Downregulation of microRNA-27b-3p enhances tamoxifen resistance in breast cancer by increasing NR5A2 and CREB1 expression

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Estrogen-dependent breast cancer is often treated with the aromatase inhibitors or estrogen receptor (ER) antagonists. Tamoxifen as a major ER antagonist is usually used to treat those patients with ERα-positive breast cancer. However, a majority of patients with ERα positive fail to respond to tamoxifen due to the presence of intrinsic or acquired resistance to the drug. Altered expression and functions of microRNAs (miRNAs) have been reportedly associated with tamoxifen resistance. In this study, we investigated the role of miR-27b-3p in resistance of breast cancer to tamoxifen. MiR-27b-3p levels were remarkably reduced in the tamoxifen-resistant breast cancer cells compared with their parental cells. In addition, miR-27b-3p was also significantly downregulated in breast tumor tissues relative to adjacent non-tumor tissues. Moreover, the expression levels of miR-27b-3p were lower in the breast cancer tissues from tamoxifen-resistant patients compared with that from untreated-tamoxifen patients. Notably, tamoxifen repressed miR-27b-3p expression, whereas estrogen induced miR-27b-3p expression in breast cancer cells. Besides, we provided experimental evidences that miR-27b-3p enhances the sensitivity of breast cancer cells to tamoxifen in vitro and in vivo models. More importantly, we validated that miR-27b-3p directly targeted and inhibited the expression of nuclear receptor subfamily 5 group A member 2 (NR5A2) and cAMP-response element binding protein 1 (CREB1) and therefore augmented tamoxifen-induced cytotoxicity in breast cancer. Lastly, miR-27b-3p levels were found to be significantly negatively correlated with both NR5A2 and CREB1 levels in breast cancer tissues. Our findings provided further evidence that miR-27b-3p might be considered as a novel and potential target for the diagnosis and treatment of tamoxifen-resistant breast cancer.

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Over two-thirds of breast cancers overexpress estrogen receptor (ER), which contributes to breast cancer tumorigenesis and progression.1 Targeted inhibition of ER using selective modulators is considered the optimal treatment for breast cancer patients with ER-positive tumors. The selective ER modulator tamoxifen is an effective first-line endocrine therapy drug. It is found clinically that tamoxifen can significantly improve overall and relapse-free survival rates of all stages of patients with ER-positive breast cancer.2 Studies showed tamoxifen can reduce the incidence of contralateral breast cancer.3 Therefore, the drug has been approved as a prophylactic agent to prevent breast tumor.4 Despite their documented benefits in the management of patients with potentially endocrine-responsive breast cancers, an intrinsic or acquired resistance to tamoxifen is common in a significant proportion of those patients treated with the drug.4,5

MicroRNAs (miRNAs) are a class of small non-coding RNAs that post-transcriptionally block expression of the target genes by directly interacting with mRNA of the genes. Numerous miRNAs have been discovered in human cells; however, their targets and function remain largely unknown. Dysregulated miRNA expression is frequently involved in the development of many human tumor types.6,7 In addition, studies show involvement of miRNA in resistance of cancer cells to chemotherapeutic drugs.8–10 Moreover, upregulation of miRNAs expression causes tamoxifen resistance in breast cancer cells, such as miR-221/222,11 whereas miR-342 and miR-378a confer tamoxifen sensitivity by inhibiting their target genes.12,13

MiRNA-27b-3p is one of the few miRNAs differentially expressed between tamoxifen-sensitive and -resistant breast cancer cell lines.14 It has reported that marked downregulation of miRNA-27b-3p in tamoxifen-resistant cells compared with...
Recently, several studies have identified miR-27b-3p (also known as miR-27b) promotes cell proliferation and invasion in glioma and breast cancer, 15,16 and blocks paclitaxel-induced apoptosis in cervical cancer. 17 MiR-27b-3p also reportedly plays a cancer-promoting role and is associated with poor prognosis in triple-negative breast cancer patients.17 On the other hand, miR-27b-3p functions as a tumor suppressor to inhibit cells growth, tumor progression and the inflammatory response by inhibiting the expression of PPARγ in neuroblastoma.18 Moreover, miR-27b-3p attenuates the acquisition of cancer stem cell properties in luminal-type breast cancer by repression of ENPP1.19 These findings suggest that the functions of miR-27b-3p are diverse and may be dependent on the specific cancer types.

