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Original Paper

miR-449a Suppresses Tamoxifen Resistance in Human Breast Cancer Cells by Targeting ADAM22

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Key Words
miR-449a • ADAM22 • Tamoxifen resistance • Estrogen receptor • Breast cancer

Abstract
Background/Aims: Most of estrogen receptor positive breast cancer patients respond well initially to endocrine therapies, but often develop resistance during treatment with selective estrogen receptor modulators (SERMs) such as tamoxifen. Altered expression and functions of microRNAs (miRNAs) have been reportedly associated with tamoxifen resistance. Thus, it is necessary to further elucidate the function and mechanism of miRNAs in tamoxifen resistance. ***Methods:*** Tamoxifen sensitivity was validated by using Cell Counting Kit-8 in tamoxifen-sensitive breast cancer cells (MCF-7, T47D) and tamoxifen-resistant cells (MCF-7/TAM, T47D/TAM). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to detect the expression level of miR-449a in tamoxifen-sensitive/-resistant cells and patient serums. Dual-luciferase assay was used to identify the binding of miR-449a and predicted gene ADAM22. The expression level of ADAM22 was determined by qRT-PCR and western blotting in miR-449a +/- breast cancer cells. Subsequently, rescue experiments were carried out to identify the function of ADAM22 in miR-449a-reduced tamoxifen resistance. Finally, Gene ontology (GO) and Protein-protein interaction analyses were performed to evaluate the potential mechanisms of ADAM22 in regulating tamoxifen resistance. ***Results:*** MiR-449a levels were downregulated significantly in tamoxifen-resistant breast cancer cells when compared with their parental cells, as well as in clinical breast cancer serum samples. Overexpression of miR-449a re-sensitized the tamoxifen-resistant breast cancer cells, while inhibition of miR-449a conferred tamoxifen resistance in parental cells. Luciferase assay identified ADAM22 as a direct target gene of miR-449a. Additionally, silencing of ADAM22 could reverse tamoxifen resistance induced by miR-449a inhibition in ER-positive breast cancer cells. GO analysis results showed ADAM22 was mainly enriched in the biological processes of cell adhesion, cell differentiation, gliogenesis and so on. Protein-protein interaction analyses appeared that ADAM22 might regulate tamoxifen resistance through PPARG, LGI1, KRAS and LYN. ***Conclusion:*** Decreased miR-449a

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causes the upregulation of ADAM22, which induces tamoxifen resistance of breast cancer cells. These results suggest that miR-449a, functioning by targeting ADAM22, contributes to the mechanisms underlying breast cancer endocrine resistance, which may provide a potential therapeutic strategy in ER-positive breast cancers.

Introduction

Breast cancer is the most common type of cancers and one of the leading causes of cancer-related death in women globally. The estrogen receptor (ER), which has a prominent role in breast cancer, is a member of the nuclear receptor superfamily of ligand activated transcription factors. Up to 70% of all breast cancers are ER-positive, and the majority fully depends on estrogen signaling, making it an excellent candidate for endocrine therapy [1]. Since the 1980s, death rates from breast cancer have been steadily decreasing with better screening methods and improved treatment methods. The major breakthrough in the treatment of breast cancer is the development of the selective estrogen receptor modulators (SERMs), such as tamoxifen, which blocks estrogen signaling. This therapeutic approach has been successfully used to treat approximately two-thirds of ER-positive breast cancers.

Although these drugs are initially effective, overall clinical benefit from use of them is often eventually limited by the development of endocrine resistance. Tamoxifen, for instance, is not effective in approximately 30% of patients and resistance is observed in 50% of patients eventually after treatment. Thus, studies have been performed to clarify the molecular mechanisms underlying endocrine resistance. Various mechanisms have been proposed to explain tamoxifen resistance, including loss or mutations of ER, deregulation of the ER pathway, high levels of so-called antiestrogen binding sites, and alterations in cell cycle and survival molecules [2-4]. For example, Lee MH et al. demonstrated that MTA1 induced AMPK activation and subsequent autophagy that could contribute to tamoxifen resistance in breast cancer [5]. CYP3A4-mediated EET pathway was found to represent a potential therapeutic target for the treatment of tamoxifen-resistant breast cancer [6]. Besides, ER phosphorylation was found to be important in breast cancer initiation and tamoxifen resistance [6]. Since tamoxifen resistance is complex and multifactorial, the underlying mechanisms are not fully understood yet.

Recent studies have reported that abnormal expression of miRNAs plays an important role in tamoxifen resistance [2, 7-10]. MiRNAs belong to a novel class of regulatory determinants which are small non-coding RNAs, about 20-23 nucleotides in length. They bind to complementary sequences in the 3'UTR of multiple target mRNAs, usually resulting in their silencing, and thus playing a vital role in regulating gene expression of target genes involved in different cellular functions including proliferation, differentiation and apoptosis [11-13]. A METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) study has provided a systematic analysis of miRNA expression profiles across a large number of human breast tumors and revealed oncogenic and tumor-suppressive roles of differential miRNAs [14]. More than 10 miRNAs have been found to target ERα and its co-regulators that regulate ERα transcriptional activities [15]. Reports in the recent years have indicated the involvement of miRNAs in tamoxifen resistance as well. Specifically, miR-221/222 enhanced tamoxifen resistance in recipient cells by reducing the target genes expression of P27 and Era [16]. MiR-148a and miR-152 were able to reduce tamoxifen resistance in ER-positive breast cancer via downregulating ALCAM [10]. Downregulation of microRNA-27b-3p enhanced tamoxifen resistance in breast cancer by increasing NR5A2 and CREB1 expression [8]. Therefore, miRNAs may be a novel therapeutic approach to sensitizing and suppressing the growth of tamoxifen-resistant breast tumors. Although new technical developments such as small RNA high-throughput sequencing have revealed series of cancer-related miRNAs [17, 18], their contribution to endocrine-resistance is not well elucidated.

