Published in final edited form as:

Int J Cancer. 2010 February 1; 126(3): 669–683. doi:10.1002/ijc.24814.

Isolation of circulating epithelial and tumor progenitor cells with an invasive phenotype from breast cancer patients

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Abstract

Recent research advances show that tumor cell intravasation (entry into the circulation) and metastasis occur very early in breast cancer progression. Clinical studies also illustrate the potential importance of detection of circulating tumor cells (CTCs) in outcomes of patients with metastatic breast cancer. Whether these cells exhibit the invasiveness and express tumor stem or progenitor markers, hallmark of the metastatic phenotype, is less well characterized. To detect CTCs with the invasive phenotype and to explore their molecular features, we applied a functional cell separation method, called collagen adhesion matrix (CAM) assay, as enrichment and identification steps. The CAM-coated device successfully recovered tumor cells spiked in one mL of blood with a 54%±9% (n=18) recovery rate and 0.5-35% purity, and detected invasive tumor cells in 10/10 blood samples (100% yield) from patients with metastatic breast cancer with a range of 18 to 256 CTCs/mL and average of 126±25 (mean±SD) CTCs/mL. CTCs were detected in blood samples of 28/54 (52%) stage I-III breast cancer patients with a mean count of 61 CTCs/mL. Furthermore, the relative frequency of these cells correlated to the staging, lymph node-status and survival of patients with early stage breast cancer. CAM-captured cells were capable of propagation in culture. Gene expression and multiplex flow cytometric analyses on CAM-captured cells demonstrated the existence of distinct populations of CTCs including these of epithelial lineage and stem or progenitor cells. Thus, CAM-initiated CTC detection provides advantages for examining invasiveness and tumor progenitor phenotypes.

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Statement of novelty and impact – This work advanced our understanding of function of circulating tumor cells in breast cancer progression. Specifically, we showed that these cells express tumor-associated markers and are invasive when exposure to a collagenous matrix. The functional enrichment method reported here helps defining different subpopulations of CTCs and improves the detection sensitivity in early stage breast cancer. It has broad implications in advancing both cancer biology research and utility of CTCs in treatment selection.

Authors' Disclosures of Potential Conflicts of Interest: W-T Chen, patent applications filed on detection of CTCs, is the founder & President of Vitatex Inc that commercializes the CAM technology. T. Fan was a consultant of Vitatex Inc from September 2006 to June 2007 and her spouse maintains a position in the company. Other authors indicated no potential conflicts of interest.

Keywords

tumor invasion; breast cancer; metastasis; circulating tumor cells

Introduction

Recent studies show that entry of tumor cells into the circulation and subsequent metastasis occur early in breast cancer progression (1;2). Although effective detection of “rare” tumor cells (a few CTCs in 1-mL of blood) remains technically challenging, populations of CTCs, identified by various technologies focusing on the epithelial cell surface marker EpCAM (also known as ESA/Epi/TADST) (3;4), have been shown to correlate with cancer progression (5; 6). Likewise, CTC detection based on multiple epithelial lineage mRNAs using sensitive PCR-based molecular assays (7;8) appear useful for establishing clinical correlations with early breast cancer prognosis.

Tumor cells expressing invasive phenotypes lose many types of epithelial antigens in a transformation process called epithelial-mesenchymal transition (EMT) (9-11). The transition, which occurs early in cancer progression, involves tumor progenitor (TP) cells with stem/invasive cell properties (12;13). Such cells may enter the circulation to generate multiple populations of CTCs with specific phenotypes (2;4) that could contribute to aggressive growth of tumor cells in the lung and to early metastatic colony formation (14;15). However, the majority of current technologies for CTC detection involve either use of antibody against EpCAM for complex analytic approaches such as immuno-affinity micropost (CTC-chip™) or immuno-magnetic particles (CellSearch™), or multiplex qPCR approaches for tumor-associated mRNAs (5;6). Low EpCAM expression by hematopoietic micrometastases has been reported, and attempts to isolate CTCs using antibodies against EpCAM result in loss of most of the CTCs in some cases (16-19). In addition, whether the epithelial cells isolated by these methods exhibit the invasive phenotype and express stem cell markers is not well understood.

Since the proclivity of a tumor cell to invade collagenous matrices is one of the hallmarks of metastasis (20;21), we hypothesized that populations of CTCs that adhere and invade collagenous matrices would be invasive and exhibit the propensity of progenitor cells to metastasize. We reasoned that an enrichment step based on function of invadopodia, which are subcellular structures involved in cell invasion into collagenous matrices (22;23), would serve to separate CTCs from the majority of cell types found in the blood. Recently, we demonstrated the principle that metastatic tumor cells derived from peritoneal effusions of ovarian cancer patients were invasive when in contact with collagenous matrices (24). We further developed CTC enrichment and identification prototypes using collagen adhesion matrix (CAM) for uses in cellular analysis of ovarian cancer (25) and molecular analysis of prostate cancer (26). The CAM-coated prototypes enrich tumor cells in blood and peritoneal fluid based on their unique avidity to the CAM enabling sensitive cellular and molecular analyses of ovarian and prostate cancers. A particular feature is that CAM surfaces allow direct assessment of tumor cell invasiveness by their ability to remove and ingest CAM fragments, called CAM uptake, and facilitate an independent means of identifying a tumor cell using the CAM uptake criterion.

Considering the wide range of sensitivity (0.7-176 CTCs per mL of blood) and yields (14-100%) of CTC detection in patients with metastatic breast cancer (5;6), in this study we aimed: (a) to determine if the CAM method generates an effective and sensitive detection of CTCs in breast cancer, and (b) to examine if populations of CTCs with novel phenotypic expression exist. The CAM method achieved effective enrichment and identification, and successfully detected CTCs in whole blood of an experimental setting in 18 of 18 (100%) samples and that of metastatic breast cancer patients in 10 of 10 (100%) samples, with a range

of 10–1,000 CTCs per mL of blood. The high sensitivity and specificity of the CAM method encouraged us to perform a pilot investigation on the prognostic significance of circulating epithelial cells with the collagen-invasive phenotype in early stage breast cancer patients. We demonstrate that the relative frequency of invasive CTCs correlates to the staging, lymph node-status and survival of patients with early stage breast cancer. CAM-captured cells derived from blood of breast cancer patients are capable of propagation in culture. Concomitantly, we showed by gene expression profiling that CAM-enriched circulating cells retained the expression of distinct sets of epithelial and tumor progenitor or EMT stem cell (*CD44* and *TWIST1*) genes. Furthermore, we showed by multiplex fluorescent activated cell sorting (FACS) that CAM-captured CTC populations consist of epithelial lineage cells (Epi⁺ cell: EpCAM⁺CD34[−]CD45[−]7AAD[−]) and tumor progenitor cells (TP⁺ cell: CD44⁺CD34[−]CD45[−]7AAD[−]), in which 50-75% of the two populations overlap (Epi⁺TP⁺ cell: CD44⁺EpCAM⁺CD34[−]CD45[−]7AAD[−]). We conclude that the CAM method may provide an effective novel tool for sensitive identification and characterization of subtypes of CTCs in patients with breast cancer.

Materials and Methods

Patients and clinical samples

This study was conducted at Stony Brook University Medical Center after approval from the Committee on Research in Human Subjects was obtained. Breast cancer patients receiving care at the medical oncology practice from January 1, 2000 through October 30, 2007 were recruited. Eligibility criteria included patients who had not yet undergone chemotherapy. Patients were staged according to the American Joint Committee on Cancer TNM system. Disease recurrence and progression were diagnosed by clinical examination, radiological findings, and/or biopsy.

Peripheral blood was obtained from 54 patients with stage I-III breast cancer, 10 patients with stage IV breast cancer, 1 patient with ductal carcinoma in situ (DCIS) and 53 healthy women. Five to twenty milliliters of blood were collected during the patient's regularly scheduled clinical visit. In the cases of healthy participants, 53 healthy women (age range 21-72) were recruited from the community and blood collected to test the reproducibility of the CAM assay. Three mL aliquots were used for quantification of CTCs. Blood was collected in VacutainerTM tubes (Becton Dickinson, Franklin Lakes, NJ; green top, lithium heparin as anticoagulant) and processed within four hours from collection.

