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Original article

MiRNA-27a sensitizes breast cancer cells to treatment with Selective Estrogen Receptor Modulators

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ABSTRACT

Background: MicroRNA-27a (miR-27a) is a small non-coding RNA, shown to play a role in multiple cancers, including the regulation of ERα expression in breast cancer. Most ERα positive tumors are treated with Selective Estrogen Receptor Modulators (SERMs) and thus the role of miR-27a expression in response to SERM treatment is of interest.
Methods: Tamoxifen resistant cells were generated by molecular evolution with six cycles of tamoxifen treatment. MCF7 and T47D luminal A breast cancer cell lines were either treated with miR-27a mimics, or ER-signaling was modulated ectopically. The changes were analyzed with RT-qPCR, western blotting and transcriptional activity ERE-reporter assays. Moreover, the response to SERM treatments (tamoxifen, endoxifen and toremifen) was investigated by cell viability and apoptosis measurements. An *in silico* analysis of survival data from the METABRIC study was performed in order to assess the prognostic value of miR-27a for response to SERM treatment.
Results: Tamoxifen-resistant cells showed decreased expression of ERα and miR-27a. The overexpression of miR-27a increased the levels of ERα, while modulation of ERα decreased miR-27a expression. High miR-27a expression increased the sensitivity of MCF7 and T47D cells to SERM treatments and re-sensitized the cells to tamoxifen. Patient survival of luminal A breast cancer patients that underwent endocrine therapies was better in groups with high miR-27a expression.
Conclusion: MiR-27a sensitizes luminal A breast cancer cells to SERM treatments based on a positive feedback loop with ERα. An increased overall-survival of ER-positive breast cancer patients that underwent endocrine treatments and displayed high miR-27a levels was found.

Breast cancer is one of the leading causes of cancer deaths worldwide [1]. The group of breast cancers is highly heterogeneous in its prevalence, short-as well as long-term mortality. Therefore, the tumors are characterized and clustered according to different characteristics. The most common approach is based on determination of receptor expression, like the estrogen- and progesterone receptor (ER and PR) or HER2. While luminal A breast cancer, with positive expression of ER and PR, are the most prevalent tumors, they are also considered least harmful, as they tend to be less aggressive and in general well treatable with endocrine therapies, like estrogen receptor modulators (SERMs) [2,3].

Nevertheless, the ATLAS trial showed that therapies with anti-endocrine agents, e.g. the SERM tamoxifen (TAM), should be considered for a full treatment period of at least 5 years, ideally even 10 years and longer for best effects [4]. Previous reports show that as much as 40% of all ER-positive breast cancers treated with adjuvant tamoxifen undergo relapse, with eventually fatal outcomes [5]. It is unclear whether this poor response is based on initial (*ab initio*) resistance to the therapies or due to acquired (*de novo*) resistance.

Moreover, different side effects of SERMs, like an increased risk for cervical carcinoma or osteoporosis, becloud their positive effects. In order to attenuate long-term adverse effects of SERM treatments, the ESMO guidelines suggest a switch of treatment to a newer class of drugs, i.e. selective estrogen receptor down regulators (SERDs) like fulvestrant. SERDs are often considered in long-term second line treatments (after 5 years or more). Also transitions to aromatase-inhibitors like anastrozol are made [6]. While some of these approaches may circumvent adverse effects and resistance, the survival of luminal A breast cancer patients is declining over time, indicating that the current treatment approaches for luminal A tumors cannot be considered optimal for all

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luminal A tumors, in regard to their long-term outcome [4].

Thus, there is a practical need for further diversification of breast cancer in general and especially for ER-positive tumors. Additional sub-classifications of breast cancer tumors were suggested, e.g. the screening for GATA3 mutations [7–9]. While multiple protein-markers are already considered during the treatment of triple negative breast cancer (TNBC) [10], the main reference point for endocrine treatments is ER expression [11]. Additional surrogate markers could be used to identify high risk populations, which would profit from switches from standard therapies to suitable chemotherapies already from the onset of the treatment, as is already part of the ESMO guidelines for patients with high tumor burden [3].

MicroRNAs (miRNA) are small non-coding RNAs which are important in transcriptional and translational regulation of cellular processes, making them promising prognostic markers. As the detection in tissue, as well as in blood samples, is getting easier, alterations in expression levels could be used to assess aggressiveness of tumors and in certain cases even predict treatment response [12]. A miRNA of special interest is miR-27a, which was shown to play a role in multiple metabolic processes and different cancer types. In general, miR-27a is considered tumor promoting, i.e. increasing cancer progression and resistance to chemotherapeutic agents, as observed in different cancer types including breast cancer [13–15]. Therefore, miR-27a is considered a potent oncomiR, whose high expression is unfavorable for patients' survival in many settings, like osteosarcoma and gastric cancer [16,17]. Previous studies suggest that miR-27a is regulating the ERα expression indirectly via ZBTB10 and the sp-protein family [18], hence the role of miR-27a in ER-positive breast cancers is of interest.

Our findings, while not objecting miR-27a's tumor promoting effects, suggests that high expression of miR-27a may serve as an indicator of functional ER-expression in luminal A breast cancer and therefore act as a positive marker for SERM response *in vitro*, which could result in a survival benefit as observed *in vivo*.

1. Materials and methods

1.1. Reagents

Puromycin dihydrochloride (cat. P8833), Tamoxifen (cat. T5648), Endoxifen (cat. E8284), Toremifen (cat. T7204) and Estradiol (E1024) were obtained from Sigma-Aldrich.

1.2. Cell culture

MCF7 were acquired from cell line service (Eppelheim, Germany), grown at 37 °C and 5% CO_2 in high glucose DMEM (Sigma) supplemented with 10% fetal calf serum (FCS/Gibco). TAM6 as resistant clone were generated from parental MCF7 by six rounds o treatment with tamoxifen as described before [19] and cultured like MCF7. T47D were acquired from ATCC, grown at 37 °C and 5% CO_2 in RPMI-media (Sigma). All cells were routinely tested and confirmed as mycoplasm free.

1.3. Overexpression of miR-27a

Overexpression experiments were performed by transfection of a miR-27a mimic (miRIDIAN Human hsa-miR-27a 3p, Dharmacon) and miRIDIAN Mimic Negative Control #1 (Dharmacon) with K2 transfection reagent (Biontex, Germany) according to the manufacturer's protocol. Cells were seeded in 6-well plates to 80% confluence depending on the experiment kept in 6-well or seeded 24 h after transfection for following experiments.