MiR-449a is often dysregulated in various cancers, and its downregulation has been linked with increased proliferation, migration, invasion, extravasation and metastasis in

various cancer types, such as breast tumor [19], gynecologic clear cell carcinoma [20], non-small cell lung cancer [21], hepatocellular carcinoma [22], gastric carcinoma [23], prostate [24] and endometrial cancer [25], where miR-449a acts as a tumor suppressor. In breast cancer, miR-449a has been found to be a potent player in regulating the viability of breast cancer cells. Zhang, Z., et al. demonstrated downregulation of miR-449 promoted migration and invasion of breast cancer cells by targeting TPD52 [19]. Besides, miR-449a was found to be downregulated in human breast cancer tissues and negatively associated to aggressiveness and progression [26]. These findings suggest that miR-449a plays an important role in breast cancer and may be associated with the endocrine resistance. However, the role of miR-449a involved in the resistance of breast cancer has not been investigated.

In this study, we constructed tamoxifen-resistant breast cancer cell models and investigated the potential roles of miR-449a in the acquisition of tamoxifen resistance. Herein, we previously predicted ADAM22 as a target gene of miR-449a by bioinformatics analysis. We further investigated whether miR-449a regulates tamoxifen resistance by targeting ADAM22 in breast cancer cells.

Materials and Methods

Clinical serum samples

Serum samples were obtained with informed consent from patients at Nanjing Maternity and Child Health Care Hospital (Nanjing, China). The patients were biologically unrelated, but all belonged to the Han Chinese ethnic group from the Jiangsu province in China. Patients with disease progression or recurrence 6 months or less after tamoxifen therapy were defined as tamoxifen resistance, whereas those without recurrence or with recurrence 48 months after tamoxifen therapy were defined as tamoxifen sensitivity. After systemic tamoxifen treatments, 21 tamoxifen-sensitive patients and 33 tamoxifen-resistant patients contributed sera. All of the serum samples were retrieved after diagnosis and before surgery, and were immediately snap-frozen in liquid nitrogen, then stored at -80 °C until used for miRNA expression analysis.

Cell lines, cultures and reagents

The human breast cancer cell lines (tamoxifen-sensitive) MCF-7, T47D obtained from ATCC (the American Type Culture Collection, Manassas, VA, USA), were maintained in Dulbecco's Modified Eagle Medium (Thermo Scientific Hyclone, MA, USA), supplemented with 10% fetal bovine serum (Thermo Scientific Hyclone, MA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin and incubated with 5% CO2 at 37°C. Tamoxifen-resistant breast cancer cell lines MCF-7/TAM, T47D/TAM were respectively produced from parental MCF-7 and T47D cells by persistence gradient exposure to tamoxifen for about 12 months, which were subjected to increasing concentrations from 0.05 μM until the cells acquired resistance to 1 μM of tamoxifen (Sigma-Aldrich, St. Louis, MO, USA). The MCF-7/TAM and T47D/TAM drug-resistant cell lines were maintained in Roswell Park Memorial Institue (RPMI) 1640 (Gibco, CA, USA) with the same conditions above. Cells were seeded at a density of 0.5×10^6 viable cells per 100-mm plate, and the medium was changed every other day for 5 days.

Transfection

All miRNAs mimics and hairpin inhibitor form Ribobio (Guangzhou, China) were used for the overexpression and inhibition of miRNA activity in cells. After cultured in 6-well plates, 50 nM miRNAs mimics and 100 nM miRNAs inhibitor were transfected using Lipofectamine 3000 (Invitrogen, CA, USA) as described by the manufacturers. MCF-7/TAM and T47D/TAM cells were transfected with either siADAM22 (Ambion 4390824; S28739) or scrambled nontargeting siRNA (Ambion, AM4635). MCF-7 and T47D cells were transfected with either ADAM22 overexpression vector or empty vector. After transfection, the expression of miR-449a was determined by qRT-PCR, and the expression of mRNA and protein of ADAM22 was respectively determined by qRT-PCR and western blotting.

CCK-8 assay

Cell viability was assessed using Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) assay according to the manufacturer's instructions. Cells (5×10^3) in different transfected groups were cultured and placed into 96-well plates for 24 h and then were treated with varying concentration of tamoxifen (0, 0.05, 0.1, 0.5, 1, 5, 10 μM) for 48 h. As the assay began, 10 μl of CCK-8 solution was added to the medium in each well and then incubated at 37°C for 2 h. The absorbance of each well was measured at 450 nm by Synergy 2 Absorbance Microplate Hybird Reader (Biotek Instrument Inc., Highland Park, VT, USA). Cell viability was expressed as a fraction of absorbance compared to control.

Target genes analysis of miR-449a and bioinformatics analysis of ADAM22

We predicted the potential targets of miR-449a by using online miRNA target bioinformatics prediction databases (TargetScan, miRcode, miRDB) [27]. The 3′ untranslated region (3′-UTR) sequences were retrieved from the Entrez Nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore). To investigate the possible biological functions of ADAM22, we used the Gene Ontology (GO) and Protein-protein interaction analyses.

Dual-luciferase assay

The wild type and mutant ADAM22 3'-UTR dual-luciferase reporter vectors were constructed by subcloning the human ADAM22 mRNA 3'-UTR and mutant 3'-UTR sequences into the pGL3 Dual-Luciferase Reporter Vectors (Promega, Madison, USA). HEK293 cells were transfected with 80 ng luciferase reporter vectors and miR-449a mimics (final concentration, 50nM) using the Lipofectamine 3000 (Invitrogen, CA, USA), according to the manufacturer's protocol. After 24 h, luciferase activities were measured using Dual-Luciferase Reporter System (Berthold), according to the manufacturer's instructions [28].

RNA isolation and qRT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. CDNAs were synthesized from 1 μg of total RNA with RevertAidTM First-strand cDNA Synthesis Kit (Thermo Scientific, MA, USA) in a 25 μl volume containing 1 μg total RNA, 5 nM reverse transcription primer (Bulge-LoopTM miRNA primer from Ribobio, Guangzhou, China), 0.8 U/μl reverse transcriptase, 4 U/μl RNase Inhibitor and 0.2 mM dNTPs. Eluted RNA samples were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA was extracted from 4 cell lines MCF-7, T47D, MCF-7/TAM and T47D/TAM and the clinical breast cancer sera.

qRT-PCR was carried out with the reagents of SYBR Green Master (ABI, CA, USA) in 20 μl reaction volume (10 μl SYBR Green Mix, 0.4 μM forward primers, 0.4 μM reverse primers and 2 μl cDNA template) by using 7500 real-time PCR system (Applied Biosystems, CA, USA). The primer for miR-449a was purchased from Ribobio. U6 was used as the internal control. For mRNA detection, GAPDH was used as an internal control to normalize gene expression values for ADAM22 gene expression analysis. Primers used for amplifcation were shown in Table 1. PCR was run in triplicate at 95 °C for 2 min followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s and 72 °C for 30 s. Comparative quantification was performed using the $2^{-\Delta\Delta Ct}$ method as previously described [29].