Tumor cell enrichment and labeling

Blood samples were subjected to enrichment and identification for invasive CTCs using a two-step CAM method described recently (25;26). Briefly, Ficoll density gradient centrifugation was used to obtain the mono-nucleated cells (MNCs). To capture and label tumor cells that remove and ingest fluorescent CAM fragments (CAM⁺), MNCs from 0.5 mL whole blood aliquots were seeded onto one well of a red fluorescent CAM-coated 16-well chamber slide (Vita-AssayTM, Vitatex Inc., Stony Brook, NY), and cultured with Cancer Cell Culture (CCC) media (1:1 mixture of Dulbecco's modified Eagle's medium and RPMI1640 medium supplemented with 10% calf serum, 10% Nu-serum, 2 mM L-glutamine, 1 unit/mL penicillin, and 10 µg/mL streptomycin) for 12 hours. Non-adherent cells were washed away with PBS. To stain cells, cells were fixed with 3.5% paraformaldehyde/PBS, permeabilized with 0.1% Triton X-100, and stained with an anti-CD45 antibody (clone T29/33, DakoCytomation, Carpinteria, CA) and followed by red color alkaline-phosphatase-anti-alkaline-phosphatase (APAAP) conjugated secondary antibodies (DakoCytomation, Carpinteria, CA) for internal control of cells with common leukocyte markers. The cells were then stained for CTCs using a mixture of fluorescein-conjugates of antibodies against standard epithelial cell lineage

markers (Epi⁺), including EpCAM (clone Ber-Ep4, DakoCytomation, Carpinteria, CA); ESA (clone B29.1, Biomedex, Foster City, CA); and pan-cytokeratins 4,5,6,8,10,13 and 18 (CK) (clone C11, Sigma, St. Louis, MO). A Nikon E-400 inverted fluorescence microscope equipped with Microfire digital camera system and Image Pro Plus software was used to examine and record the images. Any sample that had at least one CTC counted was considered “CTC-positive”. Counts were performed by trained personnel and confirmed by a second observer.

Cell-line experiments

The human breast carcinoma cell line MDA-MB-231 and its lines derived from genetic transfection with green fluorescent protein (GFP) were maintained and grown in CCC medium at 37°C in 5% CO₂. Growth medium was aspirated and cells were suspended using treatment of monolayer with trypsin/EDTA. The cell concentration in whole blood was determined by counting with a hemacytometer. Non-fluorescent or GFP-tagged cells that were pre-selected by collecting CAM-avid cells, 10-1,000 tumor cells, were spiked into 1-mL of whole blood. Tumor cells in the MNC fraction were seeded to adhere on the CAM-coated bottom surface in a well of the 16-well chamber slide (Vita-Assay™, Vitatex Inc., Stony Brook, NY). Alternatively, CAM-adherent cells were directly isolated from 1-mL of whole blood using a CAM-coated tube (Vita-Cap™, Vitatex Inc., Stony Brook, NY). Non-fluorescent tumor cells were made fluorescent by staining with fluorescein-conjugates of antibodies against standard epithelial cell lineage markers (Epi⁺), including EpCAM, ESA and pan-cytokeratins.

Clinical data collection

All clinical data was abstracted from patients' medical chart and collected through April 15, 2008. Survival length was calculated from the date of blood sample collection to date of death (for overall survival) or recurrence/progression (for disease-free survival), or most recent documented contact as of April 15, 2008, if the patient was still alive with no evidence of disease.

Statistical analysis

Continuous variables, such as CTC counts and age, were summarized by means and standard errors of the mean. Categorical variables, such as stage, lymph node status, tumor size category, tumor histological type and grade, ER/PR/Her-2 status, surgery type, and chemotherapy regimen, were listed as frequencies and percentages. Chi-square tests were used to compare categorical data. For continuous data, because the distribution was not normal, non-parametric statistics (Kruskal-Wallis test for multiple groups and Mann-Whitney U test for two groups) were used. Mean CTC counts prior to and after completion of chemotherapy were also evaluated using the Wilcoxon Signed-Rank test. Kaplan-Meier curves for overall and disease-free survival were estimated. Survival differences between patients who had high versus low CTC counts were compared using log-rank tests. The median CTC count for stage I-III patients (median = 10 CTCs/mL in current study population) was used to distinguish patients with high versus low CTC counts. CTC detection rate was defined as the percentage of patients who had at least 1 detectable CTC. All analyses were performed using SPSS version 16.0. Results with two-tailed P-values less than 0.05 were considered statistically significant.

Cell viability and culture

Cell viability was determined with the LIVE/DEAD viability assay kit #4 (Invitrogen, Carlsbad, CA: dead cells stain red fluorescence with ethidium homodimer-1 and live cells stain green fluorescence with calcein AM). This assay is based on intracellular esterase activity of live cells and plasma membrane integrity of dead cells. Briefly, cells were incubated at 25°C for 30 min in a solution of 2 µM calcein AM and 4 µM ethidium prepared in PBS. At the end of the incubation period, the well was washed with 0.1-mL PBS and examined under the

microscope. To culture CAM-captured cells from breast cancer patients, MNCs from 0.5 mL whole blood aliquots were seeded onto one well of a CAM-coated 96-well microtiter plate (Vita-Assay™, Vitatex Inc., Stony Brook, NY) with the CCC media and incubated in a CO₂ cell incubator for 12 hours, followed by removal of old media and non-adherent cells and replacement of fresh media every 3 days for continued culture.

Microarray data analysis

CAM-adherent cells that were directly isolated from 1-mL of whole blood using a CAM-coated tube (Vita-Cap™, Vitatex Inc., Stony Brook, NY) were used in microarray and flow cytometric analyses. Total RNA from cells bound to CAM tube (Vita-Cap™) were purified by RNeasy Mini Kit (Qiagen, Valencia, CA) and then subjected to DNA microarray analysis. Generation of cRNA, labeling, hybridization, and scanning of the Affymetrix high-density oligonucleotide microarray HG_U133_Plus_2 chip (containing 54,675 gene probes) were performed according to the manufacturer's specifications (Affymetrix, Santa Clara, CA). Analysis of each chip was performed using the Affymetrix Microarray Suite 5.1 Software to generate raw expression data. GeneSpring 7.2 software (Silicon Genetics, Redwood City, CA) was used to assist in the statistical analysis and the selection of genes specific for CAM-enriched circulating cells.

Cell staining for flow cytometry

CAM-enriched cells were suspended using CAM-degrading enzymes, and washed twice with PBS with 0.2% BSA. Cells in suspension and washes were transferred to a 15-mL tube, and cells were collected by low speed-centrifugation for 5 min at 1,000 rpm, then resuspended in 100-μL (per $<1 \times 10^4$ cells) of the PBS/0.2% BSA. Antibodies (appropriate dilution per antibody; phycoerythrin (PE)-conjugated anti-CD44 antibody (rat mAb C37 from Vitatex Inc., Stony Brook, NY), fluorescein-conjugated anti-epithelial-specific antigen (ESA clone B29.1, Biomedex, Foster City, CA., and EpCAM clone Ber-Ep4, DakoCytomation, Carpinteria, CA), and allophycocyanin (APC)-conjugated anti-CD45 (clone T29/33, DakoCytomation, Carpinteria, CA) and CD34 (clone 581, BD Pharmingen) antibodies, and 7-aminoactinomycin D (7AAD, BD Biosciences, 1-μg/mL final concentration) were then added and incubated for 30 min on ice. Fixation and RBC lysis were then performed by using the FACSlyse buffer (BD Biosciences) to lyse the RBCs and mildly fix the stained cells.

Circulating Epi⁺, TP⁺ and Epi⁺TP⁺ cells, 10-400 cells/mL blood, were recovered from blood samples (5×10^9 cells per mL) by Vita-Cap™. CAM-captured cells were sorted by FACS using the following staining and gating: (a) 7AAD to negatively select out dead cells; (b) APC-CD45 and APC-CD34 to negatively select out hematopoietic stem cells and leukocytes; (c) PE-Stem Cell marker (CD44) to positively label CAM-captured progenitor cells; (d) FITC-Epi (EpCAM and ESA) to positively label CAM-captured cells with the epithelial lineage markers; (e) Improved gating was factored in: (i) events of normal adult (peripheral) blood samples as the baseline measurement; (ii) additional positive events to include the spiked tumor cells in experimental model settings.

