Accession of Tumor Heterogeneity by Multiplex Transcriptome Profiling of Single Circulating Tumor Cells

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BACKGROUND: Transcriptome analysis of circulating tumor cells (CTCs) holds great promise to unravel the biology of cancer cell dissemination and identify expressed genes and signaling pathways relevant to therapeutic interventions.

METHODS: CTCs were enriched based on their EpCAM expression (CellSearch®) or by size and deformability (Parsortix™), identified by EpCAM and/or pan-keratin–specific antibodies, and isolated for single cell multiplex RNA profiling.

RESULTS: Distinct breast and prostate CTC expression signatures could be discriminated from RNA profiles of leukocytes. Some CTCs positive for epithelial transcripts (EpCAM and KRT19) also coexpressed leukocyte/mesenchymal associated markers (PTPRC and VIM). Additional subsets of CTCs within individual patients were characterized by divergent expression of genes involved in epithelial–mesenchymal transition (e.g., CDH2, MMPs, VIM, or ZEB1 and 2), DNA repair (RAD51), resistance to cancer therapy (e.g., AR, AR-V7, ERBB2, EGRF), cancer stemness (e.g., CD24 and CD44), activated signaling pathways involved in tumor progression (e.g., PIK3C4 and MTOR) or cross talks between tumors and immune cells (e.g., CCL4, CXCL2, CXCL9, IL15, IL1B, or IL8).

CONCLUSIONS: Multimarker RNA profiling of single CTCs reveals distinct CTC subsets and provides important insights into gene regulatory networks relevant for cancer progression and therapy.

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Detection and molecular characterization of circulating tumor cells (CTCs)† could provide valuable information for the clinical management of cancer patients because CTCs represent a real-time snapshot of the current tumor burden (1). Mutation analysis of CTCs might help to investigate alterations in genes involved in the response to targeted therapies (2, 3), which supports the use of CTCs as a “liquid biopsy.” However, to identify signaling pathways and splice variants relevant to cancer biology and therapies, transcriptome analysis of viable cancer cells is essential (4). Quantitative real-time PCR (qPCR) targeting keratin19 mRNA (KRT19)§ has been widely used to detect and monitor CTCs in cancer patients (5, 6). However, single marker analysis is not sufficient to understand the broad and complex nature of tumor biology. So far, multiplex qPCR targeting a few transcripts of interest has been applied (7, 8). However, most reports have relied on CTC-enriched fractions that still contain substantial numbers of contaminating leukocytes. Moreover, analy-
several of pooled CTC fractions miss the information on intrapatient heterogeneity of CTC populations.

Isolation of single CTCs and subsequent RNA analysis is challenging but the information obtained is much more extensive with regard to tumor cell heterogeneity in individual patients. A dual colorimetric RNA in situ hybridization assay was recently used to examine individual CTCs and tumor cell clusters for the expression of epithelial transcripts and mesenchymal markers (9). Heterogeneous RNA expression of single CTCs has also been demonstrated in patients with prostate cancer using the CTC-iChip for CTC isolation that is not commercially available (10).

In this study we performed single cell analysis after EpCAM-dependent (CellSearch®, Janssen Diagnostics) or size-based (Parsortix™, ANGLE Plc) enrichment. We established a workflow to screen up to 84 genes specific for different tumor entities, genes involved in DNA repair, epithelial–mesenchymal transition (EMT), tumor suppressor genes, therapy-related targets, stem cell-related markers, transcripts of activated signaling pathways, or genes mediating cross talk between tumors and immune cells.

Materials and Methods

CELL CULTURE
All cells [MDA-MB-468 (MDA-468), MDA-MB-231 (MDA-231), SKBR-3 and MCF7] were obtained from the American Type Culture Collection proposed by the vendor. Cells were harvested when they reached a confluence of approximately 80%.

