Anti-Epithelial Cell Adhesion Molecule Antibodies and the Detection of Circulating Normal-Like Breast Tumor Cells

Anieta M. Sieuwerts, Jaco Kraan, Joan Bolt, Petra van der Spoel, Fons Elstrodt, Mieke Schutte, John W. M. Martens, Jan-Willem Gratama, Stefan Sleijfer, John A. Foekens

Identification of specific subtypes of circulating tumor cells in peripheral blood of cancer patients can provide information about the biology of metastasis and improve patient management. However, to be effective, the method used to identify circulating tumor cells must detect all tumor cell types. We investigated whether the five subtypes of human breast cancer cells that have been defined by global gene expression profiling—normal-like, basal, HER2-positive, and luminal A and B—were identified by CellSearch, a US Food and Drug Administration–approved test that uses antibodies against the cell surface–expressed epithelial cell adhesion molecule (EpCAM) to isolate circulating tumor cells. We used global gene expression profiling to determine the subtypes of a well-defined panel of 34 human breast cancer cell lines (15 luminal, nine normal-like, five basal-like, and five Her2-positive). We mixed 50-150 cells from 10 of these cell lines with 7.5 mL of blood from a single healthy human donor, and the mixtures were subjected to the CellSearch test to isolate the breast cancer cells. We found that the CellSearch isolation method, which uses EpCAM on the surface of circulating tumor cells for cell isolation, did not recognize, in particular, normal-like breast cancer cells, which in general have aggressive features. New tests that include antibodies that specifically recognize normal-like breast tumor cells but not cells of hematopoietic origin are needed.

J Natl Cancer Inst 2009;101:61–66

Circulating tumor cells are cells that have detached from the primary tumor or metastatic tumor sites and entered the peripheral circulation. A limited number of markers are used for the isolation (ie, cell surface antigens) or detection (ie, various antigens or mRNAs) of circulating tumor cells. These markers include epithelial cell surface markers, such as the epithelial cell adhesion molecule (EpCAM; also known as CD326, ESA, HEA125, and TACSTD1); cytokeratins 7, 8, 18, 19, and 20; and more cancer-specific markers, such as HER2-neu and mucin 1 for breast carcinoma (1–3). Commercially available tests for isolation and detection of circulating tumor cells include the CellSearch circulating tumor cell test (Veridex LLC, San Diego, CA) and other tests (1–3). These tests use combinations of specific antibodies against these molecules and generally include antibodies against EpCAM for cell isolation.

However, it is unclear whether such tests can detect all tumor subtypes. We investigated whether the CellSearch test could recognize all subtypes of breast cancer, including normal-like, basal, HER2-positive, and luminal A and B tumor cells.

The CellSearch circulating tumor cell test is the only diagnostic test that is currently approved by the US Food and Drug Administration as an automated test to detect and enumerate circulating tumor cells (4). Briefly, a blood sample that contains many leukocytes and few circulating tumor cells is drawn into 10-mL CellSave Preservative Tubes (Veridex LCC), which contain EDTA as an anticoagulant and a cellular preservative. The blood is maintained at room temperature and subsequently processed within 72 hours of collection by use of the CellSearch system (Veridex LLC), which consists of the CellTracks Autoprep (an automated sample

preparation system), the CellSearch epithelial cell kit (to enrich for cells expressing EpCAM and to label nuclei, leukocytes, and epithelial cells), and the CellSpotter Analyzer (a semiautomated fluorescence-based microscopy system that permits computer-generated reconstruction of cellular images). By use of the CellSearch epithelial cell kit, circulating tumor cells are isolated with anti-EpCAM antibodies coupled to microscopic iron particles, and complexes of circulating tumor cells bound to anti-EpCAM–coupled iron particles are "pulled" out of the blood sample by use of powerful magnets. Unbound cells and the remaining plasma are aspirated, and the immunomagnetically isolated cells are permeabilized and stained with 4',6-diamidino-2-phenylindole (to detect nuclei); anti-CD45 antibodies labeled with allophycocyan (to detect leukocytes); and anti-cytokeratin 8, 18, and 19 antibodies labeled with phycoerythrin (to detect epithelial cells). After incubation with staining reagents, the magnetic separation is repeated and excess staining reagents are removed by aspiration. In the final processing step, the immunomagnetically isolated cells are resuspended in a MagNest Cell Presentation Device (Veridex LLC). This device consists of a chamber and two magnets that orient the immunomagnetically labeled cells for analysis by use of the