In the present study, we developed a tamoxifen-resistant breast cancer cell model and investigated the potential roles of miR-27b-3p in the acquisition of tamoxifen resistance. We found that miR-27b-3p expressions were remarkably reduced in tamoxifen-resistant cells compared with their parental cells. In addition, miR-27b-3p was also significantly downregulated in the breast tumor tissues relative to their adjacent non-tumor tissues. Moreover, the expression levels of miR-27b-3p were lower in the breast cancer tissues from tamoxifen-resistant patients compared with that from untreated-tamoxifen patients. Additionally, we provided experimental evidences that miR-27b-3p enhances sensitivity of breast cancer cells to tamoxifen in vitro and in vivo models. Furthermore, we validated that miR-27b-3p directly targeted and inhibited the expression of nuclear receptor subfamily 5 group A member 2 (NR5A2) and cAMP-response element binding protein 1 (CREB1) and therefore augmented tamoxifen-induced cytotoxicity in breast cancer. Lastly, miR-27b-3p levels were found to be significantly negatively correlated with both NR5A2 and CREB1 levels in breast cancer tissues.

**Results**

**Downregulation of miR-27b-3p in tamoxifen-resistant breast cancer cells and tissues.** To evaluate the expression of miR-27b-3p in breast cell lines, RT-PCR was...
performed in the five breast cancer cell lines, including MCF-7, T47D, BT-549, SK-BR-3 and MDA-MB-231 cells, and a noncancerous breast epithelial cell line MCF-10A cells. We found miR-27b-3p was significantly downregulated in the five cancer cell lines compared with MCF-10A. Notably, the levels of miR-27b-3p in the two ER-positive (ER-positive) breast cancer cells MCF-7 and T47D showed significant upregulation compared with the ER-negative BT-549, SK-BR-3 and MDA-MB-231 cells (Figure 1aA). Furthermore, we modeled the development of acquired tamoxifen resistance in patients by treating MCF-7 and T47D cells with 1 μM of 4-hydroxytamoxifen (TAM) for 12 months to select the tamoxifen-resistant cells (named MCF-7/TAM-1, MCF-7/TAM-2, T47D/TAM-1 and T47D/TAM-2). Our results indicated that all these tamoxifen-resistant cells were significantly more resistant (for MCF-7: 4+20-folds; for T47D: 4+4-folds) to TAM treatment relative to the parental cells (Supplementary Figure S1). We found that tamoxifen-resistant MCF-7 and T47D cells exhibited lower miR-27b-3p levels compared with the parental cells (Figures 1aB and C).

To further confirm the association between miR-27b-3p and breast cancer, we detected the expression of miR-27b-3p in 19 paired breast cancer and adjacent normal tissues. We found tumor tissue specimens exhibited generally lower miR-27b-3p levels compared with their adjacent normal tissues (Figure 1b). Moreover, miR-27b-3p expression was significantly higher in the tamoxifen-resistant breast cancer tissues from patients with tamoxifen-treated than the tumor tissues from patients who were not treated using any chemotherapy drugs (Figure 1c).

**MiR-27b-3p is repressed by tamoxifen and induced by estrogen in breast cancer cells.** To examine whether tamoxifen regulated miR-27b-3p expression, MCF-7 and T47D cells were treated with increasing doses of TAM for 48 h, and then the levels of miR-27b-3p were determined by RT-PCR. We indicated that miR-27b-3p expression was remarkably inhibited by TAM in a concentration-dependent manner (Figure 2a). Additionally, we detected the levels of miR-27b-3p in both MCF-7 and T47D cells treated with the indicated dose of TAM for different time (2–32 h). We observed that the levels of miR-27b-3p gradually decreased in a time-dependent manner during 4–32 h (Figure 2a). Next, to determine whether estrogen induced miR-27b-3p expression in breast cancer cells, MCF-7 and T47D cells were
treated with increasing doses of 17β-estradiol (EST) for 48 h, and then the levels of miR-27b-3p were determined by RT-PCR. To avoid the effects of estrogen-derived medium, MCF-7 and T47D cells were cultured in estrogen-free medium for 3 days before incubation with EST. In contrast to TAM, a trend of induction of miR-27b-3p expression by EST in a dose-dependent manner was observed in both breast cancer cells (Figure 2c). Moreover, we also showed EST increased the expression of miR-27b-3p in a time-dependent manner (Figure 2d).

