European Review for Medical and Pharmacological Sciences 2019; 23: 1563-1573

Downregulated lncRNA ADAMTS9-AS2 in breast cancer enhances tamoxifen resistance by activating microRNA-130a-5p

Y.-F. SHI[1,2], H. LU[3], H.-B. WANG[4]

[1]Center of Diagnosis and Treatment of Breast Disease, Qingdao University, Qingdao, China
[2]Department of Breast and Thyroid Surgery, Affiliated Hospital of Jining Medical University, Jining, China
[3]Collage of Basic Medicine, Jining Medical University, Jining, China
[4]Center of Diagnosis and Treatment of Breast Disease, The Affiliated Hospital of Qingdao University, Qingdao, China

Yafei Shi and Hai Lu contributed equally to this work

Abstract. – OBJECTIVE: The aim of this study was to elucidate the regulatory effect of long non-coding RNA (lncRNA) ADAMTS9-AS2 on Tamoxifen (TAM) resistance in breast cancer (BC), and to explore its underlying mechanism.

PATIENTS AND METHODS: TAM-resistant BC cell lines were first verified. Subsequently, cell proliferation and apoptosis were detected using cell counting kit-8 (CCK-8), 5-ethynyl-2'-deoxyuridine (EdU) assay and flow cytometry, respectively. Protein levels of ABCB1, ABCC1, and ABCG2 in TAM-treated MCF-7 and MCF-7R cells were determined by Western blot. ADAMTS9-AS2 expression in BC tissues, para-cancerous tissues, as well as MCF-7 and MCF-7R cells, was accessed by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The correlation between ADAMTS9-AS2 expression with pathological grade and tumor size of BC was explored. Chromatin fractionation was conducted to elucidate the subcellular distribution of ADAMTS9-AS2. The binding condition between ADAMTS9-AS2 and microRNA-130a-5p, as well as microRNA-130a-5p and PTEN, was verified by the dual-luciferase reporter gene assay. Furthermore, regulatory effects of ADAMTS9-AS2, microRNA-130a-5p, and PTEN on the proliferation and apoptosis of MCF-7R cells were determined.

RESULTS: MCF-7R cells were identified with TAM resistance. ADAMTS9-AS2 was lowly expressed in BC tissues and MCF-7R cells. Particularly, ADAMTS9-AS2 expression in BC tissues with grade III-IV or tumor size ≥2 cm was significantly lower than that of controls. Dual-luciferase reporter gene assay confirmed the binding condition between ADAMTS9-AS2 and microRNA-130a-5p, showing a negative correlation indicated by Pearson correlation analysis. PTEN was positively correlated with ADAMTS9-AS2. Overexpression of ADAMTS9-AS2 reversed the increased viability and decreased apoptosis induced by microRNA-130a-5p mimics transfection. In addition, PTEN knockdown reversed the decreased viability and accelerated apoptosis caused by ADAMTS9-AS2 overexpression.

CONCLUSIONS: We found that ADAMTS9-AS2 is lowly expressed in BC tissues and drug-resistant BC cells. Low expression of ADAMTS9-AS2 inhibits PTEN expression and enhances tamoxifen resistance through targeting microRNA-130a-5p.

Key Words:
ADAMTS9-AS2, MicroRNA-130a-5p, Breast cancer (BC), Tamoxifen resistance.

Introduction

The global incidence and mortality of breast cancer (BC) rank first among female solid malignancies[1]. With the continuous development of national economy, the incidence of BC in China has increased each year[2]. In recent years, advanced early diagnostic techniques and comprehensive therapeutic methods have greatly improved the prognosis of BC patients. Tamoxifen (TAM) is the first-line drug used for endocrine therapy of BC, which effectively reduces the recurrence and metastasis rate of ER-positive BC. In the past few decades, TAM treatment has remarkably prolonged the survival of BC patients[3]. However, the emergence of TAM resistance results in tumor recurrence and metastasis, seriously affecting the prognosis of these patients[4]. Multiple researches have been performed to elucidate the mechanism of TAM resistance. However, it is still a difficulty in clinical application. Hence, searching for new

Corresponding Author: Haibo Wang, MD; e-mail: hbwang66@126.com

targets and intervention methods is of great significance for the treatment of GC.

Long non-coding RNA (lncRNA) contains over 200 nt in length, and it cannot encode protein. LncRNAs are capable of regulating cellular physiological processes, such as epigenetic modification, gene transcription, and post-transcriptional modification. Meanwhile, they participate in the biological interaction of various types of cells[5]. In recent years, lncRNA has been well recognized in oncology researches. A large number of studies have reported the functions of lncRNAs in tumor occurrence, development, metastasis, and drug resistance. Moreover, it has great potential as a new prognostic indicator or therapeutic target[6]. Several lncRNAs have been identified to be closely related to the occurrence and prognosis of BC. Besides, lncRNAs relative to TAM resistance have also been reported[7,8]. In this study, we aim to explore relative lncRNAs that are involved in TAM resistance in BC, so as to provide potential therapeutic targets for BC.

LncRNA ADAMTS9-AS2 (ADAMTS9 antisense RNA 2) is an antisense transcript of tumor suppressor ADAMTS9. Recently, it has been confirmed to inhibit glioma cell migration and influence glioma survival[9]. In addition, microarray analysis has shown that ADAMTS9-AS2 is abnormally expressed in gastric cancer, colon cancer, liver cancer, and lung cancer, which may serve as a tumor biomarker[10]. However, the exact role of lncRNA ADAMTS9-AS2 in the pathogenesis and drug resistance of BC has not been studied.

Patients and Methods

Cell Lines

BC cell line MCF-7 was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA), and MCF-7R cells were gifted from Dr. Stephen Ethier (University of Michigan, Ann Arbor, MI, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Hyclone, UT, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). All cell lines were preserved in the laboratory of the Affiliated Hospital of Jining Medical University.

Cell Proliferation Assay

Cells were inoculated into 96-well plates at a density of 2×10^3 cells/well. After cell adherence, 10 μL of Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) solution was added to each well, followed by incubation at 37°C for 1 h in the dark. The absorbance of each well at 450 nm was recorded by a microplate reader.

Western Blot

Total protein in cells was lysed with radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China). The concentration of extracted protein was quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Protein expressions of genes in cells were detected in strict accordance with standard protocols of Western blot.

Flow Cytometry

Cells were washed with phosphate-buffered saline (PBS) twice and digested with Ethylene Diamine Tetraacetic Acid (EDTA)-free trypsin. After resuspension, the cells were transferred to a flow cytometry tube, incubated with buffer and antibodies for 15 min in the dark. Apoptosis was determined within 1 hour by flow cytometry.

Tissue Collection

BC tissues and para-cancerous tissues (5 cm away from tumor lesions) were surgically resected and preserved in liquid nitrogen. Samples were harvested from Breast Surgery Department, the Affiliated Hospital of Jining Medical University, labeling with collection date and type. General information of BC patients was recorded, including hospital number, age, pathological grade, and lymph node metastasis. The informed consent was obtained from each patient before sample collection. This study was approved by the Ethics Committee of the Affiliated Hospital of Jining Medical University.

