High miR-26a and low CDC2 levels associate with decreased EZH2 expression and with favorable outcome on tamoxifen in metastatic breast cancer

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Abstract For patients with metastatic breast cancer, we previously described that increased EZH2 expression levels were associated with an adverse outcome to tamoxifen therapy. Main objective of the present study is to investigate miR-26a and miR-101 levels, which both target EZH2, for their association with molecular pathways and with efficacy of tamoxifen as first-line monotherapy for metastatic breast cancer. Expression levels were measured using quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) in primary breast cancer specimens of 235 estrogen receptor-α (ER)-positive patients. Pathway analysis was performed on microarray data available for 65 of these tumors. Logistic regression and Cox uni- and multivariate analysis were performed to relate expression levels with clinical benefit and time to progression (TTP). Increasing levels of miR-26a were significantly \((P < 0.005)\) associated with both clinical benefit and prolonged TTP, whereas miR-101 was not. Cell cycle regulation and CCNE1 and CDC2 were the only significant overlapping pathway linked to miR-26a and EZH2, respectively. In addition, increasing mRNA levels of CCNE1 \((P < 0.05)\) and CDC2 \((P < 0.001)\) were related to poor outcome. Multivariate analysis revealed miR-26a and CDC2 as an optimal set of markers associated with outcome on tamoxifen therapy, independently of traditional predictive factors. To summarize, only miR-26a levels are related with treatment outcome. Cell cycle regulation is the only overlapping pathway linked to miR-26a and EZH2 levels. Low mRNA levels of EZH2, CCNE1, and CDC2, and high levels of miR-26a are associated with favorable outcome on tamoxifen.

Keywords CDC2 · EZH2 · miR-26a · miR-101 · Metastatic breast cancer · Tamoxifen

Introduction

The anti-estrogen tamoxifen has been used for more than three decades for the treatment of estrogen receptor-α (ER)-positive breast cancer in both adjuvant and metastatic settings. The majority of breast tumors express ER, however, half of the patients with metastatic disease initially fail to respond to endocrine therapy, while the remaining patients will develop resistance during therapy. More insight into factors underlying tamoxifen resistance as well biomarkers to identify patients likely to benefit from tamoxifen is therefore needed.

We identified and validated an 81-gene signature that predicts tamoxifen resistance in patients with metastatic breast cancer [1, 2]. This signature included a member of the Enhancer of Zeste Homolog (EZH) family, which consists of EZH1 (OMIM 601674) and EZH2 (OMIM 601573). EZH2 is one of the polycomb proteins, a highly conserved group of chromatin modifiers known for their...
role in epigenetic memory and preservation of cellular characteristics [3]. Our in vitro studies showed that knockdown of EZH2 upregulates ER as a consequence of which sensitivity to anti-estrogen therapy increases [4]. In line with this, we have validated the predictive value of EZH2 and showed that low EZH2 levels were associated with favorable outcome on tamoxifen treatment in breast cancer patients with metastatic disease [4].

MicroRNAs (miRs) consist of a family of endogenously expressed small noncoding RNAs that target coding mRNAs to repress translation or induce degradation of their target mRNAs [5]. There is accumulating evidence that misregulation of miRs plays an important role in cancer. In breast cancer, miRs have been related with metastatic behavior, clinical outcome and ER status [6, 7]. Expression of several miRs in ER-positive breast cancer have also been associated with response to tamoxifen in cell lines (miR-221 and -222) [8], and in patients with metastatic disease treated with first-line tamoxifen (miR-30a, -30c, and -182) [9].

With respect to EZH2, miR target prediction tools have indicated that several miRs can target EZH2, but only two miRs, i.e., miR-26a and miR-101, have actually been shown to regulate EZH2 expression in different tissues [10, 11]. In the present study, we examined whether miR-26a and miR-101 were associated with EZH2 mRNA levels in breast cancer and with outcome on first-line tamoxifen therapy. In addition, using available whole genome mRNA data from a subset of tumors, the global testing approach (GTA) was performed to identify molecular pathways correlated with expression levels of miR-26a, miR-101, and EZH2 and to reveal genes, within these pathways, that associate with outcome on tamoxifen.

