Article

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Targeting LncRNA *ADAMTS9-AS1* is a Promising Therapeutic Strategy to Inhibit the Progression of Bladder Cancer

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Background: Bladder cancer (BC) is a malignant tumor that begins in the cells of the bladder, characterized by poor cell differentiation and strong invasion capacity, with a high incidence rate. Identifying key molecules that enhance BC cells' cisplatin sensitivity can help improve the clinical efficacy of BC treatment. Hence, this study aimed to determine the expression level of long non-coding RNA (lncRNA) ADAM Metallopeptidase with Thrombospondin Type 1 Motif 9 Antisense RNA 1 (*ADAMTS9-AS1*) in BC and explore its related mechanism underlying the amplification of cisplatin sensitivity.

Methods: Cancer tissues and para-cancerous tissues of 10 BC patients treated in The 908th Hospital of Joint Logistic Support Force of PLA were collected retrospectively and analyzed for the expression of the lncRNA *ADAMTS9-AS1* and fused in sarcoma (*FUS*) in this tissue. Normal bladder epithelial cell line SV-HUC1, and BC cell lines such as T24, J82, 5637, KU-19-19, and EJ were cultured for *in vitro* experimentation. Then, the expression levels of *ADAMTS9-AS1*, *FUS* mRNA, and FUS protein were detected by means of reverse-transcription quantitative polymerase chain reaction (RT-qPCR), Western blotting, and immunohistochemistry. pcDNA3.1 vector, pcDNA3.1-ADAMTS9-AS1, or pcDNA3.1-ADAMTS9-AS1 and *FUS* overexpression plasmid was transfected into the cultured T24 and 5637 cells. A series of tests were performed to detect cell proliferation, migration capacity, apoptosis, and cisplatin half-effective concentration (IC50) values of BC cells using Cell Counting Kit-8 (CCK-8) assay, colony formation assay, wound healing assay, flow cytometry, and gradient cisplatin culturation.

Results: Compared with SV-HUC1 cell line and adjacent normal tissues, *ADAMTS9-AS1* levels were significantly decreased in T24, J82, 5637, KU-19-19, EJ cell lines, and BC tissues, while *FUS* mRNA and protein expression levels were up-regulated ($p < 0.05$). After transfection with pcDNA3.1-ADAMTS9-AS1, the colony number, cell viability, wound healing ratio, and cisplatin IC50 value, were remarkably reduced ($p < 0.05$), but apoptosis ratio, cleaved-caspase3 and cleaved-poly-ADP-ribose polymerases (*PARP*) expressions were increased ($p < 0.05$). *ADAMTS9-AS1* was found to directly target *FUS*, and overexpression of *FUS* reversed *ADAMTS9-AS1* effects on BC cells.

Conclusions: *ASAMTS9-AS1* can inhibit the proliferation and migration, and promote apoptosis and cisplatin sensitivity of BC cells through regulating *FUS*, thus providing a theoretical basis for *ADAMTS9-AS1* as a potential therapeutic target in BC treatment.

Keywords: bladder cancer; ADAMTS9-AS1; FUS; cisplatin sensitivity

Introduction

Bladder cancer (BC) is a malignant tumor of the bladder characterized by poor cell differentiation and strong invasion capacity, with a high incidence rate. Since the early symptoms of BC are not conspicuous, most patients are only diagnosed when the cancer has progressed to an advanced stage [1,2]. Currently, chemotherapy remains an irreplaceable treatment for BC, improving patients' prognosis and extending their lifespan [3,4]. Among them, cisplatin, as the first-line chemotherapy drug, induces DNA damage and mitochondrial apoptosis and promotes tumor cell apoptosis [5]. Nevertheless, due to patients' resistance to cisplatin, BC recurrence occurs in approximately 40% of patients within 5 years, presenting complexities in BC treatment and resulting in poor overall prognosis [6]. Therefore, identifying key molecules that can enhance BC cells' cisplatin sensitivity will help improve the clinical efficacy of BC treatment.

