Long Non-Coding RNA TFAP2A-AS1 Inhibits Cell Proliferation and Invasion in Breast Cancer via miR-933/SMAD2

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Background: It is well documented that long non-coding RNAs (lncRNAs) are involved in the progression of multiple human tumors by sponging microRNAs (miRNAs). However, whether lncRNA TFAP2A-AS1 plays a role in the tumorigenesis of breast cancer (BC) remains undetermined.

Material/Methods: Real-time PCR (qRT-PCR) assay was performed to detect the relative mRNA expression of TFAP2A-AS1 and miR-933. Flow cytometry analysis, CCK-8 assay, and Transwell assay were applied to detect the effects of TFAP2A-AS1 overexpression on cell cycle, apoptosis, viability, and invasion of BC cells. In vivo proliferation assay was performed to evaluate the effects of TFAP2A-AS1 overexpression on tumor growth. Bioinformatics methods, dual-luciferase reporter, RNA immunoprecipitation (RIP), and RNA pull-down assays were used to predict and validate the interaction between TFAP2A-AS1 and miR-933, as well as SMAD2 and miR-933. Western blot assay was performed to examine the protein expression of SMAD2 in treated BC cells.

Results: TFAP2A-AS1 expression was significantly lower in BC tissues and cell lines, and patients with high TFAP2A-AS1 expression exhibited a better prognosis than those with low TFAP2A-AS1 expression. Overexpression of TFAP2A-AS1 in BC cells caused cell cycle arrest, promoted cell apoptosis, suppressed cell ability, and attenuated cell invasion in vitro, and inhibited tumor growth in vivo. TFAP2A-AS1 was revealed to act as a miRNA sponge for miR-933 and then regulated the expression of Smad2.

Conclusions: Results from the present study suggest that TFAP2A-AS1 acts as a tumor suppressor in BC via the miR-933/SMAD2 axis.

MeSH Keywords: Breast Neoplasms • MicroRNAs • RNA, Long Noncoding • Smad2 Protein

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/912421
Background

Breast cancer (BC), derived from breast tissues, is one of the most common types of cancers in women [1,2]. BC can occur at any age, but it tends to develop more readily in older individuals [3]. Although the causes of BC are not fully understood, multiple risk factors of BC have been identified, including lack of physical exercise, alcohol intake, obesity, and being female [4,5]. Because of the development of early-stage cancer diagnosis technologies, BC screening has significantly reduced the mortality rate of BC; however, BC still ranks as the second leading cause of cancer-related death worldwide [6]. The conventional treatment for BC is surgical resection followed by chemotherapy, but this can cause damage aesthetic appearance, and the recurrence rate remains high [7]. Elucidating the mechanisms of tumorigenesis of BC not only could contribute to understand this disease, but could also help develop new effective therapeutic targets for BC patients.

Long non-coding RNAs (lncRNAs), characterized by a length of more than 200 nucleotides, and microRNAs (miRNAs), characterized by length less than 22 nucleotides, are the 2 most important non-coding RNAs (ncRNAs), which lack protein-encoding capacity [8]. Previous studies have demonstrated that lncRNAs and miRNAs participate in multiple physiological processes, such as cell proliferation, tissue differentiation, and metabolic regulation [8,9]. Dysregulation of lncRNAs and miRNAs can cause a series of dysfunctions and diseases, including various human cancers [9]. Recently, emerging evidence has suggested a cross-modulation between lncRNAs and miRNAs [10,11]. lncRNAs may serve as competing endogenous RNA (ceRNA) or an RNA sponge in regulating the expression and functions of miRNAs [12–14].

TFAP2A-AS1/miR-933/SMAD2 axis involved in the tumorigenesis of breast cancer

In the present study, we explored the functions and mechanisms of TFAP2A-AS1 in BC. TFAP2A-AS1 expression was first examined in BC tissues and cell lines, and gain-of-function assays were performed to evaluate the TFAP2A-AS1 effects on tumor growth in vitro and in vivo. To explore the possible mechanisms involved, bioinformatics analysis was performed to predict the targeted miRNA of TFAP2A-AS1. TFAP2A-AS1 was identified as a miRNA sponge for miR-933, which targeted the 3’-UTR of SMAD2. Our study revealed that lncRNA TFAP2A-AS1 can act as a tumor suppressor in BC by sponging miR-933 to release SMAD2.