Patients and methods

Patients

Frozen breast tumor tissue specimens from female patients with primary operable breast cancer, who entered the clinic between 1981 and 1996 were analyzed. Follow-up, tumor staging, and response to therapy were performed as defined by standard International Union Against Cancer (Geneva, Switzerland) classification criteria [12]. This retrospective study was performed in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands (http://www.fmwv.nl), and reported following the REMARK recommendations [13], wherever possible. The study has been approved by the medical ethics committee of the Erasmus MC Rotterdam, The Netherlands (MEC 02.953).

Tumor protein expression levels of ER and progesterone receptor (PgR) were determined and used to classify tumors as ER- and/or PgR-positive as described previously [4, 14]. The following criteria were applied to include breast tumor specimens for final analysis in this study: (1) sufficient frozen tumor material, (2) more than 30% epithelial tumor cell nuclei in haematoxylin/eosin-stained sections, and (3) specimen of good RNA quality according to predefined criteria [15]. After applying these criteria, 235 patients with ER-positive tumors, who had metastatic disease treated with tamoxifen as first-line therapy, were included in this study. From these 235 patients, 89 patients (38%) underwent breast-conserving lumpectomy and 146 patients modified mastectomy (62%). The median follow-up time of patients alive was 89 months, range 10–165 months. Hundred and sixty five patients (70%) did not receive prior adjuvant systemic therapy, while 42 patients (18%) were previously treated with adjuvant chemotherapy [25 patients (11%) with non-anthracycline-based (CMF) and 17 patients (7%) with anthracycline-based (FAC/FEC) regimens].

Twenty eight patients (12%) presented with distant metastases at initial diagnosis (M1 patients). Clinical benefit on first-line tamoxifen monotherapy, defined as a complete or partial response according to standard International Union Against Cancer (Geneva, Switzerland) classification criteria [12] or no change longer than 6 months after treatment initiation (stable disease), was observed in 148 patients (63%). Eleven patients (5%) showed a complete response, 33 (14%) a partial response, and 104 patients (44%) had stable disease. No clinical benefit occurred in 87 patients (37%). Time to progression (TTP) was defined as the time elapsed between initiation of tamoxifen therapy and first detection of disease progression.

Methods

Details of applied methodologies are available at Supplementary Methods. In brief, tissue processing, RNA isolation, cDNA synthesis, quantitative Real-Time Polymerase Chain Reaction (qRT-PCR), and expression data generation were performed as described previously [15]. For pathway analysis, samples with whole genome mRNA expression profiles available, measured on Affymetrix HG-U133A and Plus2 chips, were selected (N = 65, 28%) and only reliable, i.e., quality checked, probes (N = 10,520) were evaluated. Samples were grouped according to median expression levels of miR-26a, miR-101 or EZH2. The Global Test Approach (GTA) was used to identify KEGG/BioCarta biological pathways in genes co-expressed with the biomarker of interest [41]. Pathways were taken into account when P-values, after correction for multiple testing.
and resampling, were below 0.05 and genes with z-scores >1.96 were considered significant contributors to the pathways. The GTA package version 4.14.0 was run in the R version 2.9.0. Data analysis and statistics were performed as previously described [4]. Expression levels of miR-26a, miR-101, and EZH2, CCNE1, CDC2, ER, and PgR mRNA levels were transformed to reduce distribution skewness. Logistic regression analysis was used to compute the odds ratio (OR) for clinical benefit and the Cox proportional hazards model to calculate the hazard ratio (HR) for TTP. Computations were done with the STATA statistical package, release 11.1 (STATA Corp., College Station, TX). All P-values were two-sided, and P < 0.05 was considered as statistically significant.

Results

Associations with clinicopathological factors

In this study, we determined the miR-26a, miR-101, and EZH2 mRNA expression levels in 235 primary breast carcinomas. The median and interquartile ranges of expression levels for miR-26a were 0.99 and 0.41, for miR-101 were 1.03 and 0.81 and for EZH2 were 0.10 and 0.07. The miR-26a and miR-101 levels correlated with each other (rs = 0.43, P < 0.001) and showed an inverse relation with EZH2 mRNA levels (rs = −0.21 and rs = −0.15, respectively, P < 0.05). Expression levels of both miRs were not significantly related with age, tumor grade, tumor size, or nodal status (Table 1). Only miR-101 levels were associated with postmenopausal status (P = 0.036). The ER and PgR mRNA levels showed a significant positive correlation with those of miR-26a (rs = 0.21 and rs = 0.34, for both P < 0.002) and miR-101 (rs = 0.13, P = 0.04 and rs = 0.27, P < 0.001).

