relevant CTCs; the ability to only capture EpCAM positive cells has led to speculation that it captures limited subsets of CTCs. In contrast, alternative approaches to CTC isolation are often cited as capturing large numbers of CTCs from patient blood. Not surprisingly the number of cells isolated by alternative approaches show poor correlations when compared to CellSearch, even when accounting for EpCAM presence or absence. In an effort to address this discrepancy, we ran an exploratory method comparison study to characterize and compare the CTC subgroups captured from duplicate blood samples from 30 breast and prostate cancer patients using a microfiltration system (CellSieve™) and CellSearch. We then categorized the CellSieve Cytokeratin(CK)+/CD45−/DAPI+ cells into five morphologically distinct subpopulations for correlative analysis. Like other filtration techniques, CellSieve isolated greater numbers of CK+/CD45− cells than CellSearch. Furthermore, analysis showed low correlation between the total CK+/CD45− cells captured by these two assays, regardless of EpCAM presence. However, subgrouping of CK+/CD45−/DAPI+ cells based on distinct cytokeratin staining patterns and nuclear morphologies elucidated a subpopulation correlative to CellSearch. Using method comparison analyses, we identified a specific CTC morphology which is highly correlative between two distinct capture methods. These data suggests that although various morphologic CTCs with similar phenotypic expressions are present in the blood of cancer patients, the clinically relevant cells isolated by CellSearch can potentially be identified using non-EpCAM dependent isolation.

• Key terms

Circulating Tumor Cells; liquid biopsy; cancer phenotype characterization; CTC cytometry; CellSearch; CellSieve

CIRCULATING tumor cells (CTCs) are cancer cells that originate from primary/metastatic solid tumors and are found transiting the circulatory system (1–4). It has been postulated that CTCs represent a noninvasive method for treatment monitoring, subtyping, and tracking tumor progression in cancer patients (5–7). However, isolation of CTCs is challenging because of their extreme rarity, 1–10 CTCs among 10^9 total blood cells, and compounded by the inherent heterogeneity of tumor cells (2–4,7,8). CTC isolation was first reported in 1869 and although great strides were made in increasing the efficiency of CTC isolation, a clinically validated prognostic assay was not developed until the advent of affinity-based isolation(1–4,7,9). This clinical immunoassay, the CellSearch® CTC Test, captures CTCs from blood samples using

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ferrofluid nanoparticles conjugated with antibodies against the epithelial cell adhesion molecule (EpCAM). Often called the "standard" CTC Test, CellSearch is the only US Food and Drug Administration (FDA) approved clinically validated CTC assay proven to serve as an independent prognostic indicator of patient survival for breast, prostate, and colorectal cancer patients(1–4,9).

CellSearch captures cells using a monoclonal antibody specific to EpCAM, and identifies CTCs using differential fluorescent antibodies to detect the presence of CK within a nucleus-containing intact cell, and absence of CD45, as defining characteristics of CTCs (1–4,8–10). Although CellSearch® has the sensitivity to capture 1 CTC in 7.5 mL of blood, it only captures cells in <78% of metastatic carcinomas. As such, concerns have been raised as to whether the assay definition of CTCs is too restrictive and underestimates the number of true CTC events (1–7,10–14). To account for this underestimation, a number of techniques are being developed to increase capture efficiency by either altering the capture antibodies, or by forgoing affinity capture all together (5–7,11–13,15). Although to date, these techniques have failed to identify the CellSearch CTC population based on presence of CK, or EpCAM, and have shown neither correlation nor equivalency (6,7,9,11,13). Often, it is theorized that the inability to correlate these two techniques is a result of tumor cells losing their EpCAM expression, or cytokeratin expression, possibly through epithelial to mesenchymal transition (EMT) processes (6,7,11,12).

Size exclusion, using microporous filters, is a technique for isolating CTCs irrespective of their surface marker expression (5,12,13,16) that has been shown to capture far greater numbers of CTCs than CellSearch, at times, into the thousands per millilitre (5,12,13,15). This approach was first used over 50 years ago (15) and was recently refined for greater clinical utility (12,13). However, commercial filters used for isolating CTCs can be quite imprecise and highly variable (5). Recent advances in microfabrication have allowed for the commercial production of precision microporous filters, which overcome some previous issues, such as low porosity and high pressure (5). We describe one such microfilter, CellSieve™, made with precision pores arranged in arrayed patterns, giving the filters high porosity under low pressure (5,16). It has been shown that a low-pressure filtration system can isolate circulating cells while preserving fine intracellular architecture, such as cytoskeletal structures, for in depth analysis (5,16).