Long non-coding RNAs (lncRNAs) are endogenous RNA molecules, each with a length of more than 200 nucleotides. The dysregulated expression of lncRNA is closely related to the progression of various tumors, including BC [7]. For example, lncRNA phosphatase and tensin

homolog (PTEN) pseudogene 1 (*PTENP1*), whose expression is significantly reduced in BC, mediates PTEN to influence the biological functions of BC cells by competitively binding with miR-17 [8]. Both lymph node metastasis-associated transcript 2 (*LNMAT2*) and bladder cancer-associated transcript 2 (*BLACAT2*) have been found to promote lymphatic metastasis of BC [9,10]. In addition, *KMU15* [11], urothelial cancer-associated 1 (*UCA1*) [12], low expressed in bladder cancer stem cells (*LBCS*) [13], and hypoxia inducible factor 1α-antisense RNA 2 (*HIF1A-AS2*) [14] are reportedly implicated in BC chemoresistance. LncRNA ADAM Metallopeptidase with Thrombospondin Type 1 Motif 9 Antisense RNA 1 (*ADAMTS9-AS1*), located at chromosome 3p14.1, is the antisense transcript of the protein-coding gene *ADAMTS9* and is abnormally expressed in tumors such as colorectal cancer [15], glioma [16], non-small cell lung cancer [17], and BC [18]. Research demonstrated that the low level of *ADAMTS9-AS1* is associated with a good prognosis in BC patients [19], but its effects on cisplatin sensitivity and biological functions of BC cells remain deciphered.

The fused in sarcoma (*FUS*), located at chromosome 16, is an RNA-binding protein. Previous studies have shown that *FUS* is the pathogenic factor of various diseases, such as amyotrophic lateral sclerosis [20], myocardial infarction [21], and atherosclerosis [22]. Moreover, abnormal expression of *FUS* is highly related to human cancers. It has been demonstrated that the deletion of *FUS* inhibits the migration and proliferation of cervical cancer cells [23], while the overexpression of *FUS* is conducive to the development of the malignant phenotype of lung cancer [24]. Nevertheless, the relationship between *FUS* and BC remains to be clarified.

In the current study, we found that both BC cell lines and tissues exhibited low expression of *ADAMTS9-AS1*. By targeting *FUS*, *ADMTS9-AS1* inhibited the proliferation and migration of BC cells, while promoting their apoptosis and cisplatin sensitivity. These findings provide a valid theoretical foundation supporting *ADAMTS9-AS1* as a potential therapeutic target for BC.

Materials and Methods

Sample Acquisition

Cancer tissues and peritumoral tissues were collected from ten BC patients who were treated. The treatment context of the 10 patients with BC are summarized in Table 1. The tissue specimens were stored in liquid nitrogen for subsequent experiments such as reverse-transcription quantitative polymerase chain reaction (RT-qPCR), Western blotting, and immunohistochemical detection. The clinicopathological characteristics of the BC patients are summarized in Table 2.

Table 1. Treatment context of patients with bladder cancer.

Form	Patients (n = 10)
Types	
NMIBC	6
MIBC	4
Treatment modality	
TURBT	5
LND	2
Chemotherapy	2
RARC	1

Note: NMIBC, non-muscle invasive bladder cancer; MIBC, muscle invasive bladder cancer; TURBT, transurethral resection of bladder tumor; LND, lymph node dissection; RARC, robotic-assisted radical cystectomy.

Table 2. Clinicopathological characteristics of the patients with bladder cancer.

Variable	Patients (n = 10)
Age (years)	
<60	5
≥60	5
Gender	
Female	4
Male	6
Tumor size (cm)	
<5	6
≥5	4
TNM stage	
I–II	7
III–IV	3
Tumor grade	
G1	3
G2–G3	7
Lymph node metastasis	
N0	2
N1/N2/N3	8

Note: TNM, tumours, nodes and metastases.