**MiR-27b-3p enhances tamoxifen-induced apoptosis in breast cancer cells.** To investigate the role of miR-27b-3p in the sensitivity of the breast cancer cells to tamoxifen, breast cancer cells were treated with miR-27b-3p mimics and inhibitors, respectively, and the cytotoxicity induced by TAM...
NR5A2 and CREB1 are direct targets of miR-27b-3p. The mRNA expression (a–d) and protein levels (e–h) of NR5A2 and CREB1 were downregulated by miR-27b-3p in breast cancer cells. MCF-7 (a and e), MCF-7/TAM-1 (b and f), T47D (c and g) and T47D/TAM-1 (d and h) cells were transfected with miR-27b-3p mimics or inhibitors and the negative control (NC), respectively. RT-PCR was performed to detect the mRNA expression of NR5A2 and CREB1. Western blot was performed to detect the protein expression of NR5A2 and CREB1. Actin was used as a loading control. Data were from three independent experiments. (e–h) (B) and (C) Relative protein levels of NR5A2/Actin and CREB1/Actin were quantified using Image J software. Data are mean ± S.D. from three independent experiments. **P < 0.01; ***P < 0.001, compared with the control group. (i) The predicted miR-27b-3p target sites in the 3′ UTR of NR5A2 and CREB1 mRNA and their mutated version. (j–m) Luciferase activity assays in MCF-7 and 293T cells showed that miR-27b-3p inhibited the expression of NR5A2 and CREB1. MCF-7 and 293T cells were cotransfected with pGL3 vector containing the wild type or mutated 3′ UTR of NR5A2 and CREB1, pGL3-Control vector, along with miR-27b-3p mimics or inhibitors or NC. After 48 h, luciferase activity was detected. Data were normalized to luciferase activity in the corresponding cells transfected with NC and are represented as the mean ± S.D. of three replicates.
was examined. Forced expression of miR-27b-3p mimics in MCF-7/TAM-1 and T47D/TAM-1 cells significantly increased miR-27b-3p levels (Figure 3aA and Supplementary Figure S2Aa). MiR-27b-3p mimics significantly increased the cytotoxicity of TAM in both tamoxifen-resistant breast cancer cells (Figure 3bA and Supplementary Figure S2Ba). On the contrary, miR-27b-3p inhibitors apparently decreased miR-27b-3p levels in MCF-7 and T47D cells (Figure 3aB and Supplementary Figure S2Ab). Moreover, downregulation of miR-27b-3p reduced cell toxicity of TAM in both MCF-7 and T47D cells (Figure 3bB and Supplementary Figure S2Bb).

Figure 4  Continued

On the contrary, miR-27b-3p inhibitors apparently decreased miR-27b-3p levels in MCF-7 and T47D cells (Figure 3aB and Supplementary Figure S2Ab). Moreover, downregulation of miR-27b-3p reduced cell toxicity of TAM in both MCF-7 and T47D cells (Figure 3bB and Supplementary Figure S2Bb).
To further determine the role of miR-27b-3p in increasing the sensitivity of cancer cells to TAM, the mimics and inhibitors-transfected cells were treated with TAM, and apoptosis was detected by annexin V and propidium iodide (PI) staining assay. Our data indicated that miR-27b-3p mimics observably increased TAM-induced cell apoptosis in MCF-7/TAM-1 and

Figure 5 Overexpression of NR5A2 and CREB1 reverses reduction of cell viability and induction of apoptosis by miR-27b-3p mimics, and depletion of NR5A2 and CREB1 reverses induction of cell viability and reduction of apoptosis by miR-509-5p inhibitors in tamoxifen-treated cells. (a–h) MCF-7/TAM-1 (a) and T47D/TAM-1 (b) cells were cotransfected with negative control (NC) or miR-27b-3p mimics along with control (Ctr) or NR5A2 or CREB1 vectors. MCF-7 (c) and T47D (d) cells were cotransfected with NC or miR-27b-3p inhibitors along with NC or NR5A2 or CREB1 siRNA. After 8 h, cells were treated with indicated dose of 4-hydroxytamoxifen (TAM) for additional 48 h. (a–d) MTT assay was performed to examine cell viability. (e–h) Cell apoptosis was assessed by Annexin-V-FITC/PI staining assay by flow cytometry. Columns, means of three determinations; bars, S.D.; **P < 0.01; ***P < 0.001, compared with NC-treated cells.
miRNA-27b-3p sensitizes breast cancer to tamoxifen

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Identification of NR5A2 and CREB1 as direct targets of miR-27b. Since miRNAs perform biological functions through negatively regulating their target genes, we predicted the potential targets of miR-27b-3p by using online miRNA target bioinformatics prediction databases (TargetScan, PicTar4, miRDB, miRWalk, and miRanda). We initially predicted 126 target genes of miR-27b-3p (Supplementary Table S1). Based on the biological functions of these 126 target genes, we selected NR5A2, CREB1, VAV3, GOLM1, EGFR, FOXO1, and IRS1 as candidate target genes. Subsequently we tested the expression of these seven genes in tamoxifen-sensitive cells MCF-7 and tamoxifen-resistant cells MCF-7/TAM-1 and MCF-7/TAM-2. Results showed NR5A2, CREB1 and GOLM1 expression levels were significantly higher in tamoxifen-resistant breast cancer cells (Supplementary Figure S3). To further validate targeting of NR5A2, CREB1 and GOLM1 by miR-27b-3p, the mRNA levels of all three genes were detected in MCF-7/TAM-1 cells transfected with miR-27b-3p mimics and MCF-7 cells transfected with miR-27b-3p inhibitors. We found that miR-27b-3p inhibitors significantly increased expression of NR5A2 and CREB1 in MCF-7/TAM-1 cells, whereas miR-27b-3p mimics led to obvious reduction of NR5A2 and CREB1 levels (Supplementary Figures S4A and B). However, there were no significant changes in the mRNA levels of GOLM1 (Supplementary Figures S4A and B).