In this exploratory study, we isolated and enumerated CK+/CD45− cells, with 4′,6-diamidino-2-phenylindole positivity (DAPI+), from 30 breast and prostate cancer patients. We used duplicate samples run in parallel at different locations, using both CellSearch and CellSieve platforms. It was found that CellSieve filters capture greater numbers of CK+/CD45−/DAPI+ cells than CellSearch, findings that are consistent with other studies using size exclusion (6,12,13). After identifying CK+/CD45−/DAPI+ cells and EpCAM+ CK+/CD45−/DAPI+ cells on CellSieve, neither of which showed correlation to CellSearch, and realizing that many previous studies focusing on EpCAM positivity in CTCs have failed to resolve the enumeration discrepancies versus CellSearch, we looked into characterizing the distinct morphological features of the CK+/CD45−/DAPI+ cells. Starting with the cytology-based FDA definition of CTCs (e.g., positive fluorescent staining of CK 8, 18, and 19, CD45−, a diameter >4 × 4 μm, and a DAPI+ nucleus 50% of which is contained within the CK border), we found the CTCs isolated by CellSieve™ express three distinct CK histologically definable staining patterns (e.g., filamentous, diffuse, and punctate) (10,11,14,16–19). Additionally, the nuclear staining patterns of CTCs isolated by CellSieve could be distinguished histologically as either apoptotic or highly abnormal (e.g., high pleomorphism, nonuniform margins, and unusually large size) (10,18,20). Using these criteria, we identified five distinct CK+/CD45−/DAPI+ subpopulations isolated by CellSieve. Comparison analyses found that one main CK+/CD45−/DAPI+ population was highly correlative to the CellSearch Test ($R^2 = 0.91$, $P = 3.18^{*}10^{-16}$), and this correlation was not dependent on EpCAM positivity. These findings suggest that microfiltration of blood samples from cancer patients are indeed capturing a larger variety of CK+ expressing circulating cells (epithelial-like) than the CellSearch system and, furthermore, the clinically prognostic CTC population enumerated by CellSearch may be characterized using a microfiltration approach followed by detailed cytometric analysis. Unlike previous studies on this subject, which have never found correlations to the CellSearch subtype, we do not attempt to determine the underlying functional biology of these CK+ expressing cells by comparing the expression of levels of various biomarkers. Here, we describe that characterization and categorization of CK+/CD45−/DAPI+ cells captured by microfiltration based on their CK and nuclear morphologic patterns numerically correlate to the prognostically valuable CellSearch CTC subtype, which interestingly, does not seem dependent on EpCAM staining.

Materials and Methods

Blood Sample Collection

In total, 30 patient peripheral blood samples from breast ($n = 21$) and prostate ($n = 9$) anonymized cancer patients

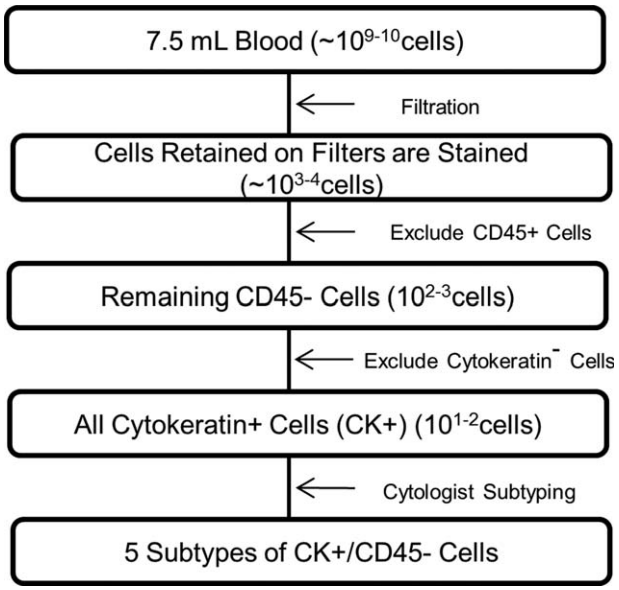

Figure 1. Flow chart of CTC identification using the CellSieve™ system. 7.5 mL peripheral blood containing $\sim 10^{9-10}$ cells is filtered. $\sim 10^{3-4}$ cells are retained on the filters and are stained with DAPI, anti-CK and anti-CD45 antibodies. Stained cells on the filter are scanned for CD45 signal. $\sim 10^{2-3}$ of the cells are CD45−. Remaining cells are then scanned for CK+. ~10–100 of the cells are CD45− and CK+. CK+ CTCs are imaged and subtyped by a trained cytologist into five distinct subpopulations based on cytokeratin and DAPI staining patterns.

were supplied through a collaborative agreement with Fox Chase Cancer Center (FCCC) and University of Maryland Baltimore (UMB), with written informed consent and according to the local Institutional Review Board (IRB) approval at each institution. In addition, 30 nonblinded healthy volunteer blood samples were collected in CellSave preservative tubes™, with written informed consent and IRB approval by Western Institutional Review Board. Anonymized blood samples were drawn in tandem into two CellSave tubes (~9 mL). Within 72 h, one tube (7.5 mL) was used to enumerate CTCs using CellSearch at FCCC. The second tube (7.5 mL) was used to enumerate CTCs using CellSieve microfiltration at UMB or Creatv MicroTech. Results and patient identification from institutions were not shared or communicated until completion of the study.

