


ORIGINAL ARTICLE

Long noncoding RNA ZBED3-AS1 restrains breast cancer progression by targeting the microRNA-513a-5p/KLF6 axis

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Abstract

Breast cancer (BC) is the most commonly occurring malignancy in women. This study aimed to investigate the functions of the long noncoding RNA ZBED3-AS1 (ZBED3-AS1) in BC and its molecular mechanisms. qRT-PCR was conducted to access the expression of ZBED3-AS1, microRNA-513a-5p (miR-513a-5p), and Kruppel like factor 6 (KLF6) in BC. Additionally, BC cell viability and proliferative capacity were measured by MTT and 5-Ethynyl-20-deoxyuridine (EdU) assays. A transwell assay was used for evaluating BC cell migration and invasion. The interactions among ZBED3-AS1, miR-513a-5p, and KLF6 in BC were confirmed by dual-luciferase reporter assay. Furthermore, feedback approaches were performed to determine whether ZBED3-AS1 influences BC cell behaviors by regulating the miR-513a-5p/KLF6 axis. The murine xenograft model was established to assess the effect of ZBED3-AS1 on tumor growth. The expression of ZBED3-AS1 and KLF6 was reduced, while miR-513a-5p expression was elevated in BC. ZBED3-AS1 elevation attenuated the malignant behaviors of BC cells, including viability, proliferative capacity, migration, and invasion. Mechanical experiments revealed that ZBED3-AS1 targeted miR-513a-5p, and miR-513a-5p targeted KLF6 in BC. Feedback approaches validated that miR-513a-5p overexpression or KLF6 depletion reversed the inhibitory effects of ZBED3-AS1 upregulation on viability, proliferative capacity, migration, and invasion of BC cells. Furthermore, ZBED3-AS1 elevation attenuated the tumor growth in the murine xenograft model. ZBED3-AS1 hindered the malignant development of BC cells by regulating the miR-513a-5p/KLF6 axis, providing a novel therapeutic target in BC.

KEYWORDS

breast cancer, KLF6, long noncoding RNA ZBED3-AS1, microRNA-513a-5p, viability

INTRODUCTION

Breast cancer (BC) ranks second among causes for cancer-related death in women.¹ Early BC is considered potentially curable.² However, it is diagnosed in their advanced stages due to the negligence of women regarding the self inspection and clinical examination of the breast.³ Despite advances made in diagnostic and clinical therapeutic methods, the prognosis of BC patients is still unsatisfactory.⁴ In recent years, novel targeted therapies have been used in the treatment of BC⁵ and there is an urgent need to explore new genetic targets for BC therapy.

Long noncoding RNAs (lncRNAs) participate in diverse gynecological cancers, such as ovarian cancer,⁶ cervical cancer,⁷ and BC.⁸ It has been documented that certain lncRNAs attenuate the tumorigenesis of BC. Qin et al. have determined that lncRNA on chromosome 8p12 restrains the proliferation capacity of BC cells.⁹ Kim et al. have demonstrated that lncRNA metastasis associated lung adenocarcinoma transcript 1 retards the metastasis of BC.¹⁰ ZBED3-AS1 has been found to be involved in the regulation of stem cell chondrogenesis.^{11,12} Notably, Cui et al. have reported that ZBED3-AS1 is revealed to be associated with BC through preliminary bioinformatics

analysis.¹³ However, the precise function of ZBED3-AS1 in the progression of BC remains unclear.

Numerous microRNAs (miRNAs) have been confirmed to exert a tumor-promoting effect in BC. For instance, miR-455-5p is upregulated in BC and accelerates cell invasion and migration.¹⁴ MiR-146a enhances the proliferation and metastasis of BC cells by suppressing NM2-H1.¹⁵ Previous research has reported that miR-513a-5p often functions as an onco-miR in diverse cancers. MiR-513a-5p overexpression enhances proliferation and attenuates apoptosis of nephroblastoma cells.¹⁶ A high miR-513a-5p level inhibits glioma cell sensitivity to temozolomide.¹⁷ Notably, lncRNA ADAMTS9-AS1 attenuates the aggressive phenotypes of BC cells through downregulating miR-513a-5p.¹⁸ However, the specific regulatory relationship between ZBED3-AS1 and miR-513a-5p in BC remains undefined.

KLF6, a member of the KLFs family, plays a pivotal role in biological processes, such as proliferation, differentiation, and metabolism.¹⁹ KLF6 often acts as an anti-oncogene in diverse cancers. For instance, KLF6 attenuates oral cancer migration and invasion by inhibiting mesenchymal marker and MMP-9 activities.²⁰ LncRNA CR749391 interacts with miR-181a to enhance KLF6 expression, hampering the malignant development of gastric cancer cells.²¹ Importantly, KLF6 suppresses estrogen receptor-mediated cell growth in BC by targeting c-Scr.²² However, the relationship between ZBED3-AS1 and KLF6 in BC is still unknown.

Here, ZBED3-AS1 expression was evaluated in BC. The effects of ZBED3-AS1 on the malignant behaviors of BC cells were assessed. We explored whether miR-513a-5p is a target of ZBED3-AS1 and the relationship between miR-513a-5p and KLF6 in BC. This study may reveal molecular mechanisms and offer a new therapeutic target for BC.

MATERIAL AND METHOD

Clinical samples

A total of 17 BC patients who underwent mastectomy from January 2018 to January 2020 at our hospital were recruited to the study. BC tissues ($n = 17$, tumor group) and paired adjacent nontumor tissues ($n = 17$, normal group) were stored at -80°C . Prior to mastectomy, no radiotherapy or chemotherapy treatment was administered to the patients. This study was permitted by the Yantaishan Hospital Ethics Committee (approval no. 2016018), and informed consent was obtained from all participants.

Bioinformatics analysis

The gene expression analysis for the cancer genome atlas (TCGA) or GSE15852 cohort of BC was obtained from gene expression profiling interactive analysis 2 (GEPIA2) or the gene expression omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>), respectively. Kaplan–Meier analysis of the overall

survival of BC patients was downloaded from GENT2. StarBase and LncBase were used for predicting the miRNAs that target ZBED3-AS1. Four miRNAs were identified by both prediction tools. The expression of miR-513a-5p was significantly higher in both MDA-MB-468 and MCF7 cells than in MCF-10A cells. Thus, miR-513a-5p was selected as the candidate miRNA for further experiments. TargetScan was employed to predict the target of miR-513a-5p, one of which was Kruppel like factor 6 (KLF6). KLF6 is a well-known anti-oncogene and is downregulated in BC cells.²² Thus, KLF6 was selected for following research.

Cell culture

Human breast epithelial cell line MCF-10A and four BC cell lines, MCF7, MDA-MB-231, MDA-MB-468, and MDA-MB-453 (American Type Culture Collection, Manassas, VA, USA), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplied with 10% fetal bovine serum (FBS, Invitrogen) at 37°C with 5% CO_2 .

