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LncRNA ZFPM2-AS1 aggravates the malignant development of breast cancer *via* upregulating JMJD6

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Abstract. – OBJECTIVE: The purpose of this study was to explore the expression pattern of long non-coding RNA (lncRNA) ZFPM2-AS1 in breast cancer (BC) tissues, and its biological influence on clinical features and prognosis in BC patients.

PATIENTS AND METHODS: ZFPM2-AS1 levels in 52 paired BC tissues and adjacent normal ones were detected. Then, the relationship between ZFPM2-AS1 level and clinical features in BC patients was analyzed. Regulatory effects of ZFPM2-AS1 on proliferative and migratory abilities in MCF-7 and SKBR3 cells were assessed. In addition, *in vivo* regulation of ZFPM2-AS1 in nude mice bearing BC was evaluated. Finally, the interaction between ZFPM2-AS1 and JMJD6 and the involvement of ZFPM2-AS1 in the development of BC were illustrated.

RESULTS: The results showed that ZFPM2-AS1 was upregulated in BC tissues, and its high level was linked to advanced tumor stage, high rates of lymphatic metastasis, and distant metastasis, as well as poor prognosis in BC. The knockdown of ZFPM2-AS1 suppressed proliferative and migratory abilities in BC cells. In addition, JMJD6 was verified to be the downstream gene binding to ZFPM2-AS1, which was highly expressed in BC tissues and positively regulated by ZFPM2-AS1. *In vivo* knockdown of ZFPM2-AS1 in nude mice bearing BC showed a smaller tumor volume and lower tumor weight than controls. In addition, JMJD6 was downregulated in BC tumors extracted from mice with silenced ZFPM2-AS1.

CONCLUSIONS: LncRNA ZFPM2-AS1 is upregulated in BC and linked to tumor stage, metastasis, and prognosis in BC patients. It aggravates the malignant development of BC via upregulating JMJD6.

Key Words:
ZFPM2-AS1, JMJD6, Breast cancer, Malignant development.

Introduction

Breast cancer (BC) is a worldwide public health problem[1]. It is estimated that there were 2.1 million newly onsets of BC (11.6% of total cancer onsets) and 627,000 death cases of BC (6.6% of total cancer deaths) globally in 2018[1-3]. In China, the incidence and mortality of BC account for 12.2% and 9.6%, respectively[4,5]. BC screening reduces its mortality by 25-31% in included patients in Europe due to the high detective rate of early-stage BC. In 1990-2010, the mortality of BC was reduced by 38-48% in people who were screened[6-8]. Biological hallmarks are vital in early diagnosis, pathological classification, and individualized treatment of BC[9,10]. As a complex disease, the pathogenesis of BC involves environmental, genetic, and reproductive factors. However, the susceptibility to BC varies in people even exposed to the same environmental and reproductive factors because of individual differences[11,12]. Therefore, studies on cancer susceptibility are of significance in screening and treatment of BC[12].

Long non-coding RNAs (lncRNAs) barely encode proteins because of the absence of the open reading frame[13,14]. Genome-wide association studies have identified a large number of cancer-associated lncRNAs[15,16]. Through transcriptional and post-transcriptional regulations, lncRNAs interfere with stability of cell environment[17,18]. Mutant or abnormally expressed lncRNAs are involved in tumor growth and metastasis[18]. In particular, HOTAIR is overexpressed in many types of tumors[19,20]. ANRIL is highly expressed in leukemia and breast cancer[21]. Due to the tissue specificity and stable expression, lncRNAs may be promising biological hallmarks and therapeutic targets in hu-

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man diseases[15,18]. LncRNA ZFPM2-AS1 has been reported to be upregulated in cancers, which is involved in cancer cell metastasis and closely linked to pathological staging and prognosis[15,18,22,23].

Functionally, lncRNAs can regulate protein functions by directly interacting with them, mediating protein-protein interaction or guiding subcellular distribution[24]. Through bioinformatics analysis, it was found that ZFPM2-AS1 could bind to JMJD6. Their functions in the malignant development of BC were mainly explored in this paper.