PRIMER VALIDATION FOR SINGLE CELL RNA KIT COMPARISON
Total RNA (500 ng) from MDA-468, MDA-231, SKBR3, and MCF7 cells was isolated using the RNasy Micro Kit (QIAGEN) according to the protocol of the vendor. To generate first-strand cDNA, 0.5 μg of total RNA was reverse transcribed using the First-Strand cDNA Synthesis Kit (Thermo Scientific). Qualitative PCR was performed in a peqStar Thermocycler (Peqlab) under the following conditions: after 10 min denaturation at 95 °C, 35 cycles were carried out by denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation for 30 s at 72 °C. After a final elongation step at 72 °C for 10 min the samples were stored at 4 °C. Ethidium bromide stained-agarose gel electrophoresis was used to confirm the formation of the expected PCR products. (See Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue11.)

Primers were designed using the Primer3 software [epithelial cell adhesion molecule (EpCAM); forward 5′-GCTGGTGTGTGAACACTGCT-3′ and reverse 5′-ACGCGTTGTGATCTCCTTCT-3′; epidermal growth factor receptor (EGFR); forward 5′-CAG CGCTACCTTGTATTCA-3′ and reverse 5′-TG CACTCAGAGGCTCAGG-3′; erb-b2 receptor tyrosine kinase 2 (ERBB2); forward 5′-TGCGCTGT CCCTACAATACC3′ and reverse 5′-GACG CATAGACACTCGG; KRT19; forward 5′-CGCTACCTTGTACTTCA-3′ and reverse 5′-GAT CTGACATCCAGGTCCG-3′; and β-Actin (ACTB); forward 5′-CCACCCGAGAGATGA-3′ and reverse 5′-CCAGAGCGGTACAGGGATG-3′].

ENRICHMENT OF TUMOR CELLS FOR SINGLE CELL RNA ANALYSIS

CellSearch Epithelial Cell Profile Kit. The CellSearch Epithelial Cell Profile Kit (Janssen Diagnostics) contains a ferrofluid-based capture reagent coated to antibodies targeting the EpCAM antigen. EDTA blood samples were processed without fixation or permeabilization.

Parsortix System. The Parsortix (ANGLE Plc) is a marker-independent system for tumor cell enrichment capturing intact and viable tumor cells based on their size (≥10 μm) (11).

STAINING AND ISOLATION OF THE TUMOR CELLS
To distinguish tumor cells from the leukocytes, an epithelial-cell specific staining protocol was established targeting EpCAM and/or pan-keratins (EpCAM and pan-keratins for CTCs isolated by Parsortix and pan-keratins for tumor cells isolated by CellSearch). Briefly, enriched tumor cells were eluted into Eppendorf tubes, stained, and isolated by micromanipulation for single cell analysis.

The EpCAM antibody (Novocastra) was diluted in 10% AB-Serum/PBS (1:100) and incubated for 45 min on a rotor. Next, the sample was washed and a fluorescently labeled secondary antibody (Thermo Fisher) was added (1:150) for another 30 min in the dark. For subsequent pan-keratin staining, samples were washed and the CellSearch monoclonal pan-keratin antibody cocktail (included in the CellSearch Circulating Epithelial Cell Kit) was added (1:10; incubation time 20 min in the dark). Stained cells were washed and carefully transferred onto a glass slide for single cell isolation by micromanipulation (2).

TESTING OF DIFFERENT KITS FOR SINGLE CELL ANALYSIS
We tested the NucleoSpin RNA II Kit® (Macherey-Nagel), the Picopure RNA Isolation Kit® (Life Technologies), the MessageBooster Kit™ (Epicentre), and the CelluLyser™ Micro Lysis and cDNA Synthesis Kit (TATAA Biocenter) for single cell analysis. Kits were applied according to the instructions of the vendors.
MULTIMARKER EXPRESSION PROFILING OF SINGLE CELLS

**TATAA PreAMP and qPCR.** To analyze a multimarker profile of single cells, cells were lysed using the CelluLyser Micro Lysis and cDNA Synthesis Kit. cDNA was produced using the GrandScript cDNA Synthesis Kit and was subsequently preamplified with the CTC Grand-Performance assays using the PreAmp GrandMaster® Mix (TATAA Biocenter). PCR was done according to the manufacturer's instructions using the Bio-Rad CFX96 cycler (Bio-Rad).