Moreover, inhibition of NR5A2 and CREB1 by miR-27b-3p was further validated in MCF-7, T47D, MCF-7/TAM-1 and T47D/TAM-1 cells. Our results showed that miR-27b-3p inhibitors significantly attenuated both protein and mRNA levels of NR5A2 and CREB1, whereas miR-509-3p inhibitors observably enhanced the protein and mRNA levels of the two genes in all the four breast cancer cells (Figure 4a–h). Additionally, the conserved miR-27b-3p target sites in the 3′-untranslated regions (3′-UTRs) of NR5A2 and CREB1 were predicted, respectively (Figure 4i).

To further confirm targeting of NR5A2 and CREB1 by miR-27b-3p, luciferase activity assay was performed. The wild type (WT) or mutated (MT) 3′-UTRs of NR5A2 and CREB1 were cloned into the downstream of firefly luciferase coding region in pGL3 luciferase reporter vector (Figure 4i). The vectors were cotransfected with miR-27b-3p mimics or inhibitors into 293T and MCF-7 cells. As expected, miR-29b-3p mimics significantly decreased luciferase activity in both 293T and MCF-7 cells, whereas miR-27b-3p mimics led to obvious reduction of NR5A2 and CREB1 levels (Supplementary Figures S4A and B). However, there were no significant changes in the mRNA levels of GOLM1 (Supplementary Figures S4A and B).

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cotransfected with miR-27b-3p inhibitors and WT reporter vectors were higher than that in cells treated with negative control (NC) miRNA (Figures 4j–m). Additionally, the luciferase activities of the MT reporter vectors were not induced by miR-27b-3p inhibitors in the two cells. Above results implied that miR-27b-3p suppressed the NR5A2 and CREB1 expression by binding 3′ UTR of the two genes mRNA.

MiR-27b-3p attenuated breast cancer cell resistance to tamoxifen by repressing NR5A2 and CREB1. Previous study shows that NR5A2 promotes breast cancer cell resistance to tamoxifen involved in inducing ERα expression.20 Additionally, the luciferase activities of the MT reporter vectors were not induced by miR-27b-3p inhibitors in the two cells. Above results implied that miR-27b-3p suppressed the NR5A2 and CREB1 expression by binding 3′ UTR of the two genes mRNA.

To further determine the functional significance of NR5A2 and CREB1 suppression in miR-27b-3p-mediated tamoxifen sensitivity, MCF-7/TAM-1 and T47D/TAM-1 cells were cotransfected with miR-27b-3p mimics along with NR5A2 or CREB1 overexpression constructs, subsequently cells were treated with TAM. Our results showed TAM-inhibited cell viability was substantially decreased by miR-27b-3p, whereas NR5A2 or CREB1 overexpression significantly reversed the TAM-sensitizing effects of miR-27b-3p in both cells (Figures 5a and b). We showed NR5A2 and CREB1 overexpression vectors distinctly increased the expressions of NR5A2 and CREB1, respectively (Supplementary Figures S6A, C, E and F). Moreover, by Annexin V/PI staining to quantify the percentage of cells apoptosis, we also found NR5A2 or CREB1 overexpression remarkably attenuated the increase of TAM-induced apoptosis by miR-27b-3p in MCF-7/TAM-1 and T47D/TAM-1 cells (Figures 5e and f).

In addition, MCF-7 and T47D cells were cotransfected with miR-27b-3p inhibitors along with NR5A2 or CREB1 small interfering RNAs (siRNAs), and then treated with TAM. NR5A2 and CREB1 siRNA evidently depleted the expression of NR5A2 and CREB1 in MCF-7 and T47D cells (Supplementary Figures S6B and D). Cell viability assay indicated depletion of

Figure 7 MiR-27b-3p is negatively correlated with NR5A2 and CREB1 mRNA levels in breast cancer. (a and b) Relative expression of NR5A2 (a) and CREB1 (b) along with miR-27b-3p was determined by RT-PCR in 32 breast cancer tissues from patients with untreated-tamoxifen. (c and d) Relative expression of NR5A2 (c) and CREB1 (d) along with miR-27b-3p was determined by RT-PCR in 20 breast cancer tissues from tamoxifen-resistant patients. For NR5A2 and CREB1, β-Actin was used as an internal control; for miR-27b-3p, U6 was used as an internal control. Their expression correlation was analyzed by correlation coefficient and t-test.
NR5A2 or CREB1 obviously reversed the TAM-resistant effects of miR-27b-3p inhibitors in both cell types (Figures 5c and d). Similarly, by detection of cells apoptosis using Annexin V/PI staining assay, we also found that reduction of TAM-induced apoptosis by miR-27b-3p inhibitors was totally reversed by NR5A2 or CREB1 siRNAs in both MCF-7 and T47D cells (Figures 5g and h). In addition, we also showed that increase of TAM-induced cell death by miR-27b-3p inhibitors were totally reversed by overexpression of NR5A2 combined with CREB1 in MCF-7/TAM-1 cells (Supplementary Figures S7A and B). All these results suggested that miR-27b-3p attenuated breast cancer cell resistance to TAM by repressing NR5A2 and CREB1.

