

Knockdown of ABHD11-AS1 prevents the procession of TNBC by upregulating miR-199a-5p

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Abstract. Breast cancer (BC) has become a threat to women's health. In addition, patients with triple-negative BC (TNBC) have the worst prognosis among all patients with BC. Furthermore, long non-coding RNA ABHD11-AS1 is aberrantly highly expressed in TNBC, suggesting that RNA ABHD11-AS1 may serve as an important role in the progression of TNBC. However, the detailed function of ABHD11-AS1 in TNBC remains largely unknown. The levels of ABHD11-AS1 in MDA-MB-231 cells were assessed by reverse transcription-quantitative PCR. To investigate the effect of ABHD11-AS1 on the progression of TNBC, a xenograft animal model was established. Knockdown of ABHD11-AS1 inhibited the epithelial-mesenchymal transition and migration of TNBC cells. In addition, ABHD11-AS1 promoted the viability and migration of TNBC cells by upregulating microRNA (miR)-199a-5p. Furthermore, knockdown of ABHD11-AS1 suppressed TNBC tumor growth *in vivo* by upregulating miR-199a-5p. In conclusion, knockdown of ABHD11-AS1 suppressed the progression of TNBC via upregulation of miR-199a-5p. The data of the present study may provide novel directions and a theoretical basis for TNBC treatment.

Introduction

Breast cancer (BC) is one of the most common malignant tumors in women (1). Triple-negative BC (TNBC) is a subtype of BC (2,3). In TNBC, the results of immunohistochemical staining of BC tissues have revealed that these are negative for estrogen receptors, progesterone receptors and HER2 (4,5). In addition, TNBC is usually highly malignant, aggressive and

prone to recurrence and metastasis (6). Due to the estrogen and progesterone receptors being negative, the patient cannot receive endocrine therapy (7). Additionally, since the HER2 gene is negative, the patient cannot receive anti-HER2 targeted therapy (4,8). At present, there is no adequate treatment for TNBC. Therefore, it is important to explore novel treatments for patients with TNBC.

Long non-coding RNAs (lncRNAs) are non-coding RNAs (9,10). It has been reported that abnormal expression or function of lncRNAs is important in BC (11-13). For example, low lncRNA HOTAIR expression can induce the apoptosis of BC cells (11). Overexpression of LINC02273 promotes the migration and metabolism of BC cells (12). Furthermore, Wang *et al* (13) reported that lncRNA-ABHD11-AS1 was aberrantly highly expressed in BC. The present study demonstrated that ABHD11-AS1 may serve as an important role in the progression of BC. However, the detailed roles of ABHD11-AS1 in BC are unknown.

MicroRNAs (miRNAs/miRs) are non-coding RNAs with a size of 20-25 nucleotides (14,15). Previous studies have demonstrated that lncRNAs can interact with miRNAs as competing endogenous RNAs (16,17). For instance, knockdown of lncRNA BCRT1 could inhibit the progression of BC via upregulation of miR-1303 (16). Moreover, miR-199a-5p was considered as a vital mediator in tumor progression. For instance, miR-199a-5p could promote the tumorigenesis of oral squamous cell carcinoma through regulating SMARCA4 (18); Xu *et al* (19) suggested lnc CDKN2B-AS1 was implicated in the development and progression of liver cancer by suppressing cell autophagy through targeting miR-199a-5p. Meanwhile, miR-199a-5p was involved in lncRNA TINCR-mediated progression of BC (20). However, the detailed relation between ABHD11-AS1 and miR-199a-5p in TNBC remains unclear.

Based on the aforementioned previous studies, the detailed functions of ABHD11-AS1 in TNBC was explored. The present study aimed to confirm the mechanism underlying the function of ABHD11-AS1 in TNBC. This research could potentially lead to discovering new strategies against TNBC.

Materials and methods

Cell culture. TNBC cells (MDA-MB-231) were provided by Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd.

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(cat. no. ZQ0118). MDA-MB-231 cells were maintained in 89% L15 medium (Invitrogen; Thermo Fisher Scientific, Inc.). The medium contained 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), and 1% penicillin and 1% streptomycin, and cells were cultured at 37°C with 5% CO₂.

293T cells were obtained from American Type Culture Collection. Cells were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) and 2 mM glutamine (Sigma-Aldrich; Merck KGaA) and placed at 37°C in a humidified incubator containing 5% CO₂.

Cell transfection. First, MDA-MB-231 cells were maintained in 89% L15 medium at 37°C with 5% CO₂ for 24 h. Subsequently, cells were transfected with miR-199a-5p mimic (50 nM; Shanghai GenePharma Co., Ltd.; 5'-CCC AGUGUUAGACUACCUGUUC-3'), miR-199a-5p inhibitor (100 nM, Genepharma, 5'-GAACAGGUAGUCUGAACA CUGGG-3') or negative control (100 nM, Genepharma, 5'-CAGUACUUUUGUGUAGUACAA-3') by using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 6 h at 37°C according to the manufacturer's instruction. Next, the culture was replaced with fresh L15 medium at 37°C with 5% CO₂ for 48 h, and then reverse transcription-quantitative PCR (RT-qPCR) analysis was performed to verify the transfection efficiency of miR-199a-5p mimic or inhibitor.