RNA Extraction

Tissues (0.5×0.5×0.5 cm) were ground in liquid nitrogen. Tissue powder was transferred to an EP tube and incubated with 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA). Then, the cells were washed with PBS for three times and incubated with 1 mL of TRIzol. Tissues or cells were fully lysed on ice, extracted by chloroform and precipitated by isopropanol. The precipitation was washed with 75% ethanol, air dried and diluted in 20 μL of diethyl pyrocarbonate (DEPC) water.

Quantitative Real Time-Polymerase Chain Reaction (QRT-PCR)

Extracted RNA was quantified and reversely transcribed into cDNA for qRT-PCR detection using PrimeScript RT Master Mix. QRT-PCR was performed according to the instructions of SYBR® Green Master Mix (TaKaRa, Otsu, Shiga, Japan). GAPDH was used as an internal control. QRT-PCR parameters were: denaturation at 95°C for 15 min, followed by 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s, for a total of 40 cycles. Primers used in this study were as follows: GAPDH, F: 5′,-CACCCACTCCTCCACCTTTG-3′, R: 5′,-CCACCACCCTGTTGCTGTAG-3′,; ADAMTS9-AS2, F: 5′,-TCTGTTGCCCATTTCCTACC-3′,, R: 5′,-CCCTTCCATCCTGTCTACTCTA-3′,; PTEN, F: 5′,-TGGATTCGACTTAGACTTGACCT-3′,, R: 5′,- GGTGGGTTATGGTCTTCAAAAGG-3′,; MicroRNA-130a-5p, F: 5'-ACACTCCAGCTGGGTTCAGCTCCTATATGAT-3', R: 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAAAGGCAT-3'.

Chromatin Fractionation

Cells were re-suspended, and 200 μL of the suspension was transferred to a new EP tube for whole cell lysis. The remaining suspension was used for cytoplasm and nucleus isolation. After centrifugation, the supernatant was transferred to a RNase-free EP tube and incubated with 500 μL of RNAiso Plus Reagent for total RNA extraction. 200 μL of buffer A were added and gently mixed, and the supernatant was cytoplasmic cell. The precipitate was incubated with buffer S1, buffer S2, and RNAiso Plus Reagent. After centrifugation, the supernatant was cell nucleus.

Cell Transfection

Cells were incubated with 1.5 ml of serum-free medium and 500 μL of a mixture solution containing transfection reagent and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Complete medium was replaced 4-6 hours later. Primers of pcDNA-ADAMTS9-AS2, si-ADAMTS9-AS2 (GCCTCCTGGTTTATAGCATGA) and microRNA-130a-5p mimics were constructed by GenePharma (Shanghai, China).

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

Cells were inoculated into 96-well plates, and 100 μL of EdU reagent (50 μM) was added to each well for 2 hours of incubation. After washing with PBS, the cells were fixed in 50 μL of fixation buffer, decolored with 2 mg/mL glycine, and permeated with 100 μL of penetrant. After washing with PBS once, the cells were stained with 100 μL of Apollo in the dark for 30 min. Then, the cells were washed with penetrant and methanol. Subsequently, the cells were incubated with 100 μL of 1×Hoechst 33342, followed by viability determination under a fluorescent microscope.

Dual-Luciferase Reporter Gene Assay

According to predicted binding sites, mutant-type and wild-type plasmids of ADAMTS9-AS2 were constructed. Briefly, cells were seeded into 12-well plate. Until 70% of confluence, the cells were co-transfected with 50 pmol/L microRNA-130a-5p mimics or negative control and 80 ng mutant-type or wild-type plasmids of ADAMTS9-AS2. After transfection for 48 hours, 50 μL of 1× PLB was added in each well to lyse the cells fully. Finally, luciferase activity was determined.

RNA-Immunoprecipitation (RIP)

RIP assay was conducted in strict accordance with the instructions of Magna RIPTM RNA kit (Millipore, Bedford, MA, USA). When the confluence was up to 80-90%, the cells were lysed with RIPA containing protease inhibitor and RNA enzyme inhibitor. Cell extraction was incubated with IgG and MS2 antibodies. Protein samples were digested, and immuno-precipitated RNA was harvested. The expression level of purified RNA was detected by qRT-PCR.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 (Chicago, IL, USA) was used for all statistical analysis. Experimental data were expressed as mean ± SD ($\bar{x}\pm s$) from three independent records. The standard *t*-test was used to compare the differences between the two groups. Pearson correlation analysis was used to compare the correlation between ADAMTS9-AS2 with microRNA-130a-5p and PTEN expressions. GraphPad Prism was introduced for figure editing. $p<0.05$ was considered statistically significant.

Results

Verification of TAM-Resistant BC Cell Lines

Firstly, tumor-promoting effects of TAM-resistant BC cell line MCF-7R and non-resistant BC cell line MCF-7 were compared. All cells were assigned

