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Gene Expression Signatures in Circulating Tumor Cells Correlate with Response to Therapy in Metastatic Breast Cancer

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BACKGROUND: Circulating tumor cells (CTCs) are thought to be an ideal surrogate marker to monitor disease progression in metastatic breast cancer (MBC). We investigated the prediction of treatment response in CTCs of MBC patients on the basis of the expression of 46 genes.

METHODS: From 45 MBC patients and 20 healthy donors (HD), 2 × 5 mL of blood was collected at the time of disease progression (TP0) and at 2 consecutive clinical staging time points (TP1 and TP2) to proceed with the AdnaTest *EMT-2/StemCellSelect*™ (QIAGEN). Patients were grouped into (a) responder (R) and non-responder (NR) at TP1 and (b) overall responder (OR) and overall non-responder (ONR) at TP2. A 46-gene PCR assay was used for preamplification and high-throughput gene expression profiling. Data were analyzed by use of GenEx (MultiD) and SAS.

RESULTS: The CTC positivity was defined by the four-gene signature (*EPCAM*, *KRT19*, *MUC1*, *ERBB2* positivity). Fourteen genes were identified as significantly differentially expressed between CTC+ and CTC− patients (*KRT19*, *FLT1*, *EGFR*, *EPCAM*, *GZMM*, *PGR*, *CD24*, *KIT*, *PLAU*, *ALDH1A1*, *CTSD*, *MKI67*, *TWIST1*, and *ERBB2*). *KRT19* was highly expressed in CTC+ patients and *ADAM17* in the NR at TP1. A significant differential expression of 4 genes (*KRT19*, *EPCAM*, *CDH1*, and *SCGB2A2*) was observed between OR and ONR when stratifying the samples into CTC+ or CTC−.

CONCLUSIONS: *ADAM17* could be a key marker in distinguishing R from NR, and *KRT19* was powerful in identifying CTCs.

Although major improvements in the diagnosis and treatment of breast cancer (BC)[7] have been achieved, about 20%–30% of patients will develop recurrent disease *(1)*. This phenomenon is explained commonly by the dissemination of tumor cells into the blood vessels as circulating tumor cells (CTCs) that move into secondary organs to rest there and then reappear to form metastatic disease loci *(2)*. Their prognostic value with regard to progression-free survival and overall survival has been well demonstrated in primary and metastatic BC (MBC) *(3)*.

Thus, CTCs are proposed to be an attractive source of liquid biopsy markers for monitoring disease progression *(4)*. CTCs are highly heterogeneous and can change in number and phenotype under anticancer treatment. Stem cell-like tumor cells, undergoing epithelial-mesenchymal-transition (EMT) and not displaying the classical epithelial phenotype, are discussed as an active source of metastatic spread and have been identified within the population of CTCs *(5)*. Up to now, no standard method for CTC selection and detection has been proposed *(6)*. Although CTC counts have a prognostic value, molecular methods are essential to characterize this heterogeneous cell population. Reverse transcription PCR (RT-PCR) methods have limitations because the markers used might not be expressed exclusively in CTCs but also can occur in normal blood cells. Thus, multimarker RT-PCR assays might yield better prognostic accuracy *(7)*.

Several groups, including ours, have already performed expression profiling, but studies interrelating CTC gene expression profiles and response to therapy have rarely been published *(8)*.

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[7] Nonstandard abbreviations: BC, breast cancer; CTC, circulating tumor cells; MBC, metastatic breast cancer; EMT, epithelial-mesenchymal-transition; RT-PCR, reverse transcription PCR; OR, overall responder; ONR, overall non-responder; HD, healthy donors; R, responder; NR, non-responder.

In this study, we used a 46 multimarker gene panel to (a) characterize CTCs from MBC patients undergoing palliative therapy, (b) predict treatment response on the basis of gene expression, and (c) identify differences in CTC gene expression patterns between overall responder (OR) and overall non-responder (ONR).

Materials and Methods

PATIENT POPULATION AND CHARACTERISTICS

The study was conducted at the Department of Gynecology and Obstetrics in collaboration with the Department of Internal Medicine (Cancer Research) at the University Hospital Essen. Between November 2013 and May 2015, 353 blood samples from 45 MBC patients were collected and studied. The patient population characteristics are shown in Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol63/issue10. Blood collection, isolation of CTCs, and cDNA synthesis were performed at the University Hospital Essen. cDNA was shipped on dry ice to the TATAA Biocenter (Goeteborg, Sweden) for performance of the gene panel testing.

ELIGIBILITY AND RESPONSE CRITERIA

Eligibility and response criteria of our patients have been documented in our previously published report *(8)*. Briefly, patients had either a relapse of a previously diagnosed BC and were to start chemotherapy, or had a documented progressive BC before receiving a new endocrine therapy, chemotherapy, or experimental therapy. Prior adjuvant treatment, radiation or any other treatment of the metastatic disease, were permitted. All specimens were obtained after written informed consent and collected by use of protocols approved by the institutional review board (05/2856). Before starting a new treatment, patients underwent an evaluation of metastatic sites by ultrasonography, x-ray, or computed tomography. Reevaluations of disease status were done every 8–12 weeks. Response to therapy was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST).