Affiliations of authors: Department of Medical Oncology, Josephine Nefkens Institute and Cancer Genomics Centre (AMS, JB, JWMM, JAF), Department of Medical Oncology, Daniel den Hoed Cancer Center (JK, PvdS, J-WG, SS), and Department of Medical Oncology, Josephine Nefkens Institute (FE, MS), Erasmus MC, Rotterdam, the Netherlands.

Correspondence to: Anieta M. Sieuwerts, PhD, Department of Medical Oncology, Josephine Nefkens Institute, Erasmus MC, Rm BE-400, Dr Molewaterplein 50, 3015 GE Rotterdam, the Netherlands (e-mail: a.sieuwerts@erasmusmc.nl).

CellSpotter Analyzer, a four-color semiautomated fluorescence microscope. Finally, circulating tumor cells that are defined as nucleated cells with a round to oval morphology that are positive for anti-cytokeratin antibody binding and negative for anti-CD45 antibody binding (5) are identified by an operator.

Results of the CellSearch test have been used to monitor disease progression and therapy efficacy in metastatic prostate (6), colorectal (7), and breast (8) cancer. For patients with advanced breast cancer, the number of circulating tumor cells as determined by the CellSearch test has been shown to have prognostic value (8–14). In addition, a change from the baseline number of circulating tumor cells after the first cycle of therapy appears to distinguish patients who have responded to systemic therapy (who have a decreased number of circulating tumor cells) from those who have not (who have an increased number of circulating tumor cells) (15).

Five subtypes of human breast cancers have been identified by global gene expression profiling, including normal-like, basal, HER2-positive, and luminal A and B (16). The normal-like and basal subtypes, which represented 7.8% and 25% of all breast cancers, respectively, in a cohort of 344 breast tumor samples from patients with lymph node–negative disease (17), are in general negative for estrogen receptor (*ESR1*), progesterone receptor, and HER2-neu, and so lack the molecular targets for endocrine therapy and anti-HER2–directed therapy and therefore have worse treatment options than other subtypes. Because each breast cancer subtype has distinct prognostic and therapeutic characteristics (16,18), it is important to establish whether each breast cancer subtype expresses the cell surface antigens that are used in assays to isolate and detect circulating tumor cells. It is also important to identify which breast cancer subtypes do not express an antigen and to determine which antibodies could be used instead of or in addition to antibodies that are included in the test for circulating tumor cells.

To investigate whether the CellSearch test recognized all five breast cancer subtypes that are characterized by their intrinsic gene expression profiles as previously described (16,18), we first determined the subtypes of our well-defined panel of 34 human breast cancer cell lines (19) by global gene expression profiling, as described previously (17). A previous analysis of nearly 150 polymorphic microsatellite markers in this panel of 34 cell lines has shown that each of these 34 cell lines is unique and monoclonal (20). As determined by the expression of genes in the intrinsic gene set (16,17), the complete panel contained 15 luminal cell lines (ie, no clear-cut discrimination between luminal A and B), nine normal-like lines, five basal-like lines, and five HER2-positive cell lines (Table 1). To validate the gene expression profiling data, transcript levels of 14 candidate genes were remeasured with Affymetrix GeneChip Exon 1.0 ST Arrays (Affymetrix UK Ltd., Wickham la Wooburn Grn, UK) and real-time reverse transcriptase–polymerase chain reaction. The 14 candidate genes that may as such enable discrimination between the breast cancer subgroups included genes that are more specific for cells of hematopoietic origin (*CD44* and *CD45*); epithelial cell–specific genes such as those encoding cytokeratins, EpCAM, and mucin 1; genes encoding markers specific for the breast cancer subgroups (*ESR1*, *ERBB1*, *ERBB2*, *CAV1*, and *CD24*); and genes for two well-known epithelial–mesenchymal transition markers (*TWIST1* and *VIM*). Transcript levels of these 14 genes in all the 34 cell lines were compared with those measured in whole-blood cells from 23 different blood donors before (n = 6 samples) and after (n = 23 samples) being subjected to EpCAM-based CellSearch enrichment to establish which genes were not expressed by blood cells and could be used to specifically identify the epithelial tumor cells (Table 1). The study was approved by the Erasmus MC Institutional Review Board. Blood samples were collected from healthy volunteers after written informed consent was obtained. Supplementary Table 1 (available online) lists the gene expression assays used.