To enable the enumeration of rare cells present in blood at frequencies below published rates of 500 rare cells in 5,000,000,000 blood cells of whole blood (1-mL) (27) by flow cytometry, the sample volume was reduced from 1-mL to 100 μL and total cell count from 10^9 to 10^3 without a significant loss of rare cells to pass the sample through the flow cytometer in a reasonable time period (sample flow rate = 60 μL/min). The above cell enrichment and labeling methods concentrated rare cells, reduced total number of cells to be counted to $<1 \times 10^4$, and decreased sample volume to 100 μL. To standardize FACS of such a rare event, labeled cells were transferred into a Trucount tube (BD Bioscience) and 25,000 beads were counted to determine the rare cell number in a sample. The 100 μL sample of each experiment was

analyzed on a FACSCalibur flow cytometer (Becton Dickinson) for enumeration and FACS Aria flow cytometer (Becton Dickinson) for sorting.

Results

Detection of invasive CTCs using the CAM uptake assay

CTCs are rare, comprising as few as one cell per 10^8 in the blood of patients with metastatic breast cancer; therefore, their isolation presents a substantial technical challenge. To test if invasive breast carcinoma cells present in blood samples could be enriched by their avidity to CAM, we first performed a series of model experiments using the CAM enrichment and labeling assays. Suspended individual cells, 10-1,000 cells, from the MDA-MB-231 human breast carcinoma line that were either non-fluorescent or GFP-tagged were collected and spiked into 1 mL of whole blood (5×10^9 cells) derived from a healthy donor to mimic tumor-like cells present in blood. Tumor cells in the “model” blood were then subjected to either a one-step CAM enrichment (Fig. 1-a, WB Vita-Cap™), or a two-step method (Fig. 1-a, MNC-1 Vita-Assay™) in which the blood samples were pre-processed using Ficoll density gradient centrifugation to generate the mononuclear cell (MNC) fraction. The efficiency of tumor cell isolation was examined by using fluorescence microscopy. Efficient recovery of $46.8\% \pm 12.2\%$ ($n=9$, Fig. 1-a, WB Vita-Cap™) and $77.3\% \pm 7.1\%$ ($n=9$, Fig. 1-a, MNC-1 Vita-Assay™) of tumor cells was observed (Fig. 1-a), even at the lowest concentrations of spiked cells (10 cells/mL of whole blood). Although the recovery rates were slightly higher for Vita-Cap™ than the Vita-Assay™ method, the methods were comparable ($r^2 = 0.99$). The CAM methods enriched 1,000 tumor cells in 5×10^9 blood cells (1-mL of blood) to 468-773 tumor cells per 2,000 blood cells, approximately a million-fold enrichment. These results suggest that pre-processing of blood samples is not necessary for CAM procedures.

In comparison with the CAM method, the efficiency of recovery of tumor cells was about 100-times lower with the epithelial cell immuno-affinity isolation ($n=9$ each, Fig. 1-a, WB Vita-Cap™ and WB Epi-magnetics). Only a $3\% \pm 4\%$ recovery rate was observed when 800 tumor cells were spiked into 1-mL of whole blood and recovered using epithelial antibody-magnetic beads, and only a few cells were recovered when less than 800 cells were spiked ($n=9$ each, Fig. 1-a, WB Epi-magnetics), suggesting that the CAM-initiated CTC detection is more effective than the immuno-magnetic system for detection of tumor cells in blood.

Tumor cells in the “model” blood were also enriched and identified using the two-step CAM method (Fig. 1-a, MNC-1 Vita-Assay™ and MNC-2 Vita-Assay™) in which tumor cells were either identified using GFP-tagging and CAM uptake of captured cells (Fig. 1-b: CAM G-Epi) or using immunostaining with fluorescent antibodies against EpCAM/cytokeratins and CAM uptake (Fig. 1-c: CAM Epi). Recovery rates were higher in the CAM-enrichment and GFP-targeted method than for the Epi antibody staining method, but both conditions were comparable ($r^2 = 0.99$), suggesting an insignificant loss of tumor cells identified using different means of cellular phenotyping.

To determine if the CAM enrichment and labeling method could effectively detect CTCs in breast cancer patients, we investigated its use in a training set of peripheral blood samples obtained from 53 healthy women, 1 patient with ductal carcinoma in situ (DCIS) and 10 metastatic breast cancer patients (Fig. 2). CAM-enriched cells that demonstrated uptake of fluorescent CAM fragments (CAM⁺, including surface binding and ingestion) and immunostained positively with epithelial markers (Epi⁺) were identified as CTCs, i.e., CAM⁺Epi⁺ cells (Fig. 2-a, double arrows). To normalize fluorescent intensity, CD45-positive hematologic cells (leukocytes) were used as the fluorescence baseline for positive signal (Fig. 2-a, single arrows). When split samples were analyzed under identical conditions, the reproducibility of the CAM uptake assay was found to be very high ($r^2 = 0.97$; Fig. 2-b). In the training set of blood samples,

CAM⁺Epi⁺ cells were found in 10 of 10 (100%) patients with metastatic breast cancer but none of the 54 women (including a patient with DCIS) without malignant breast cancer (Fig. 2c). The number of CTCs ranged from 18 to 256 per mL blood for metastatic breast cancer patients, with an average of 126±25 (mean±SD) CTCs/mL blood. Thus, CAM enriches CTCs in blood to a concentrated suspension that enables highly sensitive detection of the rare tumor cells by cellular assays.

Detection of CTCs in early stage breast cancer

Overall, baseline characteristics of stage I-III breast cancer patients enrolled in the study are described in Table 1. The median age of the 54 breast cancer patients enrolled in the study was 52.0 years (range, 25-87 years). Primary tumor size was less than 2.0 cm in 33.3% of patients. 57.4% of patient had undergone lumpectomy while 35.2% had undergone mastectomy. 64.8% of patients had lymph node positive disease, 68.5% estrogen receptor-positive tumors, 63.0% had progesterone receptor-positive tumors and 25.9% had Her-2 over-expressing tumors. 20.4% of patients had stage I disease, 38.9% had stage II disease and 40.7% had stage III disease.

Baseline characteristics of subjects who were CTC-negative and CTC-positive, as well as patients who had low (≤ 10 CTCs/mL) versus high (> 10 CTCs/mL) CTC counts were compared (Table 1). Among CTC-negative versus CTC-positive patients, only lymph node status, stage and rate of recurrence differed significantly. However, when CTC-low versus CTC-high subjects were compared, tumor size was also found to differ between the two groups, in addition to lymph node status, disease stage and recurrence rate. Otherwise there was no difference in age, surgery type, tumor histology or grade, ER/PR/Her-2 status (including 10 triple negative patients) or type of chemo received between the two groups.

CTC detection rates varied significantly by stage: most Stage III breast cancer patients (86.4%) had at least one detectable CTC, while only about 27.3% of Stage I patients, and 28.6% of Stage II patients had detectable CTCs (Fig. 3-a, p -value <0.001). There was also a significant difference of mean CTC counts by stage (Fig. 3-b): 10 CTCs/mL (SE=8) in stage I, 14 CTCs/mL (SE=7) in stage II and 119 CTCs/mL (SE=34) in stage III (p -value <0.001).

When patients were analyzed by their lymph node status, a significant difference was found in both their detection rate (Fig. 3-c) and mean CTC count (Fig. 3-d). Lymph node negative patients demonstrated a lower CTC detection rate of 5/19 (26.3%) and a lower mean CTC count of 14 CTCs/mL (SE=8), as compared to a detection rate of 23/35 (65.7%) and a mean CTC count of 79 CTCs/mL (SE=23) in lymph node positive patients (Fig. 3-c,d: p -values=0.006 and 0.003, respectively), suggesting a significant correlation between CTC count and lymph node status.

Among the 54 patients with early stage breast cancer, 29 patients received adjuvant chemotherapy consisting of either Adriamycin/Cyclophosphamide (AC/T), Adriamycin and Cyclophosphamide/ Taxol and Herceptin (AC/TH), Docetaxel and Cyclophosphamide (TC), or Docetaxel, Carboplatin and Herceptin (TCH). Ten patients received neo-adjuvant chemotherapy consisting of AC/T, AC/TH. Additionally, one neo-adjuvant patient received AC/T and Bevacizumab and another patient received Herceptin only. Thirteen patients received hormonal therapy only and one patient declined any therapy. Time-point of the measurements vary from within one month prior to chemo to within two months after chemo. A subset of patients ($n=21$) had CTC counts measured prior to and then upon completion of chemotherapy.