Material and Methods

Clinical patient samples and cell culture

Thirty pairs of tumor tissues and adjacent normal tissues of BC patients were collected from the Jiangsu Cancer Hospital during the period 2013–2018. Written informed consent was obtained by every patient, and methods used were approved by the Ethics Committee of Jiangsu Cancer Hospital. The normal human mammary epithelial cell line MCF-10A and BC cell lines MCF-7, MDA-MB-231, MDA-MB-435, T47D, and SKBR-3 were all obtained from the Chinese Academy of Sciences (Shanghai, China). All cell lines were maintained at 37°C with 5% CO₂ and 95% O₂ in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Sigma, USA), and penicillin (100 U/ml)/streptomycin (100 g/ml).

Cell transfection

TFAP2A-AS1 and its negative control, as well as miR-933 mimics/inhibitor and their negative control miR-NC/anti-NC, were used in the present study were all designed and obtained from GenePharma (Shanghai, China). In the in vitro cell transfection, BC cells were transfected with corresponding RNA molecules by using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instructions. In the in vivo experiments, TFAP2A-AS1 cDNA was sub-cloned into the LV5 lentiviruses (GenePharma) and then MCF-7 cells were infected with the recombinant lentiviruses.

RNA extraction and quantitative real-time PCR (qRT-PCR) assay

Total RNAs from treated BC cell lines and tissues were all prepared using TRIzol reagent (Takara, Japan), and the cDNA was produced by 50 ng total RNAs using a BestarTM qPCR RT kit (DBI Bioscience, China). The amplification was performed on the ABI PRISM 7500 Sequence Detection System (Life Technologies, USA) with the BestarTM qPCR MasterMix (DBI Bioscience) according to the instructions obtained from the manufacturers. All primers used in the present study were synthesized by Sangon (Shanghai, China), and the sequence of primers were: GAPDH: F, 5’-TGT TCA TGG TGA AC-3’, R, 5’-ATG GCA TGG ACT GTG GTC AT-3’; U1: F, 5’-GGG AGA TAC CAT GAT CAC GAA GGT-3’, R, 5’-CCA CAA ATT ATG CAG TCG AGT TTC CC-3’; miR-933: F, 5’-ATT ATA TGT GCG CAG TGA GC-3’, R, 5’-GCG AGC ACA GGA TTA ATA CCA CTC ACT ATG G-3’; TFAP2A-AS1: F, 5’-GTT GAG GCC TCC AGC ATT-3’, R, 5’-GCT GGA AGG CGC CCA CAC-3’, CDK6 F, 5’-GAC TCT ACC AGG AGC C-3’, R, 5’-CTG ACC TGG TCC AAA AAA-3’, cyclin D1 F, 5’-GCT GGC AAG TGG AAA CCA TC-3’, R, 5’-GTT GAG GCC TCC AGC ATT-3’, cyclin E1 F, 5’-GCG AGC GCA GGA TTA ATA CCA CTC ACT AGT G-3’.
**Subcellular fractionation**

To determine the cellular distribution of TFAP2A-AS1 in BC cells, the nuclear fraction of MCF-7 was isolated from cytoplasm using the PARIS kit (Life Technologies, USA) following the manufacturer’s protocols. RNA was isolated from the nuclei and cytoplasm of MCF-7 cells, and the TFAP2A-AS1 expression in the nuclear and cytoplasm was measured by qRT-PCR. GAPDH and U1 were used as the cytoplasmic and nuclear controls, respectively.

**Cell apoptosis and cycle analysis**

Cell cycle and apoptosis of treated MCF-7 and MDA-MB-231 cells were evaluated using flow cytometry analysis. Briefly, 48 h after the TFAP2A-AS1 transfection, BC cells were collected and resuspended in DMEM at a concentration of 1×10^5 cells/well. Subsequently, the treated BC cells were fixed in ethanol for 30 min, and Annexin V-FITC and propidium iodide were used to stain cells for 15 min at room temperature. Finally, cell cycle and apoptosis were assessed using a flow cytometer (FACSCanto™ II, BD Biosciences).

