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Original Article

Long non-coding RNA AFAP1-AS1 was up-regulated in triple-negative breast cancer and regulated proliferation and invasion

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Abstract: Triple-negative breast cancer (TNBC) is usually associated with an unfavorable prognosis and lacks effective therapeutic targets. Long non-coding RNAs (lncRNAs) contribute to the initiation and progression of a variety of human cancers. The identification of dysregulated lncRNAs involved in TNBC might provide additional insights into its aggressive biological behavior. Here, we reported one lncRNA, actin filament associated protein 1 antisense RNA1 (AFAP1-AS1), was significantly overexpressed in TNBC and associated with lymph node metastasis, distant metastasis and stage. *In vitro* experiments demonstrated that the proliferation and invasion potential was significantly reduced in AFAP1-AS1-silenced TNBC cells. To the best of our knowledge, it was firstly reported that AFAP1-AS1 was involved in breast cancer. Our results suggested that AFAP1-AS1 represents a novel and a potential therapeutic target for TNBC. Furthermore, increased understanding of such onco-lncRNAs could provide additional insights into cancer tumorigenesis and progress.

Keywords: lncRNA, AFAP1-AS1, triple-negative breast cancer, proliferation, migration, invasion

Introduction

Breast cancer (BC) is the most common type of female cancer worldwide [1]. Triple-negative breast cancer (TNBC) is characterized by a lack of expression of estrogen receptor (ER) and progesterone receptor (PR) as well as human epidermal growth factor receptor 2 (HER-2). Overall, the prevalence of TNBC accounts for 11%-20% of the BC patient cohort [2]. TNBC is associated with the worst prognosis among all BC subtypes, partly because of a lack of effective targeted drugs such as tamoxifen in ER-positive BC and trastuzumab in HER2-positive BC [3, 4]. Although remarkable progress has been made during recent decades, the molecular mechanisms underlying the aggressive biological behavior of TNBC remain to be elucidated.

Long non-coding RNAs (lncRNAs) are a class of transcripts longer than 200 bp that lack a protein-coding capacity [5]. An increasing number of studies indicate that lncRNAs are involved in a variety of biological processes including epigenetics, transcription, post-transcription, and translation [6-8]. The dysregulation of lncRNAs has also been shown to contribute to the initiation and progression of several human cancers including BC [9-14]. Hence, identification of cancer-associated lncRNAs and investigation of their biological functions might help identify novel therapeutic targets.

LncRNA actin filament associated protein 1 antisense RNA 1 (AFAP1-AS1) is derived from theantisense strand of DNA at the coding gene locus of actin filament associated protein 1 (AFAP1) which is involved in focal adhesion formation in a TNBC cell line [15]. Besides, AFAP1-AS1 had been found to be up-regulated in pancreatic ductal adenocarcinoma, nasopharynx cancer and lung cancer [16-18]. However, whether lncRNA AFAP1-AS1 is related to the TNBC is still unclear. In this study, we compared the expression of AFAP1-AS1 in TNBC tissues and non-cancerous tissues. Moreover, we vali-

datedthe biological function of AFAP1-AS1 in TNBC.

Materials and methods

Patient samples

BC patients undergoing modified radical mastectomy were included in this study. All samples were confirmed as TNBC by postoperative histopathological examination and immunohistochemistry. Primary cancer tissues and nontumorous tissues were snap-frozen in liquid nitrogen immediately after resection and then stored at -80°C before RNA extraction. Informed consent was obtained from all individual participants included in the study. The study was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University.

RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from tissue samples using TRIzol (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. qRT-PCR was performed using a Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan) in the Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Foster City, CA). The primer sequences for PCR are as follows: AFAP1-AS1, forward 5'-AATGGTGGTAGGAGGGAGGA-3' and reverse 5'-CACACAGGGGAATGAAGAGG-3'. β-Actin was used as an internal control and thecomparative CT method (ΔΔCT) method was used to evaluate the relative quantification of AFAP1-AS1. Each sample was in triplicate.

Cell lines and small interfering RNA (siRNA)

TNBC cancer cell lines BT-549 and MBA-MD-231 were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco) in a humidified incubator with 5% CO_2 at 37°C. Cells were seeded overnight and transfected with either 100 nM siRNA or nontarget scramble control siRNA (GenePharma, Shanghai, China) using Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA) in OptiMEM medium (Gibco) for gene knockdown.