CellSieve Microfilter CTC Enumeration

Each CellSieve Microfiltration Assay isolates CTCs based on size exclusion and identifies CTCs based on the histological cell architecture of cytokeratin, and nuclear morphologies (5,16). An overview of the process is shown in Figure 1. The assay and reagents have been previously described (21). Briefly, each assay is supplied with a CellSieve microfilter (~160,000 pores each at 7 μm in diameter arrayed on a 9 mm area), Prefixation buffer, a Postfixation buffer, a Permeabilization buffer, and an antibody cocktail (5). The low-pressure system uses a filter holder assembly attached to a regulated vacuum set at 5 mL/min. Peripheral blood (7.5 mL), collected in a CellSave tube is diluted in a prefixation buffer and drawn through the filter (5). The filter is washed, postfixed, and permeabilized. The captured cells are stained with an antibody cocktail consisting of FITC-anti-Cytokeratin 8, 18, 19; Phycoerythrin (PE) conjugated EpCAM; and Cy5-anti-CD45 (5) (Fig. 2). Filters are then washed, placed onto a microscope slide and cover-slipped with Fluoromount-G/DAPI (Southern Biotech). An Olympus BX54WI Fluorescent microscope with Carl Zeiss AxioCam was used to image cells. Exposures were preset as 5 sec (Cy5), 2 sec (PE), 100–750 msec (FITC), and 10–50 msec (DAPI) for equal signal comparisons between cells. A Zen2011 Blue (Carl Zeiss) was used to process the images.

CellSearch CTC Enumeration

The CellSearch system was run following the Janssen protocols at FCCC. Immunomagnetic enrichment of CTCs using the CellTracks™ AutoPrep System has been previously described (22). Peripheral blood samples collected in CellSave Preservative tubes were maintained at ambient temperature. CellSearch Epithelial Cell kits (Janssen Diagnostics) were used for the isolation of CTCs. Isolations was performed on the CellTracks AutoPrep® System (Janssen Diagnostics). Data was collected and analyzed on the CellTracks Analyzer II® (Janssen Diagnostics).

Briefly, antipan cytokeratin (CK 8, 18, 19)-PE, anti-CD45-APC, and DAPI (CellSearch Epithelial Cell kit reagents) were used to differentially label the CTC enriched product. Ferrofluid nanoparticles conjugated with anti-EpCAM antibodies capture CTCs from 7.5 mL of blood and are magnetically separated. Cells are washed, permeabilized, labeled with fluorescent antibodies, resuspended in Cell Fixative then loaded into a cartridge held in a magnetic holder (MagNest) which aligns the ferrofluid-captured cells. The Magnest is placed into a CellTracks Analyzer II® and the fluorescently labelled cells are imaged. Images are sorted using computer-assisted software selecting and presenting CK+ and DAPI+ events. A technician selects cells meeting the FDA criteria for CTCs, for example, (1) expressing CK, (2) lacking CD45, and (3) containing a DAPI+ nucleus 50% which is contained within an intact CK+ perimeter (Supporting Information Fig. 1).

Statistical Methods

Linear regression plots were made using the enumerated counts from all subtypes of CK+/CD45− cells identified using CellSieve and the CTCs enumerated by CellSearch. Spearman correlation coefficients were calculated for each CK+/CD45− subtype using MATLAB R2013A. Power analysis for sample size was calculated using previously published CVs using MATLAB R2013A (1,5,23,24). In addition, linear regression plots, slopes, and correlative values were also calculated for breast, and prostate, cancer patient samples (Fig. 3 and Supporting Information Fig. 2).

Results and Discussion

Since the CellSearch system utilizes a highly specific EpCAM-based approach to capture CTCs, it has been argued