RT-qPCR. TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract RNA from cells and tumor samples. Cells were treated with 200 µl chloroform (Shanghai Richjoint Chemical Reagents Co., Ltd.) for 10 min. Next, isopropyl alcohol (Shanghai Richjoint Chemical Reagents Co., Ltd.) was added to the supernatant for 10 min. The supernatant was removed by centrifugation at 20 x g at 4°C for 10 min. The precipitate was washed twice with 1 ml 75% ethanol. Subsequently, miScript Reverse Transcriptase Mix (Qiagen China Co., Ltd.) was used to generate cDNA according to the manufacturer's protocol. Afterwards, 2X QuantiTect SYBR Green PCR Master Mix (Qiagen China Co., Ltd.) was used for qPCR (conditions for amplification: 95°C for 30 sec, 40 cycles of 95°C for 10 sec, 58°C for 30 sec and 72°C for 30 sec). The expression levels were normalized to those of β-actin using the 2^{-ΔΔC_q} method (21). The primers used were as follows: miR-199a-5p forward, 5'-CGCGCCAGTGTTCAGACTAC-3' and reverse, 5'-AGTGCAGGGTCCGAGGTA TT-3'; ABHD11-AS1 forward, 5'-TCCAGACAAGACTTG GTCGC-3' and reverse, 5'-CAGCTGGTTGTGTGGCTT TC-3'; β-actin forward, 5'-CTGGAACGGTGAAGGTGA CA-3' and reverse, 5'-CGGCCACATTGTGAACCTTTG-3'; and U6 forward, 5'-CTCGCTTCGGCAGCACAT-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'.

Western blotting. Total protein was extracted using the RIPA buffer (Beyotime Institute of Biotechnology) from MDA-MB-231 cells by two rounds of centrifugation at 20 x g for 10 min. Subsequently, the protein concentration was assessed using a bicinchoninic acid (BCA) kit (Beyotime Institute of Biotechnology). Total protein (40 µg/lane) was electrophoresed by 10% SDS-PAGE and

transferred onto nitrocellulose membranes. The membranes were blocked with 5 ml confining liquid (5% skim milk in TBS-0.1% Tween 20) for 1 h at room temperature and then incubated with anti-N-Cadherin (1:1,000; cat. no. ab76011; Abcam), anti-Snail (1:1,000; cat. no. ab216347; Abcam) and anti-E-Cadherin (1:5,000; 20874-1-AP; Proteintech Group, Inc.) overnight at 4°C. Subsequently, the membranes were washed with TBS-0.1% Tween 20 buffer, and then incubated with a secondary antibody (HRP-conjugated anti-rabbit IgG; 1:5,000; cat. no. ab288151; Abcam) at room temperature for 2 h. Finally, protein was visualized using an enhanced chemiluminescence kit. β-actin (1:1,000; cat. no. ab8226; Abcam) was used as the internal standard. The protein expressions were evaluated using a densitometric semi-quantitative analysis with the Image-Pro Plus 6.0 (Media Cybernetics, Inc.) software.

Transwell assay. Cells (10⁵ cells) were seeded into the upper chamber. The lower chamber contained 700 µl L15 medium supplemented with 10% FBS. After 24 h of incubation at 37°C, the migratory cells were stained with 1% crystal violet solution for 10 min at room temperature. Finally, images were captured, and migratory cells were counted in 3 random fields under a light microscope.

Cell viability assay. MDA-MB-231 cells (5x10³ cells) were seeded in 96-well plates. Subsequently, cells were treated with ABHD11-AS1 short hairpin RNA (shRNA)1, miR-199a-5p mimic, miR-199a-5p inhibitor and ABHD11-AS1 shRNA1 + miR-199a-5p inhibitor. Next, cells were treated with 10 µl of Cell Counting Kit-8 reagent (Beyotime Institute of Biotechnology) at 37°C. Finally, absorbance at 450 nm was determined using a microplate reader.