**RT² PreAMP and RT² Profiler PCR Array.** The MessageBooster Kit™ was applied in combination with the RT² PreAMP Kit (QIAGEN) and different RT² Profiler PCR Arrays (Human Breast Cancer, Prostate Cancer, or EMT PCR Array or Cancer Inflammation & Immunity Cross-talk PCR Array, QIAGEN). PCR was done according to the manufacturer's instructions using the Bio-Rad CFX96 cycler (Bio-Rad).

**SINGLE CELL qPCR DATA ANALYSIS**

Single cell qPCR data were generated as illustrated in Fig. 1. Quantification cycle (Cq) values above 35 were treated as "off-scale data." All missing data points were also treated as "off-scale data" and replaced with a Cq value of 37. This was 2 cycles above the cutoff, which was considered giving a balanced weight to the negative observation of transcripts in individual cells (12). The Cq values were converted to relative quantities and transformed to log base 2 scale. The expression data were not normalized to any endogenous markers because of the stochasticity of single cell expression (13). The measured cDNA concent-ra values were then analyzed using the Bio-Rad CFX Manager software.
trations, which are proportional to mRNA concentrations (14), are thus expressed per cell. To display all data together, principal component analysis (PCA) and hierarchical clustering were used. Each method used a different mathematical approach to separate samples into groups. PCA reduced multidimensional data into 2-dimensional charts with small loss of information. Each point in the PCA graph contained information about the expression of all the genes. For the hierarchical clustering and PCA analyses, data were mean centered to reduce the influence of the variable overall expression level of the genes (15).

**PATIENT SAMPLES**

Analysis of human samples was carried out in accordance with the guidelines for experimentation with humans by the Chambers of Physicians of the State of Hamburg (“Hamburger Ärztekammer”). Written informed consent was obtained from all patients and the experiments conformed to the principles set out in the World Medical Association (WMA) Declaration of Helsinki and the Department of Health and Human Services Belmont Report. Breast cancer samples were obtained from the Department of Gynecology, University Medical Center Hamburg-Eppendorf, Germany. Prostate cancer samples were collected at the Practice Hematology and Oncology, Hamburg, Germany. All blood samples were collected into EDTA tubes (7.5 mL) and processed within 4 h after blood withdrawal.

**Results**

**VALIDATION OF SINGLE CELL RNA ANALYSIS**

Four different kits were tested for single cell RNA analysis: the NucleoSpin RNA II Kit® (Macherey-Nagel); the PicoPure RNA Isolation Kit® (Life Technologies); the MessageBooster Kit (Epicentre); and the CelluLyser Micro Lysis and cDNA Synthesis Kit (TATAA Biocenter). To verify the kits, 1, 5, 10, and 50 MDA-468 or the SKBR3 cells were directly spiked into lysis buffer followed by qualitative PCR amplifying EpCAM, EGFR, ERBB2, KRT19, and ACTB transcripts. Only the MessageBooster Kit and the CelluLyser Micro Lysis and cDNA Synthesis Kit produced consistent RNA profiles of the tested cell lines down to single cell level (see online Supplemental Fig. 1). Hence, these kits were used for further studies.

We had to establish a specific staining protocol that was compatible with downstream single cell profiling to distinguish CTCs from leukocytes in clinical samples. In our study, EpCAM and pan-keratins were used as target proteins for tumor cell identification (Fig. 2A and online Supplemental Fig. 2A).

Having established a reliable staining protocol, single breast (MDA-468 and SKBR3; n = 3) or prostate cancer cells (LnCap and PC3; n = 3) were spiked into 7.5 mL EDTA blood of healthy donors (n = 2) and processed by CellSearch or Parsortix. Next, tumor cells or remaining leukocytes were isolated and analyzed using either the RT2 PreAmp and Profiler Human Prostate Cancer PCR Array (QIAGEN) or the TATAA PreAmp GrandMaster® Mix and TATAA Probe GrandMaster® Mix (TATAA Biocenter) (Fig. 1, blue arrow and green arrow).

Tumor cells could clearly be discriminated from leukocytes based on their expression profiles (Fig. 2, B and C). All details of the measured Cq values are listed in online Supplemental Tables 1 and 2.