MiR-27b-3p enhances sensitivity of breast tumor to tamoxifen in xenograft tumor models. To determine whether miR-27b-3p enhanced sensitivity of breast tumor to tamoxifen in vivo, MCF-7/TAM-1 cells stably expressing miR-27b-3p mimics were injected subcutaneously into the flank of female nude mice. Subsequently the nude mice were administered with tamoxifen, and the volumes of tumors as well as body weight of mice were measured. Results showed that both cell lines stably overexpressing miR-27b-3p exhibited significantly increased sensitivity to tamoxifen (Figure 6a). Notably, no significant differences of body weight in mice were found between the different treatment groups (Figure 6b). Moreover, we evaluated the antitumor efficacy of tamoxifen in mice bearing tumors originating from tamoxifen-sensitive MCF-7 cells stably expressing miR-27b-3p inhibitors (anti-miR-27b-3p). Results showed that tumors stably expressing anti-miR-27b-3p were more resistant to tamoxifen compared with these tumors stably expressing negative control (Figure 6c). Additionally, there were no significant differences of body weight in mice among the four groups (Figure 6d). Above results suggested that miR-27b-3p enhanced sensitivity of breast tumor to tamoxifen in xenograft tumor models.

MiR-27b-3p is negatively correlated with NR5A2 and CREB1 mRNA levels in breast cancer. To further examine whether miR-27b-3p levels were correlated with NR5A2 and CREB1, we analyzed NR5A2 and CREB1 mRNA levels in 32 breast cancer tissues from tamoxifen-untreated patients and 20 breast cancer tissues from tamoxifen-resistant patients by RT-PCR. The clinical information of the 52 patients is shown in Supplementary Table S2. By correlation analysis between miR-27b-3p and NR5A2, we found significantly negative correlations between miR-27b-3p and NR5A2 mRNA expression in both untreated-tamoxifen \((r = -0.537, P = 0.002\); Figure 7a) and tamoxifen-resistant tissue specimens \((r = -0.514, P = 0.014\); Figure 7c). In addition, correlation analysis was also performed between miR-27b-3p and CREB1 in the two types of tissue specimens. Similarly, significantly negative correlations between miR-27b-3p and CREB1 mRNA expression were found in both untreated-tamoxifen \((r = -0.592, P = 0.00045\); Figure 7b) and tamoxifen-resistant tissue specimens \((r = -0.574, P = 0.005\); Figure 7d). These data implied that miR-27b-3p inhibited the expression of NR5A2 and CREB1 in vivo.

Discussion

Although tamoxifen therapy is a very effective treatment for women with ERα-positive breast tumors by inhibiting the ER pathway, this therapy exerts very low effects in some patients with de novo resistance or acquired resistance. Currently there are only a few useful molecular markers to guide the use of tamoxifen for breast cancer patients with ER positive. Previous studies have revealed important roles of miRNAs in breast cancer resistance to tamoxifen.\textsuperscript{22–24} Downregulation of miR-375 and miR-873 is associated with tamoxifen resistance, whereas overexpression of miR-375 and miR-873 increases tamoxifen sensitivity in breast cancer.\textsuperscript{22,23} In this study, we identified miR-27b-3p exhibited decreased expression in tamoxifen-resistant breast cancer tissues and cells. In addition, we had shown for the first time the role of miR-27b-3p in conferring sensitivity of breast cancer to tamoxifen. This hinted that miR-27b-3p could be used as a new potential marker of tamoxifen-resistant breast cancer.

Here, we found breast tumor tissue samples examined expressed lower levels miR-27b-3p than their adjacent normal tissue. A previous study also demonstrates that miR-27b-3p is significantly downregulated in breast cancer.\textsuperscript{18} Notably, the levels of miR-27b-3p were reduced in two triple-negative breast cancer cell lines BT-549 and MDA-MB-231 compared with the ER-positive breast cancer cell lines examined. This result was consistent with recent finding that the expression levels of miR-27b-3p are downregulated in triple-negative breast cancer.\textsuperscript{25} Conversely, several other studies have shown that miR-27b-3p functions as oncogene in breast cancer and upregulated miR-27b-3p levels indicates poor prognosis of triple-negative breast cancer patients.\textsuperscript{14,26,27} All
these findings and our results suggested that the functions of miR-27b might be dependent on the specific subtype of breast tumor.