1.4. miRNA quantitative RT-PCR

Approximately 600,000 cells were harvested and total RNA isolated from cells using Total RNA Kit, peqGOLD (VWR). cDNA synthesis was carried out by a microRNA specific reverse transcription and detection with the qScript microRNA cDNA Synthesis Kit and PerfeCta SYBR Green SuperMix (Quanta Biosciences) with RT-PCR detection on a LightCycler 480 (Roche). The expression of miR-27a was normalized to miR-191, using the $2^{-\Delta CT}$ or $2^{-\Delta\Delta CT}$ method. The primers used for analysis were for miR-27a: GCCGTTCACAGTGGCTAAG and for miR-191: GCGCAACGGAATCCCAAAAG.

1.5. mRNA quantitative RT-PCR

RNA was extracted utilizing the Total RNA Kit, peqGOLD (VWR) as by manufacturer's instructions. Translation to cDNA was performed utilizing the qScript cDNA synthesis kit (Quanta Bioscience) as by manufacturer's protocol.

Analysis of expression was performed with the Lightcycler 480 (Roche) and the Universal Probe Library (Roche) with following probe and primer (forward/reverse) combinations:

ESR1 Fwd:ATCCACCTGATGGCCAAG Rev:GCTCCATGCCTTTGTTACTCA; Probe #17.

GAPDH Fwd: TCCACTGGCGTCTTCACC Rev:GGCAGAGATGATGACCCTTTT; Probe #45.

The expression of ESR1 was normalized to GAPDH, using the 2-ΔCT or 2-ΔΔCT method.

1.6. ER-signaling via ERE-luc reporter

3X ERE TATA luc was a gift from Donald McDonnell (Addgene plasmid # 11354). Transfection was performed in 6-well with cells grown to 80% confluence with K2 transfection reagent (Biontex, Germany) according to the manufacturer's instructions. After 24 h cells were seeded in 96-well plates and luc-measurements were performed as described previously [20].

1.7. Generation and stimulation of TRIPZ-shER MCF7

MCF7 cells were transducted with a 2nd generation lentiviral system generated with the plasmids pCMV-dR8.2 dvpr (Addgene plasmid # 8454) and pCMV-VSV-G (Addgene plasmid #8455), which were a gift from Bob Weinberg and a doxycycline-inducible TRIPZ-shER construct (gift from Yunus Luqmani, Kuwait). For control the TRIPZ-shCtrl construct (ThermoFisher) was used.

1.8. Western blot analysis and immunofluorescence

Cells were cultured in a 6 well plate for 48 h after transfection/stimulation, lysis, gel and blotting were performed as described previously [21], with the following primary antibodies: Estrogen Receptor-α (sc-543), Actin (sc-1616, Santa Cruz) and GAPDH (14C10, Cell Signaling). Immunofluorescence stainings were performed as described previously [22], -ES cells were cultured for the time of the experiment in phenol-red-free media with 10% charcoal stripped FCS (F6765, Sigma), +ES cells were stimulated with 3,6 μM estradiol for 1 h before fixation.

1.9. Analysis of transcription factors in promoter regions of found genes

For the analysis of the promoter region of the miR-27a locus, the sequence was retrieved from the RefSeq-Database (https://www.ncbi.nlm.nih.gov/refseq/as of January 2018) in order to identify the +1 position. Assuming the +1 position as starting site of

transcription, 500 nucleotides upstream were defined as the proximal promoter. The enhancer region was defined as the genomic sequence 30,000 base pairs upstream of the $+1$[23]. Then, for analysis of possible promoter sequences, ALGGEN [24] software was used, the analysis was performed with the highest stringency. Analysis of ERE-sites were performed by manual alignments of the consensus sequence and known variances that were previously discussed [25].

1.10. Treatment with SERMs and relative viability assays

Stock solutions of TAM, ENDO and TOR were prepared in DMSO with a concentration of 20 mM. Dilutions were prepared freshly in according media, controls contained appropriate amounts of DMSO. Treatments were performed 48 h after stimulation/transfection for 48 h. Relative viability as ATP-content was assessed by Celltiter-Glo (Promega) according to manufacturer's instructions.

1.11. Annexin V assay

The cells were cultured and treated as described above. Samples were harvested, and analyzed with the Annexin V-FiTC Apoptosis Detection Kit Plus (BioVision) according to the manufacturer's protocol. Measurement was performed using CyAn ADP Flowcytometer (Dako Cytomation/Beckmann) and FlowJo 7.6.5. (TreeStar).

1.12. In silico analysis of patient data

Patient survival data, treatment information and expression of ERα/miR-27a was acquired from the database of kmplot software (http://www.kmplot.com [26]). Analysis for miR-27a was based on data from the METABRIC study (syn1688369) [8].

1.13. Statistical analysis

Results are expressed as the mean ± SD of at least three replicas, if not stated otherwise. All experiments were conducted three times independently, one representative example is depicted. Software GraphPad Prism v6 and SigmaPlot 11 were utilized for the analysis of the data.

1.14. Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

2. Results

2.1. Induction of tamoxifen resistance leads to repression of ERα and miR-27a expression

In order to induce resistance to tamoxifen, MCF7 cells were treated with tamoxifen for six cycles each followed by recovery phases, as described previously [19].

The resulting MCF7 cells, labeled TAM6, showed an increasing IC50 of approximately 1.5-fold (Fig. 1a). As the loss of ERα expression is common in acquired resistance to tamoxifen, an analysis of ERα expression changes was performed. The resistant TAM6 cells showed significantly decreased levels of ESR1 mRNA (Fig. 1b) as well as decreased ERα protein expression (Fig. 1c). The transcriptional activity of ERα was assessed via an ERE (estrogen-receptor-response element) luciferase reporter assay which indicated a significant loss of relative signaling of about 40% in the TAM6 cells ($p \leq 0.05$) compared to wildtype MCF7 (Fig. 1d). Interestingly, the resistant TAM6 cells showed also a decreased expression of miR-27a (Fig. 1e).

2.2. The interplay of miR-27a and ERα in a positive feedback loop

To investigate whether miR-27a's is able to regulate the expression of ERα, basal miRNA expression was analyzed in two Luminal A breast cancer cell lines, T47D and MCF7. Both cell lines showed expression of miR-27a. In MCF7 the miR-27a levels were six times higher than in T47D cells (Fig. 2a). After an ectopic overexpression of miR-27a an increase of ERα mRNA (ESR1) in MCF7 and T47D of 20%–50%, respectively, was observed (Fig. 2b), and a 1.3 and 2-fold increase in protein levels (Fig. 2c). The ERE-reporter assay revealed a 40–50% increase in the luciferase signal, indicating increased transcriptional activity of ERα (Fig. 2d). These findings were supported by an immunofluorescence staining (Fig. 2e), which showed that upon stimulation with estradiol the ERα localization into the nucleus is stronger after miR-27a overexpression compared to controls.

