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CD49f-based selection of circulating tumor cells (CTCs) improves detection across breast cancer subtypes

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ABSTRACT

Circulating tumor cells (CTCs) can be enumerated using CellSearch, but not all breast cancer subtypes, specifically those with epithelial-mesenchymal transition (EMT) characteristics, sufficiently express the enrichment (EpCAM) and selection (CK8/18/19) markers used in this method. While CD146 can detect EpCAM-negative CTCs, we here evaluated the value of various cytokeratins and CD49f to detect CK8/18/19-negative CTCs. The tested cytokeratins provided no substantial benefit, but adding CD49f to CK8/18/19 as a selection marker resulted in improved recovery of normal-like cell lines.

Combined staining of CK8/18/19 and CD49f after CD146/EpCAM enrichment is likely to further improve CTC detection in breast cancer.

1. Introduction

In recent years, numerous assays for the detection of circulating tumor cells (CTCs) have been described [1]. One of these is the CellSearch™ technique (Veridex LCC, Raritan, NJ), the only FDA-approved method to detect CTCs based on their prognostic value in metastatic breast [2], colorectal [3] and prostate cancer [4]. The CellSearch™ technique consists of an EpCAM-based immunomagnetic enrichment step, followed by selection of CTCs based on cytokeratin (CK) 8/18/19 and 4,6-diamidino-2-phenylindole (DAPI) nuclear expression, and absence of the pan-leukocyte marker CD45. While CTCs according to this definition are present in 60% of metastatic breast cancer patients [5], we have recently shown that breast cancer cell lines of the normal-like type – one of the five intrinsic subtypes of breast cancer [6] – lack EpCAM expression. Consequently, CTCs of this subtype are missed when using the standard CellSearch method [7]. The normal-like subtype has been the subject of heavy debates regarding its characteristics, and has recently also been described as Claudin-low, characterized by low expression of the epithelial markers CD24 and EpCAM, and high expression of CD44 and CD49f [8]. Epithelial markers have been shown to be down regulated in circulating and disseminated tumor cells in the context of EMT, a process which tumor cells are thought to undergo upon entering the blood stream [9,10]. Evidence of EMT in CTCs, showing stem cell-like features rendering them resistant to chemotherapy, has recently been reported [11–15]. This loss of epithelial markers has been associated with the process of metastasis and poor prognosis [9,16,17], and tumors harboring more mesenchymal features frequently exhibit drug resistance. The detection of CTCs lacking epithelial markers may therefore be of crucial clinical importance.

Following up on the subtype-specific expression of EpCAM in vitro [7], we identified CD146 to be highly expressed on normal-like breast cancer cell lines, and showed that the combined enrichment of CTCs with anti-EpCAM and anti-CD146 ferrofluids improves breast cancer cell detection [18]. Additionally, CD146-positive CTCs were identified in the blood of metastatic breast cancer patients [18], and we are currently undertaking a clinical study in primary breast cancer patients in whom both EpCAM and CD146-positive CTCs are enumerated to establish the clinical relevance of these cells. Despite high CD146 expression in most cell lines with EMT features, a subset of cell lines was still not optimally recovered after combined CD146/EpCAM enrichment followed by selection based on CK8/18/19 expression [18]. We hypothesized this

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insufficient recovery to be due to lack of CK8/18/19 expression, which is a prerequisite for identification of CTCs in CellSearch, but also in other CTC detection methods [19,20]. CK expression is known to be heterogeneous among breast cancer subtypes; basal-likes commonly express CK5, 6, 14 and 17, luminals CK8, 18 and 19, and normal-likes often have low expression of all CKs [21–23].

In addition to examining the added value of selecting cancer cells with a broader array of CKs, we tested CD49f (ITGA6; integrin, alpha 6) as an alternative selection marker. CD49f is an integral cell-surface protein involved in cell adhesion which has also been described as a stem cell marker in breast cancer [24–27], making it a candidate to select EMT-like breast cancer cells with stem cell-like features.

In this study, we set out to identify a new additional marker to be used after capturing of breast cancer cells with combined anti-CD146/anti-EpCAM ferrofluids, in order to detect those cancer cells that lack CK8/18/19 expression.