To examine if samples from same patients tested in subsequent days have similar CTC counts, we measured cell numbers from five breast cancer patients in two different dates and found

comparable counts from same patients (Fig. 3-e, Correlation coefficients = 0.883). Among 24 patients analyzed prior to and after chemotherapy: 11 patients had a decrease, 7 patients had no change, and 6 patients had an increase in the number of CTCs. While not reaching statistical significance given the small sample size, a decrease in mean CTC counts after completing chemotherapy was observed (Fig. 3-f): 106 CTCs/mL prior to chemotherapy vs. 59 CTCs/mL upon completion (p-value=0.34).

Correlation of CTCs with patient survival

A significant decrease in disease-free survival was observed in patients who had CTC counts greater than the median CTC count of 10 CTCs/mL (Fig. 4-a, p-value=0.005). None of the patients who had ≤ 10 CTCs had any disease recurrence or progression; their mean follow-up time was 27.6 months (range 1-84 months). Conversely, all 6 cases of disease recurrences occurred in patients with >10 CTCs/mL detected. Their mean disease-free survival was 8.5 months (range 1-19 months). Mean follow-up of patients with >10 CTCs/mL who had no disease recurrence/progression was 21.5 months (range 1-83 months).

A significant decrease in overall survival from breast cancer-related deaths was seen in patients with CTC counts >10 CTCs/mL (Fig. 4-b, p-value=0.04). All patients who had ≤ 10 CTCs/mL were alive at the end of the study and had a mean follow-up time of 28.5 months (range 1-84 months). Furthermore, all 3 deaths occurred in the CTC-high group (>10 CTCs/mL) who demonstrated a mean overall survival of 13.3 months (range 5-25 months). The mean follow-up of patients with >10 CTCs/mL who were alive was 19.5 months (range 5-83 months). Analyses comparing disease-free survival and overall survival between CTC-negative and CTC-positive patients was also performed which revealed similar trends, although they did not reach statistical significance (data not shown).

Molecular phenotyping of circulating cells isolated by CAM from breast cancer patients

To examine that cells isolated by CAM are viable, cells of the MNC fraction (pre-CAM) and captured by Vita-Assay™ (post-CAM) were stained using a LIVE/DEAD viability kit (Fig. 5-a,b). A mixture of live green fluorescent cells and dead red fluorescent cells in the MNC fractions was seen prior to CAM enrichment but reduced dead red cells in post-CAM fraction (Fig. 5-a). Dead red fluorescent cells were frequently seen in the MNC fractions (Fig. 5-b, Pre-CAM; $66\% \pm 2\%$ viable, mean $\pm$ SE, n=10), but dead red fluorescent cells were quite infrequent after CAM enrichment (Fig. 5-b, post-CAM; $99.9\% \pm 0.1\%$ viable, n=10, $p > 0.0001$), indicating that CAM enrichment returns nearly 100% viable circulating cells.

To determine the ability of circulating cells isolated by CAM from breast cancer patients to propagate into the epithelial phenotype *in vitro*, we cultured the cells on the same CAM substrata. Freshly isolated circulating cells locally degraded the underlying CAM and exhibited CAM uptakes within one day of culture (Fig. 5-c, large white arrows). This was not observed for hematopoietic cells captured by CAM (Fig. 5-c, small arrows). The number and size of cells increased over time in culture, resulting in sizeable colonies within 10 days (Fig. 5-d, large white arrows pointing to round cells) and large spread cells with epithelial morphology after 20 days (Fig. 5-d, open white arrows pointing to spread and large epithelial cells), whereas co-purified hematopoietic cells were observed to reduce their number (Fig. 5-d, red arrows). These results indicate that the CTC phenotype includes the ability to propagate and progress to tumor cell morphology.

To examine if markers known to express in breast tumors could be found in certain populations of the circulating cells isolated by CAM from breast cancer patients, we performed gene expression profiling according to the following steps. (a) We obtained blood containing ≥ 60 invasive epithelial cells and approximately 1,000 co-purifying “normal” cells (in which most

cells were leukocytes) per mL of blood from nine breast cancer patients and none tumor cells from 7 healthy women (**blood sample set A**) and subjected blood samples to the one-step CAM method (Vita-Cap™). (b) We then used the RNA extracted from CAM-enriched cells from cancer patients and compared it with RNA extracted from CAM-enriched cells from healthy women to generate gene expression profiles of cancer-associated genes and to subtract genes upregulated in the “normal” leukocyte cells captured by CAM. Comparative gene expression profiling was performed using the Affymetrix HG_U133_Plus_2 chip (containing 54,675 gene probes, 47,000 transcripts and variants). After a global scaling procedure was performed to normalize the expression data between experiments, we screened >1,000 genes (a fold-change >2 at a probability <0.05 of that fold-change not being an actual difference) that exhibited minimal expression in the CAM-enriched fraction from normal cells but significant expression in the samples prepared from breast cancer patients. (c) We further selected seven epithelial lineage genes among the >1,000 genes that were significantly upregulated in cancer samples (for potential tumor markers) as well as 11 epithelial lineage markers, known to express in epithelial tumors, and leukocyte adhesion receptors, as internal marker controls, from the genechip (Figs. 6-a, 6-b, and supplementary Table 1S) to examine differential expression of selected genes in cancer and normal samples.

Upregulation of these selected genes in cancer samples was determined in both the training set of blood samples (**blood sample set A**) that consists of 7 normal and 9 breast cancer samples (Fig. 6-a), and the testing set (**blood sample set B**) that consists of 9 normal and 20 breast cancer samples (Fig. 6-b). In cancer samples that contained >60 CTCs/mL blood, the four cytokeratin genes (*KRT8*, *KRT16*, *KRT17* and *KRT19*) and eight tumor-associated genes (*TERT*, *MUC16/M17S2/CA125*, *CD44*, *TWIST1*, *TACSTD1/EpCAM/CD326/ESA/HEA125/GA733*, *DPP4/CD26*, *ESR1* and *PGR*), were upregulated, whereas the 3 internal control genes (*ITGA2*, *CD14* and *ITGB3*) and the six breast tumor genes (*VIM*, *MUC1*, *ERBB2*, *CD24*, *EGFR* and *DPP5/FAP/seprase/APCE*) exhibit no specific expression (Figs. 6-a, 6-b, and supplementary Table 1S). The expression data strongly suggest that circulating cells enriched by CAM from cancer samples are heterogeneous and contain subpopulations that express specific epithelial lineage, stem or progenitor markers.

To examine whether distinct populations of circulating cells isolated by CAM exist, multiplex fluorescent activated cell sorting (FACS) of CAM-captured cells was performed. The following four-five marker combinations were used to sort individual epithelial (Epi) and tumor progenitor (TP) cells: (a) Epi⁺ cell – EpCAM⁺CD34⁺CD45⁺7AAD⁺, (b) TP⁺ cell – CD44⁺CD34⁺CD45⁺7AAD⁺, and (c) Epi⁺TP⁺ cell – CD44⁺EpCAM⁺CD34⁺CD45⁺7AAD⁺.

Multiplex FACS was used to sort the circulating cells pre-enriched by CAM into homogeneous cells using the five markers, in which EpCAM and CD44 are for positive selection, but CD34 and CD45 (both conjugated with APC) negatively sort out hematopoietic cells and 7AAD to exclude dead cells (Fig. 7). FACS gating for specific expression of Epi and TP markers in cancer samples includes the use of circulating cells captured by CAM from normal blood samples as the baseline measurement, so that we obtain a cutoff value of ≤5 Epi⁺ and TP⁺ cells/mL in control normal blood samples (Fig. 7-d, e and f). Consistently, >5 Epi⁺, TP⁺ and Epi⁺TP⁺ cells/mL were identified in the blood samples from breast cancer patients (Fig. 7-a, b and c). A single-cell-sorted Epi⁺ cell is identified as a EpCAM⁺CD34⁺CD45⁺7AAD⁺ cell in blood samples (Fig. 7-a and d, number of cells/mL in red), TP⁺ cell is a CD44⁺CD34⁺CD45⁺7AAD⁺ cell (Fig. 7-b and e, number of cells/mL blood in red) and Epi⁺TP⁺ cell is a CD44⁺EpCAM⁺CD34⁺CD45⁺7AAD⁺ cell (Fig. 7-c and f, number of cells/mL blood in red). These cells could be discriminated from co-purifying circulating hematopoietic stem cells and leukocytes that were CD34⁺CD45⁺ and from dead cells that were 7AAD⁺. The latter demonstrates that both CAM-enrichment of viable cells and 7AAD exclusion of dead cells are crucial to achieve accuracy, as the remaining dead cells show high

non-specific binding of antibodies. Together, these results demonstrate that CAM enrichment of circulating cells and multiplex flow cytometry provide a powerful opportunity for phenotyping of different populations of circulating tumor cells in cancer patients.