Cell Culture

Normal bladder epithelial cell line SV-HUC1 was purchased from Wuhan Procell Life Science & Technology Co., Ltd. (CL-0274, Wuhan, China). Other BC cell lines T24 (SCSP-536), J82 (TCHu218), 5637 (TCHu1), and KU-19-19 (TCHu252) were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). BC cell line EJ was acquired from Shanghai Yaji Biological Co., Ltd. (YS1803C, Shanghai, China). HEK293T cell was acquired from Shanghai Zeye Biological Co., Ltd. (ZY-4003, Shanghai, China). Short tandem repeat (STR) identification did not show multiple alleles and no significant cross-contamination of cells and

matched completely with SV-HUC1, T24, J82, 5637, KU-19-19, EJ and HEK293T cells in the database of the American type culture collection (ATCC). No mycoplasma contamination was found in the mycoplasma testing. SV-HUC1, T24, J82, 5637, KU-19-19, and EJ cells were cultured in RPMI1640 medium (11875119, ThermoFisher, Shanghai, China) supplemented with 10% fetal bovine serum (FBS) and 100 IU/mL dual-antibiotics (streptomycin and penicillin), while HEK293T cells were inoculated in Dulbecco's Modified Eagle Medium (DMEM) (11330032, Gibco, Mountain View, CA, USA) supplemented with 5% FBS and 1% dual-antibiotics. All cell types were then cultured in an incubator (HERAcell i, ThermoFisher, Shanghai, China) infused with 5% CO_2 and set at 37 °C.

Cell Transfection

Upon reaching a confluence of 70%–80%, T24 and 5637 cells were collected and washed with phosphate buffer saline (PBS) 3 times. Separately, the cells were mixed with 50 nM Lip2000 transfection reagent solution (11668030, Invitrogen, Waltham, MA, USA), and discarded from the medium. After being washed twice with PBS, the cells were added with RPMI1640 medium supplemented with only 10% FBS and cultured for 48 h. Vectors such as pcDNA3.1 vector (V79020, Invitrogen, Waltham, MA, USA), pcDNA3.1-*ADAMTS9-AS1*, and pcDNA3.1 *ADAMTS9-AS1* and *FUS* overexpression plasmid were used in this experiment. The pcDNA3.1 vector sequence is as follows: forward primer: 5′-CTAGAGAACCCACTGCTTAC-3′, and reverse primer: 5′-TAGAAGGCACAGTCGAGG-3′. The fused in sarcoma mutant-type (*FUS-MT*) reporter plasmid sequences are as follows: forward primer: 5′-CTAGGCTTGGAGACGCTGG-3′, and reverse primer: 5′-GGGCAAATTTAGGCTAACAC-3′. The fused in sarcoma wild-type (*FUS-WT*) reporter plasmid sequences are as follows: forward primer: 5′-GGTACTCAGCGGTGTTGGAA-3′ and reverse primer: 5′-GGCCTTGCACAAAGATGGTG-3′.

RT-qPCR

BC tissues and peritumoral tissue specimens were fully ground with liquid nitrogen, and sonicated to make homogenate. At the same time, SV-HUC1, T24, J82, 5637, KU-19-19, EJ, and transfected cell lines (HEK293T) were collected. Total RNA of the tissue specimens and cultured cells was extracted using the Trizol (15596026, Invitrogen, Mountain View, CA, USA) method, and cDNA was synthesized by the reverse-transcription kit (11119ES60, ThermoFisher, Shanghai, China). Afterward, RT-qPCR was performed using the SYBR Green Master Mix kit (A46110, ThermoFisher, Waltham, MA, USA) and primers synthesized by Shanghai Sangon Bioengineering Co., Ltd. (Shanghai, China). The specific sequences of the primers used are listed as follows: *ADAMTS9-AS1* forward primer: 5′-CTCAGACCACAACTCTCCACCTTG-3′, reverse primer: 5′-CAGATGCTGCCTGGCTGATGG-3′; *FUS* forward primer: 5′-GCCAAGATCAATCCTCCATGAGTAGTG-3′, reverse primer: 5′-TCCACGGTCCTGCTGTCCATAG-3′; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) forward primer: 5′-GGACCTGACCTGCCGTCTAG-3′, reverse primer: 5′-TAGCCCAGGATGCCTTGAG-3′. Using *GAPDH* expression as an internal reference, the relative expression levels of *ADAMTS9-AS1* and *FUS* mRNA were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Western Blotting