Patients and Methods

BC Patients and Species

Paired BC species and normal breast species were surgically resected from 52 BC patients. Their clinical and follow-up data were recorded. In this study, tumor staging was conducted based on the guideline proposed by the Union for International Cancer Control (UICC). In addition, this study was in line with the declaration of Helsinki clinical practice guidelines, and obtained the approval by the Ethics Committee of The Affiliated Hospital of Shandong University of TCM and conducted after informed consent of each subject.

Cell Culture

BC cell lines (MCF-7, MDA-MB-231, SKBR3) and a normal mammary epithelial cell line (MCF-10A) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) except for SKBR3 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C. Cell passage was conducted when cells reached 80-90% confluence.

Transfection

Cells were cultured to 30-50% confluence in 6-well plates and transfected with plasmids constructed by GenePharma (Shanghai, China), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 48 hours later, the cells were collected for the following use.

Cell Proliferation Assay

Cells were inoculated in 96-well plates with 2×10^3 cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Kumamoto, Japan) for plotting the viability curves.

Colony Formation Assay

Cells were inoculated in 6-well plates with 200 cells per well and cultured for 2 weeks. Culture medium was replaced once in the first week and twice in the second week. Next, visible colonies were washed in phosphate-buffered saline (PBS), fixed in methanol for 20 min and dyed in 0.1% crystal violet for 20 min, which were finally captured and calculated.

Transwell Assay

A total of 100 μL of suspension (5×10^5 cells/ml) was applied in the upper layer of a transwell chamber (Millipore, Billerica, MA, USA) that was inserted in a 24-well plate, with 500 μL of medium containing 10% FBS in the bottom. After 48-h incubation, the bottom cells were reacted with 15-min methanol, 20-min crystal violet, and captured using a microscope. Migratory cells were counted in 5 random fields per sample.

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

RNAs extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNAs underwent qRT-PCR using SYBR® Premix Ex Taq™ (Takara, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were the internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. The primer sequences are listed as follows. ZFPM2-AS1: forward: 5'-CTAGGGGACTGGGCTGCT-3' and reverse: 5'-AGGGTCTTAGGTTCCAGGCA-3', JMJD6: forward: 5'-GACGACCTCAACGCACAGTA-3' and reverse: 5'-CACCTAATTGGGCTCCATCT-3', GAPDH: forward: 5'-TTTAACTCTGGTAAAGTGGA-3' and reverse: 5'-GAATCATATTGGAACATGTA-3'.

Western Blot

Cells or tissues were lysed for isolating proteins and electrophoresed. The protein samples were loaded on polyvinylidene difluoride (PVDF)

membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h. The membranes reacted with primary and secondary antibodies for indicated time. Band exposure and analyses were finally conducted.

Luciferase Assay

Cells were co-transfected with NC/pcDNA3.1-JMJD6 and ZFPM2-AS1-WT/ZFPM2-AS1-MUT, respectively, followed by lysis for determining the relative Luciferase activity 48 h later (Promega, Madison, WI, USA).

In Vivo Xenograft Model

This study was approved by the Animal Ethics Committee of The Affiliated Hospital of Shandong University Animal Center. A total of 10 male nude mice were randomly assigned into two groups, with 5 mice in each group. They were subcutaneously administrated with MCF-7 cells transfected with sh-NC or sh-ZFPM2-AS1, respectively. Tumor size was recorded every 5 days. Five weeks later, the mice were sacrificed for collecting BC tissues. Tumor volume = (width^2×length)/2.

Immunohistochemistry (IHC)

IHC was conducted using SP method. The result of each section was independently determined by two experienced pathologists. Then, the positive expression of JMJD6 was determined based on the staining color (unstained=negative, light yellow=weak positive, brown=positive, tan=strong positive). Positive rate=numbers of weak positive, positive and strong positive cases/total case number ×100%.

Statistical Analysis

GraphPad Prism 5 (V5.01) (La Jolla, CA, USA) was used for data analyses. Data were expressed as mean ± standard deviation (SD). The differences between groups were analyzed by the *t*-test. Chi-square test was conducted for analyzing the relationship between ZPFM2-AS1 level and clinical data of BC patients. After that, Pearson correlation test was applied for evaluating the relationship between two genes in BC species. Kaplan-Meier curves were depicted for survival analysis. $p<0.05$ suggested that the difference was statistically significant.