Western blotting

Cells were washed in phosphate buffered saline (PBS) twice before proteins were extracted, and proteins were separated on a SDS/PAGE gel, transferred onto a PVDF membrane and subjected to immunoblot analysis. Blotting for ADAM22 was carried out using Anti-ADAM22 antibody (ab227546) (Abcam, Cambridge, England), Anti-GAPDH antibody (ab9485) (Abcam, Cambridge, England). Goat Anti-Rabbit IgG H&L

Table 1. Primers sequence of qRT-PCR

Gene	Forward primer(5′ →3′)	Reserve primer(5′ →3′)
U6	TGCGGGTGCTCGCTTCGGCAGC	CCAGTGCAGGGTCCGAGGT
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
miR-449a	TGCGGTGGCAGTGTATTGTTAGC	CCAGTGCAGGGTCCGAGGT
ADAM22	GGACCTCACAGTCACGAGGT	TCAGTGCTGCATTGTGCTTC

(HRP) (ab205718) (Abcam, Cambridge, England) was used as secondary antibody. Western blotting was carried out as previously described [30].

Statistical analysis

Data were presented in terms of means and standard errors for at least three separate experiments. Statistical analysis was performed using the software SPSS 16.0 (IBM, Armonk, NY, USA) for Windows. Student's t-test was used to analyze the results from two groups. Differences were considered to be statistically significant at $P < 0.05$.

Results

Construction of tamoxifen-resistant ER-positive breast cancer cells from parental cells

Tamoxifen-resistant ER-positive breast cancer cell lines were acquired by treating MCF-7 and T47D cells with gradually increased concentration of tamoxifen until the final concentration was 1μM for about 12 months and selected the tamoxifen-resistant cells (named MCF-7/TAM, T47D/TAM). Tamoxifen resistance of cells was calculated by the viability of cells after tamoxifen treatment. CCK-8 results showed that the viability of tamoxifen-resistant cells was significantly increased as compared with parental cells after tamoxifen exposure, and IC50s of tamoxifen-resistant cells were significantly higher than those of parental cells (for MCF-7/TAM: 26-fold; for T47D/TAM: 18-fold), which indicated that the models of drug resistant cells were constructed successfully (Fig. 1A and B).

Expression levels of miR-449a in tamoxifen-resistant breast cancer sera and cell lines

To confirm the association between miR-449a and breast cancer, the expression levels of miR-449a in breast cancer sera and cells were determined by using qRT-PCR. The data showed that miR-449a expression level was significantly downregulated (-1.66-fold) in tamoxifen-resistant breast cancer sera compared with tamoxifen-sensitive breast cancer sera (p <0.05) (Fig. 2A). MiR-449a was also significantly downregulated in MCF-7/TAM (-3.71-fold) and T47D/TAM (-2.40-fold) cells compared with their parental cells MCF-7 and

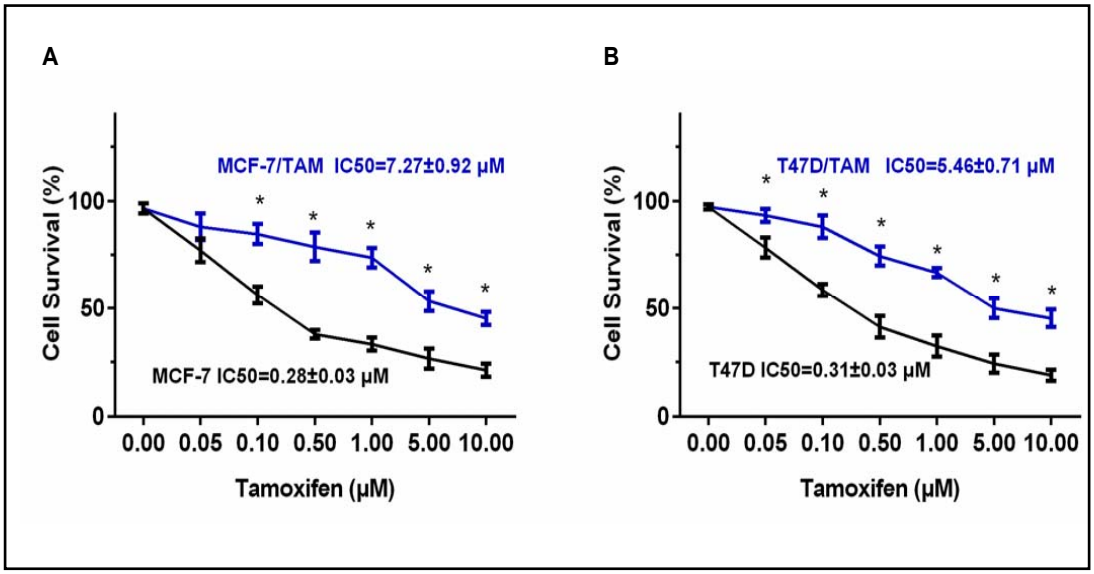


Fig. 1. CCK-8 was performed to test the vitality of acquired tamoxifen-resistant breast cancer cell lines (MCF-7/TAM, T47D/TAM) and their parental cell lines (MCF-7, T47D). IC50 of MCF-7/TAM cells is 26 folds higher than MCF-7 cells (A). IC50 of T47D/TAM cells is 18 folds higher than T47D cells (B). Data represents mean ± S.D. of triplicate independent experiments. *P<0.05.