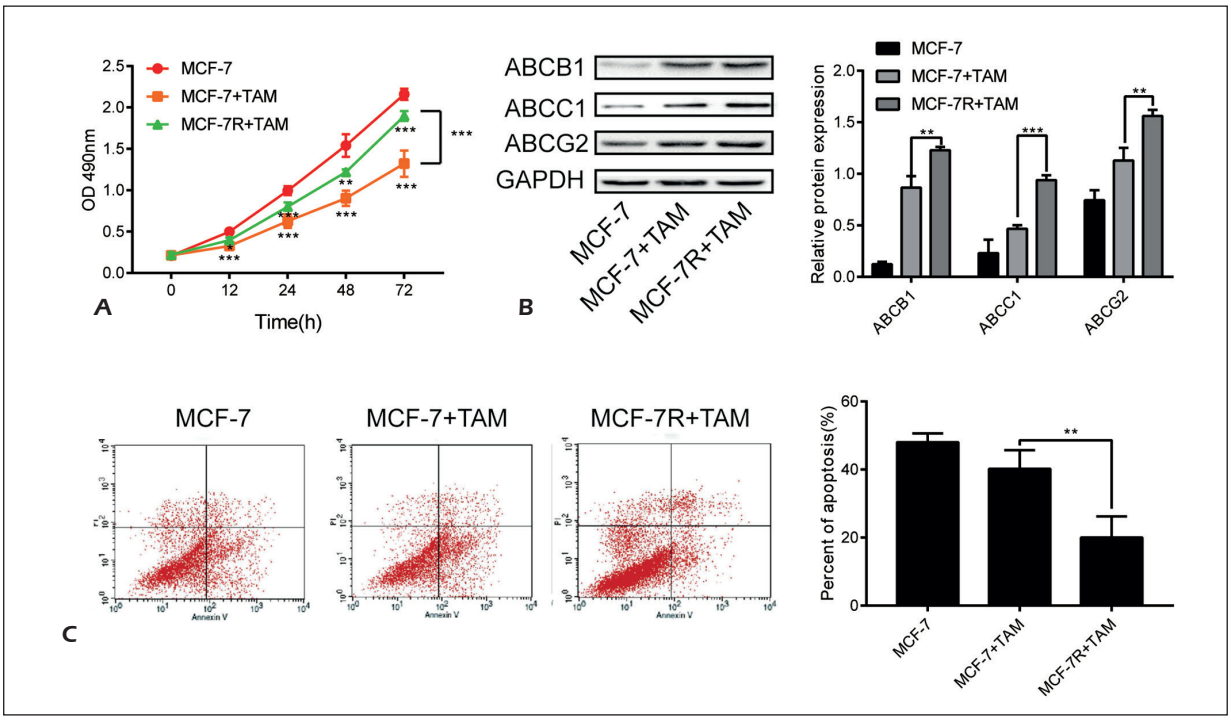

Figure 1. Verification of TAM-resistant BC cell lines. **A**, CCK-8 assay showed that MCF-7R cells had higher viability than MCF-7 cells. **B**, Western blot analysis of ABCB1, ABCC1 and ABCG2 in MCF-7, MCF-7+TAM and MCF-7R+TAM cells. **C**, Flow cytometry indicated a lower percent of apoptosis in the MCF-7R+TAM group compared with the MCF-7+TAM group. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

into three groups, namely MCF-7, MCF-7+TAM, and MCF-7R+TAM. Cell viability was accessed at 0 h, 12 h, 24 h, 48 h, and 72 h, respectively. Results indicated that cells in the MCF-7+TAM group showed significantly decreased viability. Although cell viability was reduced in the MCF-7R+TAM group, it was still higher than that of the MCF-7+TAM group (Figure 1A). Protein expressions of ABC transporter proteins (ABCB1, ABCC1, ABCG2) involved in drug transport or redistribution were detected by Western blot. Results showed that the expression levels of these proteins in the MCF-7R+-TAM group were significantly upregulated than the MCF-7+TAM group (Figure 1B). Furthermore, flow cytometry data demonstrated that the percent of apoptosis in the MCF-7R+TAM group was remarkably lower when compared with the MCF-7+TAM group (Figure 1C). These results all demonstrated the TAM resistance in MCF-7R cells. The cells were utilized for subsequent experiments.

Low Expression of ADAMTS9-AS2 in BC

The expression level of ADAMTS9-AS2 in 52 pairs of BC tissues and para-cancerous tissues was detected by qRT-PCR. ADAMTS9-AS2 was lowly expressed in BC tissues (Figure 2A). Besides, ADAMTS9-AS2 expression in MCF-7R cells was significantly decreased when compared with MCF-7 cells (Figure 2B). Significantly lower expression of ADAMTS9-AS2 was observed in BC tissues with pathological grade III-IV than those with grade I-II (Figure 2C). Furthermore, ADAMTS9-AS2 expression in BC tissues with tumor size ≥ 2 cm was remarkably lower than those with tumor size < 2 cm (Figure 2D). The above results suggested that ADAMTS9-AS2 might be closely related to drug resistance in BC.

ADAMTS9-AS2 Enhanced TAM-Sensitivity of MCF-7R Cells

To elucidate the potential mechanism of ADAMTS9-AS2 in regulating drug resistance, we first detected cytoplasmic and nuclear expressions of ADAMTS9-AS2 through chromatin fractionation assay. ADAMTS9-AS2 was mainly distributed in the cytoplasm, indicating that it might be involved in post-transcriptional regulation (Figure 3A). Subsequently, ADAMTS9-AS2 expression was effectively downregulated or upregulated by transfection of si-ADAMTS9-AS2 or pcNDA-ADAMTS9-AS2, respectively (Figure