STUDY DESIGN AND STRATIFICATION OF PATIENTS

Two 5-mL blood samples from each of the 45 MBC patients were evaluated for CTCs at the time of progressive disease (PD) when patients were about to start a new therapy line (TP0) and every 8–12 weeks on average thereafter (TP1, TP2) (Fig. 1). Blood collection was performed before the application of therapy and with adjustments to the ongoing therapy. Twenty healthy donors (HD) were used as controls. Patients were stratified into responder (R) and non-responder (NR) at TP1, and into overall responder (OR, response to therapy at TP1 and TP2) and overall non-responder (ONR, no response to therapy at TP1 and TP2) at TP2. According to the RECIST criteria, patients with stable disease, complete remission, or partial remission are defined as R, whereas patients with progressive disease are NR.

IMMUNOHISTOCHEMICAL ANALYSIS

Tumor type, TNM staging, and grading were assessed according to the WHO classification of tumors of the breast and the sixth edition of the TNM Classification System. The estrogen (ER) and the progesterone (PR) receptor status were determined by immunohistochemistry, the expression of *ERBB2* with the HercepTest® (Dako) by use of FISH analysis for cases with 2+ staining as described elsewhere *(8)*.

SAMPLING OF BLOOD

Two 5-mL samples of EDTA blood were collected at each time point in S-Monovettes® (Sarstedt AG) for "in-house" or in *AdnaCollect*™ tubes (QIAGEN) for "overnight" samples. Spiking experiments using the AdnaTest *ColonCancer*™ (QIAGEN) to compare EDTA vs *AdnaCollect*™ showed that the specificity and recovery rates were very similar (see Table 2 in the online Data Supplement). The stabilization effect of the *AdnaCollect*™ tubes over 24-h transportation time could be demonstrated by use of T84 cells and the AdnaTest *ColonCancer*™ to determine specificity and recovery at the time of blood sampling and after 24 h of storage at 4–10 °C (see Table 3 in the online Data Supplement). In total, gene expression of 353 cDNA samples, originating from 177 duplicates, was analyzed.

ENRICHMENT AND DETECTION OF CTCs

CTCs were isolated by use of the immunomagnetic AdnaTest *EMT-2/StemCellSelect*™, targeting *EPCAM*, *EGFR*, and *ERBB2*. The resulting cell lysate was stored at −80 °C until further use applying the AdnaTest *EMT-2/StemCellDetect*™ (both QIAGEN) according to the manufacturer's instructions (130122 EN). mRNA was isolated from the pre-enriched cell lysate with oligo(dt)25-coated magnetic beads. Reverse transcription was performed by use of the Sensiscript Reverse Transcription Kit™ (QIAGEN). The test procedure has been described in detail elsewhere *(8)*. Sensitivity and specificity were analyzed by use of 20 HD samples and 20 blood samples of HD enriched with 30 cells (20 cells PC3 and 10 cells MiaPaca) for *TWIST*, *AKT2*, and *PIK3CA* (see Table 4 in the online Data Supplement).

LIST OF TARGET GENES

The GrandPerformance CTC Assay panel comprises 46 transcripts of cancer-related genes, including BC, stem cell, EMT, and reference markers that were designed and optimized for a work flow based on preamplification and high-throughput profiling (TATAA Biocenter). Each sample was profiled in duplicates for expression of