Colony formation assay and MTT assay

For colony formation assay, 5000 cells were plated in each well of a six-well plate. When there was visible colony by naked eye, cells were fixed with 4% formaldehyde and were stained with crystal violet (0.25%). Colonies were then counted. For MTT assay, 2000 cells from each group were plated in each well of five 96-well plates. To analyze cell proliferation, 20 μL of MTT substrate at a concentration of 2.5 mg/mL in PBS was added to each well. The plates were then returned to a standard tissue incubator for an additional 4 h. The medium was then removed, and the cells were solubilized in 150 μL of dimethylsulfoxide for the colorimetric analysis (wavelength, 490 nm). One plate was analyzed immediately after the cells adhered. Then, one plate per day was examined for the next 4 consecutive days.

Wound healing assay

Cells were grown to 90% confluency in six-well culture plates. A p200 pipette tip was used to scratch the cell monolayer. Images were captured immediately and 24 h and 48 h after wounding, and wound closure was monitored by microscopy. Wound sizes were verified with an ocular ruler.

Transwell assay

Cell migration and invasion were measured on Transwell plates (Costar, New York, NY) and Matrigel chamber plates (BD Biosciences, Bedford, MA), respectively. A total of 1×10^5 cells were seeded onto Transwell or Matrigel insert membranes on day 2 following transfection. Growth medium containing 20% FBS was used as a chemoattractant. After incubation at 37°C for 24 h, cells that did not migrate or invade through the pores of the Transwell inserts were manually removed with a cotton swab. Cells that had invaded through the filter pores were fixed with methanol, stained with 0.1% crystal violet, and observed under a microscope. The number of invasive tumor cells was counted from five randomly selected 20 × fields for each experiment and averaged.

Statistical analysis

Comparisons of genes expression levels between the TNBC group and the normal tissue group was made usingthe Student's *t*-test. Clinicopathological characteristics were evaluated using the Chi-square test. One-way analysis of variance (ANOVA) was used to evaluate significant differences for multiple compari-

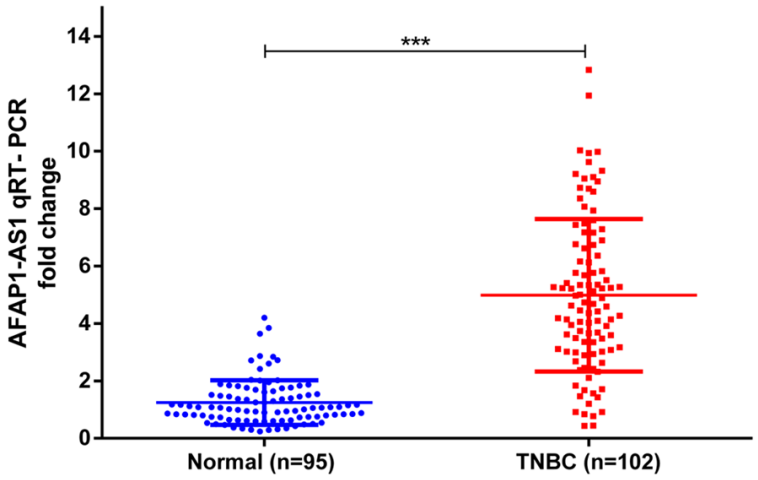

Figure 1. AFAP1-AS1 is overexpressed in TNBC. AFAP1-AS1 expression levels in 102 TNBC tissues and 95 non-cancerous tissues were confirmed by qRT-PCR. ***P<0.001; Student t-test.

Table 1. Relationship between AFAP1-AS1 expression and patient clinicopathological characteristics

Clinicopathologic characteristics	N	AFAP1-AS1 expression		X^2	P
		High (%)	Low (%)		
Age				3.923	0.074
≤60	52	31 (60.7)	21 (41.2)		
>60	50	20 (39.3)	30 (58.8)		
Tumor				3.116	0.211
≤2 cm	29	11 (21.7)	18 (35.3)		
2-5 cm	69	37 (72.5)	32 (62.7)		
>5 cm	4	3 (5.8)	1 (2.0)		
Lymph node metastasis				6.039	0.024*
No	38	13 (25.5)	25 (49.0)		
Yes	64	38 (74.5)	26 (51.0)		
Distant metastasis				6.725	0.031*
No	93	43 (84.3)	50 (98.0)		
Yes	9	8 (15.7)	1 (2.0)		
Stage				5.607	0.030*
I-II	71	30 (58.8)	41 (80.4)		
III-IV	31	21 (41.2)	10 (19.6)		

*P<0.05; Chi-square test.

sons in cellular experiments. A P value less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 22.0 (Chicago, IL).