Cell transfection

The pcDNA3.1 (pcDNA)-ZBED3-AS1, pcDNA-negative control (NC), miR-513a-5p mimics, miR-513a-5p inhibitor, mimics NC (miR-NC), inhibitor NC, short hairpin (sh)-KLF6, and sh-NC were synthesized by GenePharma. MDA-MB-468 and MCF7 cells grown to 80% confluence were transfected or co-transfected with these agents using Lipofectamine 3000 reagent (Invitrogen). After 48 h of transfection, MDA-MB-468 and MCF7 cells were used for further studies.

qRT-PCR and western blot

Quantitative real-time polymerase chain reaction (qRT-PCR) and western blot procedures were conducted as previously

TABLE 1 Primer sequences

Name of primer	Sequence (5'-3')
ZBED3-AS1-F	TACAACTTTGCATTAACCTTCC
ZBED3-AS1-R	TGCCCTGTCTCATGTTCCG
GAPDH-F	CATGAGAAGTATGACAACAGCCT
GAPDH-R	AGTCCTTCCACGATACCAAAGT
miR-513a-5p-F	TAAATTTACCTTTCTGAGAAGG
miR-513a-5p-R	GCGAGCACAGAATTAATACGAC
U6-F	CTCGCTTCGGCAGCAC
U6-R	AACGCTTACGAATTTGCGT
KLF6-F	AGTTAACAGGCACCTTCCG
KLF6-R	CTTTTAGCCTACAGGATCCACC
β -actin-F	CGACACCAAGAAGCAGAG
β -actin-R	GAATCAATCCAACAGTAGCC

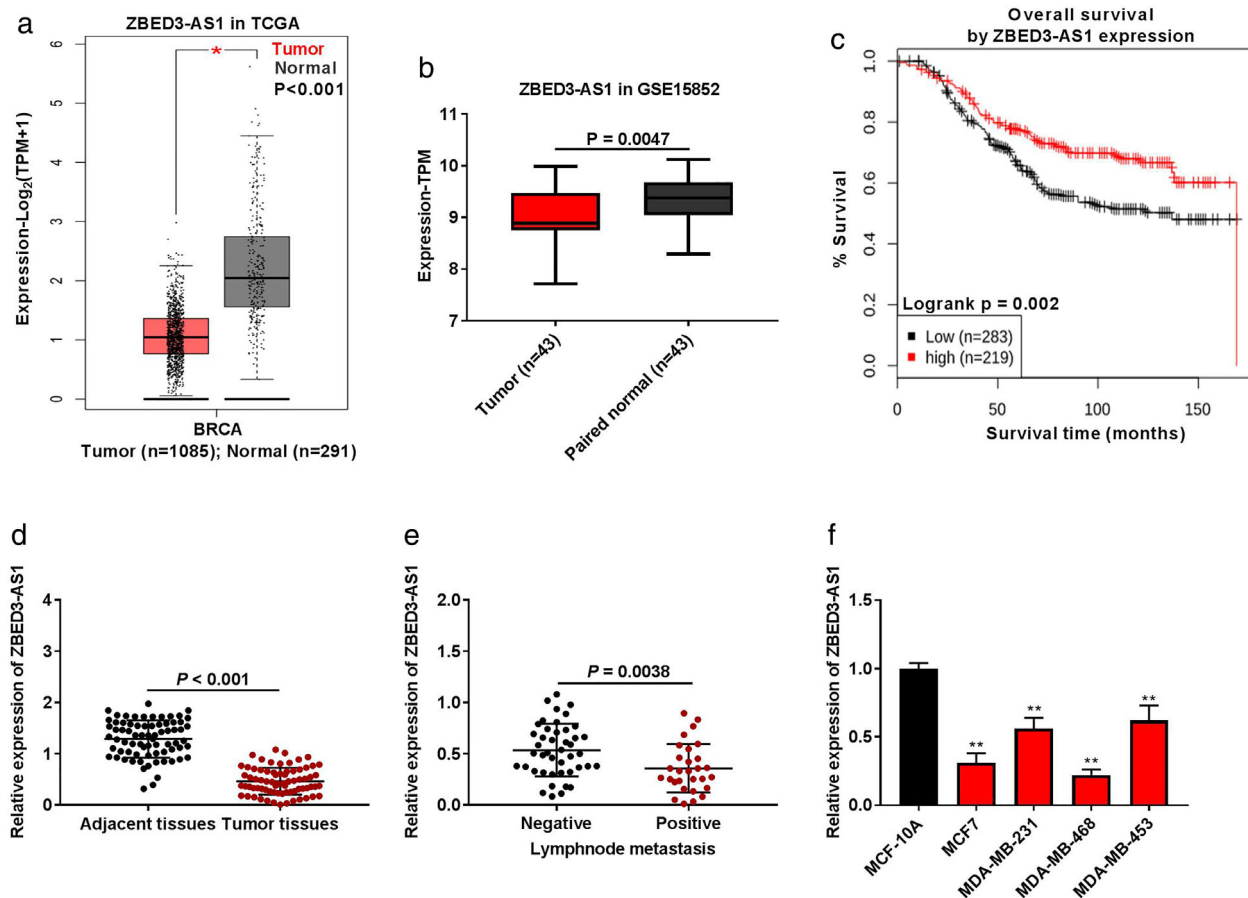


FIGURE 1 ZBED3-AS1 was considerably inhibited in breast cancer (BC). (a) ZBED3-AS1 expression analysis for the cancer genome atlas (TCGA) cohort of BC was obtained from GEPIA2 (<http://gepia2.cancer-pku.cn>). (b) the gene expression analysis for the GSE15852 cohort of BC was obtained from the gene expression omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/>). (c) Kaplan–Meier analysis of the overall survival of BC patients was obtained from GENT2 (<http://gent2.appex.kr/gent2/>). (d) qRT-PCR was implemented to detect the expression of ZBED3-AS1 in BC tissues and adjacent nontumor tissues ($n = 17$), $p < 0.001$ versus adjacent tissues. (e) The expression of ZBED3-AS1 in tissues from lymph node metastasis (LMN)-positive patients ($n = 6$) and LMN-negative patients ($n = 11$) was detected by qRT-PCR, $p = 0.0038$ versus negative. (f) qRT-PCR was performed to measure the expression of ZBED3-AS1 in MCF-10A, MCF7, MDA-MB-231, MDA-MB-468, and MDA-MB-453 cells, $p < 0.01$ versus MCF-10A

described.²³ The primers are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), RNU6-1 (U6), and β -actin were used for normalizing the expressions of ZBED3-AS1, miR-513a-5p, and KLF6, respectively. The antibodies for western blot analysis were anti-KLF6 (1:1000, av35639; Sigma) and anti- β -actin (1:1000, MABT523; Sigma) primary antibody, and HRP conjugate secondary antibody (1:5000, 12-348; Sigma). ImageLab software (Bio-Rad) was utilized to quantify the density of each band.

MTT assay

MTT assay was performed to evaluate cell viability. MDA-MB-468 and MCF7 cells (2×10^3 /well) were seeded in 96-well plates and incubated with 5% CO₂ at 37°C. At different time points (0, 24, 48, 72, and 96 h post-transfection), 10 μ l of MTT (5 mg/ml; Sigma) was added to each well and cells were cultured for another 4 h, then the absorbance was measured at 450 nm.

EdU assay

5-Ethynyl-20-deoxyuridine (EdU) assay were performed to assess cell proliferation. MDA-MB-468 and MCF7 cells (2×10^3 /well) were seeded in 96-well plates and incubated in EdU solution (50 nmol/l) for 2 h and then visualized. A fluorescence microscope was used to obtain images.

Cell migration and invasion

An invasion assay was conducted using a 24-well invasion chamber system coated with 50 μ l Matrigel (Sigma). MDA-MB-468 and MCF7 cells (1×10^5 cells/well) suspended in serum-free medium were seeded into a precoated upper chamber. Medium supplement with 10% fetal bovine serum (FBS) was plated into the lower chamber. The invaded MDA-MB-468 and MCF7 cells were dyed using crystal violet after 24 h of cultivation, and were then counted. For the assessment of migration,