Of note, also the reintroduction of miR-27a into the low-expressing tamoxifen resistant TAM6 cells reactivated ERα expression and signaling as shown by a significantly increased ERE-luc signal of more than 50% compared to controls ($p \leq 0.05$, Fig. 2f).

To investigate a possible regulation of miR-27a expression by ERα activity, MCF7 cells were depleted of estrogen stimulation in estradiol- and phenol red free media or stimulated with estradiol for 48 h. The miR-27a expression was analyzed and as shown in Fig. 3a, after the depletion of estrogen stimulation, the miR-27a expression was significantly decreased ($p \leq 0.001$) compared to control. Additionally, stimulation with estradiol showed a slight but not significant increase of miR-27a in MCF7 cells.

To further investigate the correlation, a stable MCF7 cell line with inducible expression of a short hairpin inhibitor of ERα mRNA, termed MCF7 shER, was used. Upon induction with doxycycline for 48 h, the cells showed decreasing ERα protein of one third compared to uninduced control (Fig. 3b). Long-term depletion of ERα by induction of the shER for 29 days compared to a scrambled hairpin control, showed a stable effect of 20% reduction of ESR1 mRNA (Fig. 3c). Importantly, the ERα knock-down resulted also in a highly significant decrease in miR-27a expression of 30% (Fig. 3d). These findings indicate a mutual influence of miR-27a and ERα expression in a positive feedback loop. Thus, a genomic analysis of the miR-27a locus was performed, investigating possible regulation mechanisms based on ERα transcriptional effects. Two different modes of transcriptional regulation were considered: Direct regulation of ERα via binding to known ERE structures located upstream of the miR-27a locus, or indirect influence by predicted binding of other transcription factors, which are known to be regulated by ERα. Fig. 3e shows the possible bindings and interactions in the promotor (−500/-1 bp) and the enhancer region (−30,000/-1 bp) of miR-27a: Two putative ERE sites were found, one in the proximal promotor with a match of 11/13 bases to the consensus sequence, and one in the distant enhancer with 12/13 matching bases. Additionally, a site for JUN in the promotor, as well as the co-transcription factors of ERα AP-2αA and C/EBPβ in the enhancer, were predicted with high stringency [23,27]. This analysis indicates a high probability of transcriptional regulation of miR-27a by ERα, consolidating the hypothesis of an important function of both miR-27a and ERα in the development of resistance to tamoxifen treatment.

2.3. Overexpression of miR-27a induces sensitivity towards SERM treatment in vitro

While formation of resistance to tamoxifen is correlated to loss of miR-27a, the reverse setting of miR-27a overexpression in

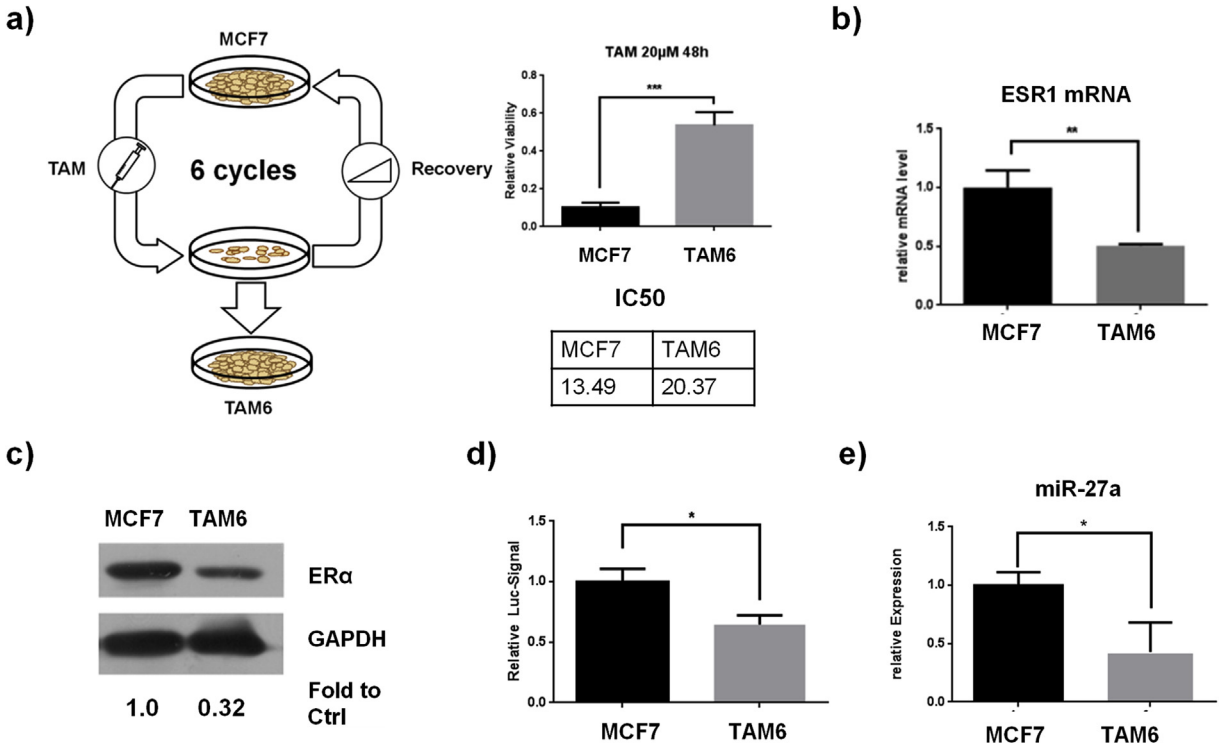

Fig. 1. Induction of tamoxifen resistance leads to repression of ERα and miR-27a expression a) A Molecular Evolution Assay of 6 cycles tamoxifen (TAM) treatment resulted in resistant MCF7 cells, the TAM6 cells, with increased resistance to 20 μM TAM ($p \leq 0.001$) as well as an increased IC50 value. b) ESR1 mRNA levels are significantly decreased by approximately 50% in the resistant cells, as shown by RT-qPCR ($p \leq 0.05$), c) as well as western-blot for protein levels, GAPDH was used as housekeeper. d) The relative luciferase signal of the ERE-reporter is significantly decreased in the resistant cells ($p \leq 0.05$). e) Expression of miR-27a is decreased to 50% in the resistant TAM6 cells compared to MCF7 wildtype ($p \leq 0.05$).

luminal A cell lines was of interest. In order to analyze the sensitivity towards the treatment, MCF7 and T47D were transfected with miR-27a mimics and subsequently treated with different SERMs: Tamoxifen, its active metabolite endoxifen as well as toremifene. As shown in Fig. 4a–c, miR-27a sensitized MCF7 cells to all tested SERM treatments. To investigate whether the changes in viability induced by tamoxifen, as determined by ATP-content, were not only based on changes in metabolic activity, an annexin V assay was performed. Here, the cell viability is regarded as the percentage of cells with negative annexin and propidium iodide staining. In line with the ATP-measurements, MCF7 with an overexpression of miR-27a showed increased sensitivity towards tamoxifen treatment (Fig. 4d).