Associations with clinical benefit and time to progression

Expression levels of miR-26a, miR-101 and EZH2 mRNA levels were evaluated in uni- and multivariate analysis for their associations with clinical benefit (Supplemental Table 2) and TTP (Table 2) in patients with metastatic breast cancer treated with tamoxifen as first-line monotherapy. The miR-101 levels were not related with clinical benefit (OR = 0.84, P = 0.40) nor with TTP (Table 2). As continuous variable, increasing levels of miR-26a were significantly associated with clinical benefit (OR = 32.1, P < 0.001) and with favorable TTP (HR = 0.13, P < 0.001; Table 2). Increasing mRNA levels of EZH2 were related to lower chance of clinical benefit (OR = 0.61, P = 0.02) and shorter TTP (HR = 1.26, P = 0.02). Analysis of miR-26a and EZH2 categorized in thirds (i.e. three quantiles) showed that the third with highest levels of miR-26a was related to clinical benefit (OR = 4.10, P < 0.001) and with prolonged TTP (HR = 0.43, P < 0.001), whereas the third with the highest EZH2 levels correlated with treatment failure (OR = 0.34, P = 0.002) and shorter TTP (HR = 1.91, P < 0.001). Kaplan–Meier curves as function of categorized expression levels of miR-26a and EZH2 visualize their association with TTP (Fig. 1). The median differences in TTP were 6.5 months between patients with high and low expression levels for miR-26a and 5.6 months for those with high and low EZH2 expression levels. In multivariate analysis, when added separately to the base model of predictive factors, miR-26a and EZH2 were significantly associated with clinical benefit and TTP, both as continuous and as categorized variables. Patients with high miR-26a levels showed clinical benefit (OR = 3.31, P = 0.005) and the longest TTP (HR = 0.52, P < 0.001), whereas those with high EZH2 levels had less benefit (OR = 0.39, P = 0.02) and shorter TTP (HR = 1.80; P = 0.001). The results of the multivariate analysis show the independence of miR-26a and EZH2 from traditional predictive factors included in the base model.

Pathway analysis for miR-26a and EZH2

In an exploratory pathway analysis with GTA, we evaluated 109 KEGG/BioCarta biological pathways and 10,520 mRNAs for differentially expressed pathways and genes. GTA identified only two pathways which significantly correlated with miR-26a, and 10 pathways with EZH2 mRNA expression (Table 3). The cyclins and cell cycle regulation pathway, and genes CCNE1 and CDC2 were the only overlapping pathway and genes between miR-26a and EZH2 that contributed significantly (Fig. 2). Increased expressions of CCNE1 and CDC2 were observed in samples with low miR-26a levels and in samples with high EZH2 levels.

To confirm this exploratory analysis, the predictive value of CCNE1 and CDC2 was evaluated by qRT-PCR. The median and interquartile mRNA levels were 0.03 and 0.03 for CCNE1 (N = 226), and 9.94 and 7.11 for CDC2 (N = 230), respectively. The mRNA levels of CCNE1 and CDC2 correlated with each other (rs = 0.44, P < 0.001) and showed a positive association with EZH2 mRNA levels (rs = 0.45 and rs = 0.57, for both P < 0.001) and an inverse relation with miR-26a (rs = −0.44 and rs = −0.30, respectively, for both P ≤ 0.001). The ER and PgR mRNA expression levels showed an inverse correlation with those of CCNE1 (rs = −0.14, P = 0.03 and rs = −0.24, P < 0.001) and CDC2 (rs = −0.07, P = 0.32 and rs = −0.27, P < 0.001). Expression levels of CDC2 and
Table 1  Associations of the expression levels for miR-26a, miR-101, CDC2, and CCNE1 with patient and tumor characteristics