Results

ZFPM2-AS1 Was Highly Expressed In BC

ZFPM2-AS1 expressions in BC cell lines and tissues were determined. Compared with that in mammary epithelial cell line, ZFPM2-AS1 was highly expressed in the three tested BC cell lines, especially MCF-7 and SKBR3 cell lines (Figure 1A). Meanwhile, ZFPM2-AS1 was upregulated in 52 collected BC tissues than normal breast tissues as well (Figure 1B). It is indicated that ZFPM2-AS1 may be an oncogene in BC development.

ZFPM2-AS1 Expression Was Linked to Tumor Staging, Metastasis and Prognosis In BC Patients

Clinical data of included 52 BC patients were recorded. Through Chi-square analysis, ZFPM2-AS1 level was positively correlated with tumor stage and rates of lymphatic metastasis and distant metastasis but not with age and tumor size in BC patients (Table I). A higher level of ZFPM2-AS1 was found in BC patients with T3-T4, or those complicated with lymphatic metastasis or distant metastasis than those with T1-T2 or without metastases (Figure 1C). Survival analysis revealed that highly expressed ZFPM2-AS1 was unfavorable to the prognosis in BC (Figure 1D).

Knockdown of ZFPM2-AS1 Inhibited Proliferative and Migratory Abilities in BC

To explore the biological function of ZFPM2-AS1 in BC, si-ZFPM2-AS1 was constructed. Transfection with sh-ZFPM2-AS1 effectively downregulated ZFPM2-AS1 in MCF-7 and SKBR3 cells (Figure 2A). After knockdown of ZFPM2-AS1, viability (Figure 2B) and colony number (Figure 2C) were decreased in BC cells, suggesting the suppressed proliferative ability. In addition, the lower migratory cell number was seen in BC cells transfected with sh-ZFPM2-AS1 than those transfected with sh-NC, suggesting the inhibited migratory ability (Figure 2D).

ZFPM2-AS1 Bound to JMJD6

Binding sequences in the 3'UTR of ZFPM2-AS1 and JMJD6 were predicted by bioinformatics analysis. Based on the predicted sequences, Luciferase vectors targeting ZFPM2-AS1 were