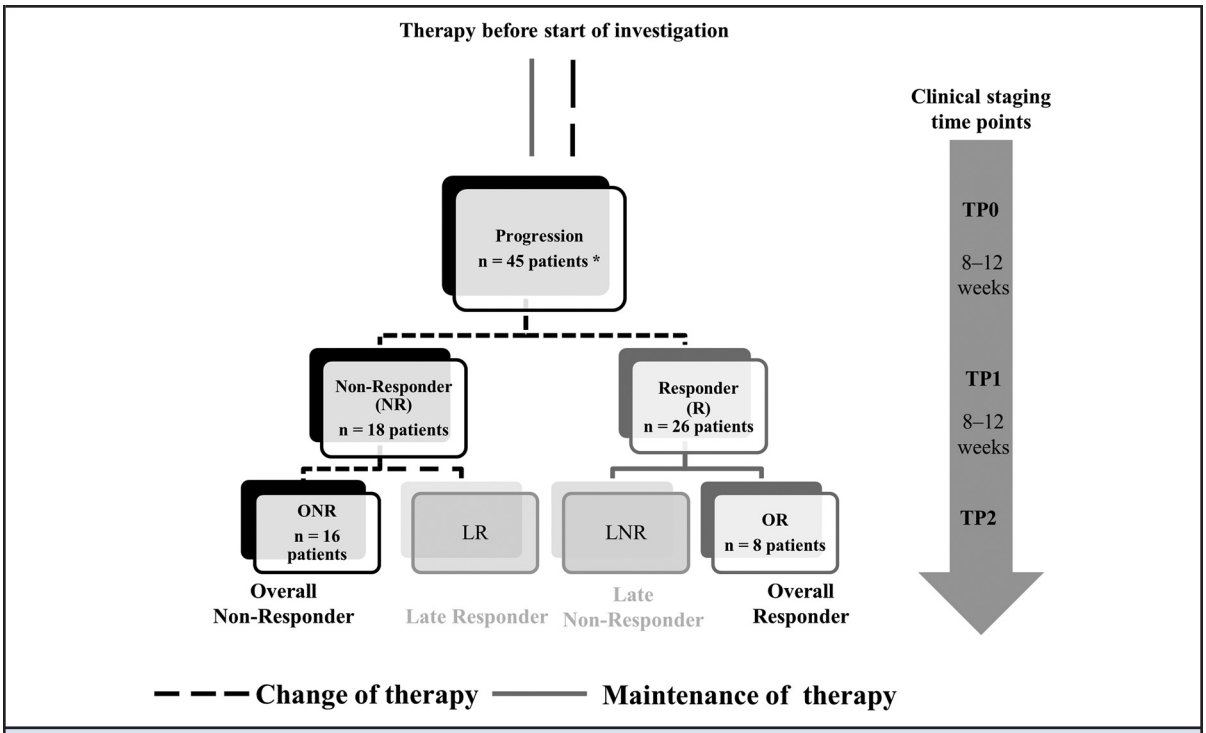

Fig. 1. Study design.

Patients were stratified into R and NR at TP1 and in OR or ONR at TP2. According to the RECIST criteria, patients with stable disease, complete remission, or partial remission were defined as R, whereas patients with progressive disease were defined as NR. *Annotation: At TP1, 1 patient was no longer eligible for the analysis.

TUBB2A,[8] *TBP*, *GUSB*, *GAPDH*, *EMP2*, *VIM*, *VEGFA*, *PLAU*, *TWIST1*, *TP53*, *TOP2A*, *SCGB2A2*, *SATB1*, *RAD51*, *PTPRC*, *PTEN*, *PIK3CA*, *PGR*, *PARP1*, *MYC*, *MUC1*, *MTOR*, *ABCC1*, *MET*, *KRT19*, *KRAS*, *KIT*, *MKI67*, *IGF1R*, *IBSP*, *FLT1*, *ESR1*, *ERBB2*, *EPCAM*, *EGFR*, *CTSD*, *CDH1*, *CD44*, *CD24*, *AURAK*, *ALDH1A1*, *AKT2*, *ADAM17*, *ACTB* (Actin β), *PPIA*, and *B2M*. Amplicon sizes and references are documented in Table 5 in the online Data Supplement.

[8] Genes: *TUBB2A,* tubulin beta 2A class IIa; *TBP,* TATA-box binding protein; *GUSB,* glucuronidase beta; *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase; *EMP2,* epithelial membrane protein 2; *VIM,* vimentin; *VEGFA,* vascular endothelial growth factor A; *PLAU,* plasminogen activator, urokinase; *TWIST1,* twist family bHLH transcription factor 1; *TP53,* tumor protein p53; *TOP2A,* topoisomerase (DNA) II alpha; *SCGB2A2,* secretoglobin family 2A member 2; *SATB1,* SATB homeobox 1; *RAD51,* RAD51 recombinase; *PTPRC,* protein tyrosine phosphatase, receptor type C; *PTEN,* phosphatase and tensin homolog; *PIK3CA,* phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; *PGR,* progesterone receptor; *PARP1,* poly(ADP-ribose) polymerase 1; *MYC,* MYC proto-oncogene, bHLH transcription factor; *MUC1,* mucin 1, cell surface associated; *MTOR,* mechanistic target of rapamycin; *ABCC1,* ATP binding cassette subfamily C member 1; *MET,* Met proto-oncogene; *KRT19,* keratin 19; *KRAS,* KRAS proto-oncogene, GTPase ; *KIT,* KIT proto-oncogene receptor tyrosine kinase; *MKI67,* marker of proliferation Ki-67; *IGF1R,* insulin-like growth factor 1 receptor; *IBSP,* integrin binding sialoprotein; *FLT1,* fms related tyrosine kinase 1; *ESR1,* estrogen receptor 1; *ERBB2,* erb-b2 receptor tyrosine kinase 2; *EPCAM,* epithelial cell adhesion molecule; *EGFR,* epidermal growth factor receptor; *CTSD,* cathepsin D; *CDH1,* cadherin 1; *CD44,* CD44 molecule (Indian blood group); *CD24,* CD24 molecule; *AURKA,* aurora kinase A; *ALDH1A1,* aldehyde dehydrogenase 1 family member A1; *AKT2,* AKT serine/threonine kinase 2; *ADAM17,* ADAM metallopeptidase domain 17; *ACTB,* actin beta; *ABCC1,* ATP binding cassette subfamily C member 1; *B2M,* beta-2-microglobulin; *PPIA,* peptidylprolyl isomerase A.