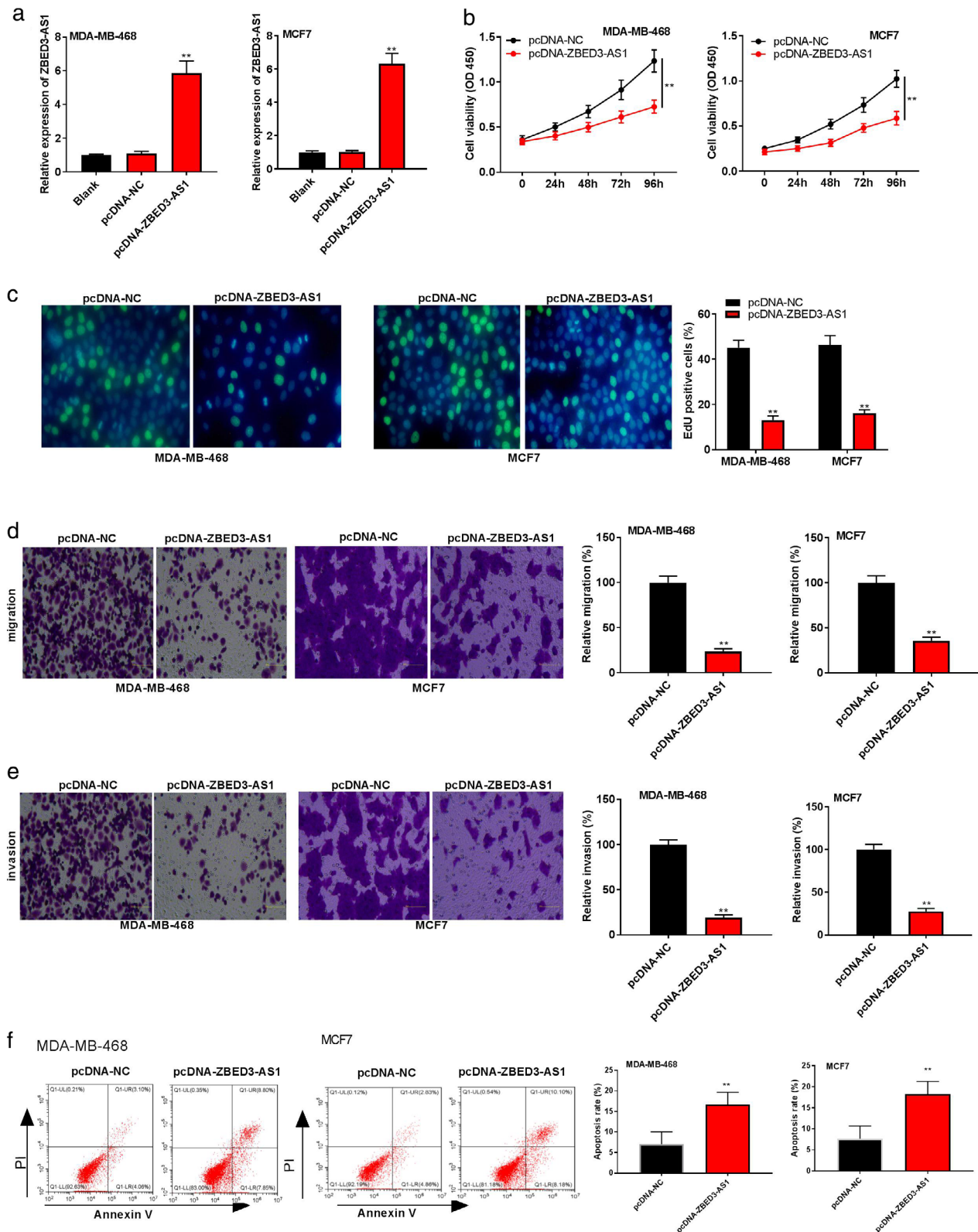


FIGURE 2 Overexpressed ZBED3-AS1 inhibited the proliferation, migration, and invasion, but promoted apoptosis of breast cancer (BC) cells. (a) The transfection efficiency of pcDNA-NC and pcDNA-ZBED3-AS1 in MDA-MB-468 and MCF7 cells was measured by qRT-PCR, ** $p < 0.01$ versus pcDNA-NC. (b) and (c) The viability and proliferative capacity of MDA-MB-468 and MCF7 cells were analyzed by MTT and EdU assays, ** $p < 0.01$ versus pcDNA-NC. (d) and (e) The migration and invasion of MDA-MB-468 and MCF7 cells were measured by transwell assay, ** $p < 0.01$ versus pcDNA-NC. (f) The apoptosis of MDA-MB-468 and MCF7 cells was measured by flow cytometry analysis, ** $p < 0.01$ versus pcDNA-NC

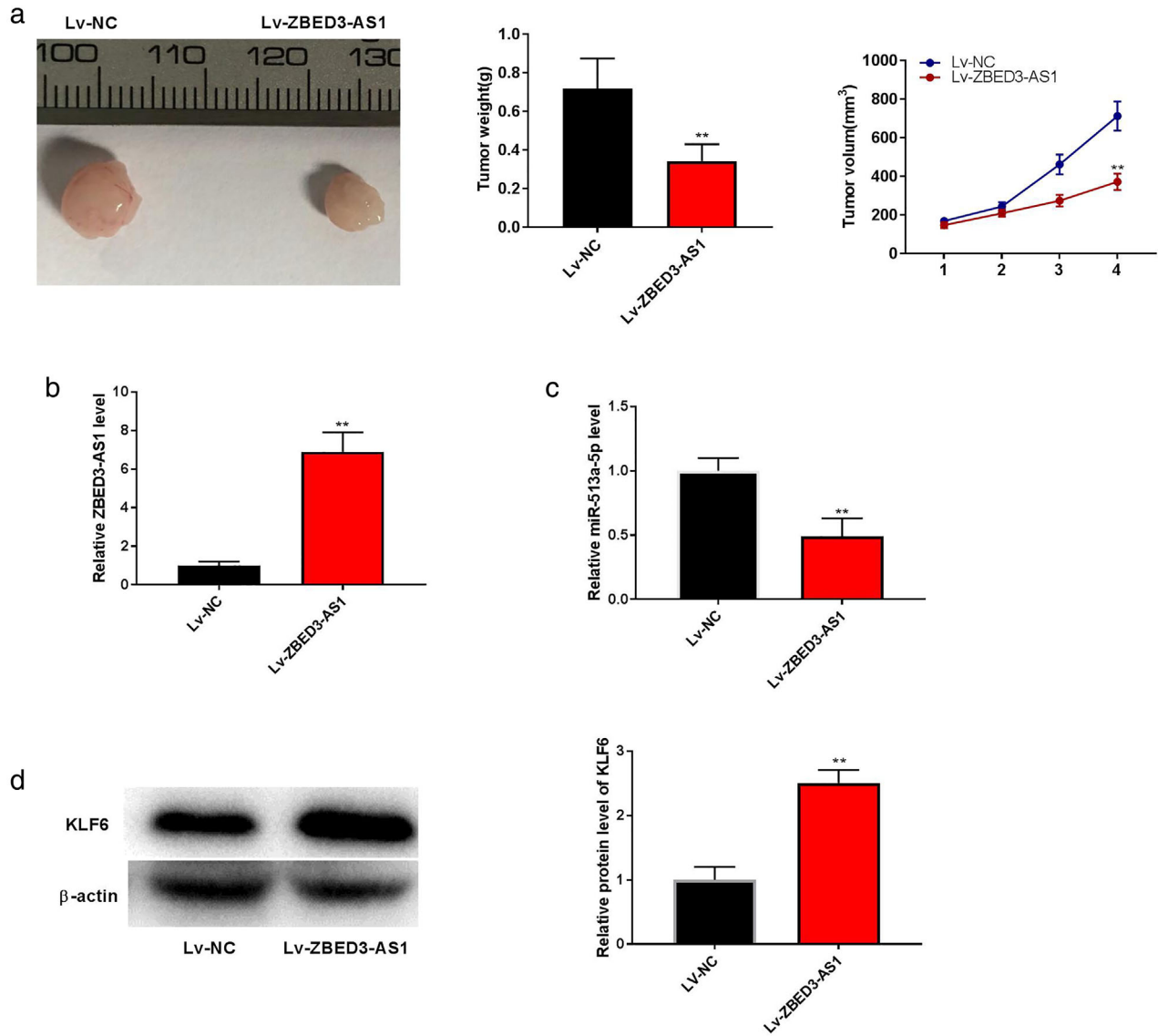


FIGURE 3 ZBED3-AS1 overexpression attenuated the tumorigenesis of breast cancer (BC). (a) The effects of ZBED3-AS1 overexpression on BC tumor volume and tumor weight, ** $p < 0.01$ versus Lv-NC. (b) The expression of ZBED3-AS1 in BC tissues of mice after injection of Lv-ZBED3-AS1 was detected by qRT-PCR, ** $p < 0.01$ versus Lv-NC. (c) The expression of miR-513a-5p in BC tissues of mice after injection of Lv-ZBED3-AS1 was detected by qRT-PCR, ** $p < 0.01$ versus Lv-NC. (d) The protein level of KLF6 in BC tissues of mice after injection of Lv-ZBED3-AS1 was determined by western blot, ** $p < 0.01$ versus Lv-NC

MDA-MB-468 and MCF7 cells were seeded into the non-coated upper chamber and the other steps were same as above.