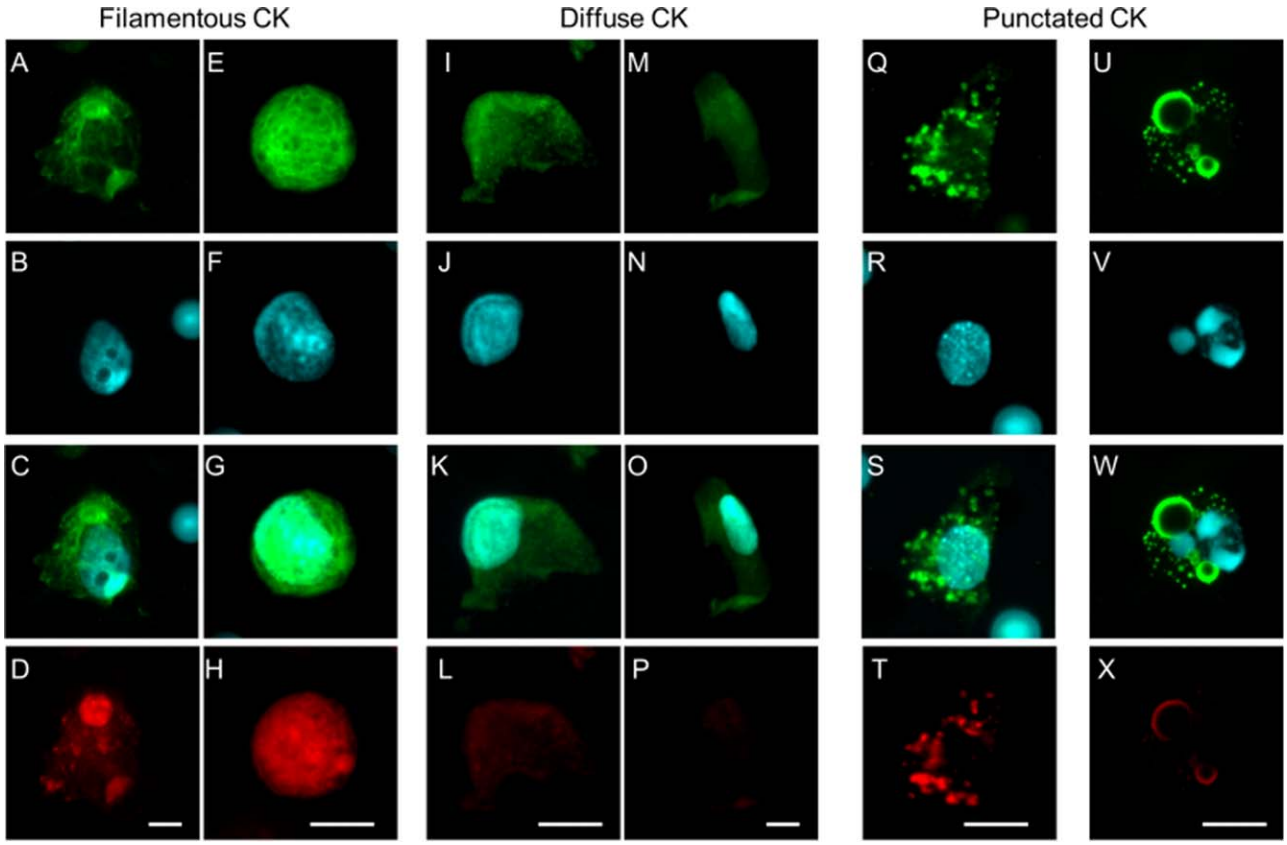

Figure 2. Subpopulations of CTCs based on cytological features of cytokeratin and DAPI on CellSieve™. (**A–H**) CTCs categorized as PDCTCs with (A, E) filamentous cytokeratin. (B, F) The nuclei are malignant, appearing with nuclear inclusions and margin irregularities. (C, G) Merged images. (D, H) Strong EpCAM+ expression. (**I–P**) CTCs categorized as EMTCTCs with (I, M) diffuse cytokeratin patterns. (J, N) Nuclei appears malignant with irregular nuclear contours but smooth margins and a regular oval shape. (K, O) Merged images. (L, P) Low/negative EpCAM expression. (**Q–T**) CTC categorized as EACTC with (Q) punctate cytokeratin. (R) Nucleus appears as malignant with an abnormal salt-and-pepper pattern. (S) Merged image. (T) EpCAM+ expression. (**U–X**) CTC categorized as LACTC with (U) punctate cytokeratin. (V) Nucleus also appears punctate, or blebbing. (W) Merged image. (X) Low/negative EpCAM expression. Scale bars, 10 μm.

that it is insensitive to circulating epithelial cells which do not express EpCAM on their cell surface. Therefore, it is concluded that this technique has limited utility on broader patient cohorts with failings in capturing and identifying cancer stem cells which have undergone EMT, a heterogeneous process with no standardized definition (11,13). Alternative techniques, such as size based isolation, whole blood cell smears, electrophoresis, so forth, attempt to increase sensitivity of CTC capture, typically while sacrificing specificity (5–7,11–13,15). Not surprisingly, less stringent techniques have been shown to capture far greater numbers of CK+ and EpCAM+ expressing cells from the blood of cancer patient samples, at times numbering thousands of CK+, or EpCAM+ expressing cells per millilitre (5–7,11–13,15). The greater number of CK+ expressing cells captured by these techniques is argued to be a result of greater efficiency of their approaches. However, the same clinically validated data provided by CellSearch has yet to be reproduced by these alternative approaches and attempts to account for these discrepancies by evaluating the functional biology of the CK+ cell types using additional biomarker information, such as EpCAM presence, have not yet yielded improved correlations with CellSearch (6,7,10,12,13).