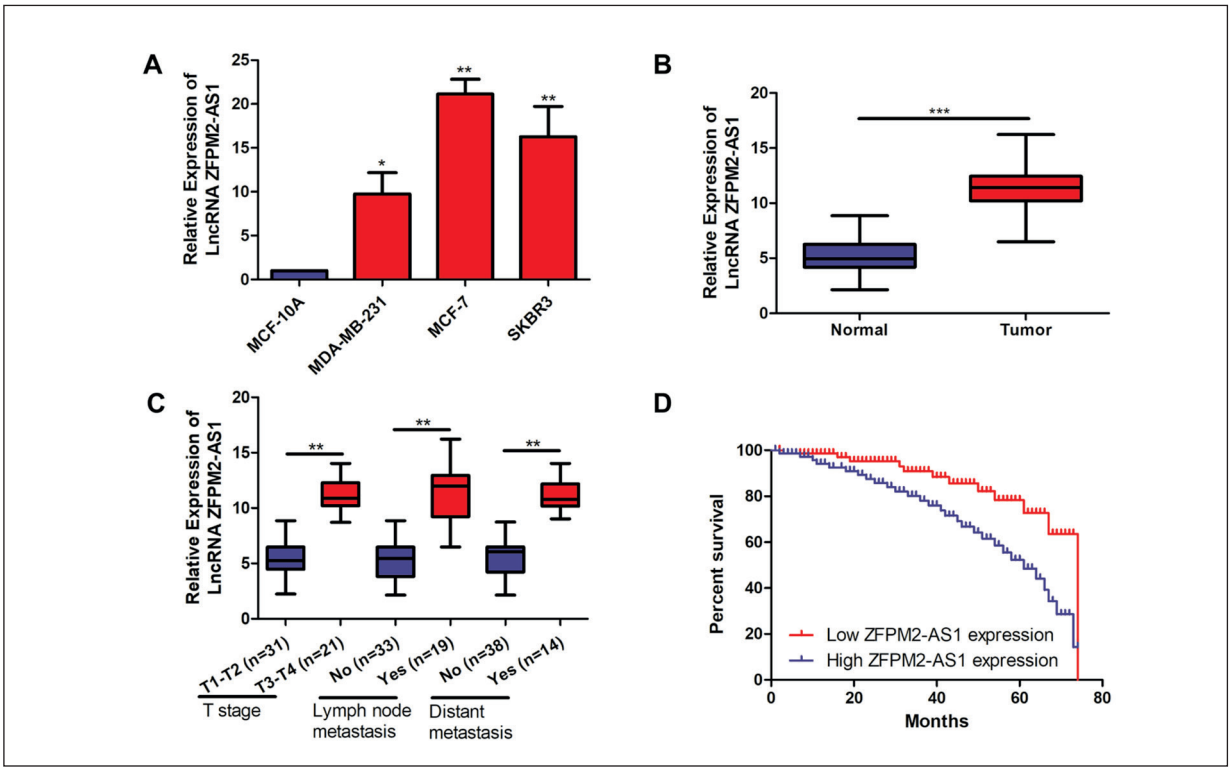

Figure 1. ZFPM2-AS1 is highly expressed in BC. **A,** ZFPM2-AS1 level in BC cell lines. **B,** ZFPM2-AS1 level in BC tissues and normal breast tissues. **C,** ZFPM2-AS1 level in BC patients in different tumor stages or either with metastasis or not. **D,** Survival rate in BC patients based on ZFPM2-AS1 level. Data are expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

constructed. It was found that the overexpression of JMJD6 markedly decreased the Luciferase activity in wild-type ZFPM2-AS1 vector, and that in mutant-type ZFPM2-AS1 vector remained unchangeable (Figure 3A). Thus, the binding between ZFPM2-AS1 and JMJD6 was verified. Protein level of JMJD6 was downregulated in BC cells with ZFPM2-AS1 knockdown (Figure 3B). As expected, ZFPM2-AS1 was upregulated in BC cells overexpressing JMJD6 (Figure 3C).

Table I. Association of ZFPM2-AS1 expression with clinicopathologic characteristics of breast cancer.

Parameters	No. of cases	ZFPM2-AS1 expression		p-value
		Low (%)	High (%)	
Age (years)				0.228
< 60	20	14	6	
≥ 60	32	17	15	
Tumor size (cm)				0.108
< 4	25	18	7	
≥ 4	27	13	13	
T stage				0.004
T1-T2	31	24	7	
T3-T4	21	8	13	
Lymph node metastasis				0.011
No	33	24	9	
Yes	19	7	12	
Distant metastasis				0.012
No	38	28	10	
Yes	14	5	9	