Flow cytometry analysis

The apoptosis of MDA-MB-468 and MCF7 cells was assessed by flow cytometric analysis. Briefly, the collected BC cells (1×10^5 cells/ml) were cultured in 96-well plates for 24 h and then stained with annexin V-fluorescein isothiocyanate and propidium iodide using an apoptosis detection kit (Thermo Fisher Scientific) at 25°C for 20 min in the dark. The apoptotic cells were measured using a flow cytometer (BD Biosciences).

Murine xenograft tumor model

Ten female BALB/c nude mice (4-week-old) were purchased from Esebio. The animal experiments and programs were approved by the Animal Care and Use Committee of Yantaishan Hospital. MDA-MB-468 cells (5×10^6 cells) infected with lentiviral vector containing ZBED3-AS1 (lv-ZBED3-AS1) or empty lentiviral vector (lv-NC) were intraperitoneally injected into mice ($n = 5$ per group). Tumor volume was detected 1 week after inoculation and calculated using the following formula: $\frac{1}{2}LW^2$ (L, length; W, width). After the last measurement at the fourth week, mice were anesthetized and sacrificed by cervical dislocation, and the tumor was weighted.

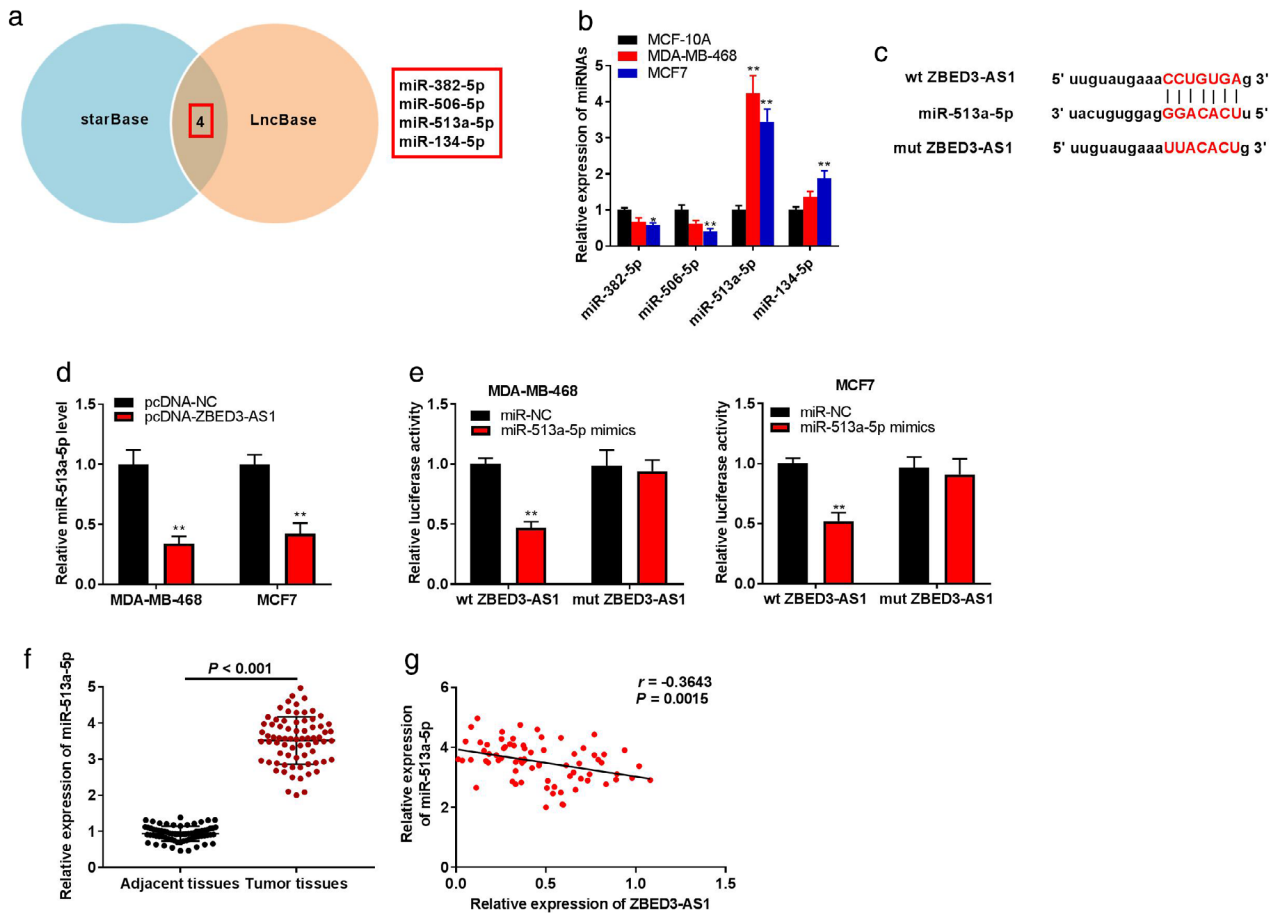


FIGURE 4 MiR-513a-5p was a target of ZBED3-AS1. (a) MiR-513a-5p was predicted to be a potential ZBED3-AS1 target via StarBase and LncBase. (b) The expression of miR-382-5p, miR-506-5p, miR-513a-5p, and miR-134-5p was detected by qRT-PCR in MCF-10A, MDA-MB-468, and MCF7 cells, $**p < 0.01$ versus MCF-10A. (c) Starbase exhibited the predicted binding site between ZBED3-AS1 and miR-513a-5p. (d) The expression of miR-513a-5p was decreased by the transfection of pcDNA-ZBED3-AS1 in MDA-MB-468 and MCF7 cells, $**p < 0.01$ versus pcDNA-NC. (e) Relative luciferase activity in MDA-MB-468 and MCF7 cells was assessed by dual-luciferase reporter assay, $**p < 0.01$ versus miR-NC. (f) qRT-PCR was performed to detect the expression of miR-513a-5p in breast cancer (BC) tissues and adjacent nontumor tissues, $p < 0.001$ versus adjacent tissues. (g) The expression of ZBED3-AS1 was negatively correlated with miR-513a-5p in BC patients

Dual-luciferase reporter assay

We generated ZBED3-AS1 and KLF6 sequences with wild type (WT) or mutant type miR-513a-5p-binding sites and cloned them in pGL3-Basic vectors (Ke Lei Biological Technology). MDA-MB-468 and MCF7 cells were co-transfected with above luciferase vectors and miR-NC or miR-513a-5p mimics by Lipofectamine 3000 (Invitrogen).

Statistical analyses

GraphPad Prism 8.0 was used for statistical analyses. Data were presented as the mean \pm SD. Variances among groups were compared by Student's *t*-test or one-way ANOVA followed by Tukey's multiple comparisons test. The significance of the correlations was evaluated by Pearson's correlation analysis. *p* values < 0.05 indicated statistically significance.