PREAMPLIFICATION

cDNA was preamplified by use of the TATAA PreAmp GrandMaster® Mix and a mixture of 46 forward and reverse primers (50 nmol/L final concentration) of each target gene including the ValidPrime™ control assay for human genomic DNA (both TATAA Biocenter) *(9)*. The preamplification was performed in a CFX96 thermocycler (Bio-Rad Laboratories) as follows: denaturation at 95 °C for 60 s, followed by 22 cycles of 95 °C for 15 s, 60 °C for 120 s, and 72 °C for 60 s. The method has been described in detail elsewhere *(10)*. Validation data are shown in Table 6 in the online Data Supplement.

HIGH-THROUGHPUT RT-QPCR

Four μl of the preamplified products were diluted 10× in 36 μl nuclease-free water (Life Technologies). Two different mixtures were prepared with a total volume of 6 μl each: (a) a sample mixture per sample and (b) an assay mixture per assay. The sample mixtures were prepared by mixing the diluted, preamplified products (2.7 μl) in-

cluding the gDNA sample plus a non amplified and undiluted template for TATAA Interplate Calibrator with TATAA Probe Low-ROX GrandMaster Mix (3 μl, both TATAA Biocenter) and 20× GE Sample Loading Reagent (0.3 μl, Fluidigm Corporation). The assay mixtures were prepared using 2.4 μl each primer pair at 4 μmol/L final concentration giving 46 assay mixtures (1 for each target gene) and 1 for TATAA Interplate Calibrator, 0.6 μl dual-labeled probe at 1 μmol/L final concentration (FAM-MGB) and 3 μl 2× Assay Loading Reagent (Fluidigm). Five μl of the sample and assay mixtures were loaded onto a 96 × 96 Dynamic Array™ IFC, and RT-qPCR was performed in a BioMark™ HD (both Fluidigm) as follows: thermal mixture: 70 °C for 30 min, followed by 25 °C for 10 min; predenaturation: 95 °C for 60 s. In total, 35 cycles at 95 °C for 5 s followed by 60 °C for 30 s were performed. Cq-values were obtained by use of the Fluidigm Real-Time PCR Analysis software v.4.1.2 (Fluidigm). All assays were validated according to the MIQE guidelines and assay disclosure *(11)*. The standard curves are provided in Table 7 in the online Data Supplement; assay list and context sequences are provided in Tables 8 and 9 in the online Data Supplement.

RELATIVE RNA AMOUNT CALCULATIONS AND STATISTICAL ANALYSIS

Cq-values were determined by GENex software by use of a ΔΔCq approach *(12)*. Analysis by GENorm and Normfinder has been provided to choose an eligible control gene set. Five control genes have been tested in total. *ACTB* was chosen to be used as control gene for all the testing settings because of its unique stability and abundance in tested RT-qPCR samples. After normalization to the control gene, the relative RNA amount was calculated by comparison to the control group gene expression profiles. The relative RNA amount is displayed in log2 values. The gene expression data were compared between predefined groups by non parametric Mann–Whitney testing (2-tailed) with $P < 0.0001$ considered as significant as automatically set by the GenEx software.

Results

IDENTIFICATION OF PATIENTS WITH CTC-LIKE CELLS

CTC positivity for the examined patient cohort was defined as the expression of at least 1 of the following 4 markers in the multimarker RT-qPCR: *EPCAM*, *MUC1*, *ERBB2*, or *KRT19*. The cluster analysis in Fig. 1 in the online Data Supplement shows that most of the CTC-positive (CTC+) samples expressed at least 2 of the CTC marker genes in parallel. At TP0, in 14 of 62 CTC+ samples (23%) *KRT19* was uniquely expressed. Out of these, 4 of 14 expressed *SCGB2A2*, 6 of 14 showed expression of *CDH1*, and 1 of 14 expressed *SCGB2A2* and *CDH1* in parallel. At TP0, 26 of the 45 patients (58%) were CTC+ and 19 of the 45 patients (42%) CTC-negative (CTC−). As illustrated in Fig. 2, CTC+ patients can be clearly distinguished from CTC− patients at TP0. The gene expression profiles of HD and CTC− samples are displayed as 1 overlapping group of points.