| Clinicopathological factors | N     | %     | miR-26a |   | miR-101 |   | CCNE1 |   | CDC2 |   |
|-----------------------------|-------|-------|---------|---|---------|---|-------|---|------|---|
|                             | Median | iqr   | P*      |   | Median  | iqr | P*    |   | Median | iqr | P*   |
| Total                       | 235    | 65    | 0.99    | 0.41 | 1.03    | 0.81 | 226   | 100 | 0.03  | 0.03 | 230  | 100 |
| Age in categories, year     |        |       |         |     |         |     |       |     |       |     |      |     |
| <40                         | 12     | 2     | 0.21b   | 0.49b | 1.19    | 0.90 | 12    | 5   | 0.60b | 0.40b |
| 41–55                       | 75     | 22    |         |     |         |     |       |     |      |     |      |     |
| 56–70                       | 89     | 26    |         |     |         |     |       |     |      |     |      |     |
| >70                         | 59     | 15    |         |     |         |     |       |     |      |     |      |     |
| Menopausal status           |        |       |         |     |         |     |       |     |      |     |      |     |
| Pre menopausal              | 56     | 17    | 0.07c   | 0.036c | 0.95    | 0.66 | 55    | 24  | 0.76  | 0.39c |
| Postmenopausal              | 179    | 48    |         |     |         |     |       |     |      |     |      |     |
| Tumor size                  |        |       |         |     |         |     |       |     |      |     |      |     |
| pT1, <2 cm                  | 63     | 32    | 0.74d   | 0.12d | 0.90    | 0.54 | 60    | 27  | 0.45d | 0.16d |
| pT2, >2–5 cm                | 140    | 60    |         |     |         |     |       |     |      |     |      |     |
| pT3, >5 cm + pT4            | 32     | 14    |         |     |         |     |       |     |      |     |      |     |
| Lymph nodes involved        |        |       |         |     |         |     |       |     |      |     |      |     |
| 0                           | 96     | 64    | 0.79d   | 0.61d | 0.99    | 0.57 | 92    | 41  | 0.61d | 0.76d |
| 1–3                         | 55     | 21    |         |     |         |     |       |     |      |     |      |     |
| >3                          | 69     | 30    |         |     |         |     |       |     |      |     |      |     |
| Grade                       |        |       |         |     |         |     |       |     |      |     |      |     |
| Poor                        | 134    | 33    | 0.15d   | 0.41d | 1.05    | 0.80 | 126   | 56  | 0.13d | 0.41d |
| Unknown                     | 72     | 24    |         |     |         |     |       |     |      |     |      |     |
| Good/moderate               | 29     | 12    |         |     |         |     |       |     |      |     |      |     |
| PgR status                  |        |       |         |     |         |     |       |     |      |     |      |     |
| PgR low                     | 44     | 16    | <0.001b | <0.001b | 0.76    | 0.63 | 43    | 19  | <0.001b | <0.001b |
| PgR high                    | 185    | 47    |         |     |         |     |       |     |      |     |      |     |
| HER2 status                 |        |       |         |     |         |     |       |     |      |     |      |     |
| HER2 low                    | 197    | 51    | 0.026c  | 0.061c | 1.03    | 0.83 | 189   | 84  | 0.003c | 0.003c |
| HER2 high                   | 34     | 10    |         |     |         |     |       |     |      |     |      |     |
CCNE1 were not related with age, menopausal status, tumor grade, tumor size, or nodal status (Table 1). In univariate analysis, increasing mRNA levels of CCNE1 were related to treatment failure (OR = 0.67, \( P = 0.005 \); Supplemental Table 2) and shorter TTP (HR = 1.27, \( P < 0.001 \); Table 2). In addition, increased expression of CDC2 was associated with poor clinical benefit (OR = 0.45, \( P < 0.001 \)) and TTP (HR = 1.53, \( P < 0.001 \)). In multivariate analysis, CCNE1 and CDC2, when added separately to the base model, were both independent from traditional predictive factors for their association with clinical benefit and TTP (Supplemental Table 2; Table 2). Categorized into thirds, Kaplan–Meier survival analysis showed that patients with higher mRNA levels of CCNE1 and CDC2 had a shorter TTP (Fig. 1). Compared to the group with low tumor levels of CCNE1, those with high levels of CCNE1 had an OR of 0.33 (\( P = 0.002 \)) and a HR of 1.87 (\( P < 0.001 \)), respectively. Patients with high tumor levels of CDC2 had an OR of 0.28 (\( P < 0.001 \)) and even a HR of 2.07 (\( P < 0.001 \)), respectively, compared with those with low tumor CDC2 levels. These results indicate that an activated cell cycle regulation pathway through increased expressions of CCNE1 and CDC2 is significantly associated with poor outcome on tamoxifen therapy. Moreover, two additional cyclins and cell cycle regulation pathway genes (E2F1 and CCNB1) were evaluated, next to CCNE2 (not in GTA because it failed quality control), to confirm the involvement of the cell cycle regulation pathway in the response to tamoxifen. All three genes showed a significant association with TTP in uni- and multivariate analyses as continuous variables, i.e., E2F1 had a HR of 1.38 (\( P = 0.013 \)), CCNE2 had a HR of 1.38 (\( P < 0.001 \)) and CCNB1 had a HR of 1.86 (\( P < 0.001 \)) (Supplemental Table 3).