RESULTS

ZBED3-AS1 was considerably inhibited in BC

As shown in Figure 1(a), the ZBED3-AS1 expression in 1085 BC tumor tissues was found to be lower than that in 291 normal breast tissues by using TCGA database. In addition, the dataset GSE15852 showed that ZBED3-AS1 expression in BC tumor tissues ($n = 43$) was considerably downregulated compared with that in paired normal tissues ($n = 43$) ($p = 0.0047$; Figure 1(b)). The Kaplan–Meier curve showed that the survival percentage of BC patients with higher ZBED3-AS1 expression ($n = 219$) was markedly better than that in the lower expression group ($n = 283$) (logrank $p = 0.002$; Figure 1(c)). The ZBED3-AS1 expression in BC tissues was evidently decreased compared with the adjacent nontumor tissues ($p < 0.001$; Figure 1(d)). ZBED3-AS1 expression in tissues from lymph node

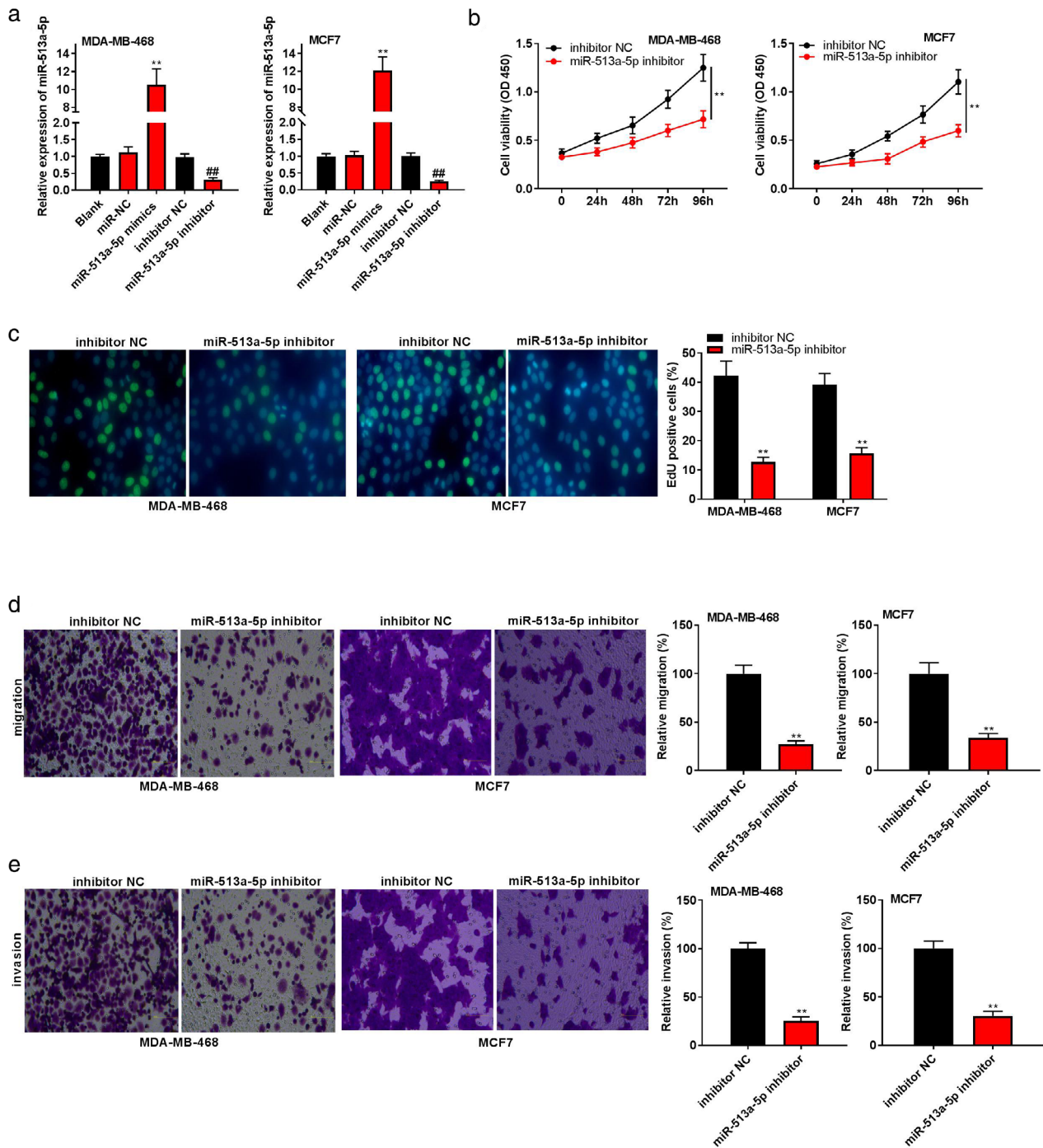


FIGURE 5 MiR-513a-5p suppression retarded the tumorigenesis of breast cancer (BC) cells. (a) The transfection efficiency of miR-NC, miR-513a-5p mimics, inhibitor NC, and miR-513a-5p inhibitor was evaluated by qRT-PCR in MDA-MB-468 and MCF7 cells, ** $p < 0.01$ versus miR-NC, ## $p < 0.01$ versus inhibitor NC. (b) and (c) MTT and EdU assays were performed to measure the viability and proliferative capacity of MDA-MB-468 and MCF7 cells after transfection with inhibitor NC or miR-513a-5p inhibitor, ** $p < 0.01$ versus inhibitor NC. (d) and (e) The effects of miR-513a-5p overexpression on the migration and invasion of MDA-MB-468 and MCF7 cells were evaluated by transwell assay, ** $p < 0.01$ versus inhibitor NC

metastasis (LMN)-positive patients was lower than that in LMN-negative patients ($p = 0.0038$; Figure 1(e)). Moreover, ZBED3-AS1 expression was markedly decreased in MCF7, MDA-MB-231, MDA-MB-468, and MDA-MB-453 cells compared with MCF-10A cells ($p < 0.01$; Figure 1(f)).

Overexpressed ZBED3-AS1 inhibited the proliferation, migration, and invasion, but promoted apoptosis of BC cells

We then explored the possible role of ZBED3-AS1 in BC progression in vitro by transfection of pcDNA-