Lentiviral infection. The lentiviral vector LV3 (H1/GFP&Puro) and shRNAs were provided by Shanghai GenePharma Co., Ltd. The sequence of ABHD11-AS1 shRNAs was as follows: ABHD11-AS1 shRNA1, ACC GGTcactgacagcaacatcaaTTCAAGAGATTGATGTTGCTG TCAGGTGTTTTTTGAATTC; ABHD11-AS1 shRNA2, ACCGGTggaccaagtctccaggaaTTCAAGAGATTCCTGGAG GACTTGGTCCTTTTTTTGAATTC; and ABHD11-AS1 shRNA3, ACCGGTggagctgcagagatgcaaaTTCAAGAGA TTTGCATCTCTGCAGCTCCTTTTTTTGAATTC. The 293T cells were maintained in serum-free L15 medium containing ABHD11-AS1 shRNA1, ABHD11-AS1 shRNA2 or ABHD11-AS1 shRNA3, plasmid (1 µg/µl), and packaging plasmid (pAX2, 1 µg/µl), envelope plasmid (pMD2.G, 1 µg/µl; 2nd-generation lentiviral packaging system) and the transfection reagent polyethylenimine (Polysciences, Inc.) for 6 h at 37°C. Next, the culture was replaced with fresh L15 medium containing 10% FBS and cells were then incubated for 72 h at 37°C. After that, the virus-containing supernatant was collected by centrifugation at 1,500 x g at 4°C for 30 min. Subsequently, MDA-MB-231 cells were incubated with the concentrated virus at multiplicity of infection (MOI)=40 and polybrene (5 µg/ml; Shanghai Yeasen Biotechnology Co., Ltd.) for 24 h. After transduction, these MDA-MB-231 cells in the second passage were incubated with puromycin (2 µg/ml, Gibco; Thermo Fisher Scientific, Inc.) for 72 h.

Xenograft tumor model. To investigate the effect of ABHD11-AS1 on the progression and development of TNBC, a xenograft animal model was established. BALB/c female nude mice (n=8; weight, 18-22 g; 6-8 weeks-old) were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. All animal experiments were approved (approval no. 20200527-SJ01) by the Ethics Committee of Huzhou University. The temperature was maintained at 18-22°C, the humidity was 50-60% and a 12-h light/dark cycle. Drinking water and a food supplement were provided 3-4 times a week. The behavior and food intake of the mice were monitored every day to maintain their health. MDA-MB-231 cells (5×10^6 cells/mouse in 100 μ l PBS) were subcutaneously injected into the right flanks of mice. When the tumor volumes reached 150 mm³, a total of 8 mice were randomly divided into two groups: ABHD11-AS1 shRNA1-control and ABHD11-AS1 shRNA1. Each group contained 4 mice. The mice were weighed, and tumor size was calculated weekly for 5 weeks. Additionally, mice were intraperitoneally injected with 50 mg/kg pentobarbital sodium for 10 min. Subsequently, the mice in the ABHD11-AS1 shRNA1 group were intratumorally injected with ABHD11-AS1 shRNA1 twice a week. After 5 weeks of treatment, all mice were euthanized using CO₂ at a displacement rate of 40% of the chamber volume/min (CO₂ flow rate, 2.5 l/min), and animal death was confirmed by cessation of heartbeat. The tumor was separated and weighed. The humane endpoints were judged by a 15% reduction of body weight, no mice were sacrificed due to meeting the endpoint.

Immunohistochemical staining. Tumor tissues were fixed in 4% paraformaldehyde for 24 h at 4°C, embedded in paraffin and then cut into 4- μ m sections. Next, the sections were heated at 60°C, dewaxed using the xylene reagent and rehydrated (100, 100, 95, 90, 80 and 70% alcohol for 5 min, respectively). Next, 3% H₂O₂ solution was dropped onto the slices for 10 min. Subsequently, the tissues were blocked in 5% BSA (Roche Diagnostics) for 20 min at room temperature, and then incubated with primary antibodies, including anti-N-Cadherin (1:100), anti-Snail (1:100; cat. no. ab224731; Abcam) and anti-E-Cadherin (1:100; cat. no. ab40772; Abcam) at 4°C overnight. Next, the tissues were incubated with secondary antibody (HRP-conjugated anti-rabbit IgG; 1:500; cat. no. ab40772; Abcam) at 37°C for 50 min. Immunostaining was detected by adding 3,3'-diaminobenzidine for 30 sec. Images were observed using a light microscope.

Statistical analysis. The statistically significant differences among multiple groups were analyzed by one-way ANOVA followed by Tukey's post hoc tests. GraphPad Prism software (version 7.0; Dotmatics) was used to analyze data. The data are presented as the mean \pm standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

Knockdown of ABHD11-AS1 inhibits the epithelial-mesenchymal transition (EMT) process and migration of TNBC cells. ABHD11-AS1 is aberrantly highly expressed in BC (13). In order to explore the role of ABHD11-AS1 in BC, ABHD11-AS1 expression in MDA-MB-231 cells was first knocked down.