**QUALITATIVE PCR AS INDICATOR FOR THE SUCCESS OF FURTHER MULTIMARKER PROFILING**

For clinical samples, the criteria for a cell to be classified as CTC were: intact morphology (bright field microscopy), nucleus larger than 4 μm in diameter, and positive staining for EpCAM and/or pan-keratins (see online Supplemental Fig. 2A). The KRT19 transcripts have been widely used to identify and study CTCs in cancer patients (5, 6). Hence, we decided to use this marker as an additional “CTC indicator” by qualitative PCR before in-depth multimarker qPCR analysis. In total, 55 single CTCs from 5 different donors were analyzed in our study. However, reliable multimarker analysis was only possible when intense signals for KRT19 were observed (n = 39). Thus, we would recommend performing additional “qualitative PCR” tests as quality control before single cell multimarker analyses in future studies (see online Supplemental Fig. 2B).

**ACCESSION OF TUMOR HETEROGENEITY BY MULTIPLEX TRANSCRIPTOME PROFILING**

In-depth analysis of CTCs from a patient with breast cancer (enriched by CellSearch) revealed that RNA expression profiles of CTCs could clearly be discriminated from leukocytes (Fig. 3, A and B, and Fig. 1, green arrows). Genes such as EpCAM, cadherin 1_1 (CDH1_1), secretoglobin family 2A member 1 (SCGB2A1), estrogen receptor 1 (ESR1), epithelial membrane protein 2 (EMP2), and KRT19 were exclusively expressed in CTCs, while protein tyrosine phosphatase, receptor type C (PTPRC; formerly called CD45) and vimentin (VIM) were predominant in the leukocytes. Although CTCs could clearly be distinguished from the leukocytes, 2 CTCs were positive for both, the epithelial transcripts (EpCAM and KRT19) and the leukocyte/mesenchymal markers (CD45 and VIM).

Principle component analysis (PCA) of the expression profiles - measured for the individual CTCs from this patient - indicated that there might be 2 clusters, suggesting 2 CTC subtypes (Fig. 3, C–E). One CTC, which had substantially lower overall transcript levels than the other cells, separated from the 2 CTC clusters and from the leukocytes, indicating it was a deviant possibly due to damage during
Fig. 2. Single cell RNA analysis of EpCAM and/or pan-keratin stained cell culture cells and leukocytes.

(A), Cells were enriched by CellSearch or Parsortix and processed as described in Fig. 1. (B), Staining of the tumor cells after enrichment. (C), Dendrogram and heat map analyses of prostate and breast cancer cells after staining [LnCap, PC3, MDA-468, and SKBR3 (n = 3 per cell line)] and single leukocytes from healthy individuals (n = 2). Data are mean centered, with mean expression responses to zero. Red and green colors represent up- and downregulation, respectively (see scale), relative to the mean of the pool.
Fig. 3. Molecular signature of CTCs and leukocytes from a breast cancer patient.

Gene expression profile of CTCs (n = 9) and leukocytes (n = 2) enriched by CellSearch and analyzed using the CelluLyser Micro Lysis and cDNA Synthesis Kit together with the GrandPerformance panel. (A), Dendrogram and heatmap presenting hierarchical clustering analysis of CTCs and leukocytes. (B), PCA classifying the CTCs and leukocytes. (C), Dendrogram and heatmap of CTCs only. (D), PCA and hierarchical clustering of CTCs subgroups. (E), Box plot showing the genes that display significantly different expression profiles between subgroup 1 and 2 of CTCs.
ACTIVATION OF INFLAMMATION AND IMMUNITY SIGNALING IN CTCs