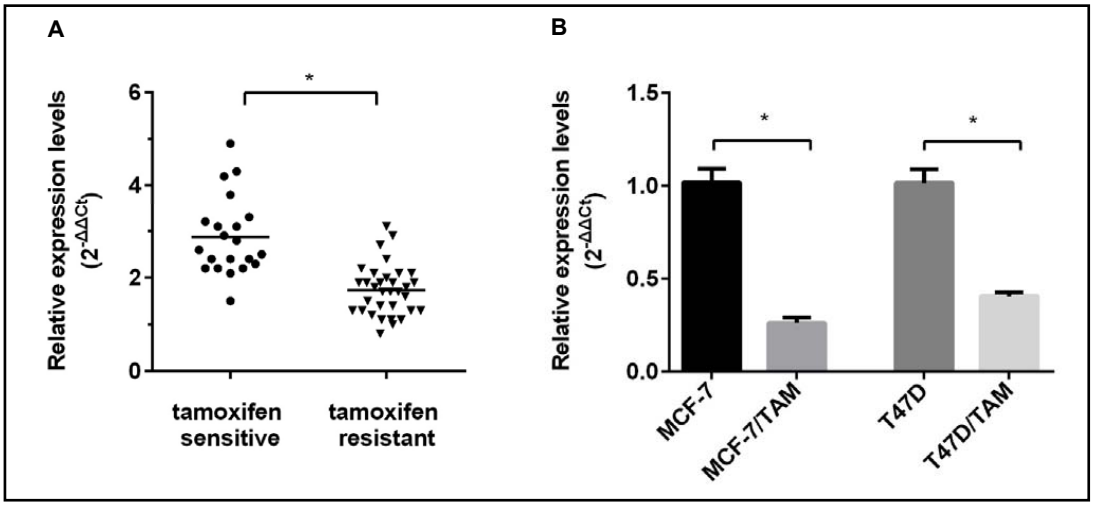


Fig. 2. MiR-449a expression is suppressed in tamoxifen-resistant breast cancer sera (A) and cell lines (B). Data represents mean ± S.D. of triplicate independent experiments. *P<0.05.

T47D ($p < 0.05$) (Fig. 2B), which is consistent with the expression pattern in clinical sera. The results indicated that miR-449a was downregulated significantly both in tamoxifen-resistant breast cancer sera and cell lines.

MiR-449a induced sensitivity of breast cancer cell lines to tamoxifen

To explore the potential role of miR-449a in tamoxifen resistance in breast cancer cell lines, we respectively transfected MCF-7/TAM cells with miR-449a mimics or mimics control, MCF-7 cells with miR-449a inhibitor or inhibitor control. Then qRT-PCR was used to determine the expression of miR-449a after transfection. The results showed that miR-449a expression was effectively enhanced about 4-fold in MCF-7/TAM cells with miR-449a mimics (Fig. 3A), and decreased in MCF-7 cells with miR-449a inhibitor (-2.39-fold) (Fig. 3B). After that, we tested the vitality of cells by CCK-8 assay. When exposing the MCF-7 cells with downregulated miR-449a to increasing concentrations of tamoxifen, we observed a dose-dependent inhibition of cell growth in control MCF-7 cells with IC50 about 0.30 μM. However, a significant resistance to tamoxifen was seen in MCF-7 cells that were transfected with miR-449a inhibitors, with IC50 increased 3.77-fold (Fig. 3D). Further, we confirmed our results in a reciprocal experimental setup where we overexpressed miR-449a in MCF-7/TAM cells. As shown in Fig. 3C, we found that control MCF-7/TAM cells are quite resistant to tamoxifen with IC50 7.29 μM. But upregulating miR-449a expression by the use of miR-449a mimics, resulted in sensitization of these cells to tamoxifen with IC50 down to 1.84 μM. Thus, overexpression of miR-449a significantly ($p < 0.05$) attenuated the tamoxifen resistance, demonstrating that overexpression of miR-449a can restore sensitivity to tamoxifen in the breast cancer cells.

Identification of ADAM22 as a direct target of miR-449a

ADAM22 was predicted to be a candidate target gene of miR-449a by using online miRNA target bioinformatics prediction databases (TargetScan, miRcode, miRDB) [27]. The paired sequence in the 3′-untranslated regions (3′-UTRs) of ADAM22 regulated by miR-449a was "CACUGCC" (Fig. 4A). To confirm the binding sites between ADAM22 and miR-449a, dual-luciferase activity assay was performed. The wild type (WT) or mutated (MT) 3′-UTRs ("ACUGC" mutated into "UUUUU") of ADAM22 were cloned into the downstream of firefly luciferase coding region in pGL3 luciferase reporter vector. The vectors were cotransfected with miR-449a mimics into HEK293 cells. Dual-luciferase assay showed that miR-449a mimics significantly decreased luciferase activity in HEK293 cells with WT reporter vectors. However, no obvious reduction of luciferase activities by miR-449a mimics was observed