Results

LncRNA AFAP1-AS1 was up-regulated in TNBC

The expression of AFAP1-AS1 was detected in 102 TNBC tissues and 95 non-cancerous tissues by qRT-PCR. As showed in **Figure 1**, the expression of AFAP1-AS1 in TNBC tissues was almost 5 folds of non-cancerous tissues (P<0.001).

The relationship between AFAP1-AS1 expression and clinical features

To analyze whether AFAP1-AS1 was associated with the development and progression of TNBC, we investigated itsrelationship with clinical features. In qPCR cohort, patients were divided into low and high AFAP1-AS1 expression groups according to the median value. The results revealed lymph node metastasis (P=0.024), distant metastasis was (P=0.031) and stage (P=0.030) were significant related with the expression of AFAP1-AS1 positively (**Table 1**).

Inhibition of AFAP1-AS1 in TNBC cells leads to reduced proliferation

To validate the function of AFAP1-AS1 in TNBC, AFAP1-AS1 siRNAs were used to inhibit AFAP1-AS1 expression in TNBC cell lines. Two siRNAs were tested for knockdown in TNBC cell lines BT-549 and MDA-MB-231, and both efficiently reduced AFAP1-AS1 expression by at least 70% in each cell line (**Figure 2A**). The results of colony formation assay revealed that siRNA1 and siRNA2 significantly inhibited the colony formation of TNBC cell lines compared with the control cells (P<0.01, **Figure 2B**). Similarly, MTT assay revealed that cells transfected with siRNA1 and siRNA2 and not control cells, had significantly inhibited growth and proliferation of TNBC cells (P<0.001, **Figure 2C**).

Inhibition of AFAP1-AS1 leads to reduced migration and invasion

Furthermore, the role of AFAP1-AS1 in TNBC cell metastasis was demonstrated. Wound