In addition, we manifested that the miR-27b-3p is repressed by tamoxifen and induced by estrogen in breast cancer cells. These results suggested that constitutive downregulation of miR-27b-3p exerted in tamoxifen-resistant breast cancer tissues and cell lines were likely initiated by prolonged tamoxifen treatment. However, the mechanism of miR-27b-3p regulated by tamoxifen and estrogen has not yet been clarified. Estrogen can stimulate the transcriptional activity of ER through promoting the recruitment of various receptor-binding coactivators.26 Tamoxifen not only prevents the combination of coactivators and ER, but increases the recruitment of corepressors and causes suppression of certain genes expressions.24 These findings raised the possibility that the transcriptional level of miR-27b-3p was directly induced by estrogen and inhibited by tamoxifen, respectively. Further work would be required to establish the mechanisms responsible for the regulation of miR-27b-3p by tamoxifen and estrogen.

Previous study has shown that miR-27b-3p is highly expressed in chemoresistant hepatocellular carcinoma cells, although whether these increase in miR-27b-3p levels directly affect the drug sensitivity of hepatocellular carcinoma cells is not determined.29 In contrast to this finding, miR-27b-3p has also been reported to sensitize gastric cancer to chemotherapy by directly targeting inhibition of CCNG1, a well-known negative regulator of p53 stability.30 Loss of miR-27b-3p provokes the generation of breast cancer stem cells that show docetaxel resistance and high tumorigenicity.18 In this study, downregulation of miR-27b-3p was found in the tamoxifen-resistant breast cancer cells and tissues. Furthermore, we demonstrated that the reduction of miR-27b-3p conferred resistance to tamoxifen in breast cancers by increasing the NR5A2 and CREB1 levels. NR5A2, also known as LRH-1, a member of the nuclear receptor subfamily 5 has been implicated in the progression of breast cancer.31 Additionally, it has been reported that a high level of CREB1 is observed in breast cancer and associated with disease progression in breast cancer patients.32,33 Our results also showed NR5A2 and CREB1 were highly expressed in the breast cancer tissues and inversely correlated to miR-27b-3p levels.

Recent study shows that NR5A2 increases breast cancer cell growth by directly binding to the promoter of EGR gene and inducing its expression.20 Interestingly, NR5A2 is transcriptionally regulated by the ERα and mediated the mitogenic effects of estrogen.34 Moreover, NR5A2 have been implicated in resistance of breast tumor to tamoxifen by promoting the expression of BCL2 and MYC, two important genes involved in inducing cellular proliferation and resisting cell apoptosis.35,36 On the other hand, although the exact mechanisms by which CREB1 confers resistance of breast cancer to tamoxifen have not yet been elucidated, CREB1 transcriptionally activates numerous critical molecules involved in antiapoptosis, such as BRCA1 and BCL2,37,38 and cellular proliferation, such as cyclin A1 and cyclin D1.39,38 Besides, emerging evidence has suggested that CREB1 enhanced aromatase transactivation in breast cancer cells.29 Aromatase as a key enzyme for the biosynthesis of estrogens enhances breast cancer cell resistance to tamoxifen.40 Indeed, upregulation of aromatase expression is found in tamoxifen-resistant human breast cancer.23 Moreover, our results also indicated that forced CREB1 expression increased aromatase expression. Notably, recent evidence suggests that miR-27b-3p enhances cytotoxicity of drugs by targeting inhibition of ENPP1, which induces ABCG2 expression and cell surface localization and increases drugs efflux.18 Therefore, we did not exclude the possibility that loss of miR-27b enhances breast cancer cells resistance to tamoxifen involved in downregulation of ABCG2 expression and activity.

A genetic regulatory network is depicted in Figure 8 and summarizes the key findings of our study. In conclusion, we had identified miR-27b-3p as a modulating factor for the tamoxifen resistance in breast cancer. Our findings will provide useful information for the development of alternative approaches to diagnose and treat tamoxifen-resistant breast cancer.

Materials and Methods

Sample information. ER-positive breast cancer samples were collected from the First Affiliated Hospital of Xi’an Jiaotong University (Xi’an, China), between 2005 and 2009, in which 19 patients contributed paired samples (i.e., both breast tumor samples and adjacent normal tissues were defined as below 1 cm from the tumor tissue) and the other 34 patients contributed breast tumor tissues only. All these samples were neoadjuvant-free and were collected before systemic chemotherapy treatments. In addition, 20 tamoxifen-resistant patients contributed breast tumor tissues after systemic tamoxifen treatments. All of the samples were retrieved within 15 min after the surgery and immediately frozen at −80 °C until used for gene expression analysis as previously described.41 This study was approved by the Clinical Ethics Review Board at The First Affiliated Hospital of Xi’an Jiaotong University and written informed consents were from all patients at their recruitment time.