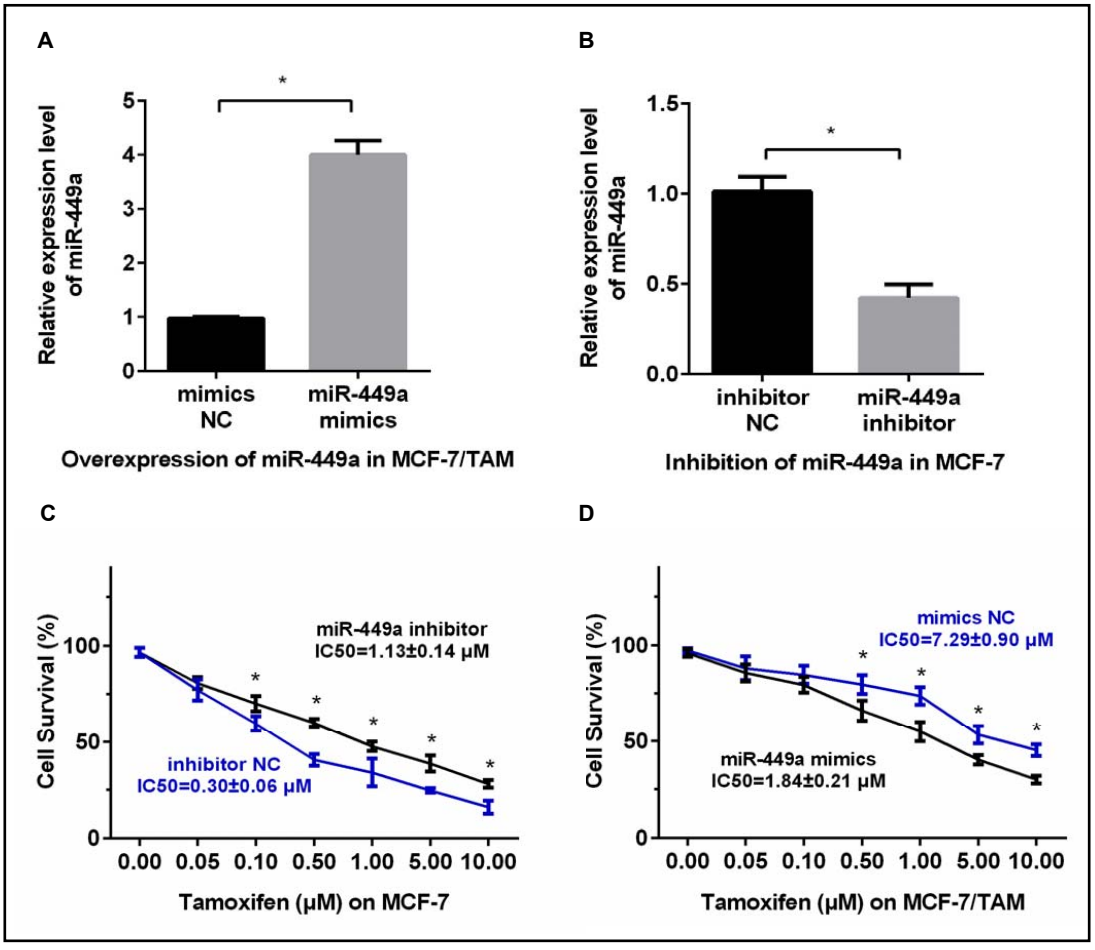


Fig. 3. Overexpression of miR-449a in MCF-7/TAM cells (A). Downregulation of miR-449a in MCF-7 cells (B). Overexpression of miR-449a induced sensitivity to tamoxifen inMCF-7/TAM cells with IC50 decreased 3.96 folds (C). Downregulation of miR-449a reduced sensitivity to tamoxifen in MCF-7 cells with IC50 inecreased 3.77 folds (D). Data represents mean ± S.D. of triplicate independent experiments. *P<0.05.

in HEK293 cells with MT reporter vectors (Fig. 4B). The results implied that miR-449a directly binds to 3 ′UTR of ADAM22 mRNA. Meanwhile, inhibition of ADAM22 by miR-449a was further validated in MCF-7 and MCF-7/TAM cells by qRT-PCR and western blotting. The results showed that miR-449a mimics significantly attenuated both mRNA ($p < 0.05$) (Fig. 4C) and protein (Fig. 4D) levels of ADAM22 in MCF-7/TAM cells, whereas miR-449a inhibitors observably enhanced mRNA ($p < 0.05$) (Fig. 4C) and protein (Fig. 4D) levels of ADAM22 in MCF-7 cells. Taken together, these data indicate that miR-449a could regulate ADAM22 expression by directly binding to 3 ′UTR of ADAM22 mRNA.

Expression levels of ADAM22 in tamoxifen-resistant breast cancer cell lines

The expression levels of ADAM22 were detected by qRT-PCR and western blotting in tamoxifen-sensitive breast cancer cells (MCF-7, T47D) and tamoxifen-resistant breast cancer cells (MCF-7/TAM, T47D/TAM). Results showed that both mRNA ($p < 0.05$) (Fig. 5A) and protein (Fig. 5B) levels of ADAM22 were significantly higher in tamoxifen-resistant breast cancer cells compared with tamoxifen-sensitive cells, which is exactly converse to the expression pattern of miR-449a.

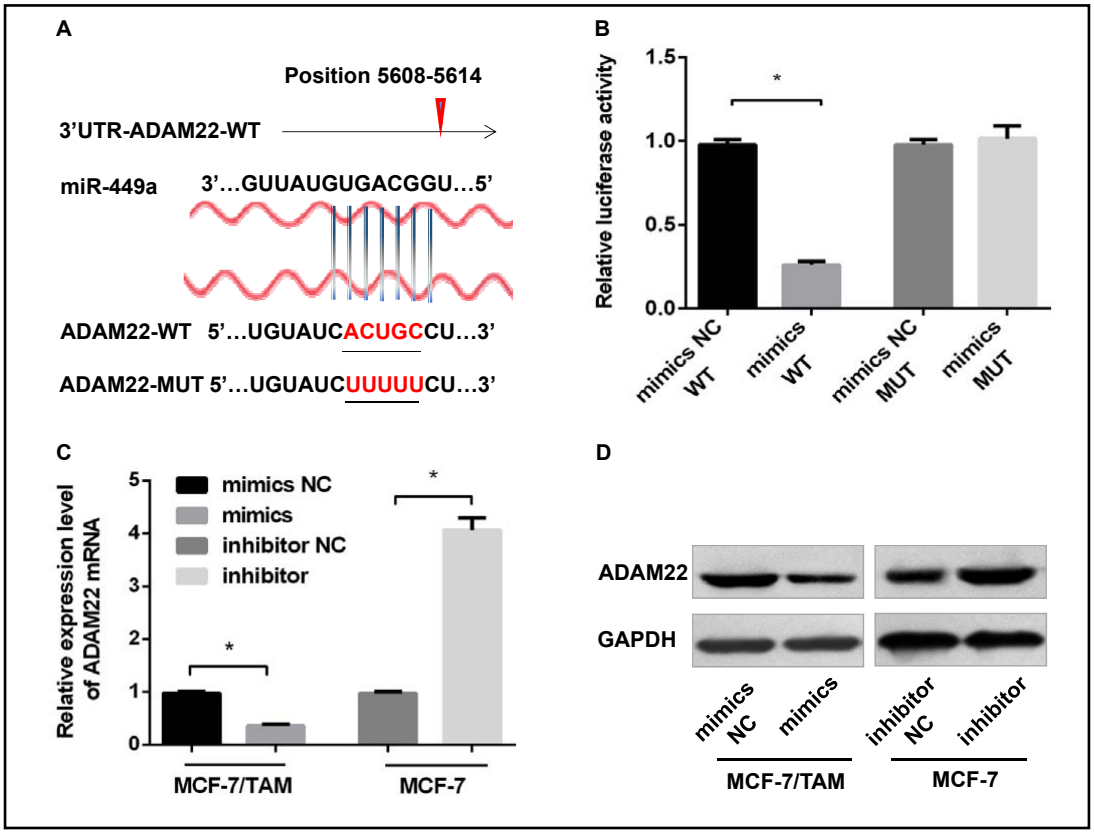
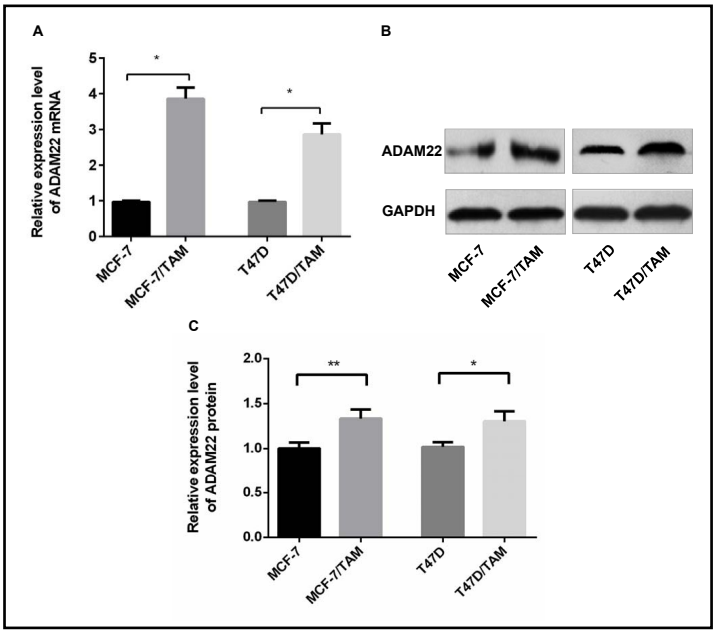