We next randomly selected 19 of the 34 cell lines; incubated each cell line with fluorochrome-conjugated antibodies against CD45, CD24, CD44, or EpCAM, which were chosen to enable a distinction between CD45-positive cells of hematopoietic origin, CD45-negative and EpCAM-positive breast cancer cells, and CD45-negative, CD24-negative, and CD44-positive breast cancer stem cells (21); and used flow cytometry to measure the expression of each marker by each cell line (Table 2). Next, an aliquot (50–150 cells per aliquot) of each of these 19 cell lines was added to a separate 7.5-mL sample of peripheral blood from a single healthy volunteer donor, and the mixtures were subjected to the EpCAM-based CellSearch assay to isolate circulating tumor cells. Gene expression data of the isolated cells showed that cells with a luminal or HER2-positive subtype were generally isolated by the assay, whereas cells with a normal-like subtype, which lack EpCAM expression, were not isolated or were only partially isolated (eg, MDA-MB-231 cells, which have marginal EpCAM expression) (Figure 1; Supplementary Table 2, available online). We found that the cell lines with the normal-like subtype

Table 1. mRNA expression in breast cancer cell lines with different intrinsic subtype characteristics*

Gene symbol (antigen)	Relative mRNA expression (95% CI)				Pt	Relative mRNA expression (95% CI)	
	Normal-like (n = 9)	Basal-like (n = 5)	Luminal (n = 15)	HER2-positive (n = 5)		Blood before enrichment (n = 6)	Blood after enrichment (n = 23)
PTPRC (CD45)	0.00 (0.00 to 0.00)	0.00 (0.00 to 0.00)	0.00 (0.00 to 0.00)	0.00 (0.00 to 0.00)	.210	13.39 (10.42 to 16.37)	6.25 (4.99 to 7.50)
CD24 (CD24)	0.40 (0.03 to 0.78)	2.31 (0.20 to 4.42)	3.84 (2.14 to 5.54)	2.69 (1.86 to 3.52)	.025	0.03 (0.02 to 0.03)	0.03 (0.01 to 0.06)