Confirmation of these results with another luminal A cell line, T47D, showed similar results for tamoxifen, endoxifen and toremifen compared to controls (Fig. 4e–g). Also the rescue of miR-27a expression in the tamoxifen resistant and miR-27a-low TAM6 cells re-sensitized the cells towards tamoxifen treatment, as seen by a significant decrease in viability after the treatments, compared to control (Fig. 4h). Further validation with the annexin V assay showed that these effects are based on apoptosis and cell death, rather than diminished metabolism (Fig. 4i).

Together, these data showed a sensitizing effect of miR-27a to SERM treatments in both tested luminal A cell lines, as well as a re-sensitizing effect in tamoxifen resistant cells.

2.4. MiR-27a is a putative prognostic marker for endocrine therapies in metastatic ER + breast cancer

To validate the *in vitro* results, the impact of miR-27a expression on the survival of patients with ER-positive tumors which underwent endocrine treatment was evaluated. An analysis of patient data derived from the METABRIC cohort was performed utilizing the tool “miR power” (http://www.kmplot.com) by Lanczky et al. [26]. In this analysis, patients were grouped according to their ER expression, as determined by immunohistochemistry and their status of node invasion. Patient groups with ER-positive tumors were narrowed down to the cohort which exclusively underwent endocrine treatment, while no further limitations were set in groups with ER-negative tumors in regard to the therapy.

As shown in Fig. 5, patients with ER-positive tumors and high miR-27a expression had beneficial overall survival (OS) of about 20 months and a lower risk of events, compared to the low expressing group ($N = 726$, HR 0.87 (0.6–1.08), $p = 0.15$; not significant). The corresponding Kaplan-Meier curves show the biggest difference between the two groups during 100–150 months of follow up, corresponding to the usual follow-up care for breast cancer patients. In contrast, ER-negative breast cancer patients with high miR-27a expression were at approximately one third higher risk and had a 1.5 years lower median OS than the low expressing cohort ($N = 266$, HR 1.33 (0.84–2.09), $p = 0.22$). When further differentiating the ER-positive group to a subgroup which is determined luminal A, the relative risk additionally decreased to 0.61 (0.39–0.94) with $p = 0.025$ (see supplemental Fig. S3).

Next, patients with more aggressive luminal A cancer were investigated. Those patients were constricted to subgroups with positive lymph node status, indicating a higher migratory ability of the tumor cells and a higher tumor burden. In this setting, the data showed highly significant difference in the ER-positive group towards a beneficial effect of high miR-27a in the OS. Patients with low miR-27a expression had an approximately 50 months shorter survival, therefore decreasing the risk in the high miR-27a to 0.65

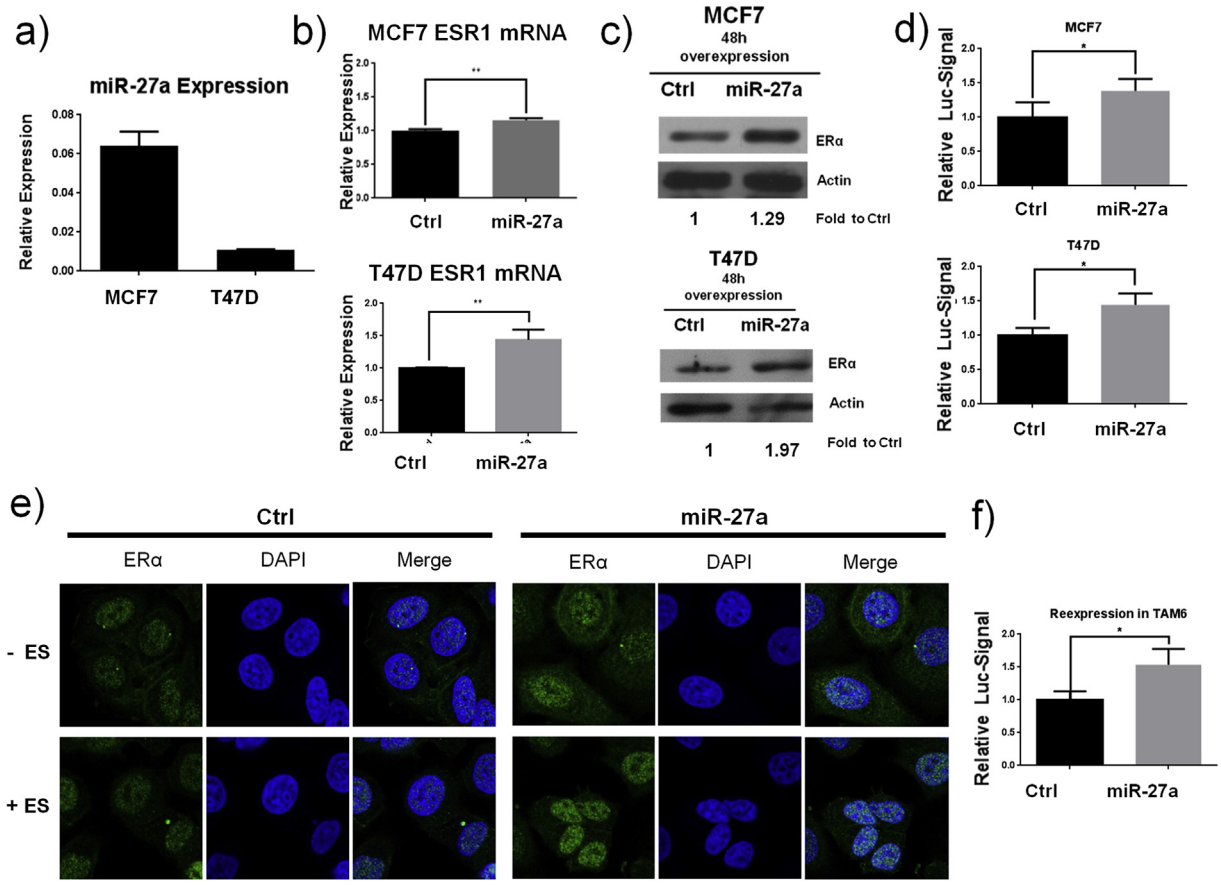

Fig. 2. The effect of miR-27a on ER-alpha signaling in luminal A breast cancer. a) Expression of miR-27a in the two luminal A breast cancer cell lines, MCF7 and T47D. b) Overexpression of miR-27a significantly increased expression of ESR1 mRNA in MCF7 and T47D ($p \leq 0.01$), b) ERα protein, as well as c) luciferase signal of the ERE-reporter compared to scrambled control ($p \leq 0.05$). e) Immunofluorescence staining of ERα and DAPI showed increased localization of ERα to the nucleus in miR-27a overexpressing cells which were stimulated with estradiol (ES). f) Re-expression of miR-27a in TAM6 cells significantly increased the relative luciferase signal of the ERE-reporter compared to scrambled control ($p \leq 0.05$).