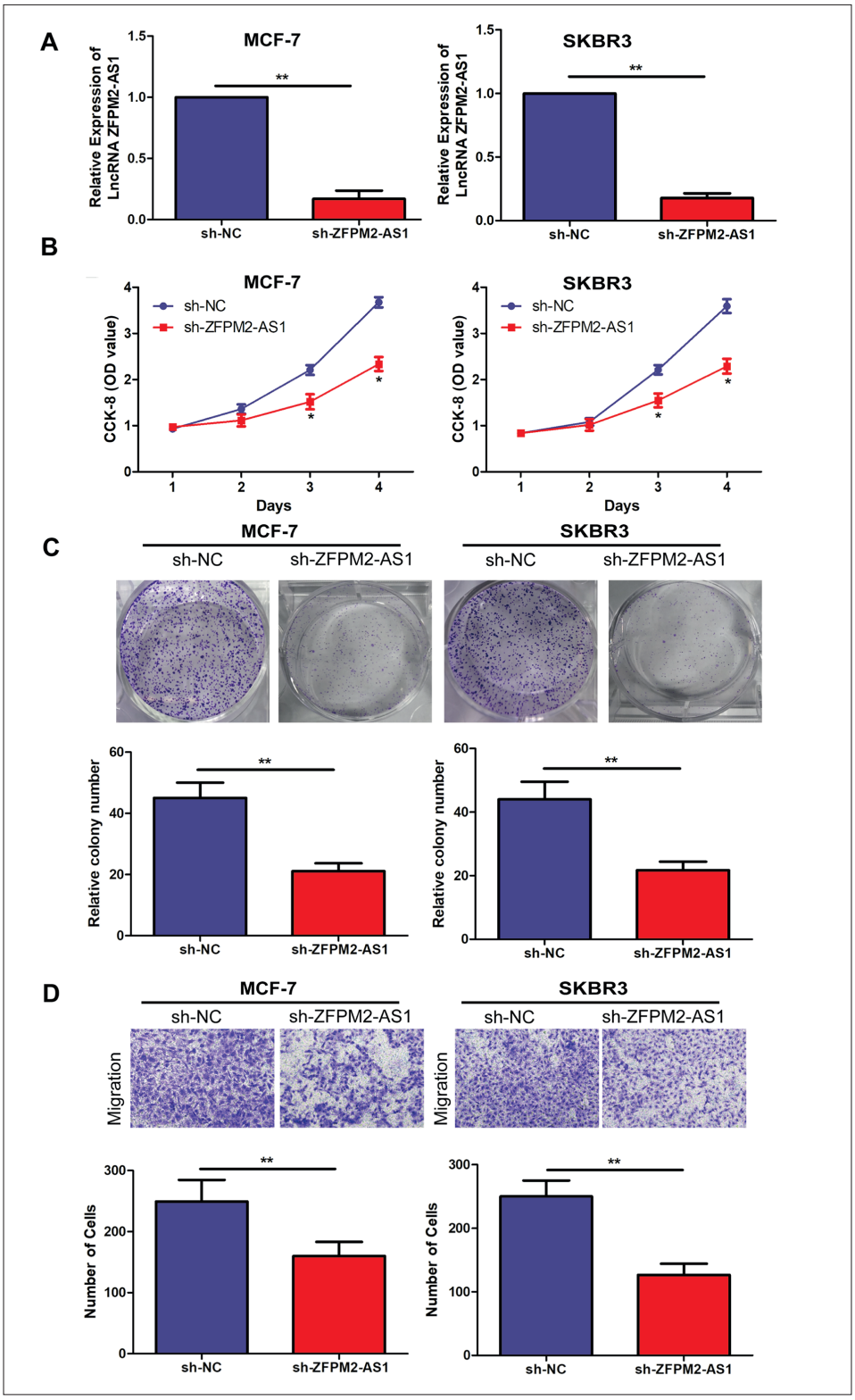

Figure 2. Knockdown of ZFPM2-AS1 inhibits proliferative and migratory abilities in BC. **A,** Transfection efficacy of sh-ZFPM2-AS1 in MCF-7 and SKBR3 cells. **B,** Viability in MCF-7 and SKBR3 cells transfected with sh-NC or sh-ZFPM2-AS1. **C,** Colony number in MCF-7 and SKBR3 cells transfected with sh-NC or sh-ZFPM2-AS1 (magnification: 10×). **D,** Migration in MCF-7 and SKBR3 cells transfected with sh-NC or sh-ZFPM2-AS1 (magnification: 40×). Data are expressed as mean ± SD, *p<0.05, **p<0.01.