**Cell viability analysis**

Cell viability of TFAP2A-AS1 transfected MCF-7 and MDA-MB-231 cells were evaluated using a Cell Counting kit-8 (CCK-8, Sigma, USA) according to the protocols provided by the manufacturer. In brief, MCF-7 and MDA-MB-231 cells were seeded into 96-well plates and incubated with TFAP2A-AS1 for 5 days. Optical density was detected using a microtiter plate reader (SpectraMax, Molecular Devices, USA) at 0, 1, 2, 3, 4, and 5 days.

**Cell invasion analysis**

Effects of TFAP2A-AS1 overexpression on the invasive ability of BC cells were evaluated by Transwell assay using the specific chamber (8-µm, Corning Incorporated, USA), coated with...
Matrigel matrix (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, 500 µl of serum-free DMEM containing TFAP2A-AS1 overexpressed MCF-7 and MDA-MB-231 cells were added into the upper chamber. DMEM containing 10% FBS was added into the lower chamber. After incubation for 48 h, the invasive BC cells were fixed and stained using 0.5% crystal violet, and the number of invasive cells was counted using ImageJ software.

**In vivo proliferation assay**

Five-week-old male BALB/c nude mice were used in this study, and the animal experiments were approved by the Institutional Animal Care and Use Committee of Jiangsu Cancer Hospital. MCF-7 cells (1×10^6 cells/ml) transfected with LV-5/TFAP2A-AS1 recombinant lentiviruses were injected into the left flank of the nude mice. The tumor volume was measured every 3 days until 21 days, then the mice were sacrificed and the tumor weight was calculated.

**Plasmid construction and dual-luciferase activity assay**

For plasmid construction, the wild-type TFAP2A-AS1 and SMAD2 3’-UTR containing the miR-933 binding sites were inserted into the luciferase vector psi-CHECK2 (Promega, Madison, USA), named as psiCHECK2-TFAP2A-AS1-WT and SMAD2-WT, respectively. The TFAP2A-AS1 and SMAD2 3’-UTR containing the mutant miR-933 binding site were also sub-cloned into psi-CHECK2 to form psiCHECK2-TFAP2A-AS1-Mut and SMAD2-Mut, respectively.

**Western blot assay**

Total proteins of treated MCF-7 cells were prepared using RIPA buffer containing 1 mM PMSF. After centrifuging at 12 000 g for 15 min at 4°C, the supernatants were collected, and the protein concentration was determined via the BCA kit (Pierce, Rockford). Total proteins were then separated by 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. The membrane was blocked with 5% non-fat milk and incubated with the primary antibodies. The secondary antibody was conjugated with horseradish peroxidase and immunocomplexes were visualized using an enhanced chemiluminescence kit.

### Table 1. Correlations between long non-coding RNA TFAP2A-AS1 expression and clinicopathologic characteristics in breast cancer.

| Clinicopathologic characteristics | No. of patients | Long non-coding RNA TFAP2A-AS1 | P value |
|----------------------------------|----------------|--------------------------------|---------|
| Age (year)                       |                |                                |         |
| >50                              | 21             | 10 (47.6%)                     | 11 (52.4%) | 0.500 |
| ≤50                              | 9              | 5 (55.6%)                      | 4 (44.4%) |
| Tumor size (cm)                  |                |                                |         |
| <3                               | 19             | 12 (63.2%)                     | 7 (36.8%) | 0.287 |
| ≥3                               | 11             | 5 (45.5%)                      | 6 (54.5%) |
| Differentiation grade            |                |                                |         |
| Well/moderately                  | 17             | 12 (70.6%)                     | 5 (29.4%) | 0.035* |
| Poorly/undifferentiated          | 13             | 4 (30.8%)                      | 9 (69.2%) |
| TNM stage                        |                |                                |         |
| 0 & I & II                       | 24             | 19 (79.2%)                     | 5 (20.8%) | 0.049* |
| III & IV                         | 6              | 2 (33.3%)                      | 4 (66.7%) |

* P<0.05, TNM stage – pathologic tumor, node, metastasis stage.

The interaction between TFAP2A-AS1 and miR-933, as well as SMAD2 and miR-933, were assessed using dual-luciferase reporter assay system following the protocols obtained from the manufacturers. Renilla luciferase activity was normalized to Firefly luciferase activity.