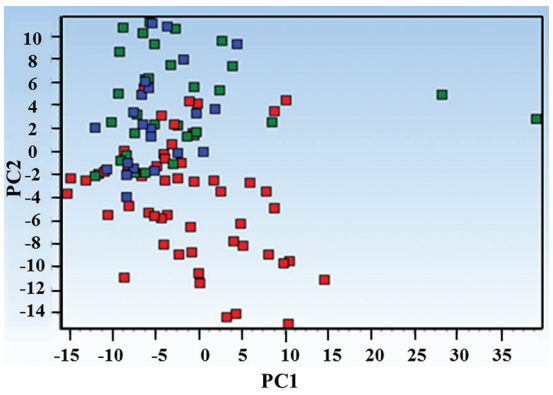

Fig. 2. Distinction of CTC+, CTC−, and HD samples.
On the basis of the expression of *EPCAM, MUC1, KRT19,* and *ERBB2,* CTC+ samples (red) can be clearly distinguished from CTC− samples (green) and HD (blue) by use of principal component analysis (PCA). PC1, projection on parameter 1; PC2, projection on parameter 2.

GENE EXPRESSION PATTERNS IN HD VS CTC+ SAMPLES

Comparison of expression patterns revealed significant differences between HD and CTC+ samples. *EPCAM*, *KRT19*, and *CD24* were significantly differentially expressed in those groups ($P = 1.17^{-05}$, $P = 8.94^{-09}$, and $P = 0.537^{-03}$). *PTPRC* (CD45) did significantly reveal the difference between gene expression signals resulting from normal cells compared to CTCs (Table 1). Homogeneity of the results and the assay validity of the 4 CTC marker genes are shown in Table 10 in the online Data Supplement as well as in the cluster analysis in Fig. 2 in the online Data Supplement, indicating an abundance of

Table 1. Differentially expressed genes in healthy donors (HD) and CTC+ samples.

Gene differentially expressed in HD vs CTC+	*P*-value
EPCAM	1.17^{-05}
KRT19	8.94^{-09}
CD24	5.37^{-03}
PTPRC	2.00^{-03}

Table 2. List of genes significantly differentially expressed in CTC+ vs CTC− samples.

Genes differentially expressed in CTC+ vs CTC− samples	*P*-value
KRT19	0
EPCAM	4.16^{-09}
FLT1	5.60^{-07}
EGFR	2.07^{-06}
MET	2.18^{-06}
CD24	6.10^{-06}
PGR	1.62^{-05}
PLAU	2.89^{-05}
KIT	1.24^{-03}
ALDH1A1	1.75^{-03}
CTSD	2.45^{-03}
MKI67	3.17^{-03}
TWIST1	7.28^{-03}
ERBB2	7.38^{-03}

the transcripts *EPCAM*, *MUC1*, *KRT19*, and *ERBB2* (raw expression data, not normalized).

DIFFERENCES IN CTC GENE EXPRESSION PROFILES IN CTC+ VS CTC− SAMPLES

As shown in Table 11 in the online Data Supplement, 93 out of 122 (76%) samples were CTC+ and 29 out of 122 (24%) samples were CTC− at TP0. At TP1, 92 out of 123 (75%) samples were classified as CTC+ and 31 out of 123 (25%) as CTC−. At TP2, CTCs were detected in 81 out of 108 (75%) and not found in 27 out of 108 (25%) samples, respectively. The overall CTC positivity rate of all cDNA samples across the 3 different time points was 75% (266 of the 353 samples) and the overall negativity rate 25% (87 of the 353 samples). Epithelial marker genes (*EPCAM*, *MUC1*, and *KRT19*) were always expressed in correlation with stemness (*ALDH1A1*, *TWIST1*, and *AKT2*) or EMT marking genes (*VIM*), but never exclusively (data not shown). Fig. 3 in the online Data Supplement shows an exemplary expression pattern of patient No.14.

As reported in Table 2, 14 genes (*KRT19*, *FLT1*, *EGFR*, *EPCAM*, *MET*, *PGR*, *CD24*, *KIT*, *PLAU*, *ALDH1A1*, *CTSD*, *MKI67*, *TWIST1*, and *ERBB2*) were evaluated as significantly differentially expressed in the tested sample cohorts between CTC+ and CTC− samples.

GENE EXPRESSION PATTERNS IN PATIENTS WITH PERSISTING CTCs

Thirty-four out of 45 (63%) patients remained CTC+ over the whole observation period (only patients with completed TP0, TP1, and TP2 blood collections were counted). Likewise, there were 5 out of 45 (9%) patients showing no sign of CTC presence in the RT-qPCR at all 3 time points tested. Those patients were called "total positive" or "total negative," respectively. Eight genes (*EGFR*, *MET*, *FLT1*, *PGR*, *PLAU*, *KIT*, *MKI67*, and *TWIST1*) were significantly differentially expressed between those 2 groups (see Table 12 in the online Data Supplement).