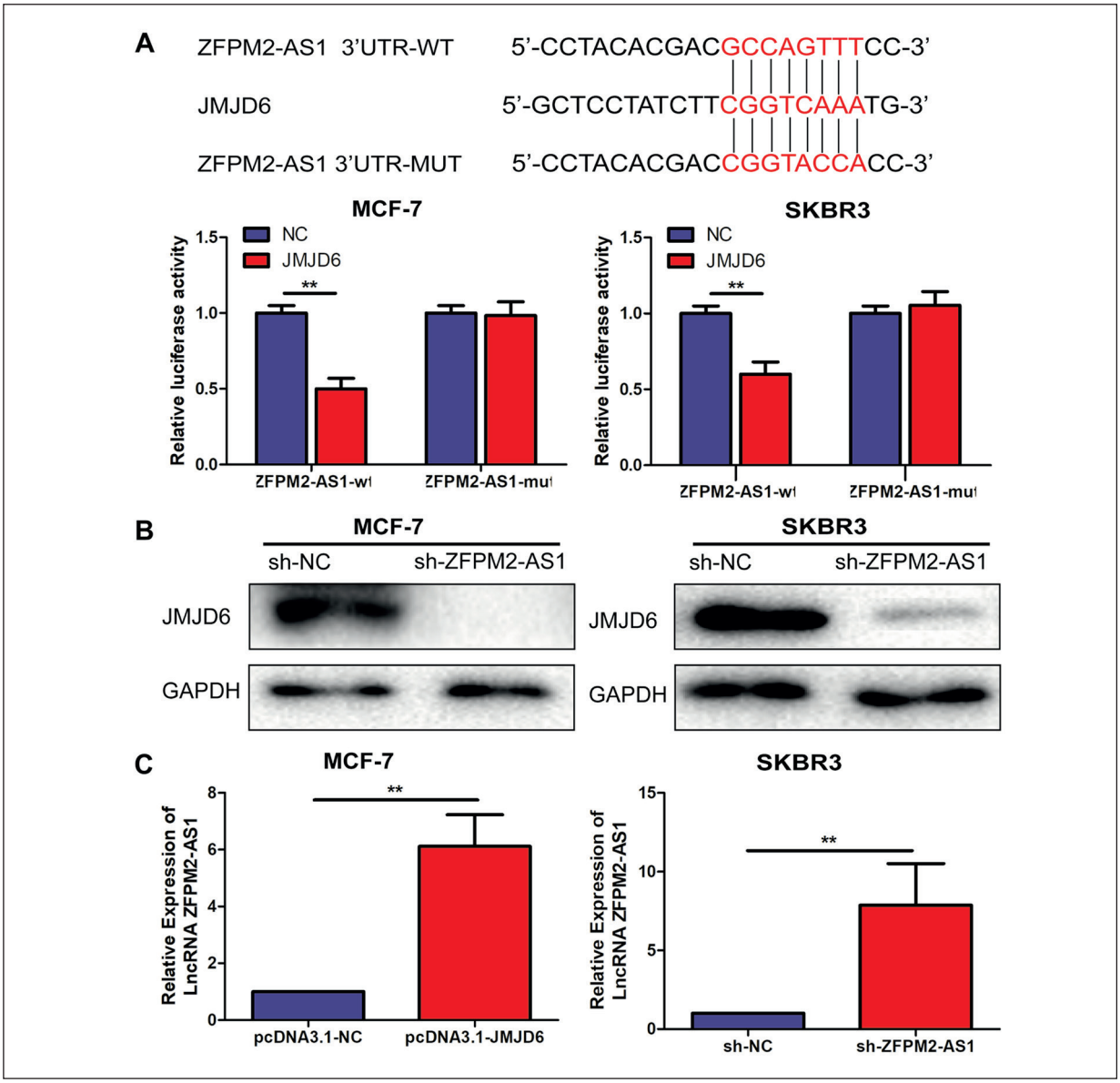

Figure 3. ZFPM2-AS1 binds to JMJD6. **A,** Binding sequences in the 3'UTR of ZFPM2-AS1 and JMJD6, and luciferase activity in co-transfected MCF-7 and SKBR3 cells. **B,** Protein level of JMJD6 in MCF-7 and SKBR3 cells transfected with sh-NC or sh-ZFPM2-AS1. **C,** ZFPM2-AS1 level in MCF-7 and SKBR3 cells transfected with pcDNA3.1-NC or pcDNA3.1-JMJD6. Data are expressed as mean ± SD, $^{**}p<0.01$.

JMJD6 Was Lowly Expressed in BC

The involvement of JMJD6 in the development of BC was further analyzed. Similar to ZFPM2-AS1, JMJD6 was highly expressed in BC cell lines (Figure 4A) and tissues (Figure 4B). In BC tissues, JMJD6 level was positively correlated with that of ZFPM2-AS1 (Figure 4C). Kaplan-Meier curves illustrated that high level of JMJD6 predicted a poor prognosis in BC patients (Figure 4D).

Knockdown of ZFPM2-AS1 Inhibited In Vivo Growth of BC

Nude mice were administrated with MCF-7 cells transfected with sh-NC or sh-ZFPM2-AS1, respectively. Compared with those of controls, mice administrated with MCF-7 cells transfected with sh-ZFPM2-AS1 presented a smaller tumor volume (Figure 5A) and lower tumor weight (Figure 5B). As expected, ZFPM2-AS1 was markedly downregulated in collected BC tissues in mice