**Validation of TFAP2A-AS1 and miR-933 interaction**

The interaction between TFAP2A-AS1 and miR-933 was verified by RNA immunoprecipitation (RIP) assay and RNA pull-down assay. For RIP assay, MCF-7 cells were collected and lysed with RIP lysis buffer, and then 50 µl of MCF-7 cell lysate was incubated with magnetic beads conjugated with a human anti-Ago2 antibody or negative control IgG in RIP buffer. Subsequently, proteinase K was used to digest proteins to RNAs were purified and validated by qRT-PCR. For RNA pull-down assay, miR-933 was transcribed with biotin-labeled RNA Mix using T7 RNA polymerase to form biotin-labeled miR-933, and RNeasy Mini Kit was used to purify targeted miR-933. After conjugation with streptavidin agarose beads, it was incubated with MCF-7 cell lysate for 2 h at room temperature. Finally, the beads were extracted using TRizol reagent and qRT-PCR was used to analyze the enrichment.

**Western blot assay**

Total proteins of treated MCF-7 cells were prepared using RIPA buffer containing 1 mM PMSF. After centrifuging at 12 000 g for 15 min at 4°C, the supernatants were collected, and the protein concentration was determined via the BCA kit (Pierce, Rockford). Total proteins were then separated by 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane. The membrane was blocked with 5% non-fat milk and incubated with the primary antibodies. The secondary antibody was conjugated with horseradish peroxidase and immunocomplexes were visualized using an enhanced chemiluminescence kit.
transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA). Non-specific binding sites were blocked in the 5% low-fat dried milk for 2 h. After washing 3 times in TBST, the membranes were incubated overnight at 4°C with the primary antibodies against SMAD2 (Rabbit, 1: 500, ab53100, Abcam) and GAPDH (Rabbit, 1: 2500, ab9485, Abcam). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated donkey-anti-rabbit secondary antibodies at room temperature for 2 h. Finally, the signals were detected with enhanced chemiluminescent reagents.

**Statistical analysis**

Data are all presented as mean ±SEM. The t test was used to compare difference between 2 groups, and one-way ANOVA was carried out to compare difference between more than 2 groups. GraphPad software (Ver. Prism 7, GraphPad Prism Software, La Jolla, CA, USA) was used to conduct the statistical analysis, and $P<0.05$ was considered as significant.
Figure 2. Effects of TFAP2A-AS1 overexpression on cell cycle and apoptosis of BC cell lines. (A, B) The cellular location of TFAP2A-AS1 in MCF-7 and MDA-MB-231 cells; the nucleus-retained U1 and cytoplasmic GAPDH was used as control. (C, D) Expression of TFAP2A-AS1 in MCF-7 and MDA-MB-231 cells transfected with TFAP2A-AS1 and its negative control were assessed by qRT-PCR. (E-H) Flow cytometry analysis was performed to analyze the cell cycle of MCF-7 and MDA-MB-231 cells transfected with TFAP2A-AS1. (I, J) The relative expression of CDK6, cyclin D1, and cyclin E1 in TFAP2A-AS1-overexpressed MCF-7 and MDA-MB-231 cells were measured by qRT-PCR. (K, L) Cell apoptosis of MCF-7 and MDA-MB-231 cells transfected with TFAP2A-AS1 and its negative control were evaluated by flow cytometry.
Results

**TFAP2A-AS1 expression was significantly downregulated in BC.**

To investigate the role of TFAP2A-AS1 in the pathogenesis of BC, we first examined the relative mRNA expression of TFAP2A-AS1 by qRT-PCR in BC tissues and cell lines. Results showed that TFAP2A-AS1 expression was significantly decreased in BC tissues compared with adjacent normal tissues (Figure 1A), and its expression was also remarkably lower in the 5 BC cell lines – MCF-7, MDA-MB-231, MDA-MB-435, T47D, and SKBR-3 – than in the normal human mammary epithelial cell line MCF-10A (Figure 1B). The correlation between the expression of IncRNA TFAP2A-AS1 and the clinicopathological features in breast cancer tissues is shown in Table 1. In addition, we analyzed the overall survival rate of BC patients with low or high TFAP2A-AS1 expression. Results indicated that the 20 patients with low TFAP2A-AS1 expression had worse prognosis than the 10 patients with high TFAP2A-AS1 expression (Figure 1C, 1D).