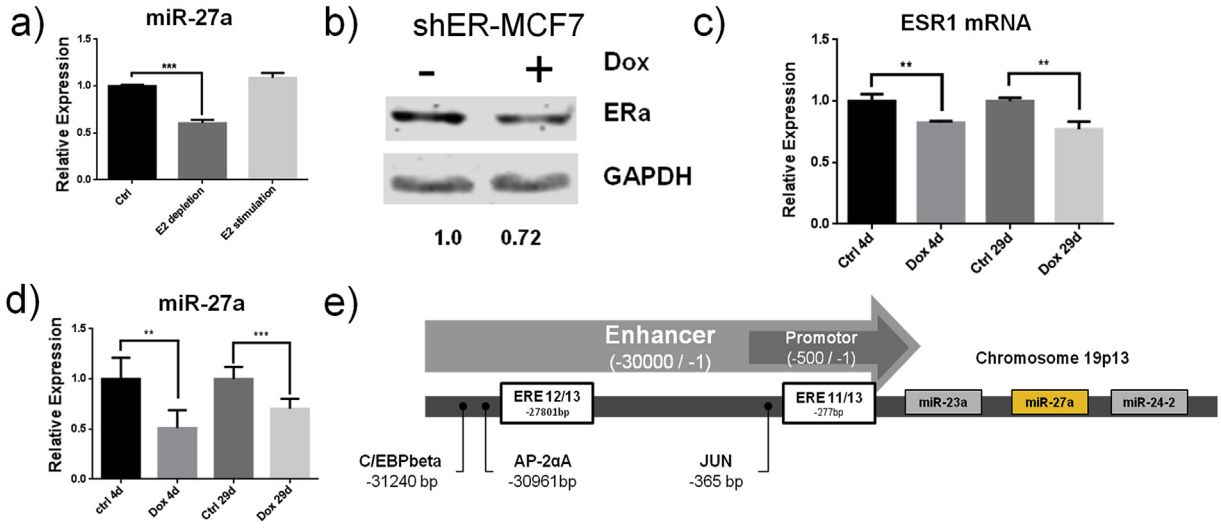

Fig. 3. The effect of ERα signaling on miR-27a expression. a) MCF7 cells that were depleted of estrogen-stimulation, showed significantly decreased expression of miR-27a ($p \leq 0.001$). b) Induction of shER-expressing MCF7 cells with 5 μg/ml doxycycline for 48 h decreased protein levels of ERα 30%. c) Long-term induction of shER for 4 and 29 days, significantly decreased ESR1 mRNA ($p \leq 0.01$), as well as d) the expression of miR-27a. e) Schematic overview of the promotor and enhancer region of the miR-27a locus. An analysis of possible transcription-factor interactions revealed three interaction partners of ERα, possibly controlling miR-27a transcription.