### Table 1

| Clinicopathological factors | \( N \) | \% | miR-101 | \% | CCNE1 | \% | CDC2 | \% | Median iqr \( a \) | \( P \) | Median iqr \( a \) | \( P \) | Median iqr \( a \) | \( P \) |
|-----------------------------|-------|---|---------|---|---------|---|------|---|----------------|---|----------------|---|----------------|---|
| EGFR low                    | 118   | 27 | 50 [42] | 48 | 1.02    | 0.44 | 0.97 | 0.44 | 0.97 [75-78] | 0.03 | 0.97 [75-78] | 0.03 | 0.97 [75-78] | 0.03 |
| EGFR high                   | 117   | 38 | 50 [58] | 50 | 0.97    | 0.40 | 0.99 | 0.40 | 0.97 [75-78] | 0.03 | 0.97 [75-78] | 0.03 | 0.97 [75-78] | 0.03 |

The number between brackets in the columns presenting the number and percentage of patients indicate the patient frequency for the 65 samples evaluated in the GTA for pathways.

\( a \) Interquartile range (q75-q25)

\( b \) Spearman rank correlation

\( c \) Mann–Whitney \( U \) test

\( d \) Kruskal–Wallis test

\( e \) Low and high serum hormone receptor protein status as defined in the ‘Methods’ section

\( f \) Nodal status PR and HER2 status were not known or determined in 15, 6 and 4 samples, respectively

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Multivariate analysis of miR-26a, EZH2, CCNE1, and CDC2

To determine a set of predictive biomarkers, the expression of miR-26a levels and of EZH2, CCNE1, and CDC2 mRNA levels were added simultaneously in a multivariate analysis to evaluate their relationship with TTP. Both CCNE1 and EZH2 mRNA levels lost their predictive value when included with miR-26a and CDC2, defining miR-26a and CDC2 levels as the set of predictive biomarkers associated with TTP. The HRs in the simultaneous analysis of miR-26a and CDC2 as continuous variables were 0.22 (\( P < 0.001 \)) and 1.38 (\( P = 0.001 \)), respectively (Table 2). Their contribution to the multivariate base model was independent from traditional predictive factors included in the model (Table 2). Converting miR-26a and CDC2 levels into a score followed by categorization into thirds resulted in a HR of 1.90 for the group with intermediate scores and
Table 2 Cox uni- and multivariate analyses for TTP in patients with metastatic disease treated with tamoxifen