Fig. 4. MiR-449a directly targeted ADAM22 in breast cancer cell lines. We constructed wild genotype (wt)/ mutant genotype (mut) of the miR-449a-binding site in ADAM22 3'-UTR (A). Luciferase activity of mimics group was inhibited significantly co-transfected with wild 3'-UTR construct compared to mutant construct groups (B). Overexpression of miR-449a decreased the mRNA (C) and protein (D) expression levels of ADAM22 compared to negative control (NC), while inhibition of miR-449a increased the mRNA (C) and protein (D) expression levels of ADAM22 compared to negative control (NC). Data represents mean ± S.D. from triplicate independent experiments. *P<0.05.

Fig. 5. Both mRNA (A) and protein (B) (C) expression of ADAM22 was overexpressed in tamoxifen-resistant breast cancer cell lines. Data represents mean ± S.D. from triplicate independent experiments. *P<0.05.



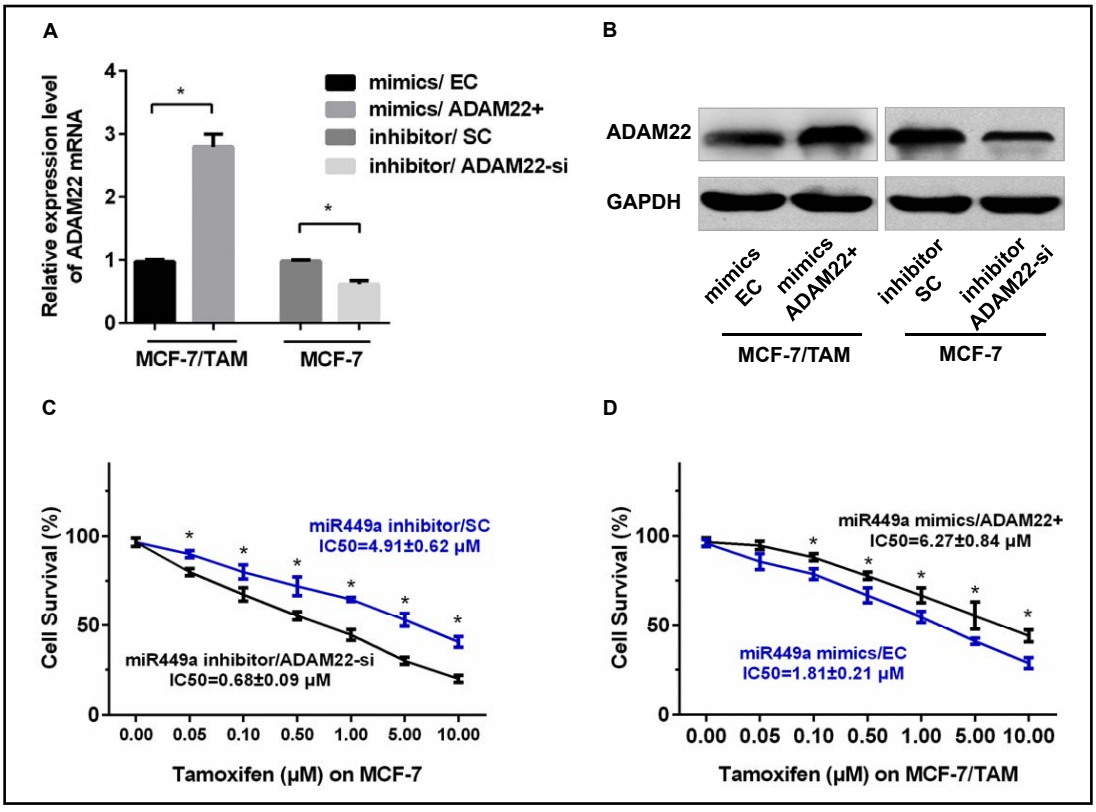


Fig. 6. MiR-449a regulated tamoxifen resistance in breast cancer cells via targeting ADAM22. MCF-7/TAM cells were co-transfected with miR-449a mimics along with ADAM22 vector or empty vector control (EC). MCF-7 cells were co-transfected with miR-449a inhibitors along with ADAM22-siRNA or scrambled control (SC). qRT-PCR (A) and western blotting (B) were performed to detect the mRNA and protein expression of ADAM22 in breast cancer cells. GAPDH was used as control. After ADAM22-siRNA treatment, inhibition of miR-449a induced tamoxifen-resistance was downregulated in MCF-7 cells significantly (C); In contrast, overexpression of miR-449a induced tamoxifen-sensitivity was reversed by overexpressed ADAM22 in MCF-7/TAM cells dominantly (D). Data represents mean ± S.D. from triplicate independent experiments. *P<0.05.