**Overexpression of TFAP2A-AS1 significantly inhibited the progression of BC in vitro and in vivo**

To assess the cellular distribution of TFAP2A-AS1 in BC cells, we examined the cellular location of TFAP2A-AS1 in MCF-7 and MDA-MB-231 cells. Results showed that TFAP2A-AS1 resided in both nucleus and cytoplasm of MCF-7 and MDA-MB-231 cells; however, the majority of TFAP2A resided in the...
Motif Name | Position | Length | Minimum free energy | Score |
--- | --- | --- | --- | --- |
hsa-miR-25-5p | 581~600 | 20 | –23.28 | 161 |
hsa-miR-99b-3p | 78~99 | 22 | –20.6 | 161 |
hsa-miR-484 | 249~275 | 27 | –29.6 | 161 |
hsa-miR-490-3p | 953~977 | 25 | –24.09 | 164 |
hsa-miR-518c-5p | 664~686 | 23 | –25.25 | 164 |
hsa-miR-516b-5p | 663~685 | 23 | –20.35 | 165 |
hsa-miR-625-5p | 953~972 | 20 | –27.25 | 166 |
hsa-miR-933 | 29~55 | 27 | –31.71 | 164 |
hsa-miR-4269 | 1233~1253 | 21 | –26.25 | 163 |
hsa-miR-3612 | 671~694 | 24 | –28.53 | 162 |
hsa-miR-4430 | 550~567 | 18 | –25.51 | 162 |
hsa-miR-4465 | 1054~1075 | 22 | –23.92 | 168 |
hsa-miR-4687-3p | 416~438 | 23 | –28.32 | 169 |
hsa-miR-4723-3p | 14~37 | 24 | –34.84 | 169 |
hsa-miR-4763-3p | 589~613 | 25 | –31.7 | 165 |

TFAP2A-AS1 acted as a competing endogenous RNAs (ceRNAs) in BC by sponging miR-933

Through bioinformatics analysis, the potential binding sites of miR-933 in TFAP2A-AS1 were predicted (Table 2, Figure 4A) and the secondary structure of miR-933/TFAP2A-AS1 match was shown (Figure 4B). Dual-luciferase reporter assay was used to verify the interaction between TFAP2A and miR-933 in MCF-7 cells. Results showed that miR-933 mimics remarkably attenuated the luciferase activity of the wild-type reporter of TFAP2A-AS1; however, miR-933 mimics failed to influence the luciferase activity of mutant reporter of TFAP2A in MCF-7 cells (Figure 4C). RNA RIP and pull-down assays were carried out in MCF-7 cells to further verified the interaction between TFAP2A-AS1 and miR-933. Results from RNA RIP showed that TFAP2A-AS1 and miR-933 bind directly to Ago2 (Figure 4D). RNA pull-down assay indicated a specific enrichment of TFAP2A-AS1 in the biotin-labeled wild-type miR-933 group (Figure 4E). Furthermore, TFAP2A-AS1-overexpressed MCF-7 and MDA-MB-231 cells showed a significant
Figure 4. Interaction between TFAP2A-AS1 and miR-933 in BC cells. (A) The putative binding site of miR-933 in TFAP2A-AS1. (B) RNA secondary structure of TFAP2A-AS1 was predicted using the Vienna RNA fold server. (C) Dual-luciferase reporter assay was carried out to evaluate the interaction of TFAP2A-AS1 and miR-933. (D) RNA RIP experiments were performed in MCF-7 cells and the co-precipitated RNA was analyzed by qRT-PCR. (E) RNA pull-down assay followed by qRT-PCR were performed to assess the interaction between TFAP2A-AS1 and miR-933 using biotin-labeled miR-933. (F) Relative mRNA expression of miR-933 was measured in TFAP2A-AS1 treated MCF-7 and MDA-MB-231 cells. (G) Relative mRNA expression of miR-933 was detected in MCF-10A, MCF-7 and MDA-MB-231 cells using qRT-PCR. (H) Relative mRNA expression of TFAP2A-AS1 in miR-933 transfected MCF-7 and MDA-MB-231 cells were examined by qRT-PCR. (I) Cell apoptosis of MCF-7 and MDA-MB-231 cells transfected with control or TFAP2A-AS1 or co-transfected with TFAP2A-AS1 and miR-933 were evaluated by flow cytometry.
Figure 5. Interaction between miR-933 and SMAD2 in BC cells. (A) Upper panel: the putative binding site of miR-933 in the 3'-UTR of SMAD2. Lower panel: the interaction between miR-933 and SMAD2 was verified by dual-luciferase reporter assay. (B) Relative protein expression of Smad2 was determined by Western blot assay in MCF-7 cells transfected with miR-933 mimics or inhibitor (anti-933). (C) Relative protein expression of Smad2 was measured in TFAP2A-AS1- and miR-933 treated MCF-7 cells by Western blot assay. (D) Correlation between miR-933 and Smad2 expression in MCF-7 cells was evaluated.