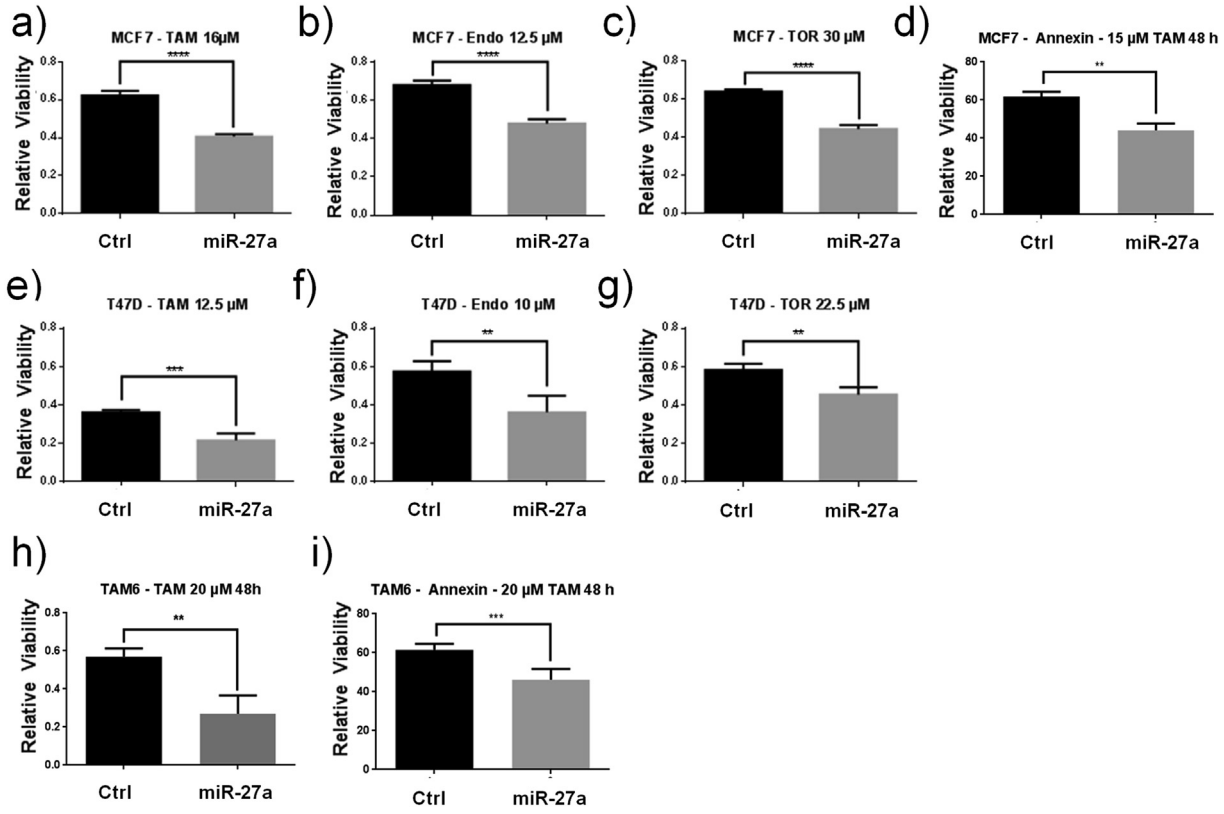

Fig. 4. Effect of miR-27a overexpression on resistance to SERMs. MCF7 cells with miR-27a overexpression showed significantly increased sensitivity towards treatments with the different SERMs as shown as viability measurement by ATP content, compared to scrambled control (Ctrl): a) 16 µM tamoxifen (TAM), b) 12.5 µM endoxifen (Endo) and c) 30 µM toremifen. d) An annexin V-FITC assay of TAM treated MCF7 cells showed a decreased number of viable cells after miR-27a overexpression. T47D cells with overexpression of miR-27a with e) 12.5 µM TAM, f) 10 µM Endo, g) 22.5 µM TOR showed significantly decreased viability, compared to scramble control. h) The resistant TAM6 cells were significantly re-sensitized to TAM treatment by overexpression of miR-27a shown as viability by ATP content, i) as also by Annexin measurements. All experiments were compared and normalized to a scrambled control transfection (**p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001).

(0.47–0.9; p = 0.0083). In line, the comparison in ER-negative patients showed the reverse picture: High miR-27a expression lead to a significant decrease in OS of about 50 months with a two-fold increased relative risk (HR 2.02, (1.09-0.023), p = 0.023). Here, in the luminal A subgroup (see supplemental Fig. S3) high miR-27a lead to significantly increased OS of about 40 months and 0.51 (0.31–0.85) relative risk (p = 0.0083). Similar results were seen in another database, where a cohort of luminal A patients with early

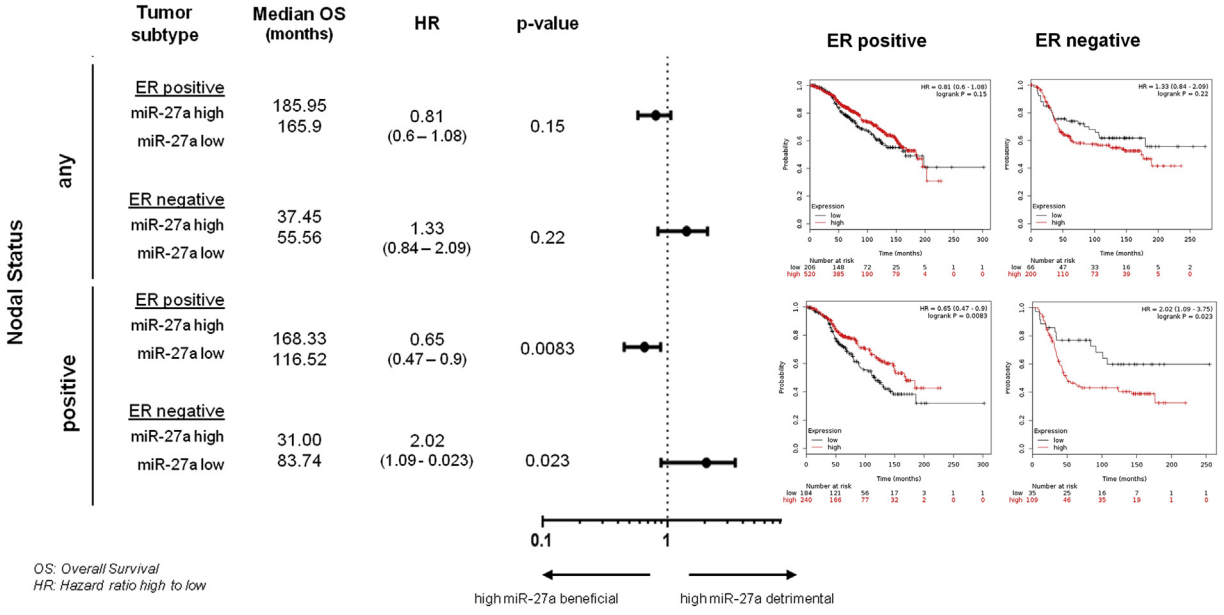

Fig. 5. Clinical data shows potential of miR-27a as prognostic marker for endocrine therapies ER+ and ER + metastatic breast cancer.

breast cancer, which underwent tamoxifen treatment, were analyzed with the MIRUMIR tool [28] (supplementary Fig. S4).

These findings showed that miR-27a expression was high in aggressive tumors and was detrimental for patients with ER-negative breast cancer, but in the setting of ER-positive tumors, which were treated with endocrine therapies, high miR-27a levels were an indication for a good response to the treatment and increased survival rates.

3. Discussion

While luminal A breast cancer is regarded as the one subtype with best prognosis and well established treatment options, current epidemiologic data suggest a need for better follow-up care of the disease, as evidently mortality of these patients is increasing after 5 years [4]. Different approaches are made to counteract bad long-term outcomes. Often SERM treatments are prolonged for a time of up to 10 years and longer or switched to different treatments with drugs of other therapeutic classes, usually aromatase inhibitors [4,29]. Many of these approaches are associated with the same adverse effects as SERM treatment, i.e. cardiovascular disease and the substantially increased risk of secondary cancers like endometrial carcinoma [30]. Hence, physicians are inclined to often discontinue therapies early to balance these risks. Still, the number of relapsing patients with therapy resistant tumors is high and therefore there is an urgent need for personalized treatment options to maximize efficacy of therapies while decreasing the adverse effects. MiRNAs are a class of potential markers, which show fine-tuned expression patterns in tissues, as well as offer the possibility to analyze their expression directly from blood samples as circulating miRNAs [12].

MiR-27 plays an ambivalent role in cancer. On the one hand, it is known to increase proliferation and metastasis in patients [13], in some cases even assumed to regulate chemoresistance [31]. This aspect makes it an interesting marker for numerous cancer types, like colon and esophageal cancer and also certain types of breast cancer, in order to adjust treatments and therapy regimes accordingly [13,14,31]. In the analysis of the METABRIC datasets, two cohorts of patients with ER-negative tumors, which consequently did not receive endocrine treatments, showed significantly worse outcomes for patients with high miR-27a expression. This result is in line with the discussed role of miR-27a as oncomir, inducing the negative effects on patient survival.

On the other hand, the *in silico* analysis of both the METABRIC and MIRUMIR datasets revealed that miR-27a expression is a predictor of beneficial breast cancer patient survival, in a defined subgroup of ER-positive tumors treated with endocrine agents.