The results of RT-qPCR indicated that ABHD11-AS1 shRNAs significantly inhibited ABHD11-AS1 expression in MDA-MB-231 cells (Fig. 1A). Since ABHD11-AS1 shRNA1 exhibited the largest effect, ABHD11-AS1 shRNA1 was used in the subsequent experiments (Fig. 1A). In addition, ABHD11-AS1 shRNA1 downregulated the expression levels of N-Cadherin and Snail, and upregulated the expression levels of E-Cadherin in MDA-MB-231 cells (Fig. 1B and C). Furthermore, ABHD11-AS1 shRNA1 significantly suppressed the migration of MDA-MB-231 cells (Fig. 1D and E). Overall, knockdown of ABHD11-AS1 could inhibit the EMT process and migration of BC cells.

miR-199a-5p mimics inhibit the EMT process of TNBC cells. Previous evidence has indicated that ABHD11-AS1 could regulate the levels of miR-199a-5p (22). miR-199a-5p mimics increased the expression levels of miR-199a-5p in MDA-MB-231 cells, while miR-199a-5p inhibitor exerted the opposite effect (Fig. 2A). Additionally, neither miR-199a-5p mimics nor miR-199a-5p inhibitor affected the expression levels of ABHD11-AS1 (Fig. 2B). By contrast, ABHD11-AS1 shRNA1 upregulated the levels of miR-199a-5p in cells (Fig. 2C). Furthermore, miR-199a-5p mimics decreased the expression levels of N-Cadherin and Snail, and increased the expression levels of E-Cadherin (Fig. 2D and E). Compared with miR-199a-5p mimics, miR-199a-5p inhibitor exerted the opposite effects on these proteins (Fig. 2D and E).

Knockdown of ABHD11-AS1 inhibits the viability and migration of TNBC cells by upregulating miR-199a-5p. To further investigate the relationship between ABHD11-AS1 and miR-199a-5p in BC cells, RT-qPCR was conducted. As indicated in Fig. 3A, ABHD11-AS1 shRNA1 or miR-199a-5p mimics significantly upregulated the expression levels of miR-199a-5p in MDA-MB-231 cells, while miR-199a-5p inhibitor suppressed the expression levels of miR-199a-5p. Additionally, the effect of ABHD11-AS1 shRNA1 on miR-199a-5p levels was completely reversed by miR-199a-5p inhibitor (Fig. 3A). ABHD11-AS1 shRNA1 or miR-199a-5p mimics inhibited the viability and migration of MDA-MB-231 cells; however, miR-199a-5p inhibitor and miR-199a-5p inhibitor + ABHD11-AS1 shRNA1 promoted the viability and migration of MDA-MB-231 cells (Fig. 3B-D).

Knockdown of ABHD11-AS1 inhibits the EMT process of TNBC cells by upregulating miR-199a-5p. As demonstrated in Fig. 4A and B, ABHD11-AS1 shRNA1 or miR-199a-5p mimics significantly decreased the expression levels of N-Cadherin and Snail, and increased the expression levels of E-Cadherin. Notably, miR-199a-5p inhibitor exerted the opposite effects on these proteins, even in the presence of ABHD11-AS1 shRNA1. Therefore, it was deduced that knockdown of ABHD11-AS1 inhibited the EMT process of BC cells by upregulating miR-199a-5p.

Knockdown of ABHD11-AS1 suppresses TNBC tumor growth by upregulating miR-199a-5p. To further investigate the effect of ABHD11-AS1 on the progression and development of TNBC, an *in vivo* animal model was established. The results of the animal experiment revealed that ABHD11-AS1 shRNA1