| Factor of base model            | N   | %   | Univariate analysis | Multivariate analysis* |
|---------------------------------|-----|-----|---------------------|------------------------|
|                                 |     |     | HR 95% CI           | P                  |
|                                 |     |     |                     |                       |
| **Age (year)**                  |     |     |                     |                       |
| ≤55                             | 87  | 37  | 1.00                | 1.00                  |
| 55–70                           | 89  | 38  | 0.82 0.60–1.11      | 0.19 0.45–1.11       | 0.13 |
| >70                             | 59  | 25  | 0.66 0.47–0.94      | 0.02 0.36–0.94       | 0.03 |
| **Menopausal status**           |     |     |                     |                       |
| Premenopausal                   | 56  | 24  | 1.00                |                       |
| Postmenopausal                  | 179 | 76  | 0.86 0.63–1.17      | 0.33                  |
| **Disease-free survival**       |     |     |                     |                       |
| ≤1 year                         | 62  | 26  | 1.00                | 1.00                  |
| 1–3 years                       | 109 | 46  | 0.66 0.48–0.91      | 0.01 0.46–0.88       | 0.006 |
| >3 years                        | 64  | 27  | 0.51 0.35–0.75      | <0.001 0.36–0.77     | 0.001 |
| **Dominant site of relapse**    |     |     |                     |                       |
| Soft tissue                     | 26  | 11  | 1.00                |                       |
| Bone                            | 127 | 54  | 1.29 0.83–2.02      | 0.26 0.79–2.07       | 0.31 |
| Viscera                         | 82  | 35  | 1.12 0.70–1.79      | 0.64 0.77–2.15       | 0.33 |
| ER mRNA                         | 235 | 100 | 0.89 0.83–0.94      | <0.001 0.84–0.96     | 0.002 |
| PgR mRNA                        | 235 | 100 | 0.90 0.84–0.96      | 0.002 0.85–0.98      | 0.02 |
| **Factors analyzed**            |     |     |                     |                       |
| miR-26a                         |     |     |                     |                       |
| Continuous variable             | 235 | 100 | 0.13 0.06–0.28      | <0.001 0.18 0.07–0.44| <0.001 |
| Low                             | 79  | 34  | 1.00                |                       |
| Intermediate                    | 78  | 33  | 0.93 0.68–1.29      | 0.68 0.83–1.66       | 0.35 |
| High                            | 78  | 33  | 0.43 0.31–0.61      | <0.001 0.52 0.36–0.76| <0.001 |
| miR-101                         |     |     |                     |                       |
| Continuous variable             | 235 | 100 | 0.87 0.70–1.07      | 0.19 0.71–1.13       | 0.37 |
| EZH2 mRNA                       |     |     |                     |                       |
| Continuous variable             | 235 | 100 | 1.26 1.06–1.51      | 0.01 1.28 1.05–1.56  | 0.02 |
| Low                             | 79  | 34  | 1.00                |                       |
| Intermediate                    | 78  | 33  | 1.58 1.14–2.19      | 0.006 1.73 1.23–2.44 | 0.002 |
| High                            | 78  | 33  | 1.91 1.37–2.68      | <0.001 1.80 1.26–2.55| 0.001 |
| CCNE1 mRNA                      |     |     |                     |                       |
| Continuous variable             | 226 | 96  | 1.27 1.12–1.45      | <0.001 1.24 1.06–1.44| 0.007 |
| Low                             | 76  | 34  | 1.00                |                       |
| Intermediate                    | 75  | 33  | 1.19 0.85–1.66      | 0.31 1.24 0.88–1.76  | 0.22 |
| High                            | 75  | 33  | 1.87 1.33–2.62      | <0.001 1.62 1.11–2.35| 0.01 |
| CDC2 mRNA                       |     |     |                     |                       |
| Continuous variable             | 230 | 98  | 1.53 1.29–1.81      | <0.001 1.54 1.27–1.87| <0.001 |
| Low                             | 77  | 33  | 1.00                |                       |
| Intermediate                    | 77  | 33  | 1.53 1.09–2.13      | 0.01 1.52 1.07–2.15  | 0.02 |
| High                            | 76  | 33  | 2.07 1.47–2.90      | <0.001 2.05 1.42–2.98| <0.001 |
| miR-26a & CDC2                  |     |     |                     |                       |
| miR-26a                         | 230 | 98  | 0.22 0.09–0.52      | <0.001 0.27 0.11–0.65| 0.004 |
| CDC2                            | 230 | 98  | 1.38 1.15–1.65      | 0.001 1.47 1.20–1.79  | <0.001 |

The expression levels of miR-26a, miR-101 and EZH2, CCNE1, and CDC2 were evaluated both as continuous, and when significant, as categorized variables in estrogen receptor-positive tumors from 235 patients recurrence of which was treated with first-line tamoxifen monotherapy. Factors were added separately to the base model in the multivariate analysis, which was stratified for menopausal status as described in our previous study [4].