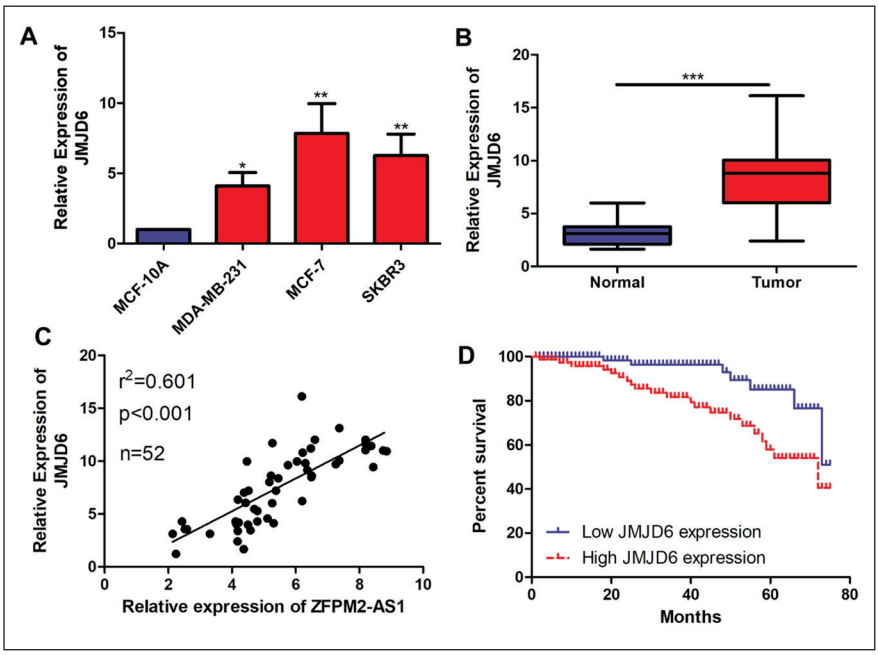

Figure 4. MJD6 is lowly expressed in BC. **A,** JMJD6 level in BC cell lines. **B,** JMJD6 level in BC tissues and normal breast tissues. **C,** A positive correlation between expression levels of ZFPM2-AS1 and JMJD6 in BC species. **D,** Survival rate in BC patients based on JMJD6 level. Data are expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

with *in vivo* knockdown of ZFPM2-AS1 (Figure 5C). Besides, protein level of JMJD6 was down-regulated in BC tissues collected from mice with *in vivo* knockdown of ZFPM2-AS1 (Figure 5D). IHC results showed the similar trend (Figure 5E). It was believed that ZFPM2-AS1 stimulated *in vivo* tumorigenesis of BC.