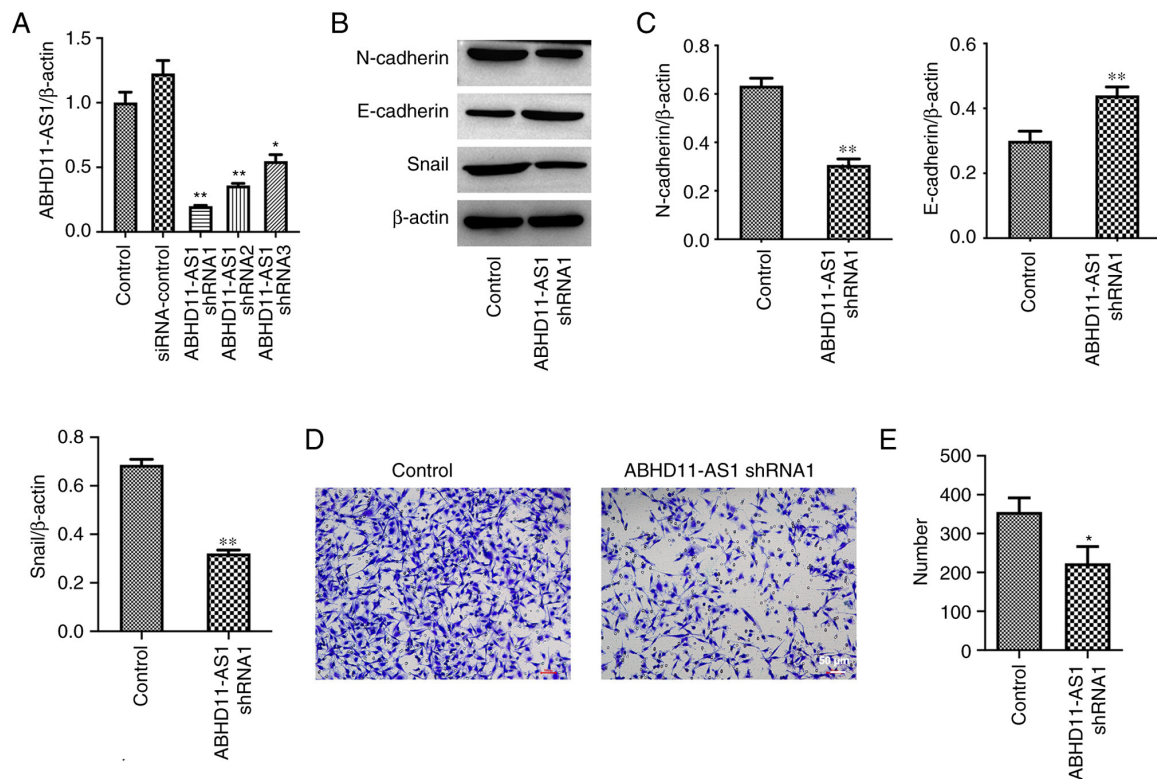


Figure 1. Knockdown of ABHD11-AS1 inhibits the epithelial-mesenchymal transition process and migration of triple-negative breast cancer cells. (A) The expression level of ABHD11-AS1 in MDA-MB-231 cells was assessed by reverse-transcription quantitative PCR. (B and C) The protein expression of N-Cadherin, Snail and E-Cadherin in MDA-MB-231 cells was assessed by western blotting. (D and E) The migration of MDA-MB-231 cells was measured by Transwell assay. * $P < 0.01$ and ** $P < 0.01$ compared with control group; $n = 3$. shRNA, short hairpin RNA.

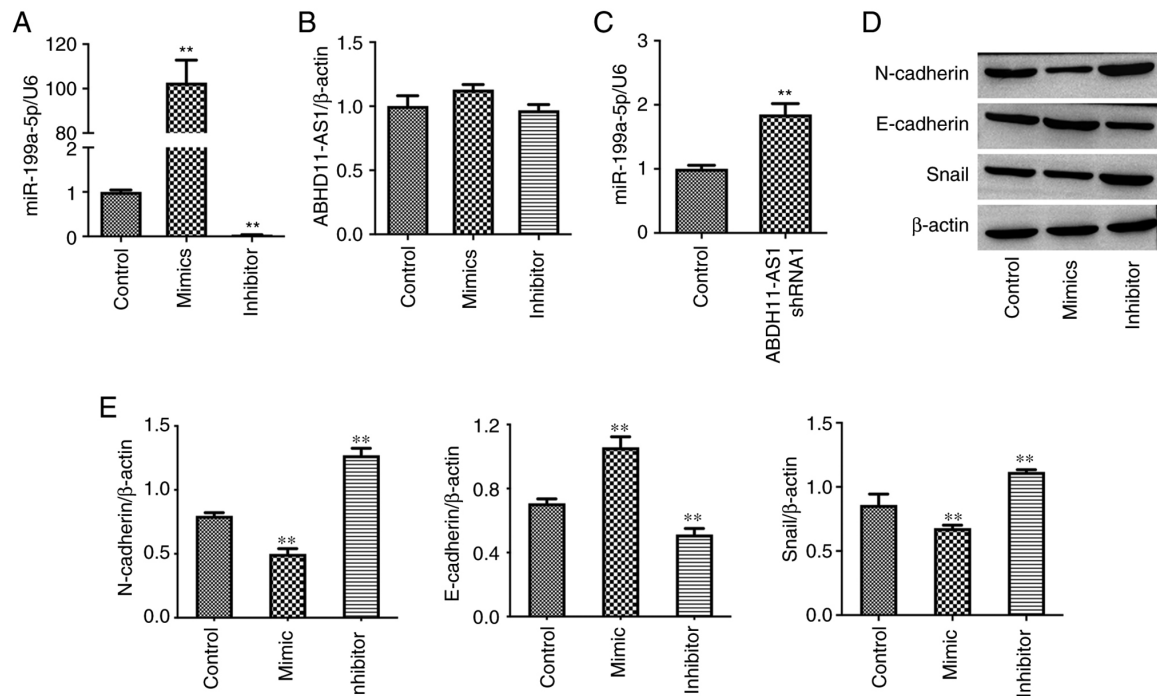


Figure 2. miR-199a-5p mimics inhibit the epithelial-mesenchymal transition process of triple-negative breast cancer cells. (A-C) The expression level of miR-199a-5p and ABHD11-AS1 in MDA-MB-231 cells was assessed by reverse-transcription quantitative PCR. (D and E) The protein expression of N-Cadherin, Snail and E-Cadherin in MDA-MB-231 cells was assessed by western blotting. ** $P < 0.01$ compared with control group. miR, microRNA.