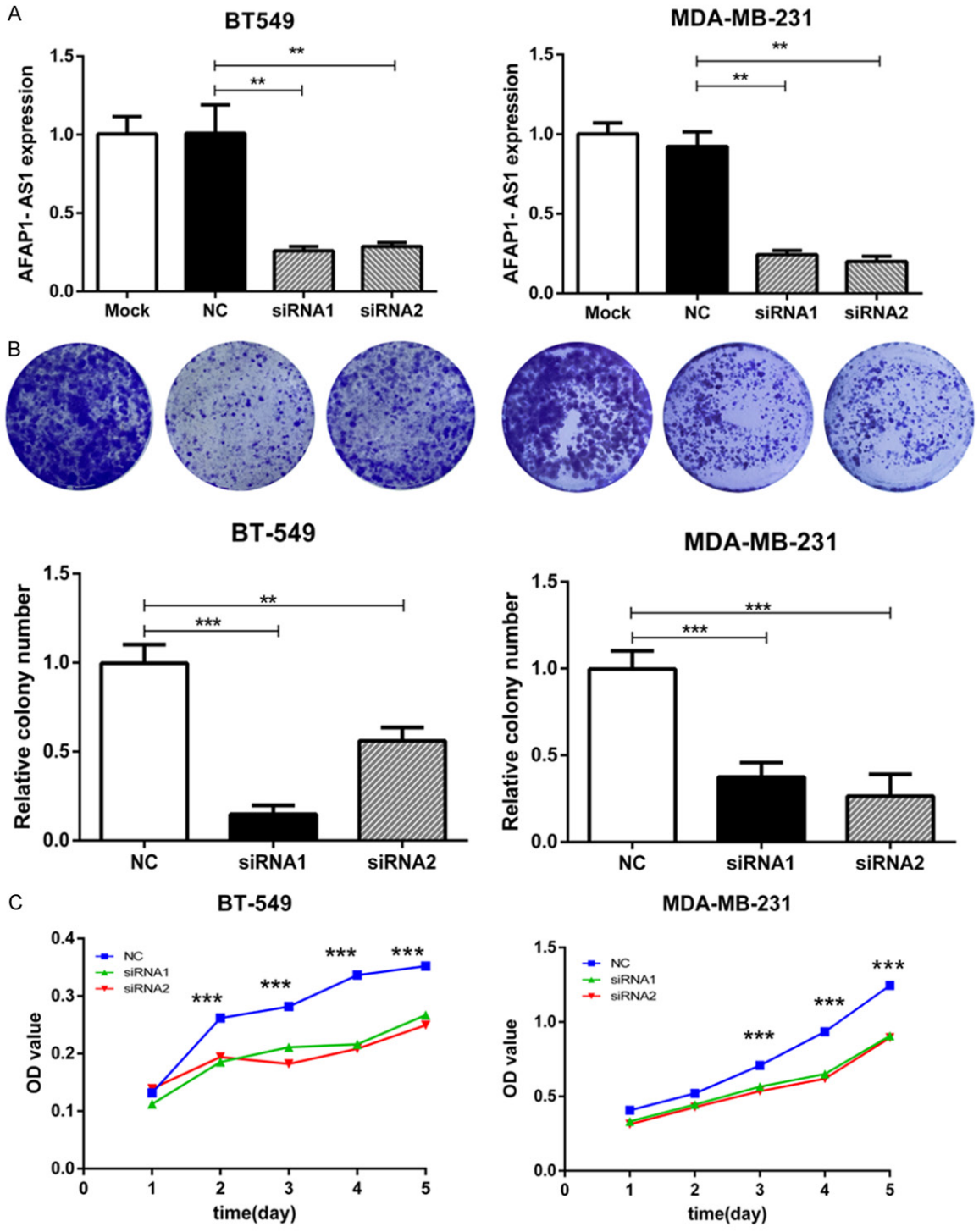

Figure 2. The effects of AFAP1-AS1 on TNBC cell proliferation. A. siRNA knockdown efficiency of AFAP1-AS1 expression in BT-549 and MDA-MB-231 cells by qRT-PCR. B. Colony formation assay. C. MTT assay. Data are shown as the mean ± standard deviation from three independent experiments. $^{**}P<0.01$, $^{***}P<0.001$; one-way ANOVA test.

recovery was significantly delayed when AFAP1-AS1 was knocked down compared with control cells ($P<0.05$, **Figure 3A**). Besides, cell migration and invasion were also significantly reduced, as assessed by a transwell assay. As shown in **Figure 3B**, migration was reduced by at least 83% ($P<0.001$) and 43% ($P<0.01$) in BT-549 and MDA-MB-231 cell lines, respectively, while invasion was reduced by 49% ($P<0.05$) and 54% ($P<0.01$), respectively (**Figure 3C**).