CD44 (CD44)	0.77 (0.59 to 0.95)	0.75 (0.40 to 1.10)	0.04 (0.02 to 0.06)	0.02 (0.00 to 0.04)	<.001	3.85 (3.13 to 4.57)	3.90 (3.29 to 4.52)
TACSTD1 (EpCAM)	0.04 (0.00 to 0.10)	2.04 (0.83 to 3.25)	8.72 (4.02 to 13.43)	4.43 (2.17 to 6.69)	.020	0.00	0.01 (0.00 to 0.02)
MUC1 (EMA, mucin 1)	0.32 (0.20 to 0.44)	0.35 (0.15 to 0.55)	1.88 (1.24 to 2.52)	0.53 (0.43 to 0.63)	<.001	0.03 (0.02 to 0.05)	0.01 (0.00 to 0.02)
CAV1 (caveolin 1)	5.04 (2.39 to 7.70)	1.70 (0.19 to 3.21)	0.05 (0.02 to 0.08)	0.03 (0.01 to 0.05)	<.001	0.02 (0.01 to 0.04)	0.05 (0.00 to 0.01)
KRT7 (cytokeratin 7)	1.53 (0.00 to 3.07)	7.90 (5.63 to 10.17)	6.62 (3.62 to 9.63)	10.46 (6.45 to 14.48)	.009	0.01 (0.00 to 0.02)	0.01 (0.00 to 0.01)
KRT18 (cytokeratin 18)	5.58 (0.96 to 10.19)	10.06 (7.88 to 12.24)	16.80 (13.23 to 20.36)	17.17 (12.11 to 22.23)	.001	0.06 (0.03 to 0.09)	0.03 (0.02 to 0.03)
KRT19 (cytokeratin 19)	0.09 (0.00 to 0.26)	4.53 (0.00 to 9.11)	28.35 (12.66 to 44.03)	11.99 (1.58 to 22.40)	.022	0.00 (0.00 to 0.00)	0.00 (0.00 to 0.00)
ESR1 (estrogen receptor)	0.00 (0.00 to 0.00)	0.02 (0.01 to 0.03)	0.76 (0.37 to 1.14)	0.06 (0.00 to 0.16)	.003	0.02 (0.01 to 0.03)	0.01 (0.00 to 0.02)
ERBB1 (EGF receptor)	0.20 (0.10 to 0.30)	0.96 (0.01 to 2.01)	0.03 (0.01 to 0.05)	0.07 (0.00 to 0.14)	.003	0.00 (0.00 to 0.00)	0.00 (0.00 to 0.00)
ERBB2 (HER2)	0.05 (0.03 to 0.06)	0.07 (0.02 to 0.12)	2.53 (0.13 to 4.94)	7.69 (2.28 to 13.09)	.009	0.02 (0.01 to 0.03)	0.01 (0.00 to 0.01)
TWIST1 (EMT marker TWIST1)	0.06 (0.01 to 0.11)	0.00 (0.00 to 0.00)	0.01 (0.00 to 0.02)	0.00 (0.00 to 0.00)	.027	0.00 (0.00 to 0.00)	0.00 (0.00 to 0.00)
VIM (EMT marker vimentin)	101.56 (36.90 to 116.23)	0.43 (0.00 to 0.90)	0.30 (0.00 to 0.67)	0.10 (0.00 to 0.27)	<.001	34.47 (22.12 to 46.82)	52.30 (44.10 to 60.50)