In an effort to reconcile the discrepancies between CK+ expressing cells captured using filtration techniques, and the prognostically significant enriched CK+ expressing cells identified as CTCs via CellSearch, we performed a detailed examination of all CK+ expressing cells captured by the CellSieve microfiltration system. To directly compare the two techniques, we only examined staining patterns of the standard CellSearch detection markers, including intact cells with cytokeratin, CD45, EpCAM, and nuclear DAPI, and not by adding additional marker systems nor including CK+ particles (10).

Cytokeratins are intermediate filament proteins expressed by epithelial derived cells and are prevalent in transformed epithelial cells (17), such as CTCs (1,9,11,14,19). These structures are extremely fine (~10 nm diameter) and their morphologies can give information regarding apoptosis, structural integrity, and anaplasia (14,17,19,25). Since the CellSieve system has been shown to preserve internal cellular structures, detailed analysis of the distinct CK+ filament architecture can be performed (5,16).

A

Comparison	Total CK+ cells vs. CellSearch®	PDCTC vs. CellSearch®	EACTC vs. CellSearch®	LACTC vs. CellSearch®	EMTCTC vs. CellSearch®	Atypical CK+ cells vs. CellSearch®
R^2	0.4427	0.9107	0.6033	0.1311	0.0108	0.0002
p-value	$6.03*10^{-5}$	$3.18*10^{-16}$	$4.50*10^{-7}$	0.05	0.58	0.95

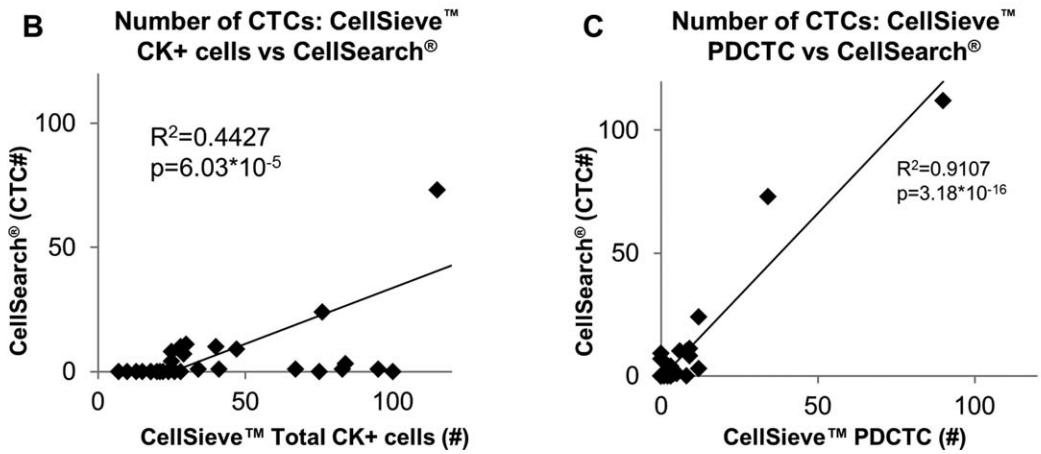

Figure 3. Correlations of CK+ subpopulations identified by CellSieve™ filters versus enumeration by CellSearch®. (**A**) Correlations between the CTC subcategories by CellSieve compared to CTCs by CellSearch. (**B**) Linear regression curve plots between Total CK+ cells versus CellSearch showing a low correlation. (**C**) Linear regression curve plots between PDCTCs versus CellSearch, showing a high correlation. Slopes are found in Supporting Information Figure 2.

The distinct CK+ staining pattern of cells captured by CellSieve can be readily identified as filamentous, diffuse, and punctate and form the basis of CTC subclassification used in this study (Fig. 2). Filamentous CK is the classical and established example of epithelial intermediate filaments, with fibril like structures traversing although the interior of a cell (Fig. 2A and 2E) (14,17,19). Diffuse CK is defined by a weak CK staining without observable filamentous patterns, usually this pattern is associated with epithelial-mesenchymal transition, although no universal definition of EMT transition currently exists (Fig. 2I and 2M) (11,14,17). Punctate CK staining can be attributed to the collapse of the cytoskeletal structure, in the early stages of apoptosis, which results in retraction of the cytokeratin filaments, referred to as blebbing (Fig. 2Q and 2U) (10,25). Cytokeratin blebbing has also been described in the CellSearch Test Analyzer and typically counted as a CTC, although disagreements in the definitions between intact "granular" CTCs and cell particles do exist (9,10).