Cell culture and reagents. Human breast cancer cell line MCF-7 (tamoxifen-sensitive), T47D (tamoxifen-sensitive), BT-549, SK-BR-3, MDA-MB-231 and MCF-10A were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The identities of cell lines were confirmed by using DNA profiling (short tandem repeat, STR). MCF-7/TAM-1 and MCF-7/TAM-2 (tamoxifen-resistant) cell lines were established from MCF-7 cells after the following continuous exposure to 1 μM of TAM (Sigma, St. Louis, MO, USA) for more than 1 year. MCF-10A were cultured in DMEM/F12 (1:1) supplemented with 5% heat-inactivated equine serum, 5 μg/ml insulin 20 ng/ml EGF, 100 ng/ml cholera toxin, 0.5 μg/ml hydrocortisone and 2 mmol l-glutamine. BT-549 cells were cultured as previously described.42 All other cell lines were maintained in DMEM medium supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) at 37 °C in a 5% CO2 humidified incubator. Cells were grown in monolayer and passaged routinely 2–3 times a week. Dimethyl sulfoxide (DMSO) and EST was purchased from Sigma.

Real-time PCR. Total RNA was extracted from breast cancer cell lines and patients tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Following DNaseI treatment, 2 μg of total RNA was reverse transcribed using cDNA synthesis kit (Bio-Rad, Richmond, CA, USA) to synthesize cDNA specimens. And then, real-time PCR (RT-PCR) analysis of gene expression was performed using 2 μl of cDNA and SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) as recommended by the manufacturer. RT-PCR was conducted by means of the SYBR on the CFX96 system (Bio-Rad). For miRNA, a Ploy-A tail was added to the miRNA, which was then transcribed into cDNA using a universal adaptor primer that included oligo-dT. The generated cDNA was then combined with the Uni-miR RT-PCR Primer (possess the binding site with universal adaptor primer, included in SuperScript III One-Step RT-PCR Kit with Platinum Taq (Invitrogen)) and a miRNA primer (sequence complementary to the miRNA) to complete the RT-PCR reaction. The miRNA expression was normalized using the endogenous U6 snRNA. For the primers, miR-27b-3p: 5′-TTCCACAGTGCTGAATGTCTGC-3′ (forward) and reverse-primer (Uni-miR RT-PCR Primer, included in SYBR Premix Ex Taq kit (Takara, Japan)). U6: 5′-CTCGAGTCGAGCAGGACCA-3′ (forward) and 5′-AACGCTTCAC
GAATTGCGGT-3' (reverse). For mRNA detection, β-actin was used as an internal control to normalize gene expression values for each gene expression analysis. For the primers, NRS2A: 5'-GGGATCCATATGGGCTCCT-3' (forward) and 5'- TGTCAATTGCGACTGCTTG-3' (reverse);45 CREB1: 5'-CCAGCAAGTGGAGATCGG-3' (forward) and 5'-GTACGTTGGACGAGTAGAT-3' (reverse);45 others primers are shown in Supplementary Data. The PCR was run in triplicate at 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s, 56 °C for 20 s and 72 °C for 20 s. Comparative quantification was performed using the 2^−ΔΔCt method as previously described.46 Each sample was analyzed in triplicate.

Plasmids and miRNAs transfection. The NRS2A and CREB1 expression vectors were purchased from Origene (Rockville, MD, USA). MiR-27b-3p mimics, inhibitors and NC were purchased from GenePharma (Shanghai, China). Cells were transipnyized, counted and seeded into six-well plates the day prior to transfection to ensure 70% cell confluence on the day of transfection. Transfection of miRNA mimics/inhibitors and plasmids into cells was performed using lipofectamine 3000 (Invitrogen) in accordance with the manufacturer's advised procedure as previously described.45 The miRNA mimics or inhibitors and plasmids were used at a final concentration of 50 nM and 2 μg, respectively. At 48 h after transfection, RF-PCR and western blotting were performed.

Determination of cell viability and apoptosis. MTT assay was conducted to assess the cell viability according to the manufacturer’s instructions (Sigma). Briefly, cells were plated into 96-well plates at a density of 0.5–1 × 10^4 cells per well and incubated for at least 8 h in a 5% CO2 atmosphere at 37 °C before exposure to vehicle and drugs. The media were then removed, and cells were treated with vehicle and drugs. After the cells were incubated for indicated time, MTT was added to the medium to a final concentration of 0.5 mg/ml. Cells were incubated with MTT for 4 h at 37 °C, and then the medium was removed and 0.2 ml DMSO was added. Absorbance of the media was then measured using a Microplate Reader (Bio-Rad) at 570 nm.46 This assay was conducted in triplicate.