downregulation of miR-933 (Figure 4F). The miR-933 expression in MCF-7 and MDA-MB-231 cells was significantly higher than in MCF-10A cells (Figure 4G), and TFAP2A-AS1 expression was significantly reduced in the MCF-7 and MDA-MB-231 cells transfected with miR-933 mimics compared to the control cells (Figure 4H). Apoptosis analysis indicated a marked rescue of apoptosis rate in TFAP2A-AS1 and miR-933 co-transfection BC cells, which further supports that TFAP2A-AS1 acts as a competing endogenous RNA (ceRNA) in BC by sponging miR-933 (Figure 4I).

**SMAD2 was directly targeted gene of miR-933**

By using bioinformatics tools, we revealed that there was a binding site of miR-933 in the 3'-UTR of SMAD2 (Figure 5A,
It is well documented that IncRNAs exhibit multiple biological functions in tumor cells by serving as miRNAs sponges to indirectly regulate the expression of targeted genes [16,23–25]. Therefore, to determine the mechanisms underlying the effects of TFAP2A-AS1 on BC, bioinformatics analysis was performed to screen the targeted miRNAs of TFAP2A-AS1. Following validation by dual-luciferase reporter and RNA RIP assays, TFAP2A-AS1 was demonstrated to sponge miR-933 in BC cells.

We further explored whether TFAP2A-AS1 exhibited its tumor-suppressive effects by sponging miR-933 to indirectly regulate the expression of targeted genes. We predicted and verified the targeted genes of miR-933 by bioinformatics tools and dual-luciferase reporter assay, respectively, showing that the 3′-UTR of SMAD2 showed bindings sites for miR-933. The transforming growth factor-β (TGF-β) signaling pathway was well documented to play important functions in cell proliferation, apoptosis, differentiation, and embryonic development [26,27], and the SMAD family was demonstrated to transduce the signaling forms TGF-β signaling pathway to participate the biological processes by activating receptor serine/threonine kinases [28,29]. The proteins of the SMAD family all have 1 conserved N-terminal Mad homology domain-1 (MH1) and 1 C-terminal Mad homology domain-2 (MH2) [29,30]. Activation of the TGF-β/SMAD signaling pathway was reported to promote tumor progression in multiple human cancers, including lung cancer, breast cancer, and renal cell cancer [31–33]. In the present study, overexpression of TFAP2A-AS1 upregulated the expression of SMAD2, whereas miR-933 reversed this upregulation of SMAD2 induced by TFAP2A-AS1. In addition, there was a negative correlation between miR-933 and SMAD2 in BC.

**Conclusions**

The most important results of the present study are: 1) IncRNA TFAP2A-AS1 was significantly downregulated in BC tissues and cell lines; 2) Silencing of TFAP2A-AS1 suppressed the tumorigenesis of BC; and 3) mechanistic analysis showed that TFAP2A-AS1 regulated SMAD2 by specifically sponging miR-933. Our study discloses a novel TFAP2A-AS1-miR-933-SMAD2 signaling pathway involved in BC cell progression. Therefore, TFAP2A-AS1 may serve as a therapeutic target for BC treatment.

**Conflict of interest**

None.