Multiple explanations can be considered for this ambivalence. First, due to the positive bidirectional feedback loop between miR-27a and ERα, the miR-27a expression may function as a surrogate marker for the ERα expression MiR-27a was described to upregulate ERα expression, as observed in the current study and was previously reported, based on miR-27a inhibition of ZBTB10 and the resulting increase of the expression of the sp-protein family [18]. In this case, higher levels of ERα, based on increased miR-27a expression, could increase the susceptibility for SERMs and therefore improve the eradication of the tumor. While an increase of drug targets is often discussed as resistance mechanism, different studies suggest that SERM antitumor effects are based not only on inhibition of the estrogen signaling, but additionally on induction of maspin or of oxidative stress [32,33].

Additionally, our data showed that stimulation with or the deprivation of estradiol, as well as a direct knock-down of ERα, showed identically directed impact on miR-27a expression, as was also reported in a genomic expression study previously [34]. The *in silico* analysis of the promotor and enhancer region revealed potential sites of transcriptional regulation of miR-27a via ERα, e.g. by direct translational effects based on EREs or upregulated transcription factor activity downstream of the ER-signaling pathway, like JUN.

Thus, high miR-27a levels may act as surrogate read-out for a high ERα transcriptional activity in the tumor, likely with crucial cancer promoting effects due to ERα's role in cell cycle and proliferation [35]. This could explain the higher impact on cancer cell survival resulting from ERα inhibition.

Both of these discussed mechanisms do not contradict miR-27a's role as oncomiR, as increase of ER-activity leads to higher proliferation and metabolic activity in the cells [35].

In fact, the *in vitro* viability data suggest that treatments with tamoxifen and toremifen are significantly more effective in eradicating these potentially more aggressive tumors, underlined by the finding that the beneficial prognostic effect of miR-27a is highest for ER-positive tumors which spread to lymph nodes.

Besides acting as predictor for an enhanced response to SERM treatments, the observed loss of miR-27a may also function as indicator of resistance to the therapy, as observed in the TAM6 cells. Treatment with TAM for six rounds in the course of multiple weeks, caused the formation of resistance, accompanied by the loss of miR-27a, as was also demonstrated by Ye et al. [36], showing increased miR-27a expression after generation of TAM resistant cells.

Many mechanisms are discussed for resistance development to tamoxifen. One obvious effect which may account for up to 17–28% of acquired resistance [37], is the loss of ERα, rendering the cancer cells estrogen independent, which could be based on the methylation of the CpG-islands of the ESR1-promotor [38]. Thus, it is possible that partial methylation of the ESR1-promotor occurred in the TAM6 cells and therefore affected changes in ERα and miR-27a expression.

In addition, the occurrence of mutations of the ER-gene were reported, which are not influencing the ERα expression, but were observed to have no estrogen mediated transcritional activity while appearing as ER-positive in immunohistological stainings [39]. In this case, miR-27a may be a valuable indicator of functional ERα expression, as ER-positive tumors with low miR-27a expression might inherit a less functional ERα translational activity and thus decreased response to SERMs.

Taken together, miR-27a expression correlates with functional ERα expression and may therefore act as surrogate read-out for a frequent resistance mechanism.

MiRNA screening can play an important part in improving patient outcomes by enabling tailored treatments and personalized medicines for cancer. A screening of different miRNAs, including miR-27a in blood plasma of breast cancer patients was performed previously [40] In the study of Jurkovicova et al., where miR-27a was shown to be one of the miRNAs with altered expression, plasma samples from the patients were analyzed. The data indicates that miR-27a expression may be used as marker for invasive breast cancers or carcinomas *in situ*. However, further studies need to be conducted to prove whether miR-27a expression is a prognostic marker for therapeutic response also in blood plasma. Our data suggests and encourages further studies of miR-27a as marker for SERM response in the clinics. Patients with ER-positive tumors with high miR-27a expression currently already receive suitable treatment with adjuvant tamoxifen, if treated according to the guidelines [3]. However, patients in the same setting with miR-27a low tumors may display resistance to the treatment, either initially or due to acquired resistance in the long-term. These patients would benefit most of an analysis of miR-27a levels.

Conflicts of interest

The authors declare that they have no conflict of interest.

Author contributions statement

BL performed the experiments and wrote the manuscript. JGR performed the analysis of transcription factor and ERE-binding sites. AS generated the TAM6 cells. EW provided conceptual advice. AR conceived the study and wrote the manuscript. All authors commented on the manuscript and conclusions of this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.breast.2018.10.007.