Discussion

Current approaches to enrich or sort tumor cells from the peripheral blood for CTC detection involve the use of a few epithelial lineage markers in specialized methods and devices. These approaches include Ficoll density gradient centrifugation-imaging system (28), flow cytometry (29), immunomagnetic particle-imaging system, CellSearch (30), microfluidic imaging system, CTC chip (31), high-throughput optical-imaging system (32), and fiber optic array scanning (33). CellSearch detects CTCs based on a cell enrichment step using anti-EpCAM antibodies and tumor cell identification using cytokeratin (*KRT8*, *KRT18* and *KRT19*) expression (30), and is currently the lead technology approved by FDA for use in the clinical setting. Using similar antibodies to enrich and cytokeratin expression to identify tumor cells, a recent report showed that CTC chip detected one to two orders of magnitude more CTCs, 79 ± 52 cells/mL, from metastatic breast cancer patients than CellSearch (31). However, there is no consensus how to compare CTC detection between these two methods and others, since blood samples and modes of cell enrichment and antigens used in other analytic approaches significantly differ (6).

For example, the CAM-initiated CTC detection method reported here reveals 126 ± 25 (mean $\pm$ SD) cells/mL blood from metastatic breast cancer patients that are CAM⁺Epi⁺ (i.e., cells with detectable expression of upregulated TACSTD1/EpCAM/ CD326/ESA/ HEA125/GA733 and KRT-4,5,6,8,10,13,18 antigens, as well as invasive/progenitor cell properties). EpCAM and cytokeratin expression is an essential positive selection criterion in major CTC detection methods. However, the CAM method identifies the cell invasiveness simultaneously while recognizing epithelial lineage antigens (Figs. 1-2), suggesting that the assay selects a subpopulation of epithelial cells that invades the extracellular matrix. Like disseminated tumor cells in bone marrow isolated by the Ficoll density gradient centrifugation method (34), CAM-captured cells are capable of propagation in culture (Fig. 5).

Although HER2, ER and PR are considered as important breast tumor markers, our immunofluorescent assay for CTC identification, involving CAM, Epi and CD45, did not find correlations between the expression of HER2, ER and PR in primary tumors and levels of CTCs detected (Table 1). However, we showed by Affymetrix DNA microarray profiling of circulating cells isolated by the single step Vita-CapTM method (Fig. 6) that some known breast tumor markers, including *CD44*, *TACSTD1/EpCAM*, *TERT*, *MUC16/CA125*, *DPP4/CD26*, *ESR1/ER* and *PGR/PR*, are likely upregulated in specific populations of the circulating cells captured by CAM from cancer blood samples, whereas others, including *MUC1*, *VIM*, *DPP5/FAP* and *ERBB2/Erb-2/HER2*, are also expressed in un-identified populations of CAM-avid cells in normal blood samples. Considering that HER2, ER and PR are important therapeutic targets for breast cancer, it will be interesting to investigate which populations of circulating cells enriched by CAM that express these targets in patients during therapeutic intervention.

Expression profiling of CAM-captured cells from breast cancer patients indeed shows the upregulation of mRNAs reflective of multiple populations of CTCs. To validate this concept, we showed by multiplex FACS (Fig. 7) that CAM-captured circulating cells from breast cancer patients consisted of three distinct populations: epithelial lineage cells (Epi⁺ cell: EpCAM⁺CD34⁻CD45⁻7AAD⁻) and tumor progenitor cells (TP⁺ cell: CD44⁺CD34⁻CD45⁻7AAD⁻), in which 50-75% of the two populations overlap (Epi⁺TP⁺ cell: CD44⁺EpCAM⁺CD34⁻CD45⁻7AAD⁻). Thus, the CAM method could detect at least three subpopulations of invasive CTCs with epithelial, progenitor and epithelial/progenitor

phenotypes. Although work is in the progress, we obtained results from a blood sample showing that CAM⁺Epi⁺ cells substantially overlapped with Epi⁺TP⁺ cells.

In spiking model experiments we found that the CAM method is about 100-times more sensitive than the epithelial cell immuno-magnetic isolation to detect MDA-MB-231 tumor cells spiked in whole blood (Fig. 1-a). Previous reports showed that, when cells derived from different tumor cell lines were used to perform epithelial cell capture using specific antibodies for the initial cell enrichment, the spiked tumor cells were recovered with different rates (30; 31). Specifically, the CAM method enriched MDA-MB-231 tumor cells spiked in whole blood to the extent that were readily detected by fluorescence microscopic assay for epithelial markers (EpCAM, ESA and cytokeratins), but CellSearch™ that targeted on EpCAM for the initial cell enrichment could not detect any MDA-MB-231 cells that had low EpCAM expression on the cell surface (4). However, the CellSearch™ test could effectively detect other tumor cell types that expressed high EpCAM on the cell surface (4). Thus, commonly used antibody-based cell enrichment methods such as the CellSearch™ test are effective to detect specific populations of CTCs that express targeted antigens, whereas the CAM method is efficient in the recovery of rare circulating cells that have strong avidity to the ECM to enable immuno-phenotyping of multiple populations of CTCs.

We have made an attempt to compare capture efficiency of tumor cells spiked in the “model” blood using a one-step CAM enrichment (Fig. 1-a, WB Vita-Cap™) and a two-step method (Fig. 1-a, MNC-1 Vita-Assay™), in which the blood samples were pre-processed using Ficoll density gradient centrifugation. Original tumor cell dose spiked in whole blood was determined by first removing their red blood cells using a red cell lysing buffer, and measuring by FACS in a Trucount tube (BD Biosciences). We found that there is 30-40% cell loss in the MNC fractionation that generates a recovery rate of $77.3\% \pm 7.1\%$ ($n=9$, Fig. 1-a, MNC-1 Vita-Assay™) of tumor cells with the two-step CAM method (Fig. 1-a). In comparison, the one-step CAM method has recovery rate of $46.8\% \pm 12.2\%$ ($n=9$, Fig. 1-a, WB Vita-Cap™, Fig. 1-a). We also found that the level of CAM⁺Epi⁺ cells resolved with the two-step CAM method correlates with expression profiling of circulating cells isolated by the single step Vita-Cap™ method (Fig. 6) that retain the upregulated expression of epithelial lineage (*KRT8*, *KRT16*, *KRT17*, *KRT19*, *TERT* and *MUC16/M17S2/CA125*) and EMT stem cell (*CD44* and *TWIST1*) genes. Together, these results suggest that pre-processing of blood samples is not necessary for CAM procedures.

In this study, we also investigated the prognostic value of epithelial cells with the invasive phenotype present in peripheral blood of patients with early stage breast cancer. We reason that detection of these invasive CTCs may offer an alternative method to assess metastatic progression that may be of prognostic value. We demonstrated that invasive CTCs were detected in 52% of patients with stage I-III breast cancer and the CTC counts significantly correlated with advanced stage disease, lymph node status, shorter disease-free survival and shorter overall survival. Also, we documented the decreased disease-free survival (p-value 0.005) and overall survival (p-value 0.04) in early stage (stages I-III) breast cancer patients who had ≥ 10 CTC counts per mL of blood. The cut-off value is higher than that of commonly used methods. We could explain that these circulating cells might represent subpopulations of “normal-like or stem” cells, which are yet to develop their aggressive behavior and could not be detected by other commonly used methods. Nevertheless, our findings indicate that as breast cancer progresses, a greater number of invasive CTCs are present.

CTCs, as defined by cytokeratin (CK) positivity and CD45 negativity, are detected in metastatic breast cancer patients and their higher number is related with worse disease-free and overall survival in these patients (35). Although the clinical significance of CTCs in patients with early stage breast cancer (28;36) was not found using the CK⁺CD45⁻ criterion, similar studies using

sensitive PCR-based molecular assays (7;8) showed encouraging clinical correlations. The detection of CTCs by PCR using CK19 and MGB1 mRNA before adjuvant chemotherapy predicts poor disease-free survival in women with early breast cancer (7). Also, the detection of CTCs by PCR using stanniocalcin-1 (STC-1), N-acetylgalactosaminyltransferase (GalNacT), and melanoma antigen gene family-A3 (MAGE-A3) offers a novel means to assess the presence of systemic disease spreading relative to lymph node status (8). Although the cellular origin of these mRNAs remains unknown, their findings that high blood mRNAs correlate with poor disease-free survival and lymph node status are similar to ours using the cellular assay. Nevertheless, CTCs can be reliably detected by the use of multiple mRNA markers, as used in these molecular assays.