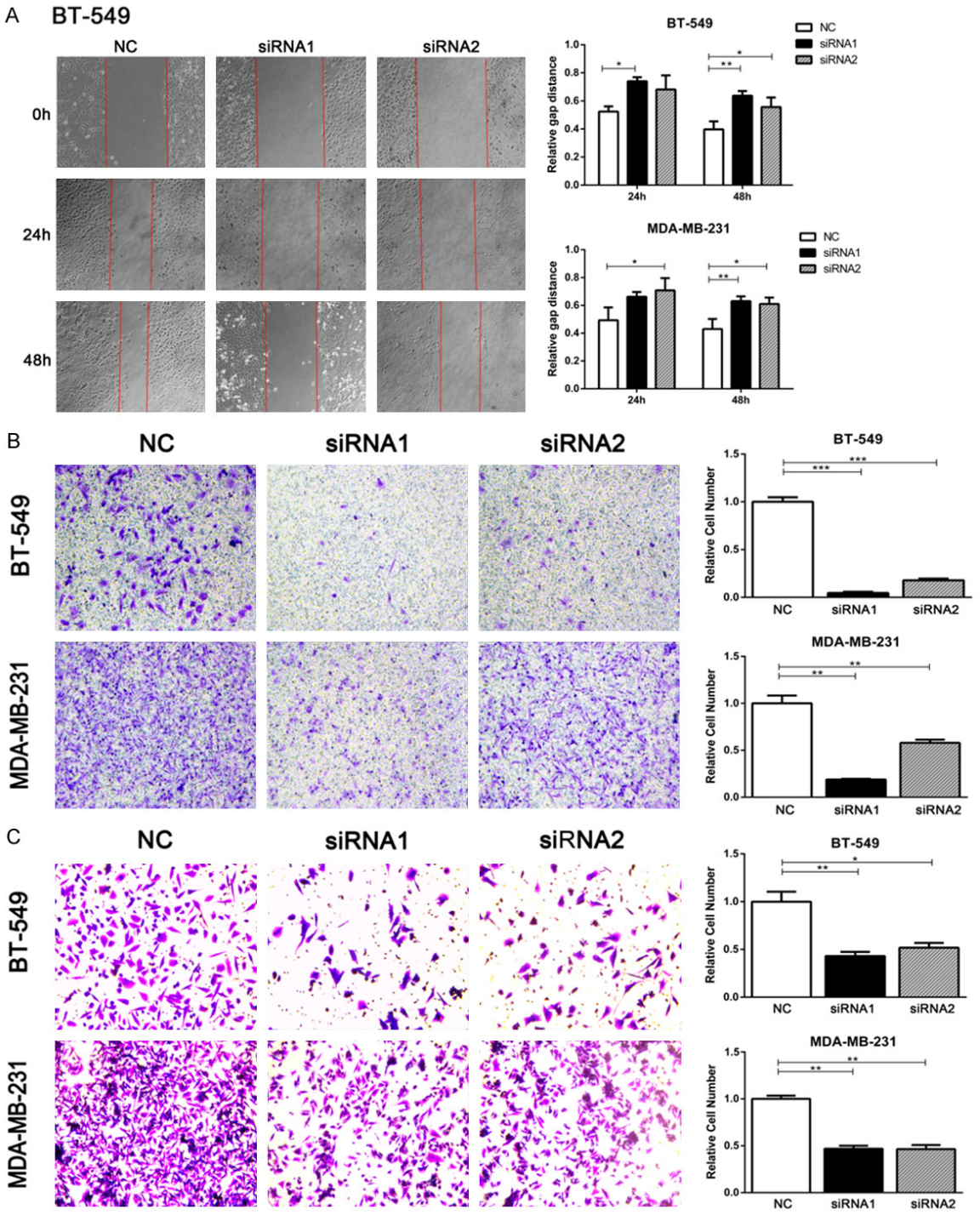

Figure 3. AFAP1-AS1 knockdown inhibited TNBC cell migration and invasion. (A) Wound healing assay (B) Migration assay (C) Invasion assay. Data are shown as the mean ± standard deviation from three independent experiments. **P*<0.05, ***P*<0.01; one-way ANOVA test.

Discussion

TNBC patients usually have significantly lower rates of disease-free and overall survival, and are more likely to have pulmonary metastasis and brain metastases than other types BC patients [19]. Given the poor outcome and lack of effective therapeutic targets, TNBC has

drawn extra attention from oncologists. Growing numbers of lncRNAs are proven to have a crucial effect in the biological regulation of a range of cancers [20]. However, considering the high number of lncRNAs, knowledge of lncRNAs and their mechanisms of action is surprisingly limited, especially with respect to TNBC. Here, we proved that lncRNA AFAP1-AS1 is significantly overexpressed in TNBC and associated with lymph node metastasis, distant metastasis and stage. *In vitro* experiments demonstrated that the proliferation and metastasis potential was significantly reduced in AFAP1-AS1-silenced TNBC cells. To the best of our knowledge, it was firstly reported that lncRNA AFAP1-AS1 was involved in breast cancer.

Wu previously reported that AFAP1-AS1 is upregulated in esophageal cancer because of extreme hypomethylation in its promoter [21]. AFAP1-AS1 silencing was shown to inhibit proliferation and colony-forming ability, induce apoptosis, and reduce migration and invasion of esophageal cancer cells [21]. Zeng reported the upregulation of AFAP1-AS1 in lung cancer, and showed it to be associated with poor prognosis [22], while Bo similarly revealed its overexpression in nasopharyngeal carcinoma and association with metastasis and poor prognosis [16]. Taken together, this indicated that AFAP1-AS1 was involved in multiple cancers and it might act as an onco-lncRNA.

The exact mechanism of AFAP1-AS1 action remains unclear. Previous studies have reported widespread sense-antisense transcripts in mammalian cells, and shown that perturbation of antisense RNA can alter the expression of the sense gene [23, 24]. AFAP1, AFAP1-AS1's coding counterpart, has been found to promote focal adhesion formation in MDA-MB-231 BC cells [15]. We therefore hypothesize that AFAP1-AS1 might exert its function by regulating AFAP1. Further studies should investigate the mechanisms by which AFAP1-AS1 modulates its sense counterpart.

In conclusion, our results suggest that AFAP1-AS1 represents a novel therapeutic target for TNBC. Furthermore, increased understanding of suchonco-lncRNAs in TNBC could provide additional insights into cancer tumorigenesis and progress.

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Disclosure of conflict of interest

None.

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