References

[1] Ferlay J, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Canc 2015;136:E359–86. https://doi.org/10.1002/ijc.29210.
[2] Blows FM, et al. Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival: a collaborative analysis of data for 10,159 cases from 12 studies. PLoS Med 2010;7:e1000279. https://doi.org/10.1371/journal.pmed.1000279.
[3] Senkus E, et al. Primary breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol : official journal of the European Society for Medical Oncology / ESMO 2015;26(Suppl 5). https://doi.org/10.1093/annonc/mdv298. v8–30.
[4] Davies C, et al. Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. Lancet 2013;381:805–16. https://doi.org/10.1016/S0140-6736(12)61963-1.
[5] Ring A, Dowsett M. Mechanisms of tamoxifen resistance. Endocr Relat Canc 2004;11:643–58. https://doi.org/10.1677/erc.1.00776.
[6] Burstein HJ, et al. Adjuvant endocrine therapy for women with hormone receptor-positive breast cancer: american society of clinical oncology clinical practice guideline focused update. J Clin Oncol 2014;32:2255–69. https://doi.org/10.1200/JCO.2013.54.2258.
[7] Guo Y, et al. Prognostic and clinicopathological value of GATA binding protein 3 in breast cancer: a systematic review and meta-analysis. PloS One 2017;12:e0174843. https://doi.org/10.1371/journal.pone.0174843.
[8] Curtis C, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature 2012;486:346–52. https://doi.org/10.1038/nature10983.
[9] Takaku M, et al. GATA3 zinc finger 2 mutations reprogram the breast cancer transcriptional network. Nat Commun 2018;9:1059. https://doi.org/10.1038/s41467-018-03478-4.
[10] Oualla K, et al. Novel therapeutic strategies in the treatment of triple-negative breast cancer. Ther Adv Med Oncol 2017;9:493–511. https://doi.org/10.1177/1758834017711380.
[11] Early Breast Cancer Trialists' Collaborative G, et al. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 2011;378:771–84. https://doi.org/10.1016/S0140-6736(11)60993-8.
[12] Hamam R, et al. Circulating microRNAs in breast cancer: novel diagnostic and prognostic biomarkers. Cell Death Dis 2017;8:e3045. https://doi.org/10.1038/cddis.2017.440.
[13] Pan W, Wang H, Jianwei R, Ye Z. MicroRNA-27a promotes proliferation, migration and invasion by targeting MAP2K4 in human osteosarcoma cells. Cell Physiol Biochem : international journal of experimental cellular physiology, biochemistry, and pharmacology 2014;33:402–12. https://doi.org/10.1159/000356679.
[14] Ba S, Xuan Y, Long ZW, Chen HY, Zheng SS. MicroRNA-27a promotes the proliferation and invasiveness of colon cancer cells by targeting SFRP1 through the wnt/beta-catenin signaling pathway. Cell Physiol Biochem : international journal of experimental cellular physiology, biochemistry, and pharmacology 2017;42:1920–33. https://doi.org/10.1159/000479610.
[15] Chae DK, et al. MIR-27a regulates the TGF-beta signaling pathway by targeting SMAD2 and SMAD4 in lung cancer. Mol Carcinog 2017;56:1992–8. https://doi.org/10.1002/mc.22655.
[16] Tang W, et al. MiR-27 as a prognostic marker for breast cancer progression and patient survival. PloS One 2012;7:e51702. https://doi.org/10.1371/journal.pone.0051702.
[17] Quan J, et al. MicroRNA-23a/24-2/27a as a potential diagnostic biomarker for cancer: a systematic review and meta-analysis. Mol Clin Oncol 2018;8:159–69. https://doi.org/10.3892/mco.2017.1492.
[18] Li X, et al. MicroRNA-27a indirectly regulates estrogen receptor {alpha} expression and hormone responsiveness in MCF-7 breast cancer cells. Endocrinology 2010;151:2462–73. https://doi.org/10.1210/en.2009-1150.
[19] Sommer AK, et al. Salinomycin co-treatment enhances tamoxifen cytotoxicity in luminal A breast tumor cells by facilitating lysosomal degradation of receptor tyrosine kinases. Oncotarget 2016;7:50461–76. https://doi.org/10.18632/oncotarget.10459.
[20] Kopp F, Wagner E, Roidl A. The proto-oncogene KRAS is targeted by miR-200c. Oncotarget 2014;5:185–95.
[21] Kopp F, Oak PS, Wagner E, Roidl A. miR-200c sensitizes breast cancer cells to doxorubicin treatment by decreasing TrkB and Bmi1 expression. PloS One 2012;7:e50469. https://doi.org/10.1371/journal.pone.0050469.
[22] Kopp F, et al. Salinomycin treatment reduces metastatic tumor burden by hampering cancer cell migration. Mol Canc 2014;13:16. https://doi.org/10.1186/1476-4598-13-16.
[23] Flach KD, Zwart W. The first decade of estrogen receptor cistromics in breast cancer. J Endocrinol 2016;229:R43–56. https://doi.org/10.1530/JOE-16-0003.
[24] Broos S, et al. PhysBinder: improving the prediction of transcription factor binding sites by flexible inclusion of biophysical properties. Nucleic Acids Res 2013;41:W531–4. https://doi.org/10.1093/nar/gkt288.
[25] Gruber CJ, Gruber DM, Gruber IM, Wieser F, Huber JC. Anatomy of the estrogen response element. Trends Endocrinol Metabol 2004;15:73–8. https://doi.org/10.1016/j.tem.2004.01.008.
[26] Lanczky A, et al. miRpower: a web-tool to validate survival-associated miRNAs utilizing expression data from 2178 breast cancer patients. Breast Canc Res Treat 2016;160:439–46. https://doi.org/10.1007/s10549-016-4013-7.
[27] Welboren WJ, Sweep FC, Span PN, Stunnenberg HG. Genomic actions of estrogen receptor alpha: what are the targets and how are they regulated? Endocr Relat Canc 2009;16:1073–89. https://doi.org/10.1677/ERC-09-0086.
[28] Antonov AV, Knight RA, Melino G, Barlev NA, Tsvetkov PO. MIRUMIR: an online tool to test microRNAs as biomarkers to predict survival in cancer using multiple clinical data sets. Cell Death Differ 2013;20:367. https://doi.org/10.1038/cdd.2012.137.
[29] Dowsett M, et al. Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. J Clin Oncol 2010;28:509–18. https://doi.org/10.1200/JCO.2009.23.1274.
[30] Katase K, Sugiyama Y, Hasumi K, Yoshimoto M, Kasumi F. The incidence of subsequent endometrial carcinoma with tamoxifen use in patients with primary breast carcinoma. Cancer 1998;82:1698–703.
[31] Zhou S, et al. miR-27a regulates the sensitivity of breast cancer cells to cisplatin treatment via BAK-SMAC/DIABLO-XIAP axis. Tumour Biol 2016;37:6837–45. https://doi.org/10.1007/s13277-015-4500-1.
[32] Bekele RT, et al. Oxidative stress contributes to the tamoxifen-induced killing of breast cancer cells: implications for tamoxifen therapy and resistance. Sci Rep 2016;6:21164. https://doi.org/10.1038/srep21164.
[33] Liu Z, Shi HY, Nawaz Z, Zhang M. Tamoxifen induces the expression of maspin through estrogen receptor-alpha. Cancer Lett 2004;209:55–65. https://doi.org/10.1016/j.canlet.2003.11.018.
[34] Klinge CM. Estrogen regulation of MicroRNA expression. Curr Genom 2009;10:169–83. https://doi.org/10.2174/138920209788185289.
[35] Frasor J, et al. Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. Endocrinology 2003;144:4562–74. https://doi.org/10.1210/en.2003-0567.
[36] Ye P, et al. Differential microRNA expression profiles in tamoxifen-resistant human breast cancer cell lines induced by two methods. Oncol Lett 2018;15:3532–9. https://doi.org/10.3892/ol.2018.7768.
[37] Viedma-Rodriguez R, et al. Mechanisms associated with resistance to tamoxifen in estrogen receptor-positive breast cancer (review). Oncol Rep 2014;32:3–15. https://doi.org/10.3892/or.2014.3190.
[38] Parl FF. Multiple mechanisms of estrogen receptor gene repression contribute to ER-negative breast cancer. Pharmacogenomics J 2003;3:251–3. https://doi.org/10.1038/sj.tpj.6500201.
[39] Mahfoudi A, Roulet E, Dauvois S, Parker MG, Wahli W. Specific mutations in the estrogen receptor change the properties of antiestrogens to full agonists. Proc Natl Acad Sci U S A 1995;92:4206–10.
[40] Jurkovicova D, et al. Down-regulation of traditional oncomiRs in plasma of breast cancer patients. Oncotarget 2017;8:77369–84. https://doi.org/10.18632/oncotarget.20484.