2. Materials and methods

2.1. Breast cancer cell lines

Expression of putative markers was assessed on the 34 cell lines of our well-defined human breast cancer cell line panel [28,29]. These cell lines have been analyzed for their global gene expression to reveal their intrinsic subtype as described previously [30]. The normal-like cell line MDA-MB-435s has been the subject of debate on its origin [31], some researchers suggesting that it is in fact a melanoma cell line. Recently however, compelling evidence has been presented on its breast cancer origin [32,33], justifying its presence in our cell line panel.

2.2. CD49f and CK mRNA expression levels

Cell line CK and CD49f transcript levels were determined with Affymetrix GeneChip Human Exon 1.0 ST Arrays (Affymetrix UK Ltd., Wooburn Green, UK) and confirmed by qRT-PCR. Total RNA was isolated using the Qiagen RNeasy kit and quality was assessed using the Agilent Bioanalyser, requiring RNA integrity >7.0. All further processing of the samples was performed according to the Affymetrix GeneChip Whole Transcript (WT) Sense Target Labeling Assay as described before [30]. Briefly, Affymetrix GeneChip Human Exon 1.0 ST Arrays were used to determine the expression levels of virtually all exons present in the human genome (1.4 million probe sets covering >1 million exon clusters). For this study, we used expression data of the core probe sets of the transcript clusters as mentioned in Supplemental Table 1 that are supported by putative full-length mRNA from e.g. the RefSeq database (Geo dataset accession number GSE9385). Signal processing was performed as described before, followed by an additional normalization on the average of the core probes of three reference genes, *HBMS*, *HPRT1* and *GUSB*.

2.3. CD49f and CK protein expression levels

Cells from cultured human breast cancer cell lines were incubated with fluorochrome-conjugated monoclonal antibodies as described before [7]. In brief, cells were incubated with CD49f conjugated with PE (clone GoH3, BD Pharmingen, San Jose, CA), CK5 (Clone XM26 [Monosan, Uden, Netherlands]), CK7 (clone OV-TL 12/30 [Millipore, Billerica, MA]), CK14 (clone LL002 [Monosan]), panCK conjugated with PE (clone C-11 [Abcam, Cambridge, UK]) and a mixture as provided by the manufacturer of CK8/18 (clone C11) and CK19 (clone A53-B/A2) conjugated with PE (Veridex LCC). For CK5, CK7 and CK14, a goat anti-mouse Ig labeled with PE was added as a second step. CK staining was preceded by a fixation and permeabilization step, using the fixation and permeabilization reagents as provided in the CellSearch Epithelial Cell kit. Cells were then analyzed on a FACSCanto flow cytometer (BD Biosciences). Unstained cells were used as a negative control.

Tissue microarrays (TMAs) were prepared in duplicate from blocks of formalin-fixed, paraffin-embedded (FFPE) breast cancer cell line cells that were cultured to near confluence, as described before [34]. These blocks were sectioned at 4 μm, mounted on StarFrost slides (Waldemar Knittel–GmbH, Braunschweig, Germany), dried, deparaffinized in xylene, and rehydrated in graded solutions of ethanol and distilled water. Specimens were pre-treated with retrieval buffer (DAKO, Glostrup, Denmark) at pH 6.0 (S1699) for CD49f, CK5, CK14 and pH 9 (S2367) for CK8/18 and CK19 for 40 min at 95–99 °C in a water bath, cooled to room temperature for 20 min and rinsed in phosphate buffered NaCl solution (PBS). This was followed by a 10-min blocking step with a 0.3% peroxide PBS solution and a 30-min blocking step with a 5% bovine serum albumin PBS solution. Slides were stained with monoclonal antibodies against CK5 (1:100, clone XM26 [Monosan, Uden, Netherlands]), CK8/18 (1:100, clone 5D3 [Thermo Fisher Scientific, Waltham, MA]), CK14 (1:20, clone LL002 [Monosan]), CK19 (1:50, clone RCK108 [DAKO]) and CD49f (1:500, clone GoH3 [Abcam]) and stained with the peroxidase-conjugated Envision technique (DAKO EnVision™ System, HRP). Nuclei were counterstained with Haematoxylin. Each core was scored as 50–100% (++++), 25–50% (+++), 10–25% (++), 0–10% (+), or 0% (−) of positive cells.