Lively debate is ongoing about communication between tumor cells and the cellular mediators of inflammation and immunity (23). Thus, we also decided to analyze the expression of genes involved in cancer inflammation and immune cross talk (Fig. 1, orange arrow). Here, both CellSearch and Parsortix enrichment strategies were applied to 2 blood samples from the same patient. The enrichment method did not bias expression of the reference genes included in the analysis (mean Cq after CellSearch enrichment: 25.31 vs mean Cq after Parsortix: 25.16), demonstrating consistent workflows of the 2 enrichment strategies. In this case, several genes involved in cross talks between immune cells and cancer cells, such as C-C motif chemokine ligand 4 (CCL4), C-X-C motif chemokine ligand 2 (CXCL2), CXCL9, interleukin 15 (IL15), IL1B, or IL8 showed different expression levels between the individual CTCs captured via EpCAM or by size (see online Supplementary Fig. 3 and online Supplemental Table 6). It is notable that tumor cell–derived CCL4 protein, which was one of the identified markers, can promote bone metastasis by interacting with CCR5-expressing nontumor cells (24). Chemokines have an important role in cancer metastasis and might mediate a signaling network that links metastasis formation and chemotherapy resistance. Chemokines, including CXCL2, CXCL8 (IL8), showed substantially higher expression in CTCs enriched with the Parsortix system. In relation to this observation, enhanced IL15 expression has previously been detected in colorectal cancer cells and shown to play an important role in cell proliferation, invasion, and metastasis formation (25). In our study, IL15 transcript levels showed large variations across CTCs independently of enrichment strategy.

Discussion

We developed optimized workflows for robust enrichment and transcriptomic profiling of single CTCs. Enrichment of CTCs was performed with 2 different platforms: the EpCAM-dependent CellSearch system...
and a novel, marker-independent size-based approach (Parsortix) (11). Our workflows allow for the characterization of different CTC single cell subpopulations depending on the markers used for the identification. Our single cell data showed high detection rates of the targeted transcripts. The GrandPerformance assays are probe based, thus avoiding false positive results due to formation of primer dimers and other aberrant products, while the RT² Profiler assays are SYBR based and generate signal also from aberrant PCR products. This is, however, minimized by careful assay optimization and by rejecting Cq values ≥35. Expression of reference genes cannot be used for normalization of single cell data due to the underlying burst kinetics (26), but their overall expression level can serve as a

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**Fig. 4. Molecular signature of CTCs from a breast cancer patient enriched by Parsortix and CellSearch.**

Gene expression profile of CTCs (n = 7 isolated by Parsortix and n = 6 isolated by CellSearch) both analyzed using the MessageBooster Kit in combination with the RT² PreAmp and Profiler Human EMT PCR Array. (A), Dendrogram and heat map presenting hierarchical clustering. (B), PCA classifying the CTCs. (C), Box plot showing the genes that display significantly different expression profiles between subgroup 1 and 2 of CTCs.
quality indicator. CTCs with exceedingly low transcript levels of reference genes are likely to have been damaged during the processing.

We found a minor subpopulation of epithelial marker-positive (e.g., KRT19 and EpCAM) and CD45-negative cells in clinical samples (Fig. 3). These cells were also strongly positive for markers such as twist family bHLH transcription factor (Twist), CD44, ATP binding cassette subfamily C member 1 (ABCC1), or ADAM metallopeptidase domain 17 (ADAM17). We assume

Fig. 5. Molecular signature of CTCs from prostate cancer patient 1.
Gene expression profile of CTCs (n = 8) enriched by Parsortix and analyzed using the MessageBooster Kit in combination with the RT² PreAmp and Profiler Human Prostate Cancer PCR Array. (A), Dendrogram and heat map, showing hierarchical clustering analysis. (B), PCA revealing 2 subgroups of CTCs. (C), Box plot showing genes that display significantly different expression profiles between subgroup 1 and 2 of CTCs.
that these cells are tumor cells but the role of CD45-positive CTCs is not clear yet (27, 28). Thus, our assay might help to identify the relevance of this specific cell type in future studies.