BC tissues and peritumoral tissues were fully ground with liquid nitrogen. Meanwhile, SV-HUC1, T24, J82, 5637, KU-19-19, EJ, and transfected cell lines (HEK293T) were collected. The tissue specimens and cultured cells were separately added with radio immunoprecipitation assay (RIPA) lysis buffer (P0013B, Beyotime, Beijing, China) and centrifuged to obtain protein supernatant. After quantification by bicinchoninic acid (BCA) kit (P0009, Beyotime, Beijing, China), the isolated proteins were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes (IPVH00010, Millipore, Boston, MA, USA). The PVDF membranes were blocked with skim milk for 1.5 h and incubated with primary antibodies (anti-FUS [ab23439, 1:2000], anti-pro-caspase3 [ab32150, 1:10,000], anti-cleaved-caspase3 [ab32042, 1:500], anti-cleaved-poly-ADP-ribose polymerases (PARP) [ab32064, 1:2000], and anti-GAPDH [ab9485, 1:10,000]; all of which were obtained from Abcam (Shanghai, China) on a shaker at room temperature for 3 h. Then, the membrane was incubated with a secondary antibody (ab7090, Abcam, Shanghai, China) for 1.5 h, developed with the enhanced chemiluminescence (ECL) kit (32106, Pierce, Waltham, MA, USA), and detected using a protein imaging system (CliNX, ChemiScope 6200, Shanghai Qinxiang Scientific Instrument Co., Ltd., Shanghai, China). The relative expression levels of FUS, pro-caspase3, cleaved-caspase3, and cleaved-PARP were detected using GAPDH as an internal reference. ImageJ software (version 1.6.0, Oracle Corporation, Redwood City, CA, USA; https://imagej.nih.gov/ij/plugins/quadrant-picking/index.html) was utilized to quantify protein levels in the Western blots obtained.

Immunohistochemistry

BC tissues and peritumoral tissues were embedded in paraffin and trimmed into sections of 5 µm thick. The paraffin-embedded sections were roasted at 60 °C for 2 h, dewaxed with xylene, hydrated with gradient alcohol, subjected to antigen retrieval, and blocked with 5% bovine serum albumin (BSA; E-IR-R107, Elabscience, Wuhan, China) for 1 h. Subsequently, the sections were incubated with an anti-FUS antibody (ab23439, 1:2000, Abcam,

Shanghai, China) overnight at 4 °C. Next, a biotin-labeled secondary antibody (ab150077, 1:1000, Abcam, Shanghai, China) was added for a 30-min incubation at 37 °C. Afterward, the sections were added with fresh Diaminobenzidine (DAB) solution (36302ES01, Yeasen, Shanghai, China), counterstained with hematoxylin (60527ES08, Yeasen, Shanghai, China) for 30 s, differentiated with hydrochloric acid alcohol, dehydrated with gradient alcohol, transparent with xylene, dried, and mounted with neutral resin. Finally, the sections were observed under an inverted fluorescence microscope (Nikon, TS100, Beijing Rongxing Guangheng Technology Co., Tokyo, Japan) and photographed.

Colony Formation Assay

T24, 5637, and the corresponding transfected cells were collected and adjusted to a density of 2×10^3 cells/mL. Subsequently, the cell suspensions were aspirated and inoculated in 24-well plates. Prior to incubating the plates in a 37 °C incubator for 5–7 days, 2 µL β2 mercaptoethanol, 200 µL FBS, and 500 µL methylcellulose were added to each well dropwise. Afterward, the cells were fixed with methanol for 0.5 h and then stained with crystal violet (60505ES25, Yeasen, Shanghai, China) for 20 min. Finally, the stained cells were observed with an inverted microscope (IX71, Tokyo, Olympus, Japan) and the cell colony number was calculated.