significantly inhibited TNBC tumor growth (Fig. 5A and B). ABHD11-AS1 shRNA1 had no significant effect on the

body weight of mice (Fig. 5C). In addition, ABHD11-AS1 shRNA1 inhibited N-Cadherin and Snail expression and

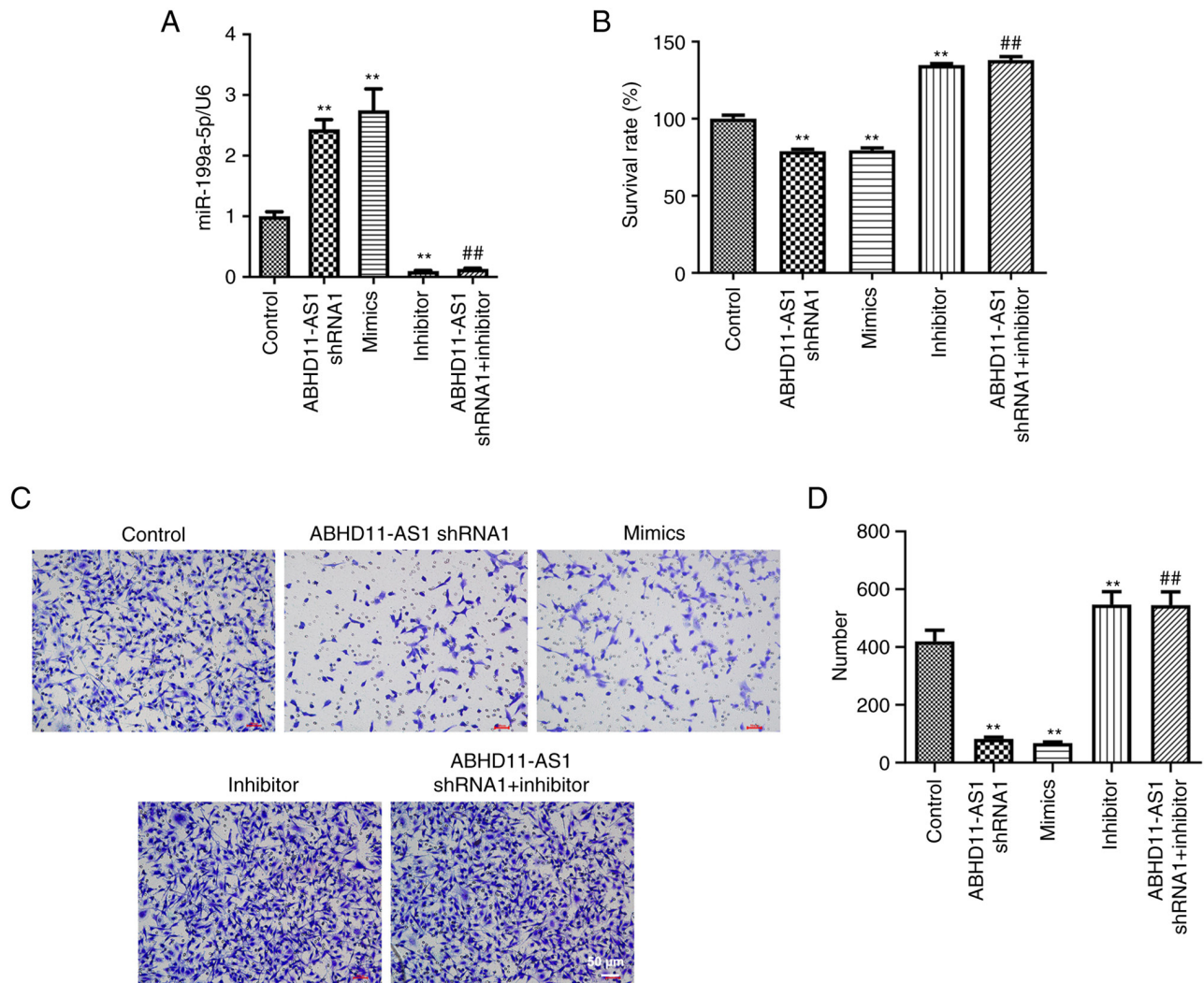


Figure 3. Knockdown of ABHD11-AS1 inhibits the viability and migration of triple-negative breast cancer cells by upregulating miR-199a-5p. (A) The expression level of miR-199a-5p was detected by reverse-transcription quantitative PCR. (B) The viability of MDA-MB-231 cells was assessed by Cell Counting Kit-8. (C and D) The migration was measured by Transwell assay. **P<0.01 compared with control group and ##P<0.01 compared with ABHD11-AS1 shRNA1. shRNA, short hairpin RNA.