DIFFERENCES IN GENE EXPRESSION PATTERNS BETWEEN R AND NR

At TP1, all patients were stratified into R and NR on the basis of the clinical staging outcome. Eleven out of 26 (42%) R were CTC+ and 15 out of 26 (58%) R were CTC−. Among the NR, 13 out of 18 (73%) patients were CTC+ and 3 out of 18 (17%) patients CTC−. Thus, CTC+ patients were more likely to be found in the group of NR, whereas CTC− patients had mostly responded to therapy. *KRT19* was highly expressed in CTC+ patients as well as in NR ($P = 0.014$), whereas *ADAM17* ($P = 0.000567$) was significantly increased in NR regardless of their CTC status (Figs. 4 and 5 in the online Data Supplement).

DIFFERENCES IN GENE EXPRESSION PATTERNS BETWEEN OR AND ONR

At TP2, patients were further classified into OR and ONR. ONR were more likely to be CTC+. Six out of 8 (75%) ONR were CTC+, whereas 2 out of 8 (25%) were CTC−. Among the OR, 6 out of 16 (38%) patients were CTC+ and 10 out of 16 (62%) CTC−. *KRT19* ($P = 1.48^{-07}$), *EPCAM* ($P = 1.14^{-06}$), *CDH1* ($P = 1.55^{-06}$), and *SCGB2A2* ($P = 4.97^{-06}$) were significantly differentially expressed in these groups (Fig. 3 and Fig. 4). There was no difference between OR and ONR, except for the 2 genes *ABCC1* ($P = 5.51^{-05}$) and *KRT19* ($P = 2.93^{-04}$) (see Table 13 in the online Data Supplement). The differential expression of *CDH1*, *EPCAM*, *ERBB2*, *ESR1*, *RAD1*, and *SCGB2A2* in patients with PD vs CR reached only borderline significance (Fig. 6 in the online Data Supplement). As shown in Figs. 7 and 8 in the online Data Supplement, two separate gene expression clusters were observed for ONR, whereas OR revealed a homogeneous expression pattern (raw expression data, not normalized). The genes *ESR*, *MUC1*, *AURKA*, *RAD51*, *TOP2A*, *ADAM17*, *SCGB2A2*, *KRT19*, and *EPCAM* were expressed in most of the ONR samples.

Discussion

Although the prognostic value of CTC counts has widely been accepted, characterization of CTCs holds great promise in monitoring therapy and guiding targeted treatment applications effectively *(13)*. Clinical trials like

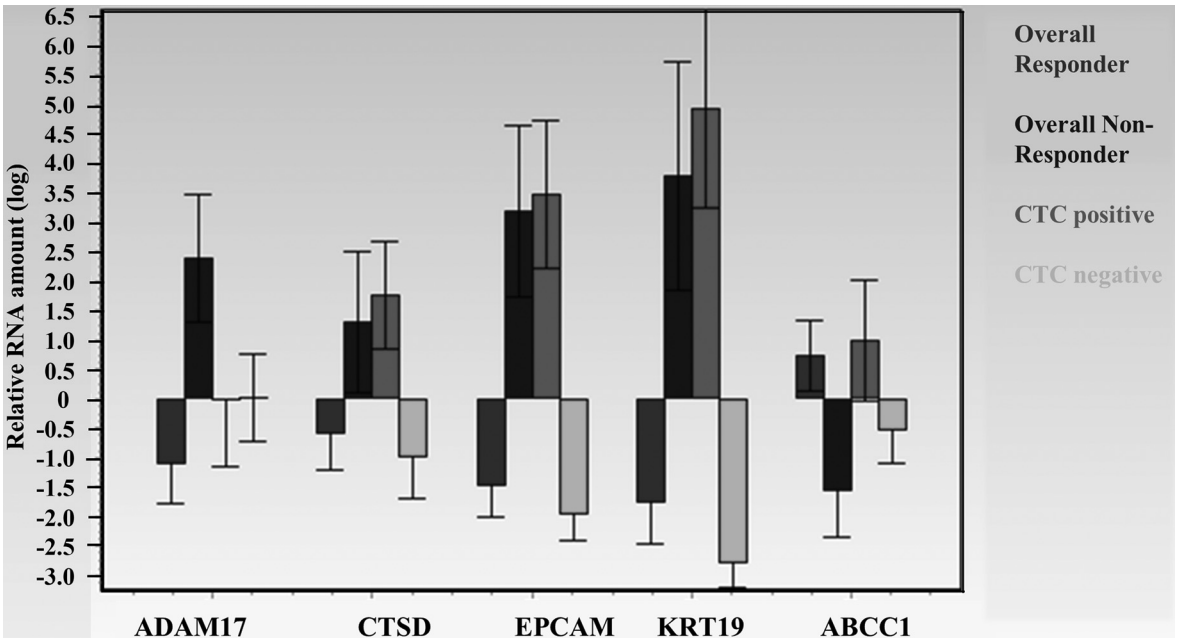

Fig. 3. Significantly differentially expressed genes in the groups of OR, ONR, CTC+, and CTC− patients.