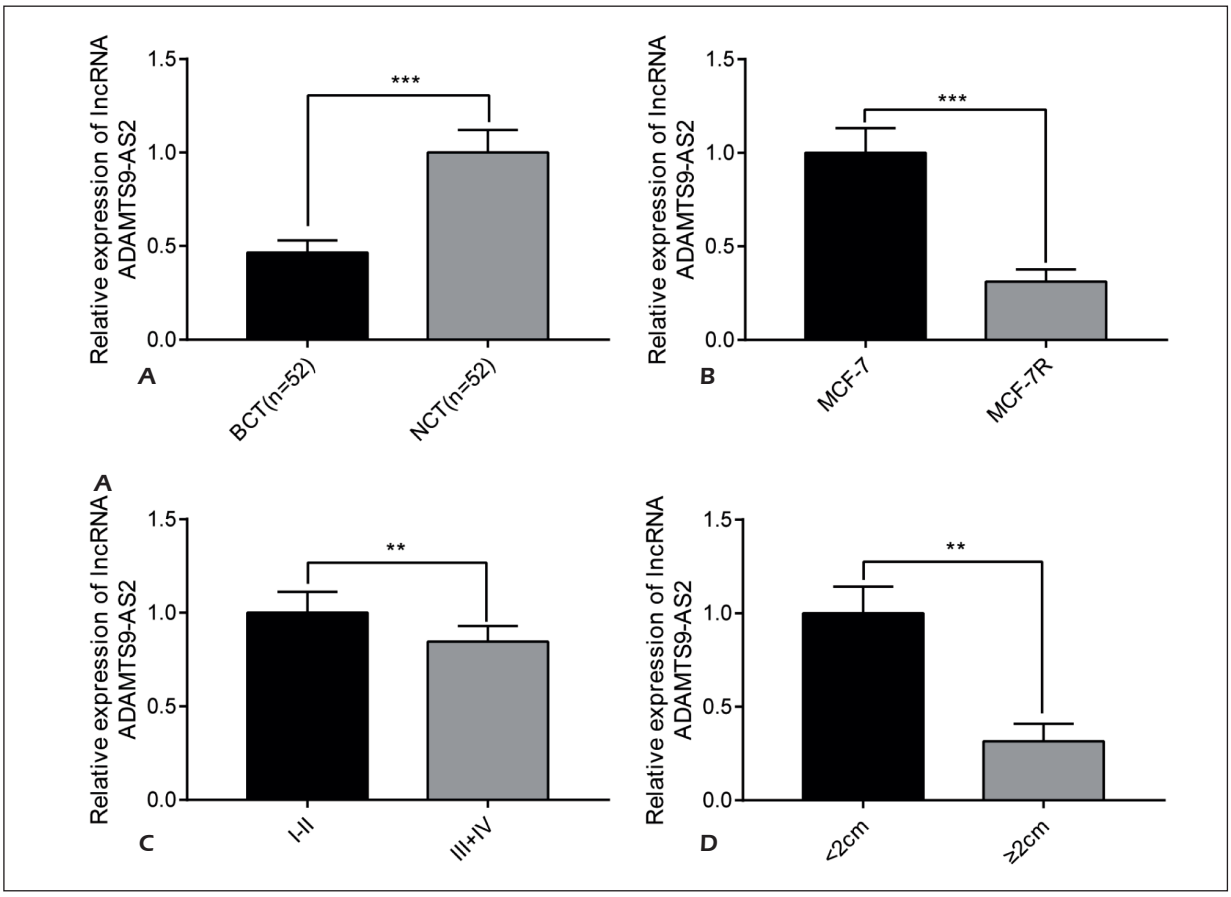

Figure 2. Low expression of ADAMTS9-AS2 in BC. **A**, ADAMTS9-AS2 was lowly expressed in BC tissues compared with para-cancerous tissues. **B**, ADAMTS9-AS2 was lowly expressed in MCF-7R cells compared with MCF-7 cells. **C**, ADAMTS9-AS2 expression was lower in BC tissues with pathological grade III-IV compared with those with grade I-II. **D**, ADAMTS9-AS2 expression was lower in BC tissues with tumor size ≥ 2 cm than those with tumor size <2 cm. *p<0.05, **p<0.01, ***p<0.001.

3B). Both CCK-8 and EdU assay demonstrated that upregulation of ADAMTS9-AS2 markedly promoted the proliferative potential of MCF-7R cells. However, ADAMTS9-AS2 knockdown significantly inhibited cell proliferation (Figure 3C and 3D). Flow cytometry indicated that ADAMTS9-AS2 overexpression remarkably accelerated the apoptosis of MCF-7R cells. However, ADAMTS9-AS2 knockdown had little effect on cell apoptosis (Figure 3E). The above data demonstrated that ADAMTS9-AS2 overexpression enhanced TAM-sensitivity of MCF-7R cells.

MicroRNA-130a-5p Was the Downstream Target of ADAMTS9-AS2

The above results proved that ADAMTS9-AS2 was mainly distributed in the cytoplasm, which might exert a post-transcriptional regulatory function. Bioinformatics predicted that microRNA-130a-5p was the target gene of ADAMTS9-AS2. According to the predicted binding sites, mutant-type and wild-type plasmids of ADAMTS9-AS2 were constructed. Subsequent dual-luciferase reporter gene assay demonstrated that the luciferase activity in cells co-transfected with wild-type ADAMTS9-AS2 and microRNA-130a-5p mimics was remarkably decreased, indicating their binding condition (Figure 4A). RIP assay showed a significantly higher abundance of microRNA-130a-5p in the ADAMTS9-AS2 group. However, no evident change was observed after the binding sites in ADAMTS9-AS2 were mutated (Figure 4B). MicroRNA-130a-5p expression was markedly upregulated by si-ADAMTS9-AS2 transfection, which was significantly downregulated after transfection of pcDNA-ADAMTS9-AS2 (Figure 4C). Moreover, microRNA-130a-5p was highly expressed in MCF-7R cells and BC tissues when compared with controls (Figure 4D and 4E). Pear-

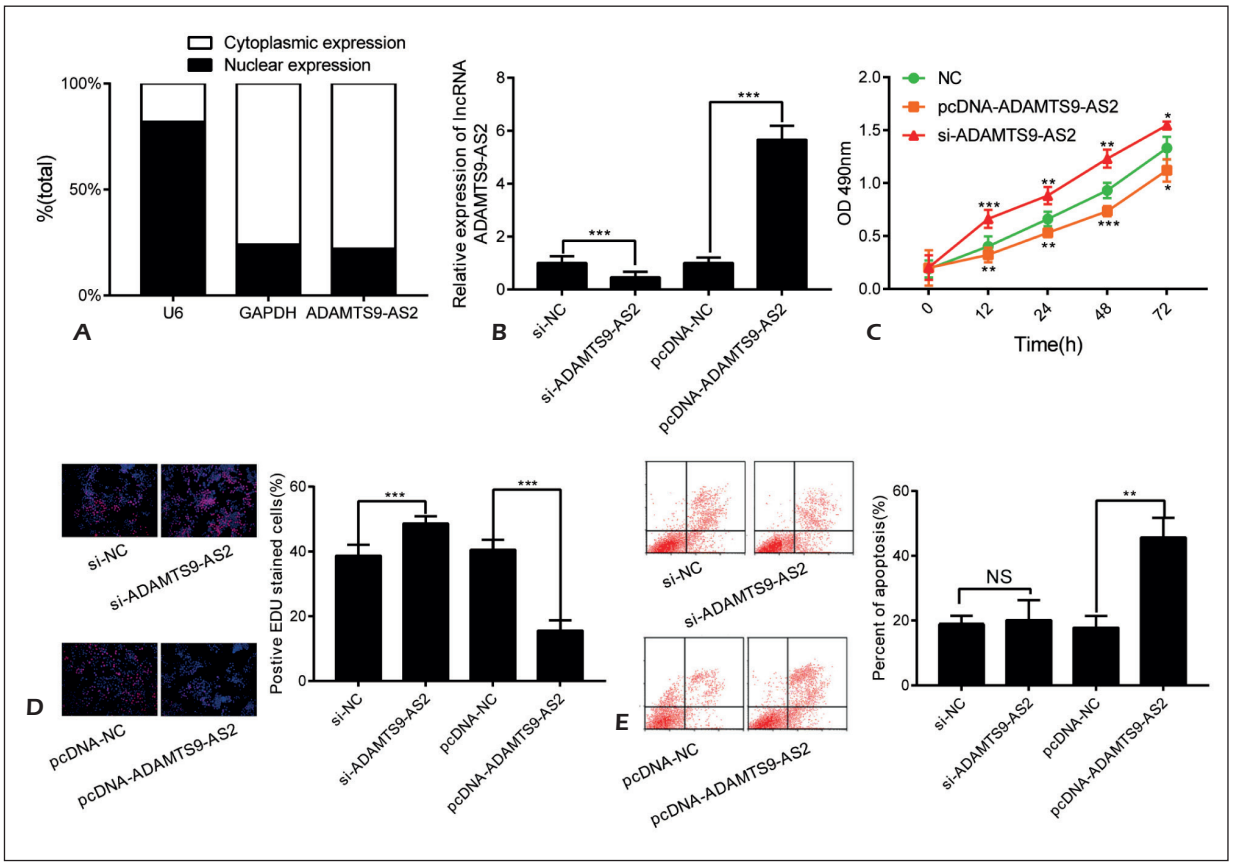

Figure 3. ADAMTS9-AS2 enhanced TAM-sensitivity of MCF-7R cells. **A**, ADAMTS9-AS2 was mainly distributed in the cytoplasm. **B**, Transfection efficacy of si-ADAMTS9-AS2, si-NC, pcDNA-ADAMTS9-AS2 and pcDNA-NC in MCF-7R cells was detected by qRT-PCR. **C**, CCK-8 assay showed that 10 μM TAM-induced MCF-7R cells transfected with si-ADAMTS9-AS2 showed higher viability than those transfected with si-NC. Transfection of pcDNA-ADAMTS9-AS2 decreased the viability of MCF-7R cells. **D**, EdU assay showed that 10 μM TAM-induced MCF-7R cells transfected with si-ADAMTS9-AS2 showed higher viability than those transfected with si-NC. Transfection of pcDNA-ADAMTS9-AS2 decreased the viability of MCF-7R cells. **E**, Flow cytometry showed that ADAMTS9-AS2 overexpression accelerated apoptosis of MCF-7R cells. However, ADAMTS9-AS2 knockdown had little effect on apoptosis. *p<0.05, **p<0.01, ***p<0.001.

son correlation analysis revealed a negative correlation between ADAMTS9-AS2 and microRNA-130a-5p (Figure 4F).