Measurement of apoptosis was conducted by Annexin V-FITC (fluorescein isothiocyanate)/PI analysis as described previously.45 Briefly, cells were seeded and treated with the drugs for 48 h. And then, cells were washed twice with PBS and 1 × 10^6 cells were resuspended in 1 ml of 1 × Annexin V-binding buffer. Cells undergoing apoptosis cell death were analyzed by counting the cells that stained positive for Annexin V-FITC and negative for PI, and late stage of apoptosis as cells positive for Annexin V-FITC and PI positive using FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Western blot analysis. Total proteins were isolated from cells with lysis buffer (50 mM Tris, pH 7.5; 150 mM NaCl; 1% NP40; 2.5 mM sodium pyrophosphate; 0.02% sodium azide; 1 mM EGTA; 1 mM EDTA; 1 mM β-glycerophosphate; 1 mM Na2VO4; 1 mM PMSF; 1 μg/ml leupeptin). The lysates were centrifuged at 12 000 r.p.m. for 30 min at 4 °C. The protein concentration was determined by Bradford dye reagent (ECL) kit (GE Healthcare, Munich, Germany), according to the manufacturer’s instructions. Blots were quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).48

miRNA target prediction. The analysis of miR-27b-3p predicted targets was performed using the algorithms TargetScan (http://targetscan.org/), PicTar (http://pictar.mdc-berlin.de/), miRDB (http://www.mirdb.org/miRDB), miRWalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk/) and miRanda (http://www.microrna.org/microrna).

Dual-luciferase activity assay. The human 3′-UTR region of NRS2A and CREB1 genes were amplified by PCR and cloned into the XbaI site of the pGL3-Control vector (Promega, Madison, WI, USA), downstream of the luciferase gene, to generate the vector pGL3-NRS2A and pGL3-CREB1. For luciferase assay, the 293T and MCF-7 cells were cultured in 24-well plates and transfected with 500 ng of pGL3-NRS2A, pGL3-CREB1 or pGL3-control vector along with 50 pmol of miR-27b-3p mimics, inhibitors or NCs, respectively. Transfection of miRNAs was carried out using Lipofectamine 3000 in accordance with the manufacturer’s procedure (Life Technologies, Carlsbad, CA, USA). At 24 h after transfection, firefly luciferase activity was measured using the Dual-Luciferase Reporter Assay (Promega) as previously described.46 The above experiment was repeated at least three times.

Animal experiment. Athymic BALB/c nude mice (4–6 weeks old) were obtained from Si-Lai-Ko-Jing-Da Experimental Animal Co. Ltd (Changsha, China). All of the procedures of animal experiments were performed according to approved protocols and in accordance with the guidelines of the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council). It was approved by the Institutional Animal Care and Use Committee of Xiamen Jiaotong University (Xiamen, China). MCF-7/TAM-1/NC and MCF-7/TAM-1/mimics cells with vector and stably overexpressed miR-27b-3p were constructed by lentivirus (GenePharma). Cells (4 × 10^5) were suspended in 0.2 ml of serum-free RPMI1640 media and were injected into the flanks of female BALB/c nude mice under isoflurane inhalation (4–6 weeks old, n = 6/group), which were maintained under pathogen-free conditions. One day after tumor cell implantation, mice were treated with tamoxifen and health of mice was checked every day. Unitary dose of tamoxifen was 30 and 120 mg/kg per day in MCF-7 and MCF-7/TAM-1 xenograft mice, respectively. In all cases, water (control) and tamoxifen were administered once a day through oral gavage during 25 days. Tumor volume was measured two times a week by using calipers (as indicated at each time point) for 25 days. The tumor volume was estimated by the following formula: length × width × width × 0.14/6. The mice whole-body weight was measured two times a week as indicated at each time point. All mice were killed by intraperitoneal injection of 200 mg/kg pentobarbital at the end of the experiment.

Statistical analysis. Results were expressed as mean ± S.D. Differences between groups were estimated using the Student’s t-Test. A level of P < 0.05 was considered to be significant. The relationships between miR-27b-3p and NRS2A or CREB1 expression level were analyzed by correlation coefficients and linear regression analysis. All analyses were performed using SPSS16.0 software (IBM, Armonk, NY, USA) and a two-tailed value of P < 0.05 was considered statistically significant.

Conflict of Interest
The authors declare no conflict of interest.

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Author contributions
JJH, JZ, ZZZ and ZJS designed the study. JZ, ZZZ, PNP, XNK and SMW performed the experiments. JZ, ZZZ and PNP analyzed the data. PPN, BYW and XNK contributed to materials. JJH, JZ, ZZZ and ZJS prepared the manuscript. JJH, ZZZ and BYW provided the financial support. All authors reviewed and approved the manuscript.

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