In summary, the CAM method is unique in the CTC field for the detection of epithelial and tumor progenitor cells with the invasive phenotype in blood, and it warrants further clinical studies to evaluate the value of invasive CTCs in guiding therapy for patients with early stage breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. B. O'Hea, N. Dacosta for help with recruiting patient subjects, Jizu Zhi for advice on expression analysis, S. Liang, Y. Hu for pathology support, and Y. Yeh, Todd Rueb for technical assistance.

Grant support: This research was supported by grants NIH R42 CA108247 and MO1RR10710 to W.-T. Chen, Carol Baldwin Breast Cancer TRO grant to J. Lu, and NYS ECRIP fellowship to T. Fan.

References

1. Husemann Y, Geigl JB, Schubert F, Musiani P, Meyer M, Burghart E, Forni G, Eils R, Fehm T, Riethmuller G, Klein CA. Systemic spread is an early step in breast cancer. *Cancer Cell* 2008 Jan;13(1):58–68. [PubMed: 18167340]
2. Podsypanina K, Du YC, Jechlinger M, Beverly LJ, Hambardzumyan D, Varmus H. Seeding and propagation of untransformed mouse mammary cells in the lung. *Science* 2008 Sep 26;321(5897):1841–4. [PubMed: 18755941]
3. Winter MJ, Nagtegaal ID, van Krieken JH, Litvinov SV. The epithelial cell adhesion molecule (Ep-CAM) as a morphoregulatory molecule is a tool in surgical pathology. *Am J Pathol* 2003 Dec;163(6):2139–48. [PubMed: 14633587]
4. Sieuwerts AM, Kraan J, Bolt J, van der SP, Elstrodt F, Schutte M, Martens JW, Gratama JW, Sleijfer S, Foekens JA. Anti-epithelial cell adhesion molecule antibodies and the detection of circulating normal-like breast tumor cells. *J Natl Cancer Inst* 2009 Jan 7;101(1):61–6. [PubMed: 19116383]
5. Pantel K, Brakenhoff RH, Brandt B. Detection, clinical relevance and specific biological properties of disseminating tumour cells. *Nat Rev Cancer* 2008 May;8(5):329–40. [PubMed: 18404148]
6. Paterlini-Brechot P, Benali NL. Circulating tumor cells (CTC) detection: clinical impact and future directions. *Cancer Lett* 2007 Aug 18;253(2):180–204. [PubMed: 17314005]
7. Ignatiadis M, Kallergi G, Ntoulia M, Perraki M, Apostolaki S, Kafousi M, Chlouverakis G, Stathopoulos E, Lianidou E, Georgoulas V, Mavroudis D. Prognostic value of the molecular detection of circulating tumor cells using a multimarker reverse transcription-PCR assay for cytokeratin 19, mamaglobin A, and HER2 in early breast cancer. *Clin Cancer Res* 2008 May 1;14(9):2593–600. [PubMed: 18451221]
8. Nakagawa T, Martinez SR, Goto Y, Koyanagi K, Kitago M, Shingai T, Elashoff DA, Ye X, Singer FR, Giuliano AE, Hoon DS. Detection of circulating tumor cells in early-stage breast cancer metastasis to axillary lymph nodes. *Clin Cancer Res* 2007 Jul 15;13(14):4105–10. [PubMed: 17634536]

9. Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol* 2006 Feb;7(2):131–42. [PubMed: 16493418]
10. Korkaya H, Paulson A, Iovino F, Wicha MS. HER2 regulates the mammary stem/progenitor cell population driving tumorigenesis and invasion. *Oncogene* 2008 Oct 16;27(47):6120–30. [PubMed: 18591932]
11. Shipitsin M, Campbell LL, Argani P, Weremowicz S, Bloushtain-Qimron N, Yao J, Nikolskaya T, Serebryiskaya T, Beroukhi R, Hu M, Halushka MK, Sukumar S, et al. Molecular definition of breast tumor heterogeneity. *Cancer Cell* 2007 Mar;11(3):259–73. [PubMed: 17349583]
12. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, Campbell LL, Polyak K, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 2008 May 16;133(4):704–15. [PubMed: 18485877]
13. Boccaccio C, Comoglio PM. Invasive growth: a MET-driven genetic programme for cancer and stem cells. *Nat Rev Cancer* 2006 Aug;6(8):637–45. [PubMed: 16862193]
14. Al Mehdi AB, Tozawa K, Fisher AB, Shientag L, Lee A, Muschel RJ. Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: a new model for metastasis. *Nature Medicine* 2000 Jan;6(1):100–2.
15. Wang H, Fu W, Im JH, Zhou Z, Santoro SA, Iyer V, DiPersio CM, Yu QC, Quaranta V, Al Mehdi A, Muschel RJ. Tumor cell $\alpha 3 \beta 1$ integrin and vascular laminin-5 mediate pulmonary arrest and metastasis. *Journal of Cell Biology* 2004 Mar 15;164(6):935–41. [PubMed: 15024036]
16. Sabile A, Louha M, Bonte E, Poussin K, Vona G, Mejean A, Chretien Y, Bougas L, Lacour B, Capron F, Roseto A, Brechot C, et al. Efficiency of Ber-EP4 antibody for isolating circulating epithelial tumor cells before RT-PCR detection. *Am J Clin Pathol* 1999 Aug;112(2):171–8. [PubMed: 10439796]
17. Thurm H, Ebel S, Kantenich C, Hensen A, Riethdorf S, Coith C, Wallwiener D, Braun S, Oberhoff C, Janicke F, Pantel K. Rare expression of epithelial cell adhesion molecule on residual micrometastatic breast cancer cells after adjuvant chemotherapy. *Clin Cancer Res* 2003 Jul;9(7):2598–604. [PubMed: 12855636]
18. Choesmel V, Anract P, Hoifodt H, Thiery JP, Blin N. A relevant immunomagnetic assay to detect and characterize epithelial cell adhesion molecule-positive cells in bone marrow from patients with breast carcinoma: immunomagnetic purification of micrometastases. *Cancer* 2004 Aug 15;101(4):693–703. [PubMed: 15305398]
19. Ring A, Smith IE, Dowsett M. Circulating tumour cells in breast cancer. *Lancet Oncology* 2004 Feb;5(2):79–88. [PubMed: 14761811]
20. Yamada KM, Cukierman E. Modeling tissue morphogenesis and cancer in 3D. *Cell* 2007 Aug 24;130(4):601–10. [PubMed: 17719539]
21. Weaver AM. Cortactin in tumor invasiveness. *Cancer Lett* 2008 Jul 8;265(2):157–66. [PubMed: 18406052]
22. Enderling H, Alexander NR, Clark ES, Branch K, Estrada L, Crooke C, Jourquin J, Lobdell NA, Zaman MH, Guelcher SA, Anderson AR, Weaver AM. Dependence of invadopodia function on collagen fiber spacing and crosslinking: computational modeling and experimental evidence. *Biophys J*. 2008 May 30;
23. Mueller SC, Gherzi G, Akiyama SK, Sang QX, Howard L, Pineiro-Sanchez M, Nakahara H, Yeh Y, Chen WT. A novel protease-docking function of integrin at invadopodia. *J Biol Chem* 1999 Aug 27;274(35):24947–52. [PubMed: 10455171]
24. Kennedy A, Dong H, Chen D, Chen WT. Elevation of seprase expression and promotion of an invasive phenotype by collagenous matrices in ovarian tumor cells. *Int J Cancer* 2008 Sep 29;124(1):27–35. [PubMed: 18823010]
25. Fan T, Zhao Q, Chen JJ, Chen WT, Pearl ML. Clinical significance of circulating tumor cells detected by an invasion assay in peripheral blood of patients with ovarian cancer. *Gynecol Oncol* 2009 Jan;112(1):185–91. [PubMed: 18954898]
26. Paris PL, Kobayashi Y, Zhao Q, Zeng W, Sridharan S, Fan T, Adler HL, Yera ER, Zarrabi MH, Zucker S, Simko J, Chen WT, et al. Functional phenotyping and genotyping of circulating tumor cells from patients with castration resistant prostate cancer. *Cancer Lett*. 2009 Jan 20;