* Data in this table are the average and its 95% CI of one representative experiment. Results from other experiments were similar. Expression data are presented relative to that of *HMBS*, *HPRT1*, and *GUSB*. Fourteen candidate genes were selected, including genes that are more specific for cells of hematopoietic origin (*CD44* and *CD45*); epithelial cell-specific genes such as those encoding cytokeratins, EpCAM, and mucin 1; genes encoding markers specific for the breast cancer subgroups (*ESR1*, *ERBB1*, *ERBB2*, *CAV1*, and *CD24*; and genes for two well-known EMT markers (*TWIST1* and *VIM*). Data of the individual cell lines are presented in Supplementary Table 3 (available online), and the gene expression assays used are shown in Supplementary Table 1 (available online). To validate the gene expression profiling data, transcript levels of candidate genes that might enable discrimination among the four breast cancer subtypes, as represented by 19 randomly selected breast cancer cell lines, were analyzed with Affymetrix GeneChip Exon 1.0 ST Arrays (Affymetrix UK Ltd., Wickham la Wooburn Grn, UK) and real-time RT-PCR and compared with levels measured in whole blood of healthy blood donors before (n = 6) or after (n = 23) EpCAM-based CellSearch enrichment for circulating tumor cells. RNA was isolated from healthy blood and cell lines with the RNeasy (Micro) kit (Qiagen BV, Venlo, the Netherlands). For the cell lines, cDNA was prepared by use of the Superscript II RNase H-kit from Invitrogen (Breda, the Netherlands). For healthy blood donors before CellSearch enrichment and the preparations from healthy blood donors and cell lines after CellSearch enrichment, cDNA was synthesized with the RevertAid H Minus First Strand cDNA synthesis kit (Fermentas, St Leon-Rot, Germany), followed by a linear preamplification for the non-[2-[N-(3-dimethylaminopropyl)-N-propylamino]-4-[2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene]-1-phenyl-quinolinium] (SYBR Green I)-based assays (TaqMan PreAmp from Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands), according to the manufacturer's instructions. The resulting cDNA preparations were analyzed by real-time PCR with TaqMan gene expression assays and TaqMan Universal PCR Master Mix No AmpErase UNG (Applied Biosystems) and Absolute qPCR SYBR Green I mixture (Abgene, Epsom, UK) for the SYBR-based assays. PCRs were performed in a 20-µL reaction volume in an Mx3000P Real-Time PCR system (Stratagene, Amsterdam, the Netherlands). Expression of *HMBS*, *HPRT1*, and *GUSB* was used as a reference to control sample loading and RNA quality, as described previously (26). To enable comparison between the different methods of measuring gene expression (Affymetrix GeneChip Exon 1.0 ST arrays and real-time RT-PCR), data were normalized by expression levels that were measured by both methods in a set of seven control cell lines (ie, MDA-MB-435s, MDA-MB-231, CAMA-1, MCF-7, T47D, SK-BR-3, and EVSA-T). EGF = epidermal growth factor receptor; EMA = epithelial membrane antigen; EpCAM = epithelial cell adhesion molecule; EMT = epithelial–mesenchymal transition; CI = confidence interval; RT-PCR = reverse transcriptase-polymerase chain reaction; cDNA = complementary DNA.

† One-way analysis of variance was used to test for differences between the four subgroups present in the cell lines, with $P < .05$ being considered statistically significant. All statistical tests were two-sided.

expressed high mRNA levels of the epithelial–mesenchymal transition markers, TWIST1 (*TWIST1*) and vimentin (*VIM*) (one-way analysis of variance, two-sided $P = .03$ and $P < .001$, respectively) (Table 1); however, MDA-MB-231 cells expressed vimentin but did not appear to express TWIST1 (Supplementary Table 3, available online).

Cells with a normal-like breast cancer subtype express high levels of genes that are characteristic of basal epithelial and adipose cells and low levels of genes that are characteristic of luminal epithelial cells (16). The normal-like breast cancer subtype is the only subtype that displays the putative tumor-initiating stem cell phenotype, which includes low expression of CD24 and high expression of CD44 (21,22) (Table 2) and has high expression of both vimentin and TWIST1 (Table 1). Expression of vimentin and TWIST1 has been used to identify cells that have undergone the epithelial–mesenchymal transition, a process that has been linked to the generation of cells with properties of stem cells and to the ability of breast cancer cells

to enter the circulation and seed metastases (23–25). Thus, the normal-like breast cancer cell subtype is an important target for the development of individualized therapy and should not be overlooked when assessing circulating tumor cells. Intrinsic breast cancer subtypes are easily missed in analyses that use only crude, standard clinical or pathological criteria such as hormone receptor and HER2 status. Therefore, our results, which are based on a distinction in breast cancer subtypes determined by global gene expression profiling, cannot be compared with those of Cristofanilli et al. (8), who did not find differences in the number of circulating tumor cells between breast cancer cell subtypes that were defined only by hormone receptor and HER2 status.