MiR-449a regulated tamoxifen resistance via ADAM22 in breast cancer cells

To further identify the role of ADAM22 in miR-449a-reduced tamoxifen resistance of breast cancer cells, a rescue strategy was adopted. MCF-7/TAM cells were co-transfected with miR-449a mimics and ADAM22 vector, or miR-449a mimics and empty vector (EC); MCF-7 cells were co-transfected with miR-449a inhibitor and ADAM22-siRNA, or miR-449a inhibitor and scrambled control (SC). The expression levels of ADAM22 were determined by qRT-PCR and western blotting. The results showed that both mRNA ($p < 0.05$) (Fig. 6A) and protein (Fig. 6B) expression of ADAM22 in MCF-7/TAM cells with ADAM22 vector was significantly higher than those with EC; simultaneously, both mRNA ($p < 0.05$) (Fig. 6A) and protein (Fig. 6B) expression of ADAM22 in MCF-7 cells was effectively inhibited by ADAM22-siRNA. Then tamoxifen resistance of ER-positive breast cancer cells was assessed by CCK-8 assay. Data showed that the viability of MCF-7 cells co-transfected with miR-449a inhibitors and ADAM22-siRNA was significantly lower than that of miR-449a inhibitors and SC ($p< 0.05$) (Fig. 6C), which demonstrated that downregulation of ADAM22 could reduce the tamoxifen resistance induced by miR-449a inhibition. Besides, the tamoxifen sensitivity induced by miR-449a overexpression was reversed by the upregulation of ADAM22 in MCF-7/TAM cells (Fig. 6D). The results suggest that ADAM22 is critically involved in miR-449a-reduced tamoxifen resistance of ER-positive breast cancer cells as a direct target of miR-449a.

GO and Protein-protein interaction analyses of ADAM22

GO and Protein-protein interaction analyses were performed to identify the biological functions and mechanisms of ADAM22. GO analysis appeared that the products of adam22 gene were primarily located in the plasma membranes, also found in axon and integral component of membrane. ADAM22 was enriched in the biological processes of metalloendopeptidase activity, proteolysis, cell adhesion, negative regulation of cell adhesion, central nervous system development, metallopeptidase activity, adult locomotory behavior, Schwann cell differentiation, myelination in peripheral nervous system, and gliogenesis (Fig. 7A). The molecular functions of ADAM22 mainly included integrin binding and metalloendopeptidase activity. Protein-protein interaction analysis showed that ADAM22 interacted with 13 other protein including PPARG, LGI1, LYN, CSNK1E, HFE, DLG4, NXF1, LSMEM2, RAC2, APP, EPS8, PCDHA11, and KRAS (Fig. 7B).

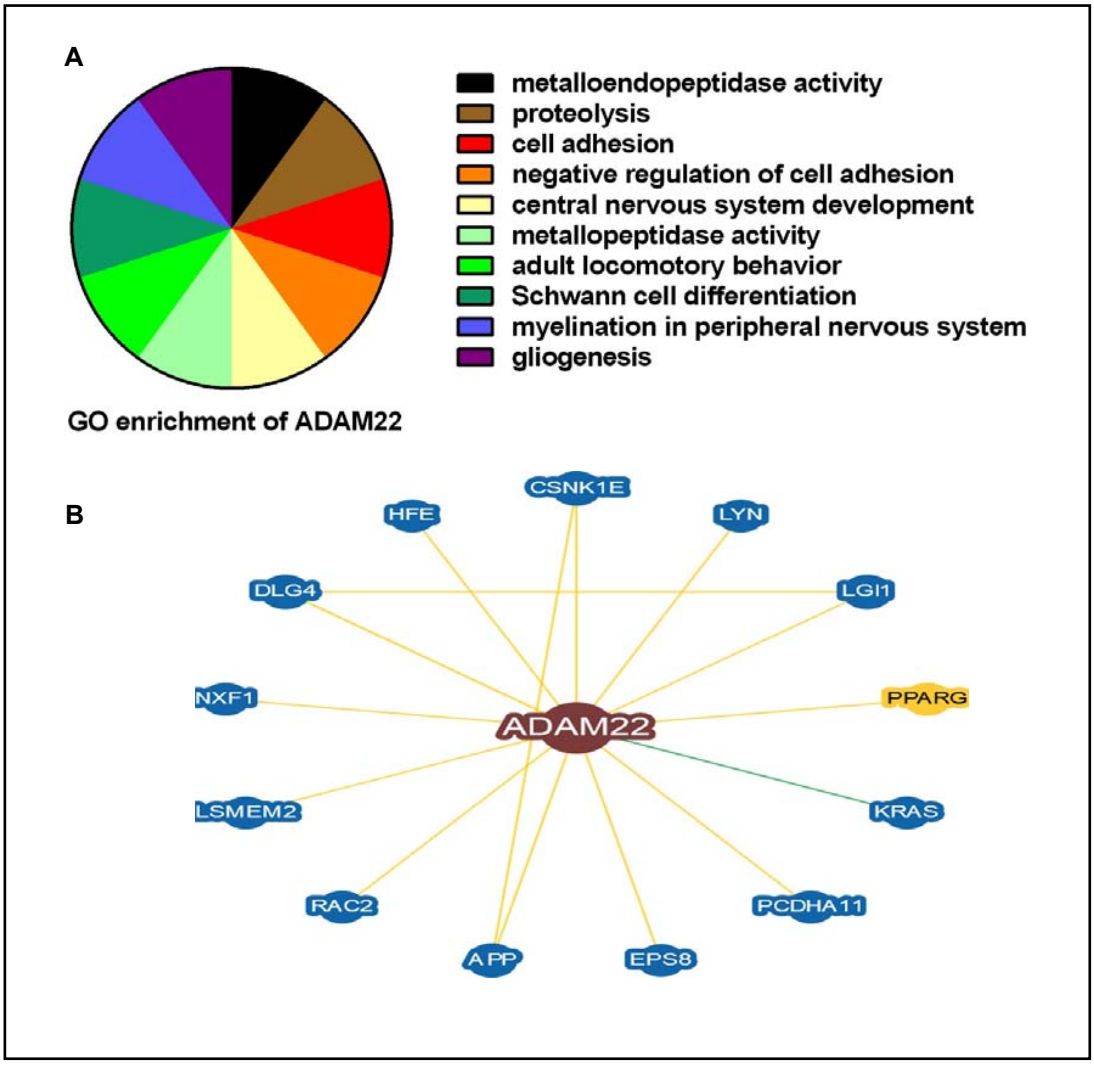


Fig. 7. GO and Protein-protein interaction analyses of ADAM22. GO analysis results showed that ADAM22 was enriched in the biological processes of cell adhesion, cell differentiation, gliogenesis and so on (A). Protein-protein interaction analysis showed that ADAM22 interacted with 13 other protein including PPARG, LGI1, LYN, KRAS, and so on (B).