ADAMTS9-AS2 Reversed the Inhibitory Effect of MicroRNA-130a-5p on TAM-Sensitivity in BC

The above findings proved that microRNA-130a-5p could bind to ADAMTS9-AS2. However, its specific role in the development of BC remained unclear. Both CCK-8 and EdU assay indicated that transfection of microRNA-130a-5p mimics significantly enhanced the viability of MCF-7R cells, which could be reversed by ADAMTS9-AS2 overexpression (Figure 5A-C). The apoptosis of MCF-7R cells was remarkably inhibited by microRNA-130a-5p overexpression, which was greatly enhanced after co-transfection of microRNA-130a-5p mimics and pcDNA-ADAMTS9-AS2 (Figure 5D). Therefore, we proposed that ADAMTS9-AS2 reversed the inhibitory effect of microRNA-130a-5p on TAM-sensitivity in MCF-7R cells.

ADAMTS9-AS2 Upregulated PTEN by Targeting MicroRNA-130a-5p

Typically, miRNAs exert their functions by binding to the 3'UTR of target genes. Here, PTEN was predicted as the target gene of microRNA-130a-5p by bioinformatics. Both the mRNA and protein levels of PTEN in MCF-7R cells were significantly downregulated than MCF-7 cells (Figure 6A and 6B). Notably, the mRNA and

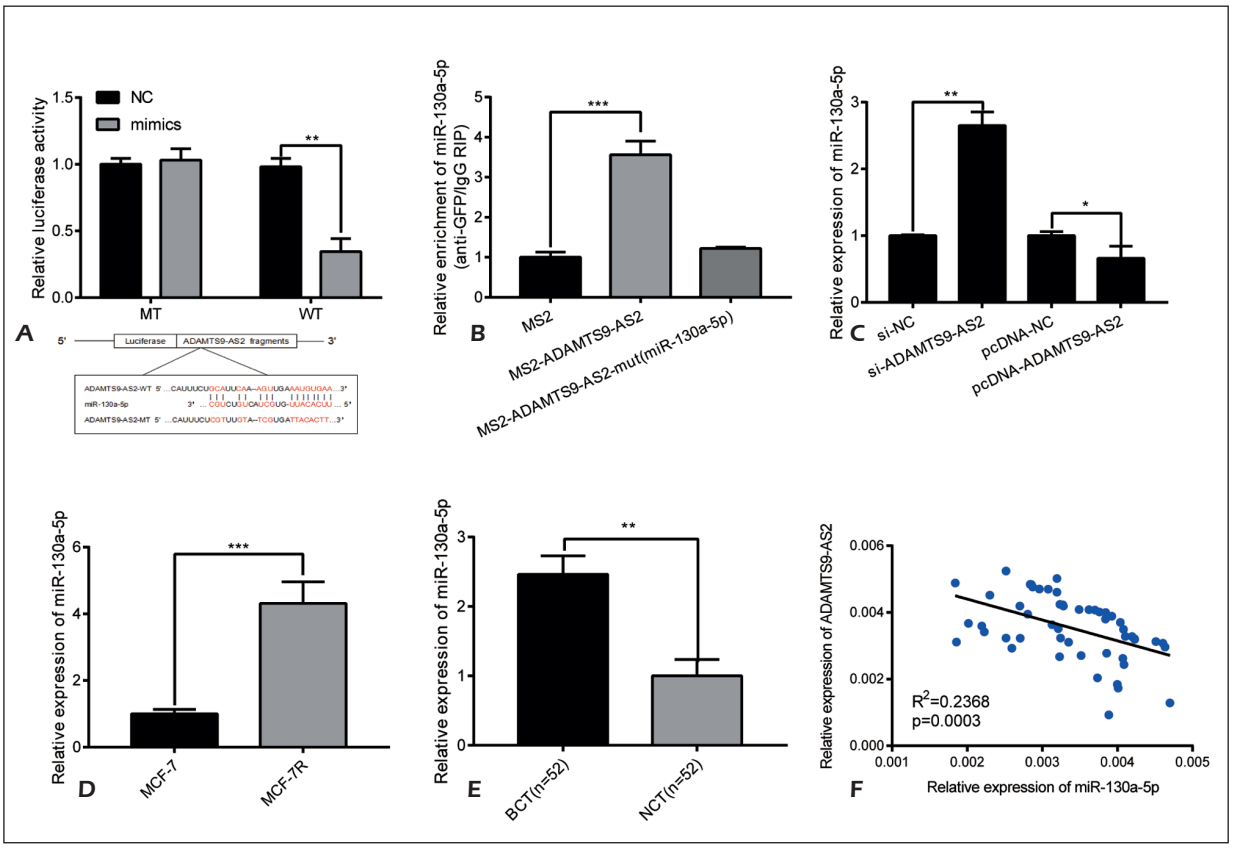

Figure 4. MicroRNA-130a-5p was the downstream target of ADAMTS9-AS2. **A**, Construction of mutant-type and wild-type plasmids of ADAMTS9-AS2. Luciferase activity remarkably decreased in cells co-transfected with wild-type ADAMTS9-AS2 and microRNA-130a-5p mimics. **B**, RIP assay showed a higher abundance of microRNA-130a-5p in the ADAMTS9-AS2 group, whereas no obvious change was observed after the binding sites in ADAMTS9-AS2 were mutated. **C**, MicroRNA-130a-5p expression was markedly upregulated by si-ADAMTS9-AS2 transfection, whereas downregulated after transfection of pcDNA-ADAMTS9-AS2. **D**, MicroRNA-130a-5p was highly expressed in MCF-7R cells than MCF-7 cells. **E**, MicroRNA-130a-5p was highly expressed in BC tissues (BCT) than para-cancerous tissues (NCT). **F**, Pearson correlation analysis revealed a negative correlation between ADAMTS9-AS2 and microRNA-130a-5p. *p<0.05, **p<0.01, ***p<0.001.

protein levels of PTEN were positively regulated by ADAMTS9-AS2 in MCF-7R cells (Figure 6C and 6D). Pearson correlation analysis further confirmed the positive correlation between the two molecules (Figure 6E). To elucidate the potential interaction between PTEN and ADAMTS9-AS2, si-PTEN and pcDNA-ADAMTS9-AS2 were co-transfected into MCF-7R cells pre-treated with 10 μM TAM. CCK-8 and EdU assay indicated that the proliferative potential in co-transfected cells was markedly enhanced when compared with that of cells transfected with pcDNA-ADAMTS9-AS2 alone (Figure 6F and 6G). Meanwhile, the apoptosis was remarkably inhibited in co-transfected MCF-7R cells than cells overexpressing ADAMTS9-AS2 (Figure 6H). Therefore, we verified that ADAMTS9-AS2 could bind to microRNA-130a-5p and upregulate PTEN expression.