27. Karczewski DM, Lema MJ, Glaves D. The efficiency of an autotransfusion system for tumor cell removal from blood salvaged during cancer surgery. *Anesthesia & Analgesia* 1994 Jun;78(6):1131–5. [PubMed: 8198270]
28. Pierga JY, Bonneton C, Vincent-Salomon A, de CP, Nos C, Blin N, Pouillart P, Thiery JP, Magdelenat H. Clinical significance of immunocytochemical detection of tumor cells using digital microscopy in peripheral blood and bone marrow of breast cancer patients. *Clin Cancer Res* 2004 Feb 15;10(4):1392–400. [PubMed: 14977842]
29. Georgakoudi I, Solban N, Novak J, Rice WL, Wei X, Hasan T, Lin CP. In vivo flow cytometry: a new method for enumerating circulating cancer cells. *Cancer Res* 2004 Aug 1;64(15):5044–7. [PubMed: 15289300]
30. Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, Tibbe AG, Uhr JW, Terstappen LW. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clin Cancer Res* 2004 Oct 15;10(20):6897–904. [PubMed: 15501967]
31. Nagrath S, Sequist LV, Maheswaran S, Bell DW, Irimia D, Ulkus L, Smith MR, Kwak EL, Digumarthy S, Muzikansky A, Ryan P, Balis UJ, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. *Nature* 2007 Dec 20;450(7173):1235–9. [PubMed: 18097410]
32. Kraeft SK, Ladanyi A, Galiger K, Herlitz A, Sher AC, Bergsrud DE, Even G, Brunelle S, Harris L, Salgia R, Dahl T, Kesterson J, et al. Reliable and sensitive identification of occult tumor cells using the improved rare event imaging system. *Clinical Cancer Research* 2004 May 1;10(9):3020–8. [PubMed: 15131038]
33. Krivacic RT, Ladanyi A, Curry DN, Hsieh HB, Kuhn P, Bergsrud DE, Kepros JF, Barbera T, Ho MY, Chen LB, Lerner RA, Bruce RH. A rare-cell detector for cancer. *Proc Natl Acad Sci U S A* 2004 Jul 20;101(29):10501–4. [PubMed: 15249663]
34. Solakoglu O, Maierhofer C, Lahr G, Breit E, Scheunemann P, Heumos I, Pichlmeier U, Schlimok G, Oberneder R, Kollermann MW, Kollermann J, Speicher MR, et al. Heterogeneous proliferative potential of occult metastatic cells in bone marrow of patients with solid epithelial tumors. *PNAS* 2002 Feb 19;99(4):2246–51. [PubMed: 11854519]
35. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LW, Hayes DF. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med* 2004 Aug 19;351(8):781–91. [PubMed: 15317891]
36. Wong NS, Kahn HJ, Zhang L, Oldfield S, Yang LY, Marks A, Trudeau ME. Prognostic significance of circulating tumour cells enumerated after filtration enrichment in early and metastatic breast cancer patients. *Breast Cancer Res Treat* 2006 Sep;99(1):63–9. [PubMed: 16541316]

Abbreviations

CAM	collagen adhesion matrix
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
DMEM	Dulbecco's modified Eagle's medium
PBS	phosphate buffered saline
CCC medium	cancer cell culture medium

Figure 1.

Detection of tumor cells in blood using the CAM enrichment and labeling assay in the experimental setting. (a), Comparison of the recovery rates of tumor cells spiked into whole blood isolated by two CAM methods (WB Vita-Cap™ and MNC-1 Vita-Assay™) and Dynal Collection™ Epithelial Enrich (WB Epi-magnetics). Approximately 10-1,000 cells derived from the MDA-MB-231 line that were either non-fluorescent or GFP-tagged were spiked into 1 mL of whole blood (5×10^9 cells) derived from a healthy donor. Tumor cells in the “model” blood were then subjected to one-step CAM enrichment (WB Vita-Cap™) or two-step method (MNC-1 Vita-Assay™), as well as an epithelial cell immuno-magnetic cell separation using Dynal Collection™ Epithelial Enrich (WB Epi-magnetics). Tumor cells captured by the two-

step method (MNC-2 Vita-Assay™) were not tagged with GFP and were post-labeled with antibodies against EpCAM and cytokeratins. Regression analysis of capture efficiency of GFP-tagged tumor cells (Samples labeled with WB and MNC-1) and antibody-stained tumor cells (Samples labeled with MNC-2) were showed in the plot. **(b)**, Fluorescence microscopic images of the captured cells derived from spiking model experiments using GFP-tagged MDA-MB-231 cells. Double arrows indicate tumor cells recovered that ingest CAM, retain GFP (G-Epi) and stain with Hoechst 33342 dye (NU). Single arrows show co-isolating leukocytes that were stained with red color substrate-conjugated antibodies against CD45 and Hoechst 33342 dye (NU). Bar = 30 μ m. **(c)**, Fluorescence microscopic images of the captured cells derived from spiking model experiments using non-fluorescent MDA-MB-231 cells. Double arrows indicate tumor cells recovered that ingested CAM, and were stained with green fluorescent dye-conjugated antibodies against epithelial antigens (Epi) and Hoechst 33342 dye (NU). Single arrows show co-isolating leukocytes that were stained with red color substrate-conjugated antibodies against CD45 and Hoechst 33342 dye (NU). Bar = 30 μ m.

Figure 2.

Detection of CTCs using the CAM-initiated immuno-phenotyping method. **(a)**, Characterization of CAM-isolated cells from a metastatic breast cancer patient. CTCs were detected as $\text{CD45}^- \text{Epi}^+ \text{CAM}^+$ cells (double arrows) and leukocytes as $\text{CD45}^+ \text{Epi}^- \text{CAM}^-$ (open single arrows). Bar = 20 μm . **(b)**, Regression analysis on reproducibility of the CAM-initiated CTC detection method using split samples ($n=19$). **(c)**, Number of CTCs per one mL of blood in healthy women and patients diagnosed with DCIS and metastatic breast cancer. The bars represent the mean values in each subgroup of blood samples.

Figure 3.

Detection of invasive CTCs in early stage breast cancer. (a), CTC detection rates varied significantly between Stage III and Stage I/II (p-value<0.001). (b), Mean CTC counts differ significantly between Stage III and Stage I/II (p-value<0.001). (c), CTC detection rates varied significantly between lymph node positive and negative patients (p-value=0.006). (d), Mean CTC counts differ significantly between lymph node positive and negative patients (p-value=0.003). (e), Repeated testing of five breast cancer patients in two different dates (Correlation coefficients = 0.883). (f), Decrease in mean CTC counts, 106 CTCs/mL prior to chemotherapy vs. 59 CTCs/mL upon completion (p-value=0.34).

Figure 4. Kaplan-Meier curves for disease outcome according to high versus low CTC counts. *(a)*, Disease-free survival is significantly decreased in patients who had CTC counts greater than the median CTC count of 10 CTCs/mL (p-value=0.005). *(b)*, Overall survival is significantly decreased in patients who had CTC counts greater than the median CTC count of 10 CTCs/mL (p-value=0.04).

Figure 5.

Viability and proliferation of circulating cells isolated by CAM from blood of breast cancer patients. **(a)**, A mixture of live green fluorescent cells and dead red fluorescent cells were seen in the cell fraction of pre-CAM enrichment. Bar = 40 μ m. **(b)**, Viability of circulating cells prior to and post to CAM enrichment. Percentages of live cells in pre- and post-CAM fractions were measured as % green cells in both green and red cells. **(c-d)**, CAM-enriched cells were cultured on the CAM scaffold for one to 33 days. Live cells were photographed under phase contrast microscopy (PCM) and fluorescence microscopy (CAM, to reveal CAM uptake/labeling of tumor cells). Tumor cells grew as time increased. On day 1 **(c)**, tumor cells were seen to associate with CAM uptakes and as round cells (large white arrows) larger than hematologic cells (small yellow arrowheads). On day 10 **(d)**, tumor cells were seen as round cells (white arrows) larger than hematologic cells (red arrows). By day 33 **(d)**, tumor cells grew in clusters with large epithelioid cells (open arrows) but hematologic cells decreased their number and not seen in the field.

