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

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BACKGROUND: Circulating tumor cells (CTC) 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 CTC 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 (TPo) 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, FLTL1, 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 SGCGB2A2) 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 CTC.

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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 CTC 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).

In this study, we used a 46 multimarker gene panel to (a) characterize CTC 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 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 CTC, and generation of cDNA 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 CTC 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 AdnaCollectTM tubes (QIAGEN) for “overnight” samples. Spiking experiments using the AdnaTest ColonCancerTM (QIAGEN) to compare EDTA vs AdnaCollectTM showed that the specificity and recovery rates were very similar (see Table 2 in the online Data Supplement). The stabilization effect of the AdnaCollectTM tubes over 24-h transportation time could be demonstrated by use of T84 cells and the AdnaTest ColonCancerTM 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 CTC
CTC 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/StemCellDetectTM (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 workflow based on preamplification and high-throughput profiling (TATAA Biocenter). Each sample was profiled in duplicates for expression of TUBB2A, TBP, GUSB, GAPDH, EMP2, VIM, VEGFA, PLAU, TWIST1, TP53, TOP2A, SCGB2A2, SATB1, RAD51, PTPRC, PTEN, PIK3CA, PGR, PARP1, MYC, MUC1, MTOR, ABCC1, GZMM, KRT19, KRAS, KIT, MKI67, IGF1R, IBSP, FLT1, ESRI, 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.

**PREAMPLIFICATION**
cDNA was preamplified by use of the TATAA PreAmp GrandMaster® Mix and a mixture of 47 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**
Preamplified products were diluted 10× in nuclease-free water (Life Technologies). Two different mixtures were prepared: (a) a sample mixture per sample and (b) an assay mixture per assay. The sample mixtures were prepared by mixing the diluted, preamplified products including the gDNA sample plus a nonamplified and undiluted template for TATAA Interplate Calibrator with TATAA Probe Low-ROX GrandMaster Mix (both TATAA Biocenter) and 20× GE Sample Loading Reagent (Fluidigm Corporation). The assay mixtures were prepared using each primer pair at 4 μmol/L final con-
centration giving 47 assay mixtures (1 for each target gene) and 1 for TATAA Interplate Calibrator, dual-labeled probe at 1 μmol/L final concentration (FAM-MGB) and 2× Assay Loading Reagent (Fluidigm). 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 nonparametric 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, KRT19, or ERBB2. 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.

| 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       |

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 CTC (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 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, CTC 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, GZMM, 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 CTC**

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, GZMM, 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 NR**

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 = 2.93−06) (see Table 13 in the online Data Supplement). The differential expression of CDH1, EPCAM, ERBB2, ESRI, 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, 2 separate gene expression clusters were observed for ONR, whereas OR revealed a homogeneous expression pattern (raw expression data, not normalized). The genes ESRI, 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 CTC holds great promise in monitoring therapy and guiding targeted treatment applications effectively (13). Clinical trials like the CirCe01 trial (NCT01349842), the DETECT V CHEVENDO trial (NCT02344472), or the STIC CTC METABREAST trial (NCT01710605) are investigating the power of CTC 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 CTC into the clinic is hindered by various existing definitions of CTC, reflected by the variety of methods available for their selection and detec-
Currently, the CellSearch System is the only FDA approved method for the identification of CTC, defining CTC as *EPCAM*+/*H11001*, *KRT8/18/19*+/*H11001*, *DAPI*+/*H11001*, and *PTPRC*+/*H11002* (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 CTC 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 preamplified DNA.  

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

**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.
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 nonselection 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 CTC of metastatic prostate and BC patients (27). In some cases, we might have detected CTC 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, CTC 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, GZMM, 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 ABCG1 were significantly differentially expressed in NR or ONR without stratifying them into CTC+/-, ADAM17 is a metalloprotease, 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 accord with our findings because ADAM17 expression was only detected in NR. ABCG1 was significantly differentially 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 CTC 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, GZMM, 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 CTC 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 CTC and found them to be a suitable marker for identifying CTC (34). The follow-up assessment of marker genes expressed in CTC 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 CTC, 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). Previously, 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 diagnostic tool for predicting response or resistance to therapy assuming our findings can be confirmed in an independent validation set.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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