New tests that include antibodies that specifically recognize normal-like breast tumor cells but not cells of hematopoietic origin are needed. As shown in Table 1, the phenotype of circulating leukocytes in the healthy blood donors before and after EpCAM-based CellSearch enrichment was characterized by the high expression of

vimentin and CD44 mRNA and the low expression of CD24 mRNAs. Because antibodies against vimentin and CD44 also bind to cells of hematopoietic origin, these antibodies would not be suitable for the detection of circulating tumor cells that have an epithelial–mesenchymal transition origin or putative breast cancer stem cells. However, current strategies for detecting circulating tumor cells can be improved to allow assessment of these normal-like cells by selecting antigens that are ubiquitously and abundantly present and that can be made accessible for isolation with immunobeads on normal-like cells but that are absent on cells of hematopoietic origin. When selecting antigens to use for isolating circulating normal-like breast cancer cells, it must be emphasized that clinical (breast) cancer samples consist of heterogeneous cell populations and always contain leukocytes and other types of blood cells; we circumvented this problem by using homogeneous cell lines. Examples of membrane antigens that might fulfill the criteria of being ubiquitously and abundantly present on normal-like cells but absent on cells

Table 2. Immunological assessment of antigens in breast cancer cell lines with different intrinsic subtype characteristics and circulating tumor cell recovery*

Intrinsic subtype	No. of cell lines	Flow cytometry,† MFI				CellSearch,‡ % of cells recovered (95% CI)
		CD45	CD24	CD44	EpCAM	
Normal-like	6	<5	<5	>1000	<5	2 (0 to 6)§
Basal-like	5	<5	5–20	200–1000	20–200	48 (36 to 61)
Luminal	5	<5	5–20	5–20	20–200	75 (62 to 89)
HER2-positive	3	<5	5–20	<5	20–200	85 (61 to 108)

* MFI = mean fluorescence intensity; EpCAM = epithelial cell adhesion molecule; CI = confidence interval; 7AAD = 7-amino-actinomycin D; FITC = fluorescein isothiocyanate.

† Cultured human breast cancer cell lines were incubated with the following fluorochrome-conjugated monoclonal antibodies: EpCAM conjugated with FITC (clone EBA-1; BD Biosciences, San Jose, CA), CD24 conjugated with FITC (clone SN3; eBioscience, Inc., San Diego, CA), CD44 conjugated with R-phycoerythrin coupled to the cyanine dye Cy7 (PE-Cy7, clone HIB19; eBioscience), and CD45 conjugated with allophycocyanin (clone 2D1; BD Biosciences). We used 7AAD (Sigma-Aldrich, St Louis, MO; 1 µg/mL) to control for viability. Cells were then analyzed on a Canto flow cytometer (BD Biosciences). To exclude nonviable cells from analysis, only 7AAD-negative (viable) cells were evaluated for antigen expression. Unstained cells were used as a negative control. Only cells that expressed EpCAM were captured by the anti-EpCAM antibody present in the CellSearch circulating tumor cell profile kit. In addition, as shown in Figure 1 and Supplementary Table 2 (available online), MDA-MB-231 cells with marginal EpCAM expression were only partially (10 of 82 added tumor cells, or approximately 12%) captured by the CellSearch antibody, indicating that anti-EpCAM antibodies from the EBA-1 clone (BD Biosciences) and from the CellSearch circulating tumor cell kit have similar sensitivities and specificities.

‡ Data are the average (95% CI) of one representative experiment performed in duplicate for the given number of cell lines. Blood samples containing EDTA (7.5-mL aliquots of blood) from a single healthy blood donor were obtained from CellSave Preservative Tubes (Veridex LCC, San Diego, CA). To each sample, 20 µL of a cell suspension containing 50–150 cultured cells from the indicated subtype of human breast cancer was added. To determine the actual viable cell number, a 100-µL aliquot of the cultured cells was incubated with 10 µL of 7AAD (1 µg/mL) and 100 µL of fluorescent beads (Beckman–Coulter, Inc., Miami, FL). After a 15-minute incubation at room temperature, 2 mL of phosphate-buffered saline was added, and samples were analyzed on a Calibur flow cytometer (BD Biosciences). At least 10 000 beads were acquired to estimate the number of 7AAD-negative (viable) cells. The efficiency of retrieving the tumor cells was controlled by counting the exact number of viable cells that were drawn in triplicate by flow cytometry and by light microscopy after serial dilution. To establish the number of circulating tumor cells recovered, samples were processed on the CellTrack AutoPrep analyzer (Veridex LLC) with the CellSearch circulating tumor cell profile kit (Veridex LLC), which uses microscopic iron particles coupled to anti-EpCAM to enrich for circulating tumor cells. The number of circulating tumor cells (ie, cells stained with the nuclear dye, 4′,6-diamidino-2-phenylindole, that are positive for cytokeratin 8, 18, and 19, and negative for CD45) were determined on the CellSpotter analyzer (Veridex LLC), according to the manufacturer's instructions (for full flow cytometry and CellSearch recovery data on these 19 cell lines, see Supplementary Table 2, available online).