Figure 6. Cytokeratin (*KRT*) and breast tumor associated genes of circulating cells isolated by CAM (Vita-Cap™) from blood of breast cancer patients. *(a)*, *KRT* genes associated with circulating cells isolated by CAM from 7 healthy women subjects and 9 patients with breast cancer that contained ≥ 60 CTCs/mL blood. Columns represent catalogues of cell samples analyzed: circulating Normal (N) cells isolated with the one-step Vita-Cap™ from healthy donors with suffix M for Male and F for Female; BCa are circulating Breast Cancer (BCa) cells isolated by the one-step Vita-Cap™ assay with suffix I-IV being stages of the disease. The number of CTCs in a blood sample is shown at the top of each column (sample analyzed). Colorgram depicts high (red) and low (blue) relative levels of gene expression. Green arrows indicate the three internal control genes that exhibited no difference between normal and cancer cell samples; black arrows for cytokeratin (*KRT*) genes known to be up-regulated in circulating tumor cells; orange arrows for genes known to be up-regulated in breast tumor cells. *(b)*, Validation of the selected genes associated with breast tumors in *(a)*. The expression of the 4-*KRT* genes, 12-breast tumor genes and 3-internal control genes was analyzed using the blood sample set B that consists of 9 normal and 20 breast cancer samples.

Figure 7.

Flow cytometric sorting of Epi⁺, TP⁺ and Epi⁺TP⁺ cells in 1-mL of whole blood of a patient with stage II breast tumor (**a – c**) and a healthy women (**d – f**). Circulating cells in blood were first isolated by incubation in a Vita-CapTM tube, followed by multiplex FACS detection. 7AAD pre-sorting for live cells is not shown. Numbers in red indicate positive events in a given multiplex sorting. (**a, d**), Live Epi⁺ cells per 1-mL blood as defined by EpCAM⁺CD34⁺CD45⁻7AAD⁻. (**b, e**), Live TP⁺ cells as defined by CD44⁺CD34⁺CD45⁻7AAD⁻. (**c, f**), Live Epi⁺TP⁺ cells as defined by EpCAM⁺CD44⁺CD34⁺CD45⁻7AAD⁻.

Table 1

Patient Characteristics and Detection of CTCs

	All patients	CTC-negative, n(%)	CTC-positive, n(%)	P-value	CTC-low (≤10 CTCs/ml), n (%)	CTC-high (>10 CTCs/ml), n (%)	P-value
Number of patients	54	26	28		28 (51.9%)	26 (48.1%)	
Mean age in yrs (range)	53.6 (25-87)	52.7	54.4	0.66	53.7 (25-83)	53.4 (30-87)	0.66
Race (n=54)							0.08
White	37 (68.5%)	21 (80.8%)	16 (57.1%)	0.26	23 (82.1%)	14 (53.8%)	
Hispanic	5 (9.3%)	2 (7.7%)	3 (10.7%)		2 (7.1%)	3 (11.5%)	
Black	8 (14.8%)	3 (11.5%)	5 (17.9%)		3 (10.7%)	5 (19.2%)	
Mixed	4 (7.4%)	0	4 (14.3%)		0 (0.0%)	4 (15.4%)	
Surgery (n=54)							0.06
Lumpectomy	31 (57.4%)	18 (69.2%)	13 (46.4%)	0.07	19 (67.9%)	12 (46.2%)	
Mastectomy	19 (35.2%)	8 (30.8%)	11 (39.3%)		9 (32.1%)	10 (38.5%)	
Other	4 (7.4%)	0	4 (14.3%)		0 (0.0%)	4 (15.4%)	
Tumor Size (n=54)							0.02*
0 - 1.9 cm	18 (33.3%)	12 (46.2%)	6 (21.4%)	0.10	14 (50.0%)	4 (15.4%)	
2.0 - 3.9 cm	22 (40.7%)	10 (38.5%)	12 (42.9%)		10 (35.7%)	12 (46.2%)	
> 4.0	14 (25.9%)	4 (15.4%)	10 (35.7%)		4 (14.3%)	10 (38.5%)	
Histology (n=54)							0.18
Invasive ductal	42 (77.8%)	18 (69.2%)	24 (85.7%)	0.19	21 (75.0%)	21 (80.8%)	
Invasive lobular	4 (7.4%)	4 (15.4%)	0		4 (14.3%)	0 (0%)	
Mixed	4 (7.4%)	2 (7.7%)	2 (7.1%)		2 (7.1%)	2 (7.7%)	
Other	4 (7.4%)	2 (7.7%)	2 (7.1%)		1 (3.6%)	3 (11.5%)	
Grade (n=52)							0.56
Low	9 (17.3%)	5 (20.8%)	4 (14.3%)	0.69	6 (22.2%)	3 (12.0%)	
Moderate	23 (44.2%)	11 (45.8%)	12 (42.9%)		12 (44.4%)	11 (44.0%)	
High	20 (38.5%)	8 (33.3%)	12 (42.9%)		9 (33.3%)	11 (44.0%)	
Lymph Node Status (n=53)				0.01*			0.003*
Negative	19 (35.2%)	14 (53.8%)	5 (17.9%)		15 (53.6%)	4 (15.4%)	

	All patients	CTC-negative, n(%)	CTC-positive, n(%)	P-value	CTC-low (≤10 CTCs/ml), n (%)	CTC-high (>10 CTCs/ml), n (%)	P-value
Positive	35 (64.8%)	12 (48.2%)	23 (82.1%)		13 (46.4%)	22 (84.6%)	
Stage (n=54)				<0.001*			<0.001*
I	11 (20.4%)	8 (30.8%)	3 (10.7%)		9 (32.1%)	2 (7.7%)	
II	21 (38.9%)	15 (57.7%)	6 (21.4%)		15 (53.6%)	6 (23.1%)	
III	22 (40.7%)	3 (11.5%)	19 (67.9%)		4 (14.3%)	18 (69.2%)	
ER Status (n=54)				0.91			0.64
Negative	17 (31.5%)	8 (30.8%)	9 (32.1%)		8 (28.6%)	9 (34.6%)	
Positive	37 (68.5%)	18 (69.2%)	19 (67.9%)		20 (71.4%)	17 (65.4%)	
PR Status (n=53)				0.18			0.24
Negative	19 (35.8%)	7 (26.9%)	12 (44.4%)		8 (28.6%)	11 (44.0%)	
Positive	34 (64.2%)	19 (73.1%)	15 (55.6%)		20 (71.4%)	14 (56.0%)	
HER2 Status (n=53)				0.59			0.38
Negative	39 (73.6%)	20 (76.9%)	19 (70.4%)		22 (78.6%)	17 (68.0%)	
Positive	14 (26.4%)	6 (23.1%)	8 (29.6%)		6 (21.4%)	8 (32.0%)	
Chemotherapy Regimen							
None (n=14)				0.16			0.10
Hormonal Therapy	13 (92.9%)	9 (34.6%)	4 (14.3%)		10 (100.0%)	3 (75.0%)	
Nothing	1 (7.1%)	0	1 (3.6%)		0 (0.0%)	1 (25.0%)	
Neoadjuvant (n=10)				0.48			0.23
AC/T	5 (50.0%)	2 (7.7%)	3 (10.7%)		3 (100.0%)	2 (28.6%)	
AC/T+H	3 (30.0%)	0	3 (10.7%)		0 (0.0%)	3 (42.9%)	
AC/T + Avastin	1 (10.0%)	0	1 (3.6%)		0 (0.0%)	1 (14.3%)	
Herceptin	1 (10.0%)	0	1 (3.6%)		0 (0.0%)	1 (14.3%)	
Adjuvant (n=29)				0.68			0.68
AC	1 (3.4%)	1 (3.8%)	0		0 (0.0%)	1 (6.7%)	
AC/T	20 (69.0%)	10 (38.5%)	10 (39.3%)		10 (71.4%)	10 (66.7%)	
TC	3 (910.3%)	1 (3.8%)	2 (7.1%)		1 (7.1%)	2 (13.3%)	
TC/H	1 (3.4%)	1 (3.8%)	0		1 (7.1%)	0 (0.0%)	
AC/TH	4 (13.8%)	2 (7.7%)	2 (7.1%)		2 (14.3%)	2 (13.3%)	

	All patients	CTC-negative, n(%)	CTC-positive, n(%)	P-value	CTC-low (≤10 CTCs/ml), n (%)	CTC-high (>10 TCs/ml), n (%)	P-value
Recurrence (n=53)							
None	47 (88.7%)	25 (100%)	22 (78.6%)	0.01*	27 (100.0%)	20 (76.9%)	0.01*
Positive recurrence	6 (11.3%)	0	6 (21.4%)		0.0 (0%)	6 (23.1%)	