Cell Counting Kit-8 (CCK-8) Assay

The proliferation of T24, 5637, and the corresponding transfected cells was detected with the CCK-8 kit (E-CK-A362, Elabscience, Wuhan, China). Briefly, 2.0×10^5 cells/well were inoculated in 96-well plates, and cultured until reaching a confluence of 80%–90%. Next, 20 µL CCK-8 reagent was added, prior to a 2-hour incubation. Optical density (OD) values at the wavelength of 450 nm were detected using a MODEL680 microplate reader (Bio-Rad, Hercules, CA, USA), and the measured values were used to calculate cell viability (%).

Cell viability = (sample OD value – blank OD value) / (control OD value – blank OD value) × 100%

Wound Healing Assay

T24, 5637, and the corresponding transfected cells were digested with trypsin (25200114, Gibco, Mountain View, CA, USA) to prepare cell suspensions, which were inoculated into 6-well plates and cultured for 24 h. The culture cell layer on the bottom of each well was scraped with a 20 µL pipette tip and then washed with PBS 1–2 times to remove cell fragments. Subsequently, a serum-free medium without double antibiotics was added to each well. At 0 h and 24 h after scratching, the wounds were observed with a microscope and photographed. The images obtained were processed with Image J 8.0 software (Oracle Corporation, Redwood City, CA, USA) for quantitative analysis of cell migration.

Flow Cytometry

According to the manufacturer's protocol of Annexin V-FITC apoptosis detection kit (C1062L, Beyotime, Beijing, China), T24, 5637, and the corresponding transfected cells were inoculated with a density of 1.0×10^6 cells/mL in 6-well plates and cultured overnight. Then, the cells were digested with 0.25% trypsin (containing 0.02% Ethylene Diamine Tetraacetic Acid (EDTA)) to prepare cell suspensions. After washing twice with PBS, 5 µL of FITC-labeled Annexin V and 10 µL of Propidium iodide (PI) were added to cell for 15-min incubation in the dark. Apoptosis cells were detected by flow cytometry (FC-500, Beckman, Fullerton, CA, USA).

Determination of Cisplatin Half-Effective Concentration (IC50) Value

T24, 5637, and the corresponding transfected cells were seeded at a density of 5×10^3 cells/well in 96-well plates, and cultured with RPMI1640 containing 0.5–2.0 µmol/L cisplatin (gradient interval of 0.1 µmol/L). After incubation for 48 h in an incubator set at 37 °C, 20 µL of CCK-8 reagent was added for a 2-hour reaction. The medium was superseded by 100 µL dimethyl sulfoxide (DMSO) prior to measuring the OD values at the wavelength of 450 nm by a microplate reader. Afterward, the IC50 values of cisplatin were determined based on the cell viability-cisplatin concentration curve.

Prediction of Downstream Target Protein

The downstream target proteins FUS of ADAMTS9-AS1 were predicted by the StarBase v2.0 database (http://starbase.sysu.edu.cn/), and the relationship between *FUS* and *ADAMTS9-AS1* was verified with a dual-luciferase reporter gene assay kit (11405ES60, Yeasen, Shanghai, China). The FUS-WT or FUS-MT reporter plasmids were co-transfected with pcDNA3.1 or pcDNA3.1-ADAMTS9-AS1, respectively, into HEK293T cells for 48 h. Subsequently, the activities of firefly luciferase and Renilla luciferase were detected, and the relative activity of luciferase was calculated by determining the ratio of the above pcDNA3.1 and pcDNA3.1-ADAMTS9-AS1 in FUS-WT and FUS-MT groups.

Statistical Analysis

GraphPad Prism 8.0 statistical software (GraphPad Software, San Diego, CA, USA) was used to process and analyze data, and each experiment was repeated 3 times. One-way analysis of variance (ANOVA) was used to analyze the differences between the three groups that met the normality requirement. Measurement data with normal distribution was compared using a *t*-test between two groups. The data are expressed as mean ± standard deviation (SD). Differences with $p < 0.05$ were considered statistically significant.