ONR were more likely to be CTC+. *KRT19* was highly expressed in the CTC+ group and *ADAM17* was highly expressed in the group of ONR regardless of their CTC status.

the CirCe01 trial (NCT01349842), the DETECT V CHEVENDO trial (NCT02344472), or the STIC CTC METABREAST trial (NCT01710605) are investigating the power of CTCs in choosing the best treatment strategy as BC evolves *(13)*. It has been proposed that the CTC marker expression profile might be related to response or resistance to therapy as well as to a worse outcome *(14)*.

The transfer of CTCs into the clinic is hindered by various existing definitions of CTCs, reflected by the variety of methods available for their selection and detection *(6)*. Currently, the CellSearch System is the only

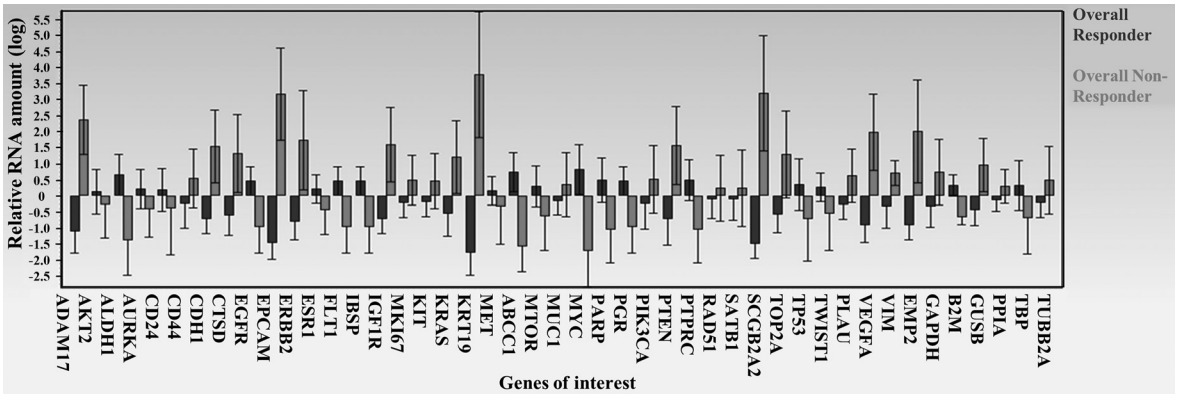

Fig. 4. CTC gene expression pattern in OR vs ONR.

When CTC+ samples were not predefined by the 4-gene signature [except for the 2 genes *ABCC1* ($P = 5.51^{-05}$) and *KRT19* ($P = 2.93^{-04}$)], no difference between the OR and ONR group was obtained.

FDA approved method for the identification of CTCs, defining CTCs as *EPCAM*+, *KRT8/18/19*+, *DAPI*+, and *PTPRC*− *(15)*. The commercially available AdnaTest *BreastCancer*™ defines a sample as CTC+ if at least 1 of the 3 markers *EPCAM*, *ERBB2*, or *MUC1* is expressed *(16)*. *KRT19* was further shown to be a specific marker to reliably identify CTCs in patients with operable BC with no expression found in HD *(17)*. Within the present study, only relative *KRT19* expression was measured, implying the limitation of evaluating *KRT19* expression signals. We detected *KRT19* expression in 2 of 20 HD at high Cq values, reaching the detection limit, taking into account that we measured preamplified and 20-fold diluted samples. This might be due to assay limitations. The detection of pseudogenes is unlikely due to the primer construction used but cannot be fully excluded. Moreover, the AdnaTest implies a PCR based on oligo(dt) captured mRNA, minimizing the risk of signals due to genomic DNA. However, owing to limitations of the AdnaTest, we did not enrich a 100% pure CTC fraction. Thus, signals in HD might have resulted from leukocyte contamination as has been shown previously *(18)*. Comparing patients and HD samples, 2 independent clusters were displayed by cluster analysis independently of *KRT19*.