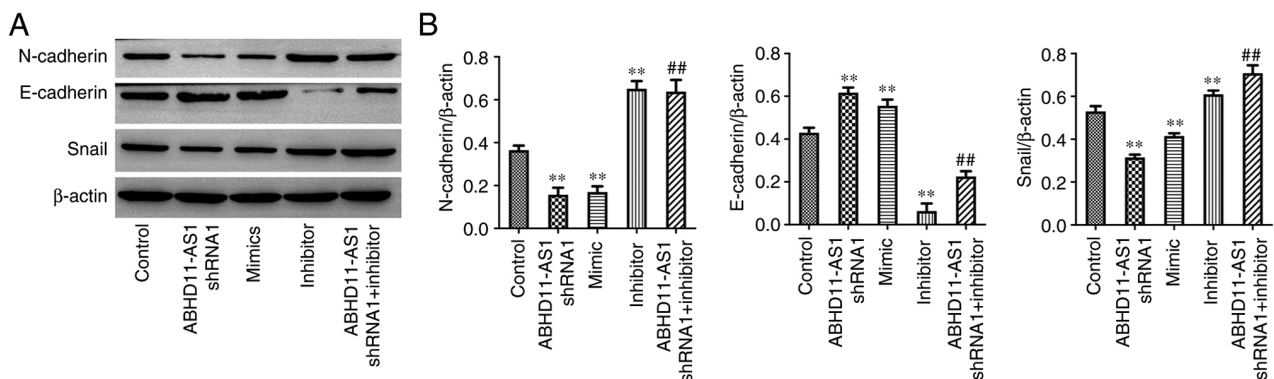


Figure 4. Knockdown of ABHD11-AS1 inhibits the epithelial-mesenchymal transition process of triple-negative breast cancer cells by upregulating microRNA-199a-5p. (A and B) The expression of N-Cadherin, Snail and E-Cadherin was assessed by western blotting. **P<0.01 compared with control group and ##P<0.01 compared with ABHD11-AS1 shRNA1. shRNA, short hairpin RNA.

promoted E-Cadherin expression in tumor tissues (Fig. 5D). Furthermore, ABHD11-AS1 shRNA1 significantly down-regulated ABHD11-AS1 expression and upregulated the

miR-199a-5p levels in tumors (Fig. 5E). These data suggested that knockdown of ABHD11-AS1 could suppress TNBC tumor growth by upregulating miR-199a-5p.