§ One-way analysis of variance was used to test for differences between the four subgroups present in the cell lines, with $P < .05$ from a post hoc Dunett t test being considered statistically significant. All statistical tests were two-sided.
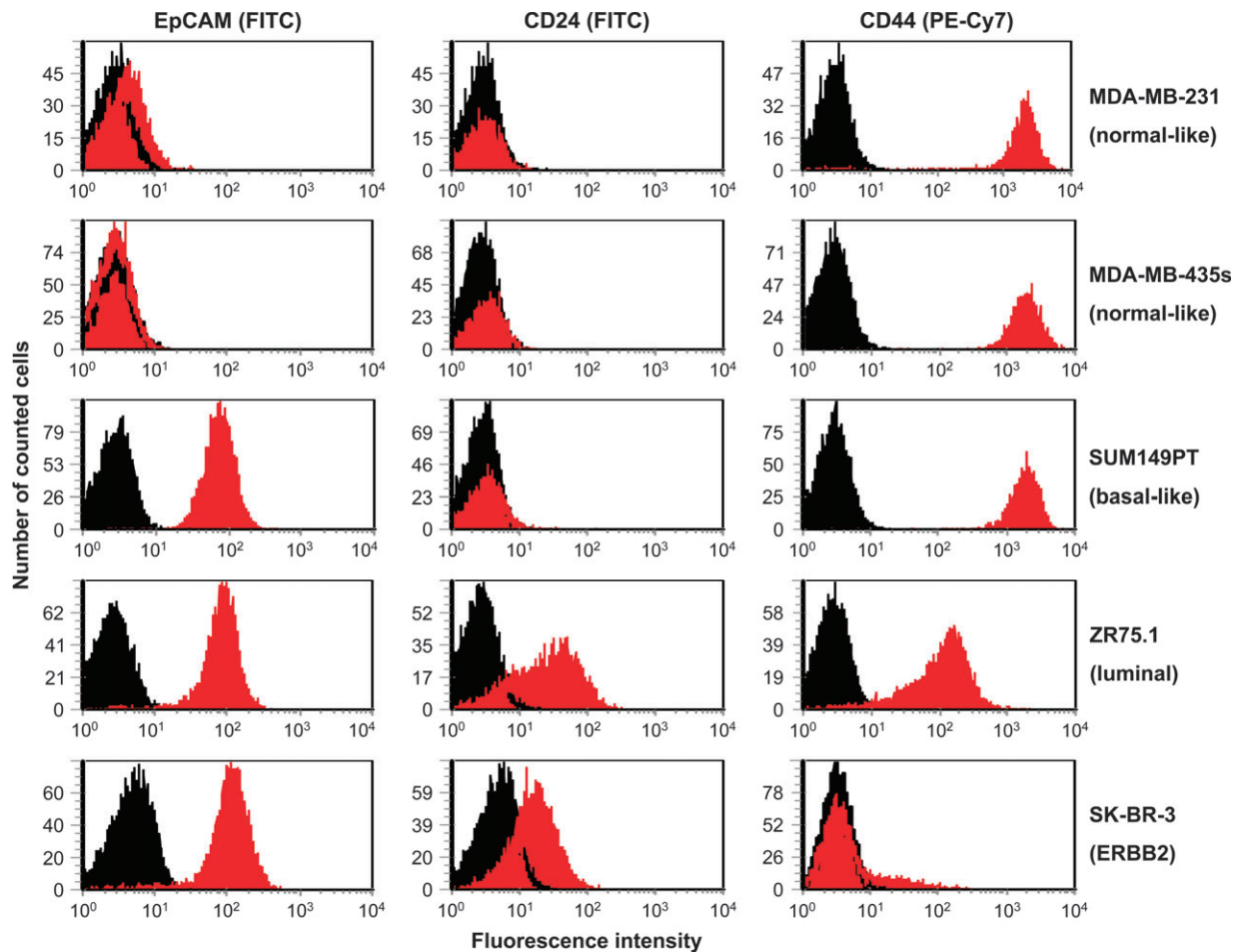
Figure 1. Immunological assessment of antigens in breast cancer cell lines representing the four intrinsic breast cancer subtypes. Data from two normal-like breast cancer cell lines, one basal-like cell line, one luminal cell line, and one HER2 (ERBB2)-positive cell line are shown. Cell surface antigens on tumor cells were assessed individually by incubating 10^6 cultured human breast cancer cells with the following fluorochrome-conjugated monoclonal antibodies: anti-EpCAM conjugated with FITC (clone EBA-1; BD Biosciences, San Jose, CA), anti-CD24 conjugated with FITC (clone SN3; eBioscience, Inc., San Diego, CA), or anti-CD44 conjugated with R-phycoerythrin coupled to the cyanine dye Cy7 (PE-Cy7, clone HIB19; eBioscience). We used 7AAD (Sigma-Aldrich, St Louis, MO) to assess viability. Only viable (7AAD negative) cells were analyzed for antigen expression (**red histograms**). Unstained cells (**black histograms**) were used as a negative control. Data are from one representative experiment that was performed three times. Results were similar for all three experiments. EpCAM = epithelial cell adhesion molecule; FITC = fluorescein isothiocyanate; 7AAD = 7-amino-actinomycin D.