Discussion

BC has gradually become the most common malignant tumor in Chinese women[2]. TAM-based chemotherapy is an important endocrine therapy for patients with ER-positive BC, which markedly improves the prognosis. However, drug resistance of TAM also exists in tumor patients. Meanwhile, the emergence of drug resistance seriously affects its therapeutic efficacy[3,4].

Multiple genes and pathways have been identified to participate in TAM resistance, including ER. In the post-genome era, the role of lncRNA has attracted great concern and some achievements have been acquired. For example, lncRNA HOTAIR has been proved to regulate TAM resistance through ER[11,12]. However, the conservatism of lncRNA largely limits relative

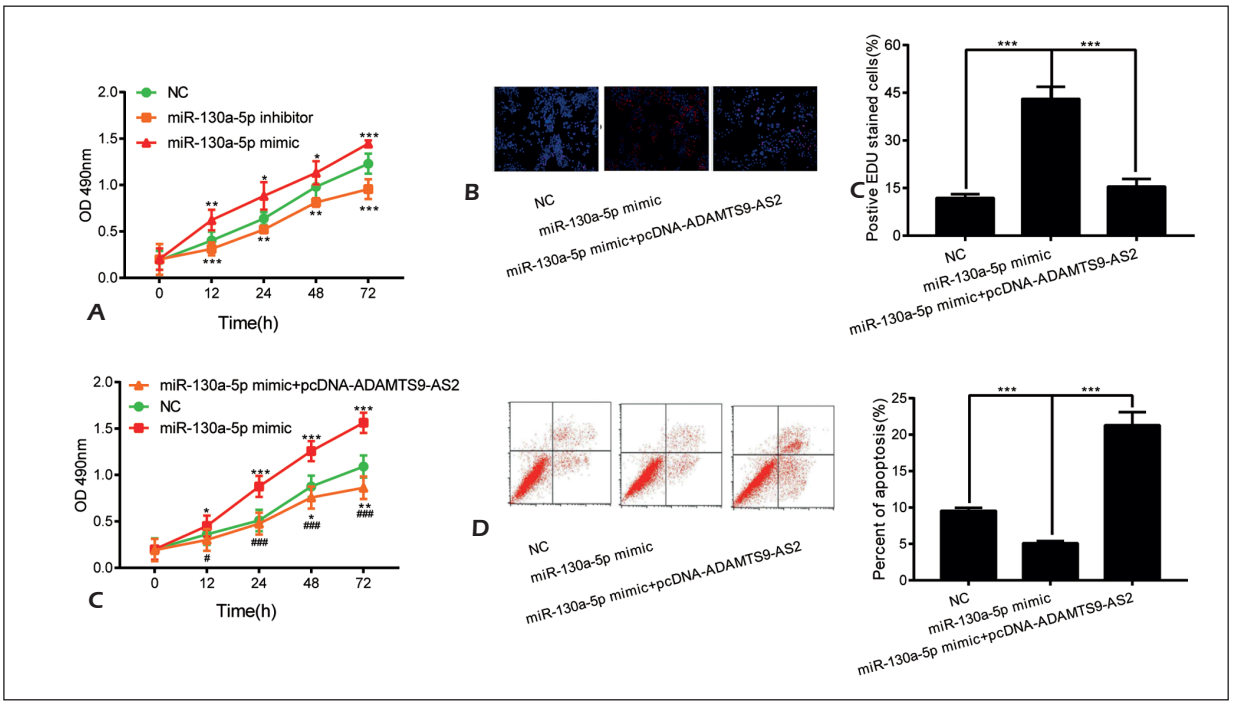

Figure 5. ADAMTS9-AS2 reversed the inhibitory effect of microRNA-130a-5p on TAM-sensitivity in BC. **A**, CCK-8 assay showed transfection of microRNA-130a-5p mimics enhanced the viability of MCF-7R cells, whereas transfection of microRNA-130a-5p inhibitor suppressed cell viability. **B**, CCK-8 assay showed that after 10 μM TAM treatment in MCF-7R cells, the microRNA-130a-5p mimics + pcDNA-ADAMTS9-AS2 group showed lower viability than the microRNA-130a-5p mimics group. **C**, EdU assay showed that the microRNA-130a-5p mimics group had higher viability than the NC group. Viability in the microRNA-130a-5p mimics + pcDNA-ADAMTS9-AS2 group was decreased compared with the microRNA-130a-5p mimics group. **D**, Flow cytometry showed that the microRNA-130a-5p mimics group had a lower percent of apoptosis than the NC group. Apoptosis in the microRNA-130a-5p mimics + pcDNA-ADAMTS9-AS2 group was enhanced compared with the microRNA-130a-5p mimics group. *p<0.05, **p<0.01, ***p<0.001.

researches in oncology. A type of lncRNAs, also known as ultra-conserved elements, are naturally highly conserved. They are of great value in regulating the occurrence and progression of tumors[13-15]. LncRNA ADAMTS9-AS2 participates in the development of various tumors. Studies have shown that the upregulation of lncRNA ADAMTS9-AS2 promotes the metastasis of salivary adenoid cystic carcinoma through PI3K/Akt and MEK/ERK pathways[16]. The downregulation of ADAMTS9-AS2 promotes the development of gastric cancer by activating the PI3K/AKT pathway[17]. ADAMTS9-AS2 regulates the progression of ovarian cancer by targeting the miR-182-5p/FOXF2 axis[18]. In this study, we analyzed the differentially expressed lncRNAs in TAM-resistant BC cell line MCF-7R and its parental cell line MCF-7. Results found that lncRNA ADAMTS9-AS2 was markedly downregulated in MCF-7R cells. Consistently, ADAMTS9-AS2 was lowly expressed in BC tissues, which was negatively correlated with the pathological grade of BC. A significantly lower expression of ADAMTS9-AS2 was observed in BC tissues with pathological grade III-IV compared with those with grade I-II. Furthermore, ADAMTS9-AS2 expression was remarkably lower in BC tissues with tumor size ≥ 2 cm than those with tumor size < 2 cm. Biological functional experiments revealed that the low expression of ADAMTS9-AS2 markedly promoted the proliferative potential of MCF-7R cells, but could not influence cell apoptosis. On the contrary, the ADAMTS9-AS2 overexpression inhibited proliferation and accelerated apoptosis of MCF-7R cells.