We combined these widely used definitions of a CTC to a 4-gene signature in the above presented analysis. According to our definition, a sample was CTC+ if at least 1 of the 4 marker genes (*EPCAM*, *ERBB2*, *MUC1*, or *KRT19*) was expressed. Interestingly, most CTC+ samples expressed at least 2 of these marker genes. Only *KRT19* was uniquely expressed in some cases, suggesting its strong role as a CTC determinant and supporting the studies of Strati et al. comparing 3 molecular detection methods *(19)*. In their comparative study, 41% of samples were positive according to *KRT19* and 55% of samples positive according to the AdnaTest *BreastCancer*. Within our CTC definition, we found 76% of MBC samples tested to be CTC+ at the start of investigation (confirms 57% of patients' positivity). This is a higher percentage compared to previously published studies showing positivity rates of 47% for *KRT19*, 28% for *MUC1*, 28% for *EPCAM*, and 22% and 16% for *ERBB2 (20)*. Others found 22% of tested samples to be positive for *EPCAM* and 14% for *KRT19*, respectively *(21, 22)*. Various groups have already performed comparison studies of molecular methods with the FDA cleared CellSearch system in different tumor entities *(23–26)*. Whereas the AdnaTest *BreastCancer* was shown to have equivalent sensitivity *(23)*, *KRT19*/mammoglobin RT-PCR showed the highest CTC positivity rate when compared to CellSearch and the AdnaTest *BreastCancer (24)*. *EPCAM* independent enrichment methods revealed a higher percentage of CTC+ non-small cell lung cancer patients as compared to the CellSearch *(25)*. CTC detection rates in metastatic castration-resistant prostate cancer patients by use of the CellSearch system, the AdnaTest *ProstateCancer*™, and a non selection PCR-based method (DDPCR) resulted in comparable sensitivity and detection rates for the AdnaTest and the DDPCR, which were superior to CellSearch *(26)*.

In CTC+ samples, epithelial marker genes were always expressed in combination with stemness or EMT associated markers, supporting the strong role of EMT in CTC characterization. This is in alignment with Armstrong et al. who found that EPCAM, cytokeratins, and E-Cadherin were mostly coexpressed in CTCs of metastatic prostate and BC patients *(27)*. In some cases, we might have detected CTCs with a mixed phenotype because these cells were still in an ongoing transition from epithelial to mesenchymal or vice versa. These findings support our and other previously published MBC studies showing EMT or stem cell marker expression related with a worse outcome *(28)* and coexpression of epithelial and mesenchymal transcripts *(29)*. Moreover, CTCs showing stem cell-like properties are believed to be the only subpopulation contributing to a relapse *(30)*.

In total, 14 genes including *KRT19*, *FLT1*, *EGFR*, *EPCAM*, *MET*, *PGR*, *CD24*, *KIT*, *PLAU*, *ALDH1A1*, *CTSD*, *MKI67*, *TWIST1*, and *ERBB2* were evaluated as significantly differentially expressed in the tested cohorts between CTC+ and CTC− samples. We observed differences in CTC gene expression only when stratifying the patients into CTC+/− in addition to their response behavior. Only *ADAM17* and *ABCC1* were significantly differentially expressed in NR or ONR without stratifying them into CTC+/−. ADAM17 is a metalloproteinase, involved in the release of EGFR ligands, acting upstream of the HER proteins and was shown to contribute to the evolvement and progression of cancer *(31)*. These data are nicely in accordance with our findings because *ADAM17* expression was only detected in NR. *ABCC1* was significantly differently expressed between OR and ONR regardless of their CTC status, a finding that already has been published by Gradilone et al. who showed that 86% of CTCs in MBC expressed at least 1 multidrug resistance related protein *(32)*. When further classifying the patients into total CTC+ and total CTC−, 8 genes including *EGFR*, *MET*, *FLT1*, *PGR*, *PLAU*, *KIT*, *MKI67*, and *TWIST1* were significantly differentially expressed between those groups. Dynamic changes in CTC gene expression patterns and their great diversity, even in the same patient, have already been described giving an insight into the complex link of disease progression and therapeutic influence *(33)*.

Presently, RT-PCR panels might be superior to other methods for the characterization of CTCs with regard to accuracy, comprehensiveness, and speed because multiple markers can be detected in a single run *(34)*. Supporting our findings, Andergassen et al. have

already analyzed the expression of 3 keratins on CTCs and found them to be a suitable marker for identifying CTCs *(34)*. The follow-up assessment of marker genes expressed in CTCs might give valuable information about therapy effectiveness. In this regard, we recently demonstrated that distinct CTC gene expression patterns were associated with not responding to therapy *(8)*. Reijm et al. identified an 8-gene signature predicting response to aromatase inhibitors *(35)*. Response to chemotherapy was retrospectively analyzed on the basis of the expression of *EPCAM*, *MUC1*, and *ERBB2* by Usiakova et al. *(36)*. Investigating 25 genes in CTCs, the expression of trefoil factor-1 was identified to be associated with the presence of bone metastasis and response to hormonal therapy *(37)*. Analyzing 197 MBC patients with a 16-gene panel, Mostert et al. were able to distinguish patients with a worse outcome from patients with a more favorable one. In addition, this gene signature complemented CTC counts in predicting patients' outcomes *(38)*. However, we have not correlated CTC gene expression patterns to sensitivity, resistance against specific treatments, or to the site of distant metastasis; determining these correlations represents the next step of our evaluations.

Conclusion

CTC gene expression profiling in the follow-up of MBC during palliative treatment seems to be a promising tool for predicting response or resistance to therapy assuming our findings can be confirmed in an independent validation set.

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