of hematopoietic origin are mucin 1 and caveolin 1. Gene transcripts of caveolin 1, an approximately 22-kDa integral membrane protein, and mucin 1, a 350-kDa glycoprotein that protects the cell surface, were ubiquitously expressed in all normal-like human breast tumor cell lines, with the apparent expression of caveolin 1 being higher (gene transcript expression range = 1.42–13.42, relative to the expression of *HMBS*, *HPRT1*, and *GUSB*) than that of mucin 1 (gene transcript expression range = 0.06–0.56, relative to the expression of *HMBS*, *HPRT1*, and *GUSB*) (Table 1; Supplementary Table 3, available online). Furthermore, in our 344 clinical breast cancer samples (17), decreased expression of EpCAM was associated with increasing

expression of caveolin 1 (Spearman $r_s = -0.26$, $P < .001$). Consequently, antibodies against antigens such as caveolin 1 might be able to specifically detect circulating tumor cells from normal-like breast tumors.

A limitation of this study is our use of homogeneous breast cancer cell lines of known subtypes instead of blood samples from patients with breast cancer that had been subtyped. Detection of normal-like breast cancer cells in clinical (breast) cancer samples, which consist of heterogeneous cell populations, will require an assay that uses a mixture of antibodies against different cell surface antigens that are present on circulating tumor cells but absent on cells of hematopoietic origin. Such an assay should be thoroughly validated before

clinical use, not only in cultured cell lines but also in clinical samples of known intrinsic breast cancer subtype, as described previously for assays relying only on anti-EpCAM antibodies (5).

In conclusion, an EpCAM-dependent assay could not detect normal-like breast tumor cells. In the future, the identification of antibodies that specifically detect normal-like breast cancer cells (which in general have aggressive features) and their inclusion in the CellSearch assay may improve the sensitivity and feasibility of that assay without a loss of specificity.

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Funding

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