Discussion

ER-positive breast cancers have high rates of metastasis and recurrence and are among the most threatening pathological types of cancer. Tamoxifen has been successfully used in clinical treatment of patients with ER-positive breast cancers. Nevertheless, the development of resistance to endocrine therapy is the biggest challenge for treatment of breast cancers with tamoxifen. By targeting oncogenes or tumor suppressors, miRNAs play divergent roles in cancer occurrence, progression, and drug resistance, and may be useful for cancer therapy by artificially counteracting the signals leading to carcinogenesis. There is an emerging interest in miRNAs as therapeutic targets in drug-resistant cancers. Recently, miR-449 has been identified in the progression of several cancers. For instance, miR-449 can suppress cell proliferation in hepatocellular carcinoma (HCC) [22]. MiR-449 can also inhibit cell migration and invasion in non-small cell lung cancer (NSCLC) [21, 31]. In breast cancer, miR-449a has been found to be a potent player in regulating the progression of breast cancer cells [19, 26]. In this study, we investigate the role of miR-449a as a suppressor in ER-positive breast cancer cells. We found that miR-449a was downregulated in tamoxifen-resistant breast cancer sera and cell lines, which is consistent with the previous reports that miR-449 expression was suppressed in breast cancer and other tumors as well as their cancer cell lines [19, 26]. Moreover, overexpression of miR-449a can restore tamoxifen sensitivity in ER-positive breast cancer cell lines, while the underlying mechanism is not clear yet.

Since miRNAs perform biological functions through negatively regulating their target genes, like inhibiting the translation or promoting the degradation of specific mRNAs by binding to the 3' UTR of target gene mRNAs, we used online miRNA target bioinformatics prediction databases to predict the potential targets of miR-449a. ADAM22 was validated to be a target gene of miR-449a, which is involved in miR-449a-mediated progression of breast cancer cell lines MCF-7 and T47D.

A disintegrin and metalloproteinase (ADAM) proteins are a family of membrane-anchored glycoproteins. The ADAM family proteins have been shown to play an important role in diverse biological processes such as neurogenesis, myogenesis, fertilization, cell signaling, inflammatory response, and cell–cell/cell–matrix interaction [32]. The structure of the ADAM22 protein suggests that it could function as an integrin ligand and play a role in cell–cell adhesion. Control of cell adhesion is important in many biological processes including embryonic development, and tumor cell invasion and metastasis. ADAM22 has been shown to interact with extracellular proteins to alter cellular processes. Previous studies have demonstrated that ADAM22 was a prognostic and therapeutic drug target that could help improve the treatment of endocrine-resistant breast cancer [33]. On the other hand, McCartan, D et al. confirmed that ADAM22 could stimulate the progress of ER-positive breast cancer patients by inducing de-differentiation and migration progression of breast cancer cells [34]. In their study, ADAM22 was identified to be a direct estrogen receptor (ER)-independent target of SRC-1, which is central to the development of the endocrine-resistant phenotype and is an independent predictor of disease-free survival in tamoxifen-treated patients. ADAM22 functioned in cellular migration and differentiation, and its levels were increased in endocrine resistant-tumors compared with endocrine-sensitive tumors in mouse xenograft models of human breast cancer. Besides, ADAM22 was found to serve as an independent predictor of poor disease-free survival clinically [34]. Consistently, our study showed that ADAM22 was overexpressed in tamoxifen-resistant breast cancer cell lines, suggesting it plays an important role in tamoxifen resistance. Then we revealed that ADAM22 was negatively regulated by miR-449a in breast cancer cells. Furthermore, miR-449a was confirmed to directly target ADAM22 and was identified as the key regulator of tamoxifen responsiveness via targeting ADAM22. To further establish the specific function of ADAM22 in miR-449a-mediated endocrine resistance to tamoxifen, we conducted the rescue strategy. Data showed that tamoxifen resistance induced by miR-449a downregulation could be reversed by the inhibition of ADAM22. All our results suggest that miR-449a attenuates tamoxifen resistance in ER-positive breast cancers by directly inhibiting ADAM22.

Since ADAM22 has been identified to improve disease progression in tamoxifen-resistant breast cancer patients, the brief mechanism is not well elucidated. GO analysis results showed that ADAM22 was mainly enriched in the biological processes of cell adhesion, cell differentiation, gliogenesis and so on. Although McCartan, D et al. demonstrated that ADAM22 could induce ER-positive breast cancer progression by LGI1-mediated migration [34], the role of ADAM22 played in de-differentiation is not clarified clearly. Protein-protein interaction analyzed data showed that ADAM22 could interact with not only LGI1, but also PPARG, KRAS, LYN and so on. Min, Y.S. et al. demonstrated that PPARγ expression at the mRNA and protein levels was higher in tamoxifen-resistant cells compared with parental MCF-7 cells, indicating that PPARγ plays an essential role in the regulation of CK20 expression in tamoxifen-resistant cells and ultimately enhances breast cancer cell invasiveness [35]. Combining whole-genome shRNA screening with massively parallel sequencing, along with subsequent validation experiments, Mendes-Pereira, A. M. et al. identified that silencing of KRAS causes sensitivity to tamoxifen in breast cancer [36]. Additionally, Liu, L.Y. et al. confirmed high expression levels of LYN to be a predictor for poor clinical outcome in ER-positive breast cancer and a novel enhancer of tamoxifen resistance [37]. As a pivotal protein in tamoxifen resistance of ER-positive breast cancer, ADAM22 may also play a role in the progression of tamoxifen-resistant breast cancer through these proteins. Further studies are warranted to explore the mechanisms of ADAM22 in regulating endocrine resistance. In conclusion, we identified that miR-449a induced tamaxifen sensitivity via ADAM22 in breast cancer cells. Our results may provide useful information for the development of alternative approaches to diagnose and treat tamoxifen-resistant breast cancer.

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Disclosure Statement

All authors declare that there is no conflict of interest regarding the publication of this article.

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