Some lncRNAs can serve as ceRNAs to sponge miRNAs, eventually leading to abnormal expressions of corresponding mRNAs. Hence, the downregulation of these lncRNAs accelerate the binding of miRNAs with their target mRNAs, which could inhibit the expressions of tumor suppressors[19-21]. Our study verified that ADAMTS9-AS2 was mainly distributed in the cytoplasm and could regulate tumor metastasis at the post-tran-

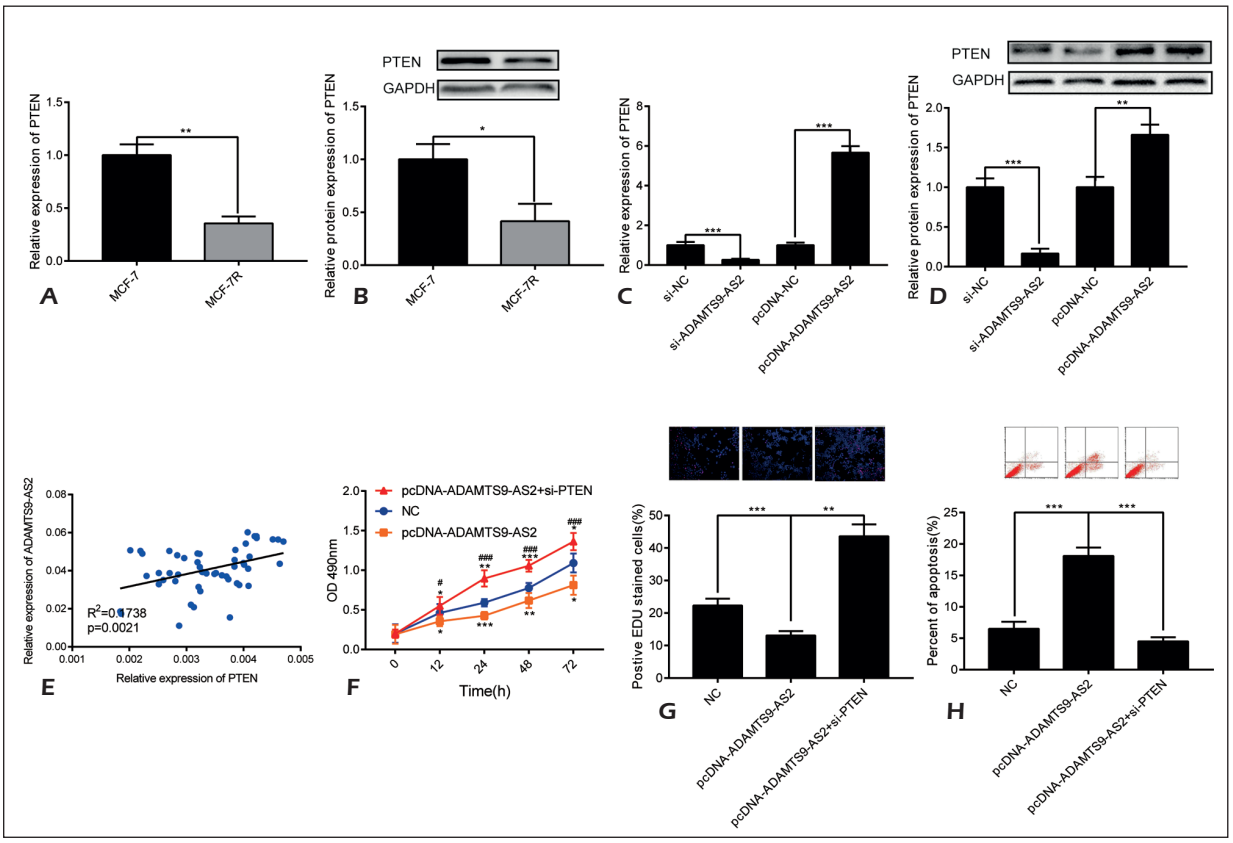

Figure 6. ADAMTS9-AS2 upregulated PTEN by targeting microRNA-130a-5p. **A**, The mRNA level of PTEN was downregulated in MCF-7R cells than MCF-7 cells. **B**, The protein level of PTEN was downregulated in MCF-7R cells than MCF-7 cells. **C**, The mRNA level of PTEN was positively regulated by ADAMTS9-AS2 in MCF-7R cells. **D**, The protein level of PTEN was positively regulated by ADAMTS9-AS2 in MCF-7R cells. **E**, Pearson correlation analysis showed a positive correlation between PTEN and ADAMTS9-AS2 in MCF-7R cells. **F**, CCK-8 assay showed higher viability in MCF-7R cells transfected with pcDNA-ADAMTS9-AS2+ si-PTEN than those transfected with pcDNA-ADAMTS9-AS2. **G**, EdU assay showed higher viability in MCF-7R cells transfected with pcDNA-ADAMTS9-AS2+ si-PTEN than those transfected with pcDNA-ADAMTS9-AS2. H, Flow cytometry showed a lower percent of apoptosis in MCF-7R cells transfected with pcDNA-ADAMTS9-AS2+ si-PTEN than those transfected with pcDNA-ADAMTS9-AS2. *p<0.05, **p<0.01, ***p<0.001.

scriptional level. Bioinformatics predicted that microRNA-130a-5p could bind to ADAMTS9-AS2 and microRNA-130a-5p could bind to PTEN.

The regulatory effect of miR-130a on the proliferative potential of tumor cells has been identified. For example, circular RNA-ZFR inhibits the proliferation of gastric cancer cells and accelerates apoptosis by sponging miR-130a/miR-107[22]. Most studies have confirmed the promotive role of miR-130a in tumor cell proliferation. It promotes the proliferation of gastric cancer cells and inhibits apoptosis by targeting RUNX3[23]. MiR-130a promotes the proliferation of cervical cancer cells by targeting PTEN[24]. It is reported that miR-130a is associated with drug resistance in ovarian cancer[25]. By detecting miR-130a expression in chemo-sensitive ovarian cancer cells and control cells, it has been found that miR-130a is highly expressed in the chemo-resistant group[26]. In the present work, the dual-luciferase reporter gene assay confirmed the binding condition between ADAMTS9-AS2 and microRNA-130a-5p. Further researches showed that ADAMTS9-AS2 knockdown could significantly upregulate microRNA-130a-5p expression. Subsequently, microRNA-130a-5p was found to be highly expressed in drug-resistant cells and BC tissues, showing a negative correlation with ADAMTS9-AS2. Functional experiments indicated that microRNA-130a-5p overexpression remarkably enhanced the proliferative potential but induced apoptosis of MCF-7R cells, which were interestingly reversed by ADAMTS9-AS2 overexpression.