2.4. Enumeration of cell line cells spiked in whole blood

Blood samples containing EDTA (7.5 mL aliquots of blood) from healthy donors were obtained from CellSave Preservative Tubes (Veridex LLC). A predefined number of 500 cultured human breast cancer cells was added to each sample. In order to determine the actual viable cell number, a 100 μL aliquot of the cultured cells was incubated with 10 μL of 7-Aminoactinomycin D (7AAD) (Sigma–Aldrich, St. Louis, MO) and 100 μL of fluorescent beads (Beckman-Coulter, Miami, FL). After 15 min incubation at room temperature, 2 mL PBS was added, and samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences) or a FACSCanto flow cytometer (BD Biosciences). At least 10,000 beads were acquired to estimate the number of 7AAD-negative (viable) cells. The efficiency of retrieving tumor cells was controlled by counting the exact number of viable cells that were drawn in triplicate by light microscopy after serial dilution. In order to establish the number of recovered breast cancer cells following spiking into blood from a healthy donor, samples were processed on the CellTracks AutoPrep analyzer (Veridex LLC) with the CellSearch Epithelial Cell kit (Veridex LLC), modified as described previously [18]. Briefly, for combined CD146 and EpCAM enrichment, EpCAM ferrofluids from the CellSearch Circulating Epithelial Cell kit and CD146 ferrofluids from the CellSearch Circulating Endothelial Cell kit were combined in even parts to a total volume of 150 μL per sample plus a standard excess volume of 300 μL. Ferrofluids coated with antibodies directed toward CD146 and EpCAM were added to the blood sample, and cancer cells bound to CD146/EpCAM coupled ferrofluid were isolated from whole blood by magnets. Unbound cells and remaining plasma were aspirated, followed by staining of the isolated cells with the nuclear dye DAPI, CK8, 18, and 19 antibodies labeled with PE and CD45 antibodies labeled with APC. As CD146 enriches for circulating endothelial cells (CECs) [35], and CECs can express CK18 [36], a marker to exclude the CK18-expressing subset of CECs was needed. As CD34 is a pan-endothelial marker [37], CD34 conjugated with FITC (clone 8G12; BD Biosciences) was added to the CellSearch Epithelial Cell kit, in a volume of 150 μL per sample plus a standard excess volume of 300 μL. After incubation, cancer cells were separated magnetically once more in order to remove unbound staining reagents. Finally, the cells were resuspended in a MagNest Cell Presentation Device (Veridex LLC), after which the number of cancer cells is enumerated according to the manufacturer's instructions.

For the detection of cells expressing CD49f, equal parts of staining reagent containing anti-CK8/18/19 conjugated to PE and CD45 conjugated to APC, and CD49f conjugated to PE were combined to a total volume of 150 μL per sample plus a standard excess volume of 300 μL. The sample was enriched for CD146 and/or EpCAM-positive cells and further characterized by staining for the presence or absence of DAPI, CK8/18/19 and CD45 as provided in the CellSearch Epithelial Cell kit and CD49f and CD34 as described above. Cancer cells were defined as DAPI+, CK8/18/19+/CD49f+, CD45−, CD34−.

2.5. Blood samples

Blood samples for spiking experiments were obtained from laboratory volunteers. Additionally, six healthy donors (age 28–58) were tested for the presence of CD146/EpCAM+, CD49f + events in 7.5 mL blood. This study was approved by the Erasmus MC Institutional Review Board (METC protocol 2007-333), and all healthy donors gave their written informed consent.

3. Results

3.1. CK8/18/19 expression

mRNA expression of *KRT8* (CK8), *KRT18* (CK18) and *KRT19* (CK19) was determined in all 34 cell lines in our well-defined breast cancer cell line panel using exon array (Table 1 and Suppl. Table 1). Generally, *KRT8, KRT18* and *KRT19* mRNA was expressed at the highest level in luminal cell lines, while the normal-like cell lines showed the lowest expression level of particularly *KRT19*.

Additionally, CK8/18/19 protein expression was determined by flowcytometry and immunohistochemistry (Table 1 and Fig. 1). For flowcytometry, the standard mixture of CK8/18 and CK19 antibodies as provided as part of the CellSearch Epithelial Cell kit was used, whereas a separate anti-CK19 monoclonal antibody and a mixture of anti-CK8/18 monoclonal antibodies were used for immunohistochemistry. According to flowcytometry, normal- and basal-like cell lines showed weak to no expression of CK8/19/19 protein.