* The multivariate analysis is stratified for menopausal status.

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Discussion

This study shows that miR-26a levels associate with outcome of metastatic disease on first-line tamoxifen monotherapy, whereas miR-101 does not. Patients with clinical benefit have high miR-26a and low EZH2 mRNA levels. Additionally, only the cell cycle regulation pathway with its genes CCNE1 and CDC2 overlap between miR-26a and EZH2 linked molecular pathways. These two genes also correlate with treatment outcome. The miR-26a and CDC2 levels that regulate EZH2 levels and activity were identified as a set of predictive biomarkers for treatment outcome.

Overexpression of EZH2 was observed in prostate and breast cancer in which it was associated with aggressive clinical behavior [16, 17]. We demonstrated that decreased EZH2 mRNA levels were predictive for favorable outcome on tamoxifen in metastatic breast cancer [4]. Both miR-26a and miR-101 repress EZH2 expression [10, 11, 18]. Although miR-26a and miR-101 expressions correlate with EZH2 levels in our current study, only miR-26a had a significant association with treatment outcome. These miRs target many other genes. Of the genes predicted to be targets of miR-26a
Table 3  miR-26a and EZH2 related pathways and genes

Global testing approach—KEGG/BioCarta pathway analysis

| Pathway                                           | Genes tested | P-value | Genes significant (z-score >1.96) |
|--------------------------------------------------|--------------|---------|---------------------------------|
| **miR-26a associated pathways**                  |              |         |                                 |
| Cyclins and cell cycle regulation                | 18           | 0.008   | CCNE1, CDK7, CDKN2D, CDC2        |
| TPO signaling pathway                            | 18           | 0.018   | HRAS, THPO, RASA1                |
| **EZH2 associated pathways**                     |              |         |                                 |
| Cell cycle G1 S check point                      | 21           | 0.002   | TGFB1, E2F1, ATM, SMAD4, CDC2, CCNE1, SKP2, ATR, ABL1 |
| Role of BRCA1 BRCA2 and ATR in cancer susceptibility | 20          | 0.003   | FANCG, RAD51, ATM, FANCA, CHEK1, ATR, RAD9A, NBN, FANCC, BRCA1 |
| **Cyclins and cell cycle regulation**            | 18           | 0.005   | CCNB1, E2F1, CDC2, CCNE1, CCND2  |
| ATM signaling pathway                            | 16           | 0.011   | RAD51, ATM, NFKB1, CHEK1, GADD45A, ABL1, NBN, BRCA1 |
| Spliceosomal assembly                            | 15           | 0.018   | SNRPD1, SNRPG, SRP40, U2AF1, SFRS2, U2AF2, SRP72, SRP54, SRP50A |
| Cytokines and inflammatory response             | 15           | 0.019   | TGFB1, HLA-DRA, IL5, CD4, CSF1, IL10 |
| Cell cycle G2 M checkpoint                       | 21           | 0.025   | CCNB1, ATM, CDC2, PLK1, CHEK1, ATR, WEE1, GADD45A, BRCA1 |
| ADPRibosylation factor                           | 15           | 0.029   | KDEL1, ARGFAP1, DDEF2, PSCD4, COPA, CENPD1 |
| Hypoxia and p53 in the cardiovascular system    | 16           | 0.038   | ATM, FHL2, CSNK1A1, GADD45A      |
| p38 MAPK signaling pathway                       | 32           | 0.044   | TGFB1, CREB1, DAXX, CDC42, DDIT3, MAPKAPK5, HMGN1, HRAS, PLA2G4A |

In 65 breast cancer samples, for which whole-genome mRNA expression profiles were available, pathways and genes were identified with the GTA of 109 KEGG/BioCarta biological pathways and 10,520 mRNAs. Only those pathways and their genes are indicated, which show a significant relationship with miR-26a and EZH2 expression levels. The number of genes tested is indicated per pathway. The P-values determine the significance of the association after correction for multiple testing and resampling.
and miR-101 (1,198 targets), only a few (66 genes, including EZH2) are targeted by both miRs (data not shown). We cannot exclude another relevant gene for endocrine therapy outcome as specific miR-26a target which is not targeted by miR-101. This certainly needs further exploration but is not within the scope of the current study.