Nuclear morphology is another criteria used in identifying, grading, and classifying cancer cells in both cancer biopsies and on the CellSearch system (9,10,18). After filtration, we identify abnormal nuclear patterns typically seen in tumor cells (e.g., pleomorphism, nonuniform margins, unusually large size) (Fig. 2B, 2F, 2J, 2N, 2R, and 2V) (18,20). These nuclear variations are a prerequisite for morphologically classifying CTCs and, in cases of punctate CK patterns, we used the presence of these variations to identify cells undergoing early apoptotic or late apoptotic events. In early apoptotic CTCs, the CK+ staining is punctate; however, the nucleus is intact (Fig. 2Q and 2R) (18,20). In late apoptotic CTCs, the CK staining is punctate and the progressive apoptotic process has broken the nucleus apart, also called nuclear blebbing or punctate (Fig. 2U and 2V) (25). In either case, a DAPI positive signal within a CK+ signal is defined as a CTC on the CellSearch Test Analyzer (9,10).

Based on the three CK+ staining patterns (filamentous, diffuse and punctate) and two nuclear staining pattern (malignant and punctate), we have identified four distinct subpopulations which make up the total CK+/CD45− expressing cells classified as CTCs isolated by CellSieve:

1. Pathologically definable CTCs (PDCTC): (1) have strong filamentous CK+ signal, (2) have a DAPI+ nuclei with malignant pathologies (Fig. 2A–2H).
2. Epithelial-Mesenchymal transition-like CTCs (EMTCTC): (1) have diffuse/nonfilamentous and weak CK+ signal, (2) have a DAPI+ nuclei with malignant pathologies (Fig. 2I–2P).
3. Early Apoptotic CTCs (EACTC): (1) have a punctate CK+ signal, (2) have intact DAPI+ nuclei with malignant pathologies (Fig. 2Q–2T).
4. Late Apoptotic CTC (LACTC): (1) have a punctate CK+ signal, (2) have a punctate nuclear DAPI+ staining (Fig. 2U–2X).

The CK+/CD45− cells in the four subpopulations ranged from high EpCAM positivity to low/negative

Table 1. CTCs enumerated by CellSearch® and CK+ subpopulations identified by CellSieve™

PATIENT	TNM	CELLSEARCH®	PDCTC CELLSIEVE™	EACTC CELLSIEVE™	LACTC CELLSIEVE™	EMTCTC CELLSIEVE™	ATYPICAL CK+ CELLS CELLSIEVE™	TOTAL CK+ CELLS CELLSIEVE™
BC1	T2/N1/M1	0	0	0	0	45	55	100
BC2	TX/NX/MX	0	0	0	5	0	10	15
BC3	TX/NX/M1	0	2	0	2	0	14	18
BC4	T2/N1/M0	0	2	0	2	2	7	13
BC5	TX/N2/M0	0	1	0	0	0	74	75
BC6	TX/N2/M0	0	3	2	3	0	20	28
BC7	T4/N3/M0	0	0	0	0	1	9	10
BC8	T3/N1/M0	0	8	0	0	6	6	20
BC9	T4/N3/M1	0	0	0	0	0	22	22
BC10	T4N3/M0	0	0	0	0	1	17	18
BC11	TX/NX/M1	0	1	0	10	0	15	26
BC12	T2/N1/M1	0	3	2	2	0	17	24
BC13	T4/N2/M0	1	5	0	18	27	33	83
BC14	T4N3/M0	1	4	3	1	0	26	34
BC15	TX/NX/M1	1	4	1	17	3	70	95
BC16	TX/NX/M1	1	5	2	2	11	21	41
BC17	TX/NX/M1	3	12	0	0	2	70	84
BC18	T2/N1/MX	8	9	1	0	4	11	25
BC19	TX/NX/M1	11	9	0	0	0	21	30
BC20	T4/N3/M0	24	12	16	0	14	34	76
BC21	T1/N2/M0	112	90	18	6	0	28	142
Average ± SD		7.7 ± 4.6	8.1 ± 19.2	2.1 ± 5.0	3.2 ± 5.4	27.6 ± 21.4	5.5 ± 11.2	46.6 ± 37.0
Median		0	3.0	0	1	21	1.0	28.0
PC1	T2/N0/M0	0	2	0	0	0	5	7
PC2	TX/NX/M1	0	0	2	0	0	19	21
PC3	TX/NX/M1	1	1	0	0	4	62	67
PC4	T3NX/MX	4	3	0	3	1	18	25
PC5	T2/N0/M0	7	0	5	0	0	24	29
PC6	T3/N1/M1	9	0	18	12	0	17	47
PC7	T3/N0/MX	10	6	6	5	0	23	40
PC8	T2/N1/M0	10	8	0	0	2	18	28
PC9	TX/NX/M1	73	34	31	23	0	27	115
Average ± SD		12.7 ± 23.0	6.0 ± 10.9	6.9 ± 10.7	4.8 ± 7.9	23.7 ± 15.7	0.8 ± 1.4	42.1 ± 32.2
Median		7.0	2.0	2.0	0.0	19.0	0.0	29.0
Total average		9.2 ± 23.8	7.5 ± 16.9	3.6 ± 7.4	3.7 ± 6.1	26.4 ± 19.6	4.1 ± 9.6	45.3 ± 35.1
Total median		1.0	3.0	0.0	0.5	20.5	0.0	28.5