Focusing on the transcript signatures of CTCs, different subgroups were identified in individual patients (independently of the enrichment strategy (see online Supplemental Fig. 4, A–C). These subgroups were characterized by divergent expression of, e.g., EMT-associated genes (CDH2, COL1A2, COL5A2, FN1, MAP1B, MMP3, SOX10, SPP1, ZEB1, and ZEB2), DNA repair genes (RAD51) (21) or targets relevant for cancer therapy. For example, EGFR or ERBB2-specific transcripts were frequently present in CTCs (Figs. 4–6). ERBB2 is an effective target in breast cancer and further-

more discussed to play also an important role in PC (29, 30). Here, ERBB2 transcripts were present in PC-derived CTCs (see online Supplemental Fig. 6). Controlling the ERBB2 pathway could be an attractive combinational therapeutic approach together with second-generation antiandrogen agents such as enzalutamide and abiraterone acetate to remove castration-resistant prostate cancer (30). We also show that ERBB2-positive CTCs might express AR-V7 (3 out of 7 CTCs in the same patient) (Fig. 6). AR-V7, a constitutively active splice variant of the AR detected in CTC pools, was recently found to drive castration-resistant growth and resistance to enzalutamide in patients with prostate cancer (4). Thus, we demonstrate the differential expression of ERBB2 and/or AR-V7 between subgroups of CTCs in

Fig. 6. Molecular signature of CTCs from prostate cancer patient 2.
Gene expression profile of CTCs (n = 7) enriched by Parsortix system and analyzed using the CelluLyser Micro Lysis and cDNA Synthesis Kit together with the GrandPerformance panel. (A), Dendrogram and heat map, showing hierarchical clustering analysis. (B), PCA revealing 2 subgroups of CTCs. (C), Box plot showing genes that display significantly different expression profiles between subgroup 1 and 2 of CTCs.

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an individual patient with prostate cancer, indicating that unraveling the transcriptome of CTCs might help to monitor the emergence of driver molecules for resistance and thereby unveil patients who would profit from optional therapies, e.g., using galeterone instead of enzalutamide and abiraterone (plus ERBB2 inhibitors).

In addition, transcripts that have been reported to be relevant for metastatic processes (EpCAM, CD44, CD24) or potential drug targets (mTOR, PI3K, VEGFA) (31–33) were also found to be significantly differently expressed among breast cancer-derived CTCs (Fig. 3). Previous reports suggested the existence of tumor stem cells that are characterized by upregulation of CD44 and the downregulation of CD24 (34). However, the role of CD24 remains controversial since both CD44high/CD24low and CD44low/CD24high cells have been shown to initiate tumor growth (35, 36). In the present study, CD44 emerged as an allocation factor between 2 subgroups of CTCs derived from the same breast cancer patient, whereas CD24 was marginally altered between the same subgroups. Interestingly, the CD44low/CD24high subgroup showed high expression levels of PI3K and mTOR. The PI3K/AKT/mTOR signaling cascade is one of the most important intracellular pathways in cancer cells (37). Numerous efforts have been made to develop targeted therapies inhibiting this cascade in hormone receptor positive, ERBB2-negative breast cancer in combination with hormonal therapy (38).

Emerging data suggests that the heterogeneity of cancer thought to be causative for the functional plasticity necessary for tumor cells to evade therapeutic approaches, as well as prosperous adoption to foreign microenvironments within the metastatic niche, is driven by transcriptional changes (39). Understanding this heterogeneity with the presented workflow allows for a better understanding of the mechanisms involved in cancer progression. RNA analysis of CTCs offers the opportunity to stratify patients with metastatic disease into phenotypical subgroups according to their expression profiles, providing an important framework for therapeutic decisions based on a simple blood test (“liquid biopsy”) (1, 28). Such information is expected to be most valuable in the selection of personalized therapies, since the most active pathways are critical for the particular CTCs present and may be their Achilles heel if suitable drugs targeting that pathway are available. Particularly when combined with mutational profiling, which reveals any critical mutations affecting the sensitivity for targeted therapy (2, 3), it might support decision-making for individualized treatments.

In summary, we established reliable workflows to study multimarker profiles of single CTCs by low cost qPCR approaches. In principle, these workflows can be combined with any CTC enrichment system that enables isolation of high quality RNA. Thus, these workflows could become a valuable new tool in biomedical research. Although the number of patients/CTCs studied in this proof-of-principle study was small, multivariate analysis of expression profiles indicated that subgroups of CTCs with different phenotypes are present within each CTC-positive patient. Intrapatient heterogeneity of CTCs might contribute to resistance to therapy. This hypothesis has to be tested in future clinical studies.

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Single Cell mRNA Profiling of CTCs

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