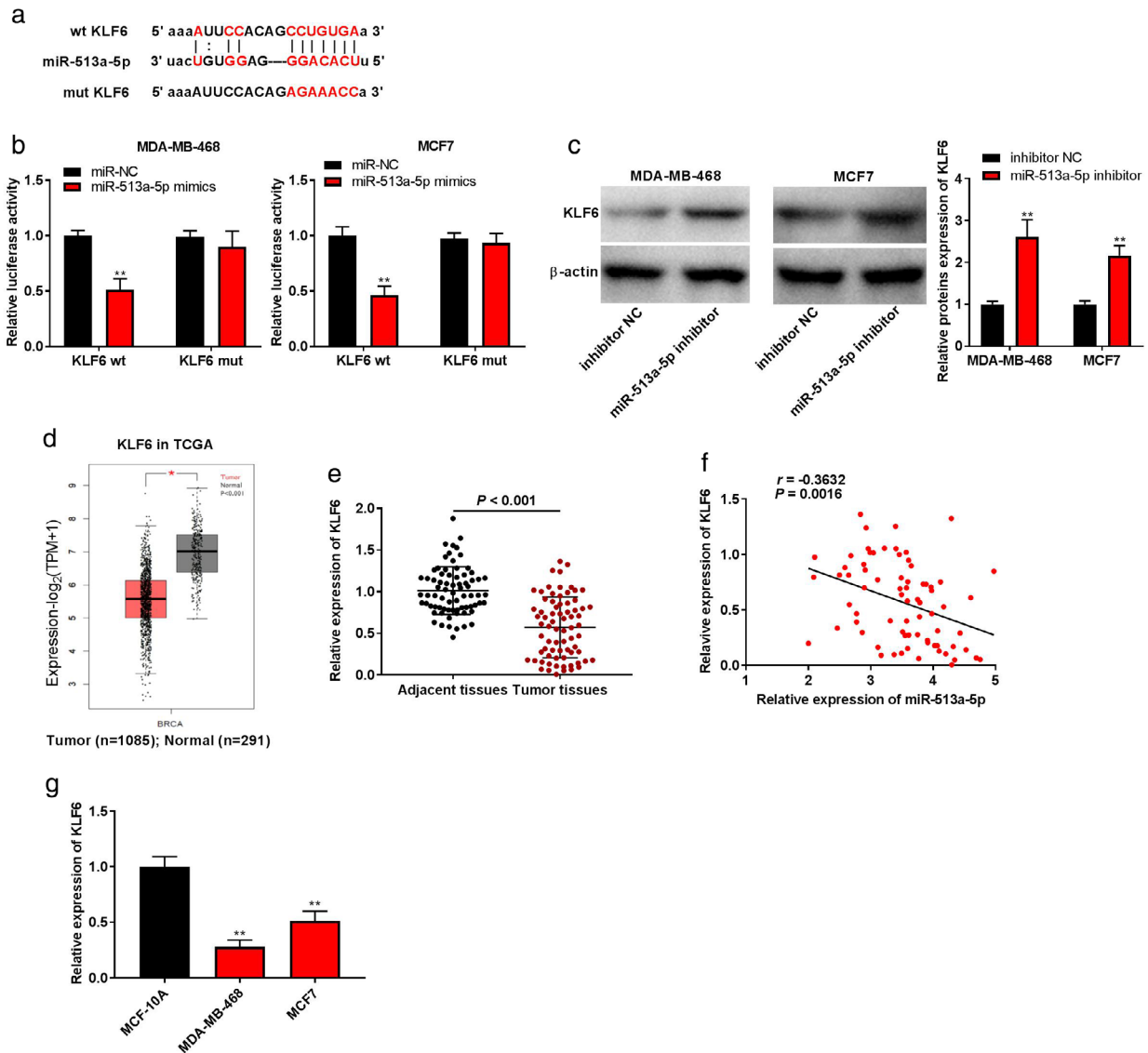


FIGURE 6 KLF6 was a target of miR-513a-5p. (a) TargetScan displayed the predicted binding site between miR-513a-5p and KLF6. (b) Dual-luciferase reporter assay was performed to assess the relative luciferase activity in MDA-MB-468 and MCF7 cells, $**p < 0.01$ versus miR-NC. (c) The protein expression of KLF6 in MDA-MB-468 and MCF7 cells was measured by western blot, $**p < 0.01$ versus inhibitor NC. (d) KLF6 expression analysis for the cancer genome atlas (TCGA) cohort of BC was obtained from GEPIA2 (<http://gepia2.cancer-pku.cn>). (e) qRT-PCR was conducted to detect the expression of KLF6 in breast cancer (BC) tissues and adjacent nontumor tissues, $p < 0.001$ versus adjacent tissues. (f) The expression of miR-513a-5p was negatively correlated with KLF6 in BC patients. (g) The expression of KLF6 was detected by qRT-PCR in MCF-10A, MDA-MB-468, and MCF7 cells, $**p < 0.01$ versus MCF-10A

ZBED3-AS1 in MDA-MB-468 and MCF7 cells. As shown in Figure 2(a), ZBED3-AS1 was effectively upregulated after pcDNA-ZBED3-AS1 transfection in MDA-MB-468 and MCF7 cells ($p < 0.01$). MTT and EdU assays revealed that ZBED3-AS1 overexpression clearly suppressed MDA-MB-468 and MCF7 cell viability and proliferative capacity, respectively ($p < 0.01$; Figure 2(b),(c)). Additionally, the transwell assay showed that ZBED3-AS1 upregulation could visibly reduce MDA-MB-468 and MCF7 cell migration and invasion, respectively ($p < 0.01$; Figure 2(d),(e)). We further found that overexpression of ZBED3-AS1 promoted the apoptosis of MDA-MB-468 and MCF7 cells ($p < 0.01$; Figure 2(f)).

ZBED3-AS1 overexpression attenuated the tumorigenesis of BC

The functions of ZBED3-AS1 on the tumorigenesis of BC in vivo were also investigated. As illustrated in Figure 3(a), ZBED3-AS1 overexpression could markedly decrease the tumor weight and volume at the fourth week after injection ($p < 0.01$; Figure 3(a)). In addition, the expression levels of ZBED3-AS1, miR-513a-5p, and KLF6 in BC tissues of mice were detected. As presented in Figure 3(b)–(d), we demonstrated that after injection with Lv-ZBED3-AS1, the expressions of ZBED3-AS1 and protein level of KLF6 were elevated, and miR-513a-5p expression was repressed ($p < 0.01$).