PTEN is a protein and lipid diphosphatase that regulates cell signaling, growth, migration,

and apoptosis[27,28]. Our study found lowly expressed PTEN in BC tissues and drug-resistant cells. Functional experiments showed that PTEN knockdown reversed the inhibited proliferation and accelerated apoptosis induced by ADAMTS9-AS2 overexpression.

Conclusions

We found that ADAMTS9-AS2 is lowly expressed in BC and drug-resistant BC cells. Low expression of ADAMTS9-AS2 inhibits PTEN expression and enhances tamoxifen resistance through targeting microRNA-130a-5p.

Funding Acknowledgements

This work was supported by The Youth Foundation of Jining Medical University (JY2016KJ009Z).

Conflict of Interests

The authors declare that they have no conflict of interest.

References

1) Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2017. CA Cancer J Clin 2017; 67: 7-30.
2) Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. Cancer statistics in China, 2015. CA Cancer J Clin 2016; 66: 115-132.
3) [No authors listed]. Tamoxifen for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group. Lancet 1998; 351: 1451-1467.
4) Ring A, Dowsett M. Mechanisms of tamoxifen resistance. Endocr Relat Cancer 2004; 11: 643-658.
5) Ulitsky I, Bartel DP. lincRNAs: genomics, evolution, and mechanisms. Cell 2013; 154: 26-46.
6) Yang G, Lu X, Yuan L. LncRNA: a link between RNA and cancer. Biochim Biophys Acta 2014; 1839: 1097-1109.
7) Godinho MF, Sieuwerts AM, Look MP, Meijer D, Foekens JA, Dorssers LC, van Agthoven T. Relevance of BCAR4 in tamoxifen resistance and tumour aggressiveness of human breast cancer. Br J Cancer 2010; 103: 1284-1291.
8) Xue X, Yang YA, Zhang A, Fong KW, Kim J, Song B, Li S, Zhao JC, Yu J. LncRNA HOTAIR enhances ER signaling and confers tamoxifen resistance in breast cancer. Oncogene 2016; 35: 2746-2755.
9) Yao J, Zhou B, Zhang J, Geng P, Liu K, Zhu Y, Zhu W. A new tumor suppressor LncRNA ADAMTS9-AS2 is regulated by DNMT1 and inhibits migration of glioma cells. Tumour Biol 2014; 35: 7935-7944.
10) Yuan J, Yue H, Zhang M, Luo J, Liu L, Wu W, Xiao T, Chen X, Chen X, Zhang D, Xing R, Tong X, Wu N, Zhao J, Lu Y, Guo M, Chen R. Transcriptional profiling analysis and functional prediction of long noncoding RNAs in cancer. Oncotarget 2016; 7: 8131-8142.
11) Hayes EL, Lewis-Wambi JS. Mechanisms of endocrine resistance in breast cancer: an overview of the proposed roles of noncoding RNA. Breast Cancer Res 2015; 17: 40.
12) Xue X, Yang YA, Zhang A, Fong KW, Kim J, Song B, Li S, Zhao JC, Yu J. LncRNA HOTAIR enhances ER signaling and confers tamoxifen resistance in breast cancer. Oncogene 2016; 35: 2746-2755.
13) Lei T, Lv ZY, Fu JF, Wang Z, Fan Z, Wang Y. LncRNA NBAT-1 is down-regulated in lung cancer and influences cell proliferation, apoptosis and cell cycle. Eur Rev Med Pharmacol Sci 2018; 22: 1958-1962.
14) Katzman S, Kern AD, Bejerano G, Fewell G, Fulton L, Wilson RK, Salama SR, Haussler D. Human genome ultraconserved elements are ultraselected. Science 2007; 317: 915.
15) Peng JC, Shen J, Ran ZH. Transcribed ultraconserved region in human cancers. Rna Biol 2013; 10: 1771-1777.
16) Xie S, Yu X, Li Y, Ma H, Fan S, Chen W, Pan G, Wang W, Zhang H, Li J, Lin Z. Upregulation of lncRNA ADAMTS9-AS2 promotes salivary adenoid cystic carcinoma metastasis via PI3K/Akt and MEK/Erk signaling. Mol Ther 2018 Aug 24. pii: S1525-0016(18)30421-0. doi: 10.1016/j.ymthe.2018.08.018. [Epub ahead of print].
17) Cao B, Liu C, Yang G. Down-regulation of lncRNA ADAMTS9-AS2 contributes to gastric cancer development via activation of PI3K/Akt pathway. Biomed Pharmacother 2018; 107: 185-193.
18) Wang A, Jin C, Li H, Qin Q, Li L. LncRNA ADAMTS9-AS2 regulates ovarian cancer progression by targeting miR-182-5p/FOXF2 signaling pathway. Int J Biol Macromol 2018; 120: 1705-1713.
19) Liu Z, Shao Y, Tan L, Shi H, Chen S, Guo J. Clinical significance of the low expression of FER1L4 in gastric cancer patients. Tumour Biol 2014; 35: 9613-9617.
20) Qiao Q, Li H. LncRNA FER1L4 suppresses cancer cell proliferation and cycle by regulating PTEN expression in endometrial carcinoma. Biochem Biophys Res Commun 2016; 478: 507-512.
21) Xia T, Chen S, Jiang Z, Shao Y, Jiang X, Li P, Xiao B, Guo J. Long noncoding RNA FER1L4 suppresses cancer cell growth by acting as a competing endogenous RNA and regulating PTEN expression. Sci Rep 2015; 5: 13445.
22) Jiang H, Yu WW, Wang LL, Peng Y. miR-130a acts as a potential diagnostic biomarker and promotes gastric cancer migration, invasion and prolifera-

tion by targeting RUNX3. Oncol Rep 2015; 34: 1153-1161.

23) Zhang J, Wu H, Li P, Zhao Y, Liu M, Tang H. NF-kappaB-modulated miR-130a targets TNF-alpha in cervical cancer cells. J Transl Med 2014; 12: 155.

24) Shen S, Guo X, Yan H, Lu Y, Ji X, Li L, Liang T, Zhou D, Feng XH, Zhao JC, Yu J, Gong XG, Zhang L, Zhao B. A miR-130a-YAP positive feedback loop promotes organ size and tumorigenesis. Cell Res 2015; 25: 997-1012.

25) Zhang X, Huang L, Zhao Y, Tan W. Downregulation of miR-130a contributes to cisplatin resistance in ovarian cancer cells by targeting X-linked inhibitor of apoptosis (XIAP) directly. Acta Biochim Biophys Sin (Shanghai) 2013; 45: 995-1001.

26) Rybstein MD, Bravo-San PJ, Kroemer G, Galluzzi L. The autophagic network and cancer. Nat Cell Biol 2018; 20: 243-251.

27) Schmid AC, Byrne RD, Vilar R, Woscholski R. Bisperoxovanadium compounds are potent PTEN inhibitors. FEBS Lett 2004; 566: 35-38.

28) Maehama T, Dixon JE. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem 1998; 273: 13375-13378.