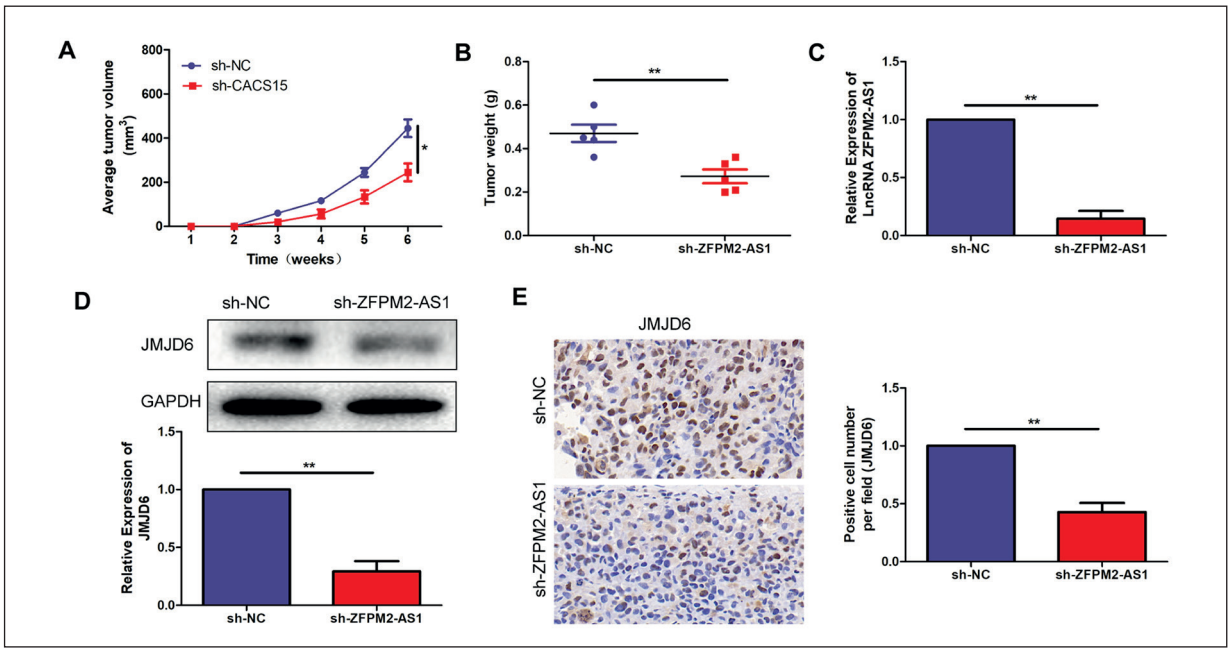

Figure 5. Knockdown of ZFPM2-AS1 inhibits *in vivo* growth of BC. **A,** Tumor volume in nude mice administrated with MCF-7 cells transfected with sh-NC or sh-ZFPM2-AS1. **B,** Tumor weight in nude mice administrated with MCF-7 cells transfected with sh-NC or sh-ZFPM2-AS1. **C,** ZFPM2-AS1 level in BC tissues collected from nude mice administrated with MCF-7 cells transfected with sh-NC or sh-ZFPM2-AS1. **D,** Protein level of JMJD6 in BC tissues collected from nude mice administrated with MCF-7 cells transfected with sh-NC or sh-ZFPM2-AS1. **E,** Positive expression of JMJD6 in BC tissues collected from nude mice administrated with MCF-7 cells transfected with sh-NC or sh-ZFPM2-AS1 (magnification: 40×). Data are expressed as mean ± SD, *p<0.05, **p<0.01.

Discussion

Breast cancer, a prevalent cancer in women[1-4], has been a global health issue with changes in living environment and lifestyle, posing a great burden on the society[10-12]. There are over 1.3 million new cases of BC and 400,000 people die of BC each year throughout the world[2-4]. It is reported that in 2018, 266,000 people were diagnosed with invasive BC and over 40,000 BC patients died in the United States[3,4]. At present, molecular target therapy and immunotherapy are promising anti-tumor treatments. However, drug resistance is an unavoidable problem that frequently occurs during clinical treatment of BC[6-9].

Genome sequences are transcribed into protein-encoding RNAs and non-coding RNAs, with the majority of the former ones[13-15]. LncRNAs are extensively involved in the regulation of locus imprinting, chromatin conformational remodeling, and allosteric regulation of enzyme activities[14-17]. Specific expression patterns of lncRNAs are related to cell phenotypes. Meanwhile, abnormally expressed lncRNAs may be indicators for human diseases[22-24]. By analyzing differentially expressed lncRNAs in BC tissues, ZFPM2-AS1 was selected. Compared with normal breast tissues, ZFPM2-AS1 was upregulated in BC tissues. After analyzing clinical data of included BC patients, it was shown that ZFPM2-AS1 level was positively correlated with advanced tumor stage and metastasis rate in BC. Moreover, ZFPM2-AS1 was an unfavorable factor of the prognosis in BC. The knockdown of ZFPM2-AS1 markedly suppressed proliferative and migratory abilities in BC cells. In addition, nude mice administrated with BC cells transfected with sh-ZFPM2-AS1 showed smaller tumor volume and lower tumor weight than controls. It is suggested that ZFPM2-AS1 is an oncogene in the malignant development of BC. The ceRNA theory proposes that lncRNAs competitively bind to corresponding proteins with target miRNAs that sharing common sequences in the 3'UTR, thus influencing tumor development[24]. Here, JMJD6 was observed to be the target of ZFPM2-AS1 since ZFPM2-AS1 vector with mutant sequences binding to JMJD6 failed to enrich JMJD6. In addition, JMJD6 was upregulated in BC species and positively correlated with ZFPM2-AS1 level. Collectively, the positive feedback loop ZFPM2-AS1 was demonstrated to facilitate the malignant development of BC *via* upregulating JMJD6.

Conclusions

These data showed that lncRNA ZFPM2-AS1 is upregulated in BC and linked to tumor stage, metastasis, and prognosis in BC patients. It aggravates the malignant development of BC *via* upregulating JMJD6.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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