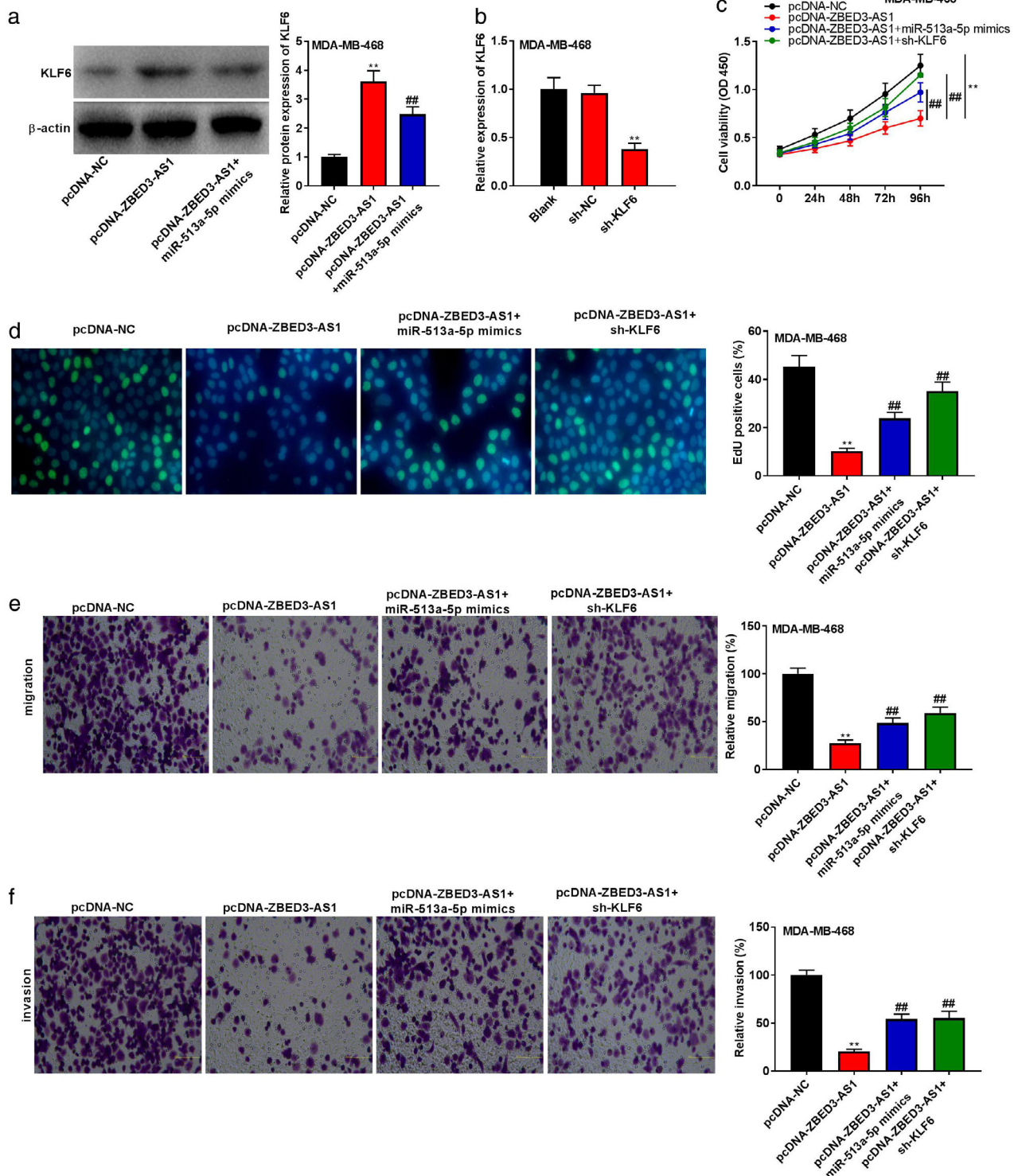


FIGURE 7 ZBED3-AS1 overexpression restrained the malignant behaviors of MDA-MB-468 cells by regulating the miR-513a-5p/KLF6 axis. (a) Western blot was performed to measure the protein expression of KLF6 in MDA-MB-468 cells, ** $p < 0.01$ versus pcDNA-NC, ## $p < 0.01$ versus pcDNA-ZBED3-AS1. (b) The transfection efficiency of sh-NC and sh-KLF6 was measured by qRT-PCR in MDA-MB-468 cells, ** $p < 0.01$ versus sh-NC. (c)–(f) Upregulation of miR-513a-5p or downregulation of KLF6 reversed the inhibitory effects of ZBED3-AS1 overexpression on viability, proliferative capacity, migration, and invasion of MDA-MB-468 cells, ** $p < 0.01$ versus pcDNA-NC, ## $p < 0.01$ versus pcDNA-ZBED3-AS1

MiR-513a-5p is a target of ZBED3-AS1

The miR-382-5p, miR-506-5p, miR-513a-5p, and miR-134-5p were predicted to be the potential ZBED3-AS1

targets according to StarBase and LncBase (Figure 4(a)). Among these potential ZBED3-AS1 targets, only the expression of miR-513a-5p was significantly higher in both MDA-MB-468 and MCF7 cells than in MCF-10A cells ($p < 0.01$;

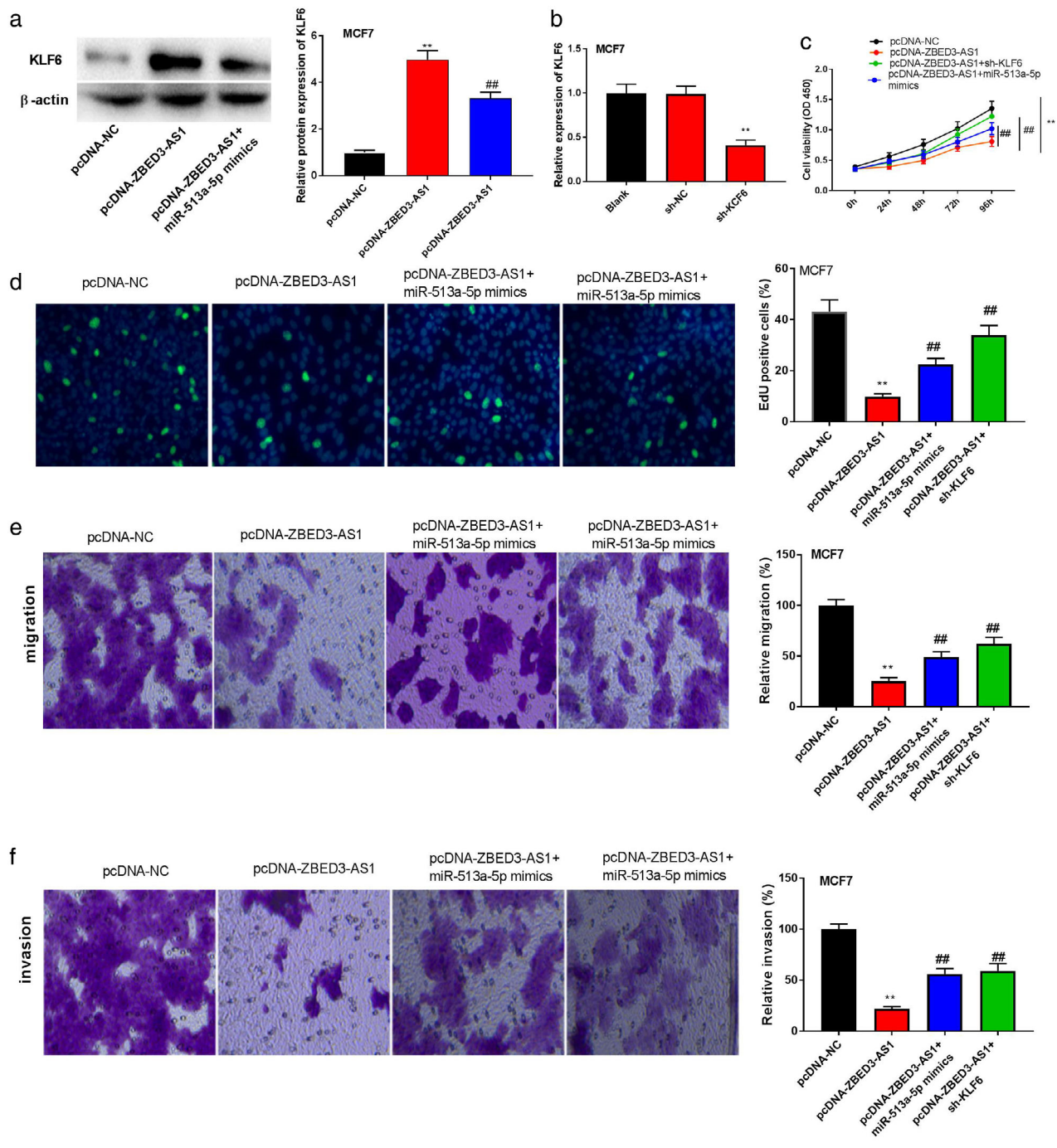


FIGURE 8 ZBED3-AS1 overexpression restrained the malignant behaviors of MCF7 cells by regulating the miR-513a-5p/KLF6 axis. (a) Western blot was performed to measure the protein expression of KLF6 in MCF7 cells, ** $p < 0.01$ versus pcDNA-NC, ## $p < 0.01$ versus pcDNA-ZBED3-AS1. (b) The transfection efficiency of sh-NC and sh-KLF6 was measured by qRT-PCR in MCF7 cells, ** $p < 0.01$ versus sh-NC. (c)–(f) Upregulation of miR-513a-5p or downregulation of KLF6 reversed the inhibitory effects of ZBED3-AS1 overexpression on viability, proliferative capacity, migration, and invasion of MCF7 cells, ** $p < 0.01$ versus pcDNA-NC, ## $p < 0.01$ versus pcDNA-ZBED3-AS1

Figure 4(b)). We therefore selected miR-513a-5p as the candidate miRNA for subsequent studies. StarBase showed that ZBED3-AS1 contained the target site of miR-513a-5p (Figure 4(c)). ZBED3-AS1 overexpression could evidently repress miR-513a-5p expression in MDA-MB-468 and MCF7 cells ($p < 0.01$; Figure 4(d)). The dual-luciferase

reporter assay revealed that miR-513a-5p upregulation effectively hindered the activity of WT-ZBED3-AS1 reporter in MDA-MB-468 and MCF7 cells ($p < 0.01$; Figure 4(e)). Furthermore, miR-513a-5p expression was dramatically elevated in BC tissues compared to adjacent nontumor tissues ($p < 0.001$; Figure 4(f)). An inverse expression correlation

between ZBED3-AS1 and miR-513a-5p was found in BC patients ($r = -0.3643$, $p = 0.0015$; Figure 3(g)).

MiR-513a-5p suppression retards the tumorigenesis of BC cells

Figure 5(a) shows that miR-513a-5p expression was clearly enhanced or attenuated following miR-513a-5p mimics or miR-513a-5p inhibitor transfection in MDA-MB-468 and MCF7 cells ($p < 0.01$). MiR-513a-5p suppression considerably weakened the viability and proliferative capacity of MDA-MB-468 and MCF7 cells ($p < 0.01$; Figure 5(b),(c)). In parallel, miR-513a-5p deficiency could visibly attenuate the migration and invasion of MDA-MB-468 and MCF7 cells ($p < 0.01$; Figure 5(d),(e)).