CTCs isolated from duplicate samples of blood from prostate (PC) and breast (BC) cancer patients. The rows from left to right show patient number, Classification of Malignant Tumors (TMN) and the number of CTCs identified by CellSearch. Right six rows show the number of CTC subpopulations, and the total number of CK+ cells identified by CellSieve.

positivity (Fig. 2D, 2H, 2L, 2P, 2T, and 2X), but were not a driving factor in concordance (Supporting Information Fig. 2). CK+/CD45− cells that could not be categorized into these four subpopulations were classified as "Atypical CK+ cells" and not counted as CTCs for this study (Supporting Information Fig. 3). These cells included CK+/CD45− cancer associated macrophage-like cells (CAMLs) (16) (Supporting Information Fig. 3, Column A) and DAPI+ and CK+ cells without visible cytoplasm (Supporting Information Fig. 3, Column B). Other CK+/CD45− events not included in this study, as they do not meet the criteria of a CTC, include (1) CK+/CD45− events with no DAPI signal (Supporting Information Fig. 3, Column C) and (2) CK+/CD45− cells which were identified as noncancerous (e.g., granulocytes, macrophages, so forth) by a pathologist (Supporting Information Fig. 3, Column D). Additionally, cell clusters/microemboli of $\geq$2 were counted as one CTC (Supporting Information Fig. 3, column E), following equivalence to CellSearch enumeration (1–4,9).

In Table 1, the 4 CK+/CD45− CTC subpopulations, Atypical CK+ cells and the total CK+/CD45− cells are shown

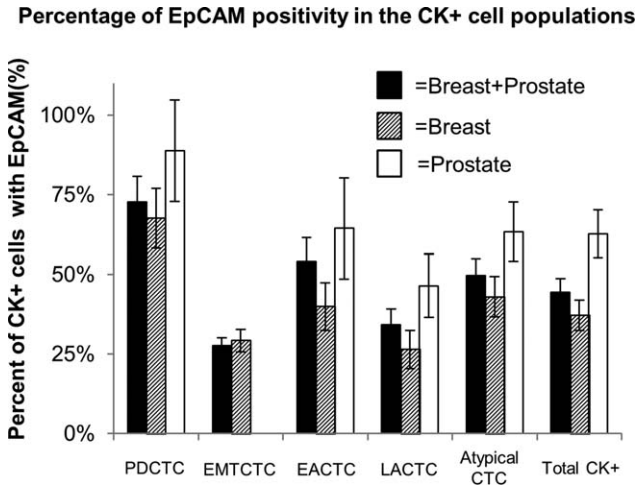

Figure 4. Percentage of EpCAM positivity in the CK+ populations. CK+ cells isolated by CellSieve™ were further categorized by visual presence of EpCAM. The percentage of EpCAM positive cells in the CK+ cells for each subcategory is listed, as is the difference in EpCAM percentage in breast samples and prostate samples. Slopes and correlations of EpCAM subcategories are found in Supporting Information Figure 2D, error bars indicate standard error of the mean.

in comparison to CellSearch enumeration, for the 30 duplicate patient samples. CellSieve captured 979 CK+/CD45− cells from 21 breast cancer patient blood samples compared to 162 CTCs captured by CellSearch. Additionally, CellSieve captured 379 CK+/CD45− cells from 9 prostate cancer patients, compared to 114 by CellSearch. No CTCs, from the 30 healthy volunteer blood samples, were found on the CellSieve system. These data support previous publications regarding greater CTC capture from patient blood samples using size exclusion (5,12,13).

To compare the two assays, we ran method comparison analyses using linear regression plots with correlation significance (23). We chose a sample size of 30 which gives the statistical power necessary to detect differences smaller than the intrinsic variability range of the CellSearch Test (1,5,23,24). When comparing regression plots between the total CK+/CD45− population isolated by CellSieve versus CellSearch we found that the cells were not equivalent, whether we included EpCAM presence or not (Fig. 3 and Supporting Information Fig. 2). This lack of equivalency matches most previous studies regarding the comparison of CellSearch to other techniques (6,7,12,13,26). However, when we compared individual CellSieve subpopulations with CellSearch, we found that the PDCTC subgroup showed significant correlation with CellSearch ($R^2 = 0.9107$, $P < 0.0001$; Fig. 3A and 3C). Additionally, the inclusion of EACTCs with the PDCTCs gave the best equivalency, higher than the inclusion of PDCTC with EpCAM+ expression (Supporting Information Fig. 2A and 2D). This data suggests that the PDCTC subpopulation, regardless of EpCAM presence, is most statistically correlated to CellSearch, while the other CK+/CD45− cells are not. Furthermore, our data suggests that although the CellSearch system relies on capturing EpCAM+ cells for isolating CTCs; correlation of this clinically relevant CTC subtype, identified using microfiltration, is primarily dependent on cytokeratin and nuclear morphologies, and not EPCAM expression (Supporting Information Fig. 2).