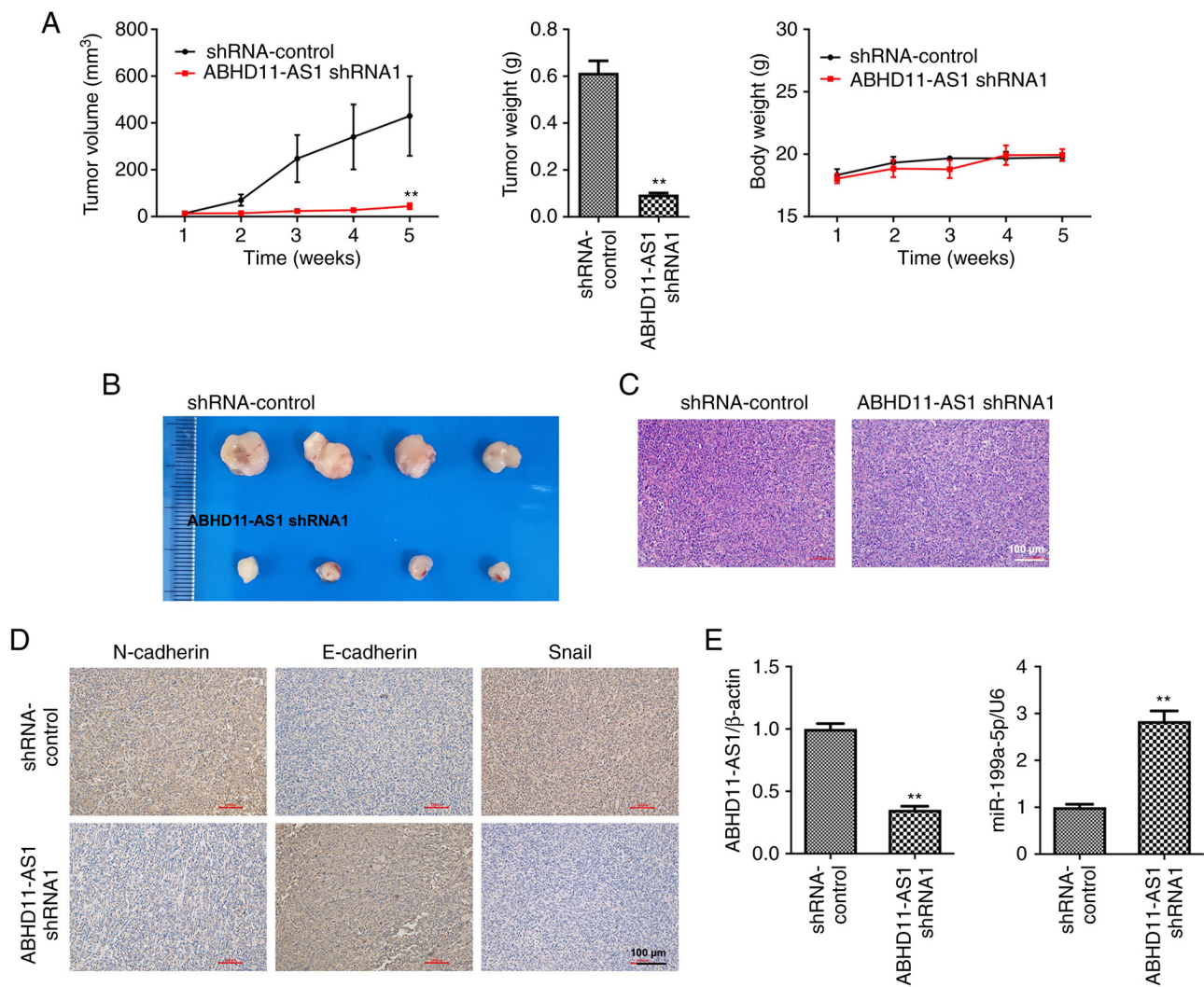


Figure 5. Knockdown of ABHD11-AS1 suppresses tumor growth of triple-negative breast cancer by upregulating miR-199a-5p. (A and B) The tumor volume and weight were measured. (C) The body volume and weight were monitored weekly. (D) The levels of N-Cadherin, Snail and E-Cadherin were assessed by immunohistochemistry. (E) The expression level of ABHD11-AS1 and miR-199a-5p was assessed by reverse-transcription quantitative PCR. **P<0.01 compared with control group. miR, microRNA; shRNA, short hairpin RNA.

Discussion

BC has the highest occurrence (29%) among all cancer types in women, and uterine cancer (8%) has the second highest incidence (23-25). TNBC is a highly malignant and aggressive subtype of BC (6,26,27). In addition, EMT is closely related to tumor invasion and metastasis (28-31). The EMT of TNBC could be inhibited by lovastatin (28). Lovastatin, a lipid-lowering drug, could suppress the development of TNBC (28). In addition, the progression of TNBC could be impaired by Dihydrotanshinone-I via obstruction of the EMT (32). DHTS is a lipophilic compound of *Salvia miltiorrhiza* Bunge (Danshen), which is a traditional Chinese medicine (32). The present study revealed that knockdown of ABHD11-AS1 markedly suppressed the progression of BC by inhibiting the EMT process. These data confirmed that BC can be suppressed by inhibiting the EMT process.

lncRNA NR2F1-AS1 could promote the proliferation and angiogenesis of TNBC cells (33). Additionally, lncRNA GATA3-AS1 could increase the immune escape in TNBC (34). Furthermore, ABHD11-AS1 is expressed in BC,

indicating that ABHD11-AS1 may serve an important role in BC (13). Therefore, the expression levels of ABHD11-AS1 in MDA-MB-231 cells were knocked down. knockdown of ABHD11-AS1 inhibited the EMT process, viability and migration of TNBC cells by upregulating miR-199a-5p. All these confirmed that lncRNAs could serve important roles in the treatment of BC.

A previous study has indicated that ABHD11-AS1 could promote the proliferation of papillary thyroid cancer cells by negatively regulating the expression levels of miR-199a-5p (22). In order to explore the mechanism by which ABHD11-AS1 regulates the genesis and development of BC, miR-199a-5p was meticulously examined and revealed that its upregulation was successful by knockdown of ABHD11-AS1 thus leading to the suppression of BC cells progression. These results revealed that miR-199a-5p could be negatively regulated by ABHD11-AS1 in different cancer types.

The true aim of this research was as follows: i) To identify the relation between ABHD11-AS1 and miR-199a-5p in TNBC; ii) the detailed function of ABHD11-AS1 in TNBC was confirmed.

There are certain limitations to the present study. For instance, whether ABHD11-AS1 can modulate other targets was not well studied. In addition, the downstream proteins of miR-199a-5p remain under investigation. Therefore, further investigations should be conducted in the future.

In the present study, knockdown of ABHD11-AS1 suppressed the progression of BC cells by upregulating miR-199a-5p. These data may provide novel directions and a theoretical basis for the treatment of BC.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DG made substantial contributions to the conception of the study. DG and YD designed the study. YD, TZ, SS, XL, PJ and YG performed the experiments. YD drafted the manuscript. DG supervised the study and revised the manuscript. All authors read and approved the final manuscript. All authors confirm the authenticity of all the raw data.

Ethics approval and consent to participate

All animal experiments were approved (approval no. 20200527-SJ01) by the Ethics Committee of Huzhou University. The National Institute of Health Guide for the Care and Use of Laboratory Animals was followed.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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