KLF6 was a target of miR-513a-5p

TargetScan showed that miR-513a-5p binds to the 3'-untranslated region of KLF6 (Figure 6(a)). The dual-luciferase reporter assay determined that miR-513a-5p overexpression effectively inhibited the activity of WT-KLF6 reporter in MDA-MB-468 and MCF7 cells ($p < 0.01$; Figure 6(b)). Additionally, miR-513a-5p deficiency could significantly enhance KLF6 protein expression in MDA-MB-468 and MCF7 cells ($p < 0.01$; Figure 6(c)). Decreased KLF6 expression was found in BC tumor tissues (1085 cases) compared to those of normal breast tissues (291 cases) using the TCGA database ($p < 0.001$; Figure 6(d)). Moreover, KLF6 expression was effectively downregulated in BC tissues compared to adjacent nontumor tissues ($p < 0.001$; Figure 6(e)). A negative correlation between KLF6 and miR-513a-5p expression was observed in BC patients ($r = -0.3632$, $p = 0.0016$; Figure 6(f)). Compared with MCF-10A cells, KLF expression was visibly reduced in MDA-MB-468 and MCF7 cells ($p < 0.01$; Figure 6(g)).

ZBED3-AS1 overexpression restrained the malignant behaviors of BC cells by regulating the miR-513a-5p/KLF6 axis

ZBED3-AS1 overexpression could clearly enhance the KLF6 protein expression in MDA-MB-468 cells, and miR-513a-5p overexpression mitigated this effect ($p < 0.01$; Figure 7(a)). After sh-KLF6 transfection, KLF6 expression was effectively suppressed in MDA-MB-468 cells ($p < 0.01$; Figure 7(b)). Feedback approaches were conducted to determine whether ZBED3-AS1 exerts its functions by targeting the miR-513a-5p/KLF6 axis. As shown in Figure 7(c)–(f), miR-513a-5p elevation or KLF6 depletion considerably reversed the suppressive effects of ZBED3-AS1 overexpression on the viability, proliferative capacity, migration, and invasion of MDA-MB-468 cells ($p < 0.01$). As expected, similarly results were observed in MCF7 cells (Figure 8(a)–(f)).

DISCUSSION

It has been documented that the expression of lncRNAs such as AY343892,²⁴ XIST,²⁵ and TUBA4B²⁶ is downregulated and associated with the tumorigenesis of BC. In the present study, ZBED3-AS1 expression was considerably suppressed in BC tissues and cells. In addition, ZBED3-AS1 expression was found to be correlated with lymph node metastasis (LNM) in BC patients, and low expression of ZBED3-AS1 brought out an adverse function in survival. Previous studies have demonstrated that certain lncRNAs are downregulated and related to poor prognosis in BC. For instance, a low lncRNA NBAT1 level is associated with LNM as well as with poor patient survival in BC.²⁷ LncRNA LINC01133 reduction is significantly correlated with LNM and advanced tumor node metastasis stage in BC patients.²⁸ The expression of LINC00641 is lower in patients with a higher stage of BC and patients with LNM.²⁹ Taken together, these data implied that ZBED3-AS1 may be involved in the progression of BC.

Numerous studies have confirmed that lncRNA takes part in the cellular processes of BC. For instances, lncRNA MAGI2-AS3 overexpression restrains BC cell migration and invasion through downregulating miR-374a and upregulating phosphatase and tensin homolog deleted on chromosome ten.³⁰ LncRNA LOXL1-AS1 negatively regulates miR-143-3p to attenuate the proliferation and enhance apoptosis of BC cells.³¹ LncRNA MIR503HG upregulation represses miR-103 to enhance OLFM4 expression, retarding the malignant development of BC cells.³² In this study, ZBED3-AS1 overexpression attenuated BC cell viability, proliferative capacity, invasion, and migration. The roles of ZBED3-AS1 were similar to those found in the aforementioned studies, indicating that ZBED3-AS1 may function as anti-oncogene in the development of BC. Additionally, certain lncRNAs act as tumor suppressors in BC, and lncRNA elevation causes the inhibition of tumor growth in vivo. For instance, lncRNA MEG3 overexpression significantly inhibits breast tumor xenograft growth in vivo.³³ Elevated of lncRNA LINC00968 reduces the BC tumor volume and weight in mice by inhibiting miR-423-5p.³⁴ Similarly, ZBED3-AS1 overexpression constrains tumor growth in mice, determining the critical role of ZBED3-AS1 in attenuating BC tumorigenesis in vivo. Above all, ZBED3-AS1 may be a promising therapeutic target for BC.

Certain lncRNAs regulate miRNAs expression to exert its anti-oncogenic effect in BC. For instances, lncRNA LINC00899 alleviates malignant tumor behavior in BC through downregulating miR-425.³⁵ LncRNA XIST upregulation exerts anti-proliferation and pro-apoptosis effects in BC cells by inhibiting miR-326-5p.³⁶ Importantly, lncRNA ADAMTS9-AS1 overexpression inhibits miR-513a-5p to attenuate the aggressive traits of BC cells.¹⁸ In the present study, we revealed that ZBED3-AS1 is a target of miR-513a-5p and is negatively related to miR-513a-5p expression, suggesting that ZBED3-AS1 may be involved in BC by restraining miR-513a-5p. Furthermore, miR-513a-5p is highly expressed and acts as an onco-miR in different cancers. For instance, miR-513a-5p

upregulation weakens the tumor suppressor function of cCNTNAP3 in esophageal squamous cell carcinoma.³⁷ MiR-513a-5p elevation accelerates the tumorigenesis of nephroblastoma cells.¹⁶ Notably, miR-513a-5p functions as an oncogene in BC by targeting progesterone receptors.³⁸ Here, we determined that miR-513a-5p deficiency attenuated the malignant development of BC cells, and miR-513a-5p elevation reversed the antitumor roles of ZBED3-AS1 in BC cells. Taken together, ZBED3-AS1 overexpression may constrain the malignant tumor behavior of BC cells by downregulating miR-513a-5p.

KLF6 is often downregulated in diverse tumors, such as colorectal cancer,³⁹ ovarian cancer,⁴⁰ and BC.⁴¹ Similarly, KLF6 expression was inhibited in BC in the present research, indicating that KLF6 may be an anti-oncogene in BC. KLF6 frequently acts as tumor suppressor in various cancers. KLF6 attenuates the malignant phenotype of glioblastoma cells by regulating neural-like genes.⁴² KLF6 exerts antigrowth and pro-apoptosis functions in osteosarcoma cells.⁴³ Interestingly, KLF6 can be involved in BC progression as a target gene for miRNAs. MiR-4262 blocks KLF6 protein expression to positively regulate proliferation and invasion of BC cells.⁴⁴ MiR-181a-5p overexpression impairs BC cell migratory and invasive capacities by targeting KLF6.⁴⁵ In this study, KLF6 was a target of and negatively regulated by miR-513a-5. We speculate that miR-513a-5p is involved in the progression of BC by modulating KLF6. Considering the targeted relationship between ZBED3-AS1 and miR-513a-5p, we suspect that ZBED3-AS1 may elevate KLF6 expression by inhibiting miR-513a-5p in BC. The feedback data revealed that KLF6 depletion reversed the antitumor effect of ZBED3-AS1 in BC cells. To sum up, ZBED3-AS1 may exert its antitumor effect by targeting the miR-513a-5p/KLF6 axis in BC.

In conclusion, ZBED3-AS1 expression was suppressed in BC tissues and cells. Additionally, miR-513a-5p is a target of ZBED3-AS1, and KLF6 is a target of miR-513a-5p. Moreover, ZBED3-AS1 restrained the malignant development of BC cells by regulating the miR-513a-5p/KLF6 axis. ZBED3-AS1 may be a potential therapeutic target for BC.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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