By analyzing the presence of EpCAM in the PDCTCs cells, our data appears to be in agreement with staining studies of primary biopsies which analyzed EpCAM expression. This study showed that 99% of prostate carcinomas and 74% of breast carcinomas were EpCAM positive (8). Our data shows that the percentage of EpCAM positivity in breast PDCTCs is 68% and 90% in prostate PDCTCs (Fig. 4). The EpCAM data also seems to agree with the theories regarding EMT cell transition as there was less EpCAM present in cells that also have diminished CK staining, the EMT-like CTCs. However, as there in no universal definition of EMT, further analysis of the CTCs exhibiting these characteristics needs to be performed when specific markers of EMT cells have been identified (6,11,12,14).

Once method correlations were established, we ran a preliminary evaluation of the prognostic significance of the CK+ categories using $\geq$5 CTCs/sample as a threshold for patient overall survival (OS). The criteria for clinical utility, for breast and prostate cancers, is the cut off value of 5 CTCs/sample, $<$5 showing longer OS than $\geq$5 CTCs (2–4). Supporting Information Figures 4 and 5 shows the survival of the 26 patients that remained on study for a 24 month period. Using the $\geq$5$>$ threshold we found that CellSieve PDCTCs and CellSearch matched in 23 of the 26 patients, and in the three instances where the methods differed, there was an observed change in the survival outcome (Supporting Information Fig. 4). Additionally, both EMTCTCs and EpCAM positive PDCTCs had some lower correlations to CellSearch, and both groups also showed some difference in overall survival for patient cohorts using the $\geq$5$>$ cell criteria (Supporting Information Fig. 5). Although this data implies differences in outcome between patient cohorts, the data set is too small to draw any statically relevant conclusions. It does, however, suggest that additional larger studies may be warranted to determine if these survival trends continue to differentiate patient populations.

For many years, the goal of CTC work has revolved around the concept of using blood as a "liquid biopsy" for cancer diagnosis, prognosis, and treatment response. Generally, histological review of biopsies define the presence of tumor cells using morphological criteria based on organ specific histopathological grading schema describing cellular features (e.g., nuclei abnormalities, mitotic proliferation, hyperactive Golgi, so forth). However, current CTC capture techniques lack the ability to provide adequate numbers of circulating epithelial cells in a format where standard histological staining can be applied, and reviewed by a pathologist. Here, we demonstrate that multiple populations of CTCs can be identified by histopathological staining patterns of CK and DAPI using filter-based isolation. These preliminary data suggest that CTCs with malignant nuclear morphologies and filamentous cytokeratin are, at least numerically, the same cells identified using CellSearch. These findings support the hypothesis that both CellSearch and CellSieve microfiltration

are capable of identifying a similar number of highly specific and clinically relevant CTC subtypes.

As CTC isolation methods have become more varied and our biological understanding become greater, the defining criteria for what cells meet the designation of a CTC has become less stringent (10,11,14). Complicating the criteria of CTCs is the knowledge that cancer cells can undergo EMT, which has no universal definition, although generally described by the down regulation of epithelial proteins, such as EpCAM and cytokeratin. As there in no scientific consensus in the EMT definition, and not within the scope of this manuscript, we did not attempt to identify the EMT processes in cells, only describe EMT-like cells by the visual loss of filamentous structure (10,11,14).

When assessing new technologies one must determine the proposed usage of the capture events. If the intent is to collect product for downstream mutational analysis, this is quite different than using a new technique as a prognostic indicator of overall survival, such as CellSearch. The primary result of many CTC capture methods is to show discordance with the clinical validity of CellSearch, by virtue of increased CTC number (23). However, a fact which is largely ignored by comparative technologies is the fact that CellSearch captures numerous cytokeratin positive particles which are known to provide prognostic value, but are discluded by the morphological identification of a trained operator (9,10,22). Groups typically bypass the morphological criteria, and explain this difference in CTC number between their techniques and CellSearch through the use of additional biomarkers, for example, EMT markers, apoptotic markers, and proliferation markers (6,7,11–13). However, to date, studies focusing on these functional biological markers have lacked the ability to correlate to CellSearch and, as such, have offered few insights into the CTC subpopulation that CellSearch enriches for (6,7,12,13). In this study, rather than focusing on the identification of the biological differences between two CTC capture technologies using differing biomarkers, we provide the first example of matched samples, using accepted markers, which can replicate the data demonstrated using the CellSearch system. Our data suggests that size exclusion techniques coupled with characterization of specific staining morphologies might be used to identify a validated and clinically relevant CTC subpopulation for breast and prostate cancer. This exploratory study reveals an opportunity to now expand and define the clinical relevance of additional CTC subpopulations captured by non-EpCAM-based techniques and better understand the CTCs CellSearch captures.

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