Our pathway analyses identified only the cell cycle regulation pathway to be correlated with miR-26a and EZH2 levels. The genes CDK7, CCNE1, CDC2, and CDKN2D for miR-26a and CCNB1, CCNE1, CDC2, CCND2, and E2F1 for EZH2 were differentially expressed within this pathway. CCNE2 and CDK2, important genes in this pathway, were not included in the analyses because their probes failed quality control. The association of EZH2 with cell cycle regulation is extensively reported [21, 22]. Moreover, the Targetscan algorithm predicted cyclins D2, E1, and E2 (CCND2, CCNE1, and CCNE2), and cyclin dependent kinase 6 (CDK6), which all play a role in the G1–S transition, as miR-26a targets [23]. Finally, estrogens that regulate G1 cyclin-dependent kinases [24] and tamoxifen has a cytostatic effect on breast cancer cells and arrest them in G0/G1 phase [25].

Based on our study, CCNE1 and CDC2 were the only overlapping genes for miR-26a and EZH2. We have shown earlier Cyclin E as prognostic marker for lymph node-negative breast cancer [26]. Now, we show that in the metastatic disease setting, high CCNE1 mRNA levels correlate with poor outcome on tamoxifen. In concordance, patients with high CCNE protein levels had less benefit from tamoxifen in an adjuvant setting [27], and the overexpression of low molecular weight CCNE isoforms was associated with resistance to fulvestrant [28] and letrozole [29]. CCNE1 is a kinase and regulatory subunit of CDK2 that accumulates at the G1–S phase [30].

Multivariate analysis of miR-26a, EZH2, CCNE1, and CDC2 to determine their associations with treatment outcome showed that the predictive values of EZH2 and CCNE1 levels were less significant than those of miR-26a and CDC2. Interestingly, not only miR-26a but also CDC2...
have a physical interaction with EZH2 (Fig. 3), although with opposite effects on EZH2 functioning. As mentioned, miR-26a binds to the 3'-UTR of EZH2 and inhibits transcription of EZH2. On the other hand, CDC2 (CDK1) and CDK2 have been shown to activate EZH2 by phosphorylation of its Thr350 residue [34–36]. This Thr350 phosphorylation is necessary for EZH2 recruitment at target loci and for maintenance of H2K27me3 levels [34]. Since EZH2 expression and activity are higher in proliferating rather than differentiating cells [22], both miR-26a and CDC2 may define endocrine-responsive or -resistant phenotypes of ER-positive breast cancer cells through their modulation of EZH2 levels and activity. In ER-negative breast cancer cells, EZH2 knockdown results in increased CDC2 and pCDC2 protein expressions [37], but recently it was suggested that EZH2 in ER-negative tumors functions as a transcriptional activator but acts as a repressor in ER-positive tumors [38].

Therapeutics that can modulate miR-26a, CDC2, or EZH2 activity might be an attractive strategy for patients resistant to tamoxifen to reestablish targets for anti-estrogen treatment. Systemic administration of miR-26a with adenovirus may regulate tumor growth in breast cancer xenografts [23]. Preclinical evaluation of CDC2 and CDK2 inhibitors revealed G2/M arrest and cell death in both anti-estrogen-sensitive and resistant cells [33]. Hydrolase inhibitors, such as D2Nep, induce EZH2 depletion in breast cancer cell lines and result in cell cycle arrest and apoptosis [39, 40]. At the end, all these treatments target EZH2 levels and activity. We hypothesize that patients resistant to tamoxifen with low miR-26a and high CDC2 and EZH2 levels in their primary tumor may benefit from these treatment strategies in order to overcome tamoxifen resistance.

In summary, we have shown that high miR-26a and low EZH2 mRNA levels associate with clinical benefit and prolonged TTP. The cell cycle regulation pathway and its genes CCNE1 and CDC2 correlate significantly with miR-26a and EZH2 levels and with outcome on tamoxifen. Multivariate analysis revealed miR-26a and CDC2 as sets of biomarkers to predict outcome on tamoxifen in metastatic breast cancer. Our findings might help one to improve the identification of individual patients resistant to tamoxifen, who may benefit from therapeutics that block EZH2 expression and activity.

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Conflict of interest None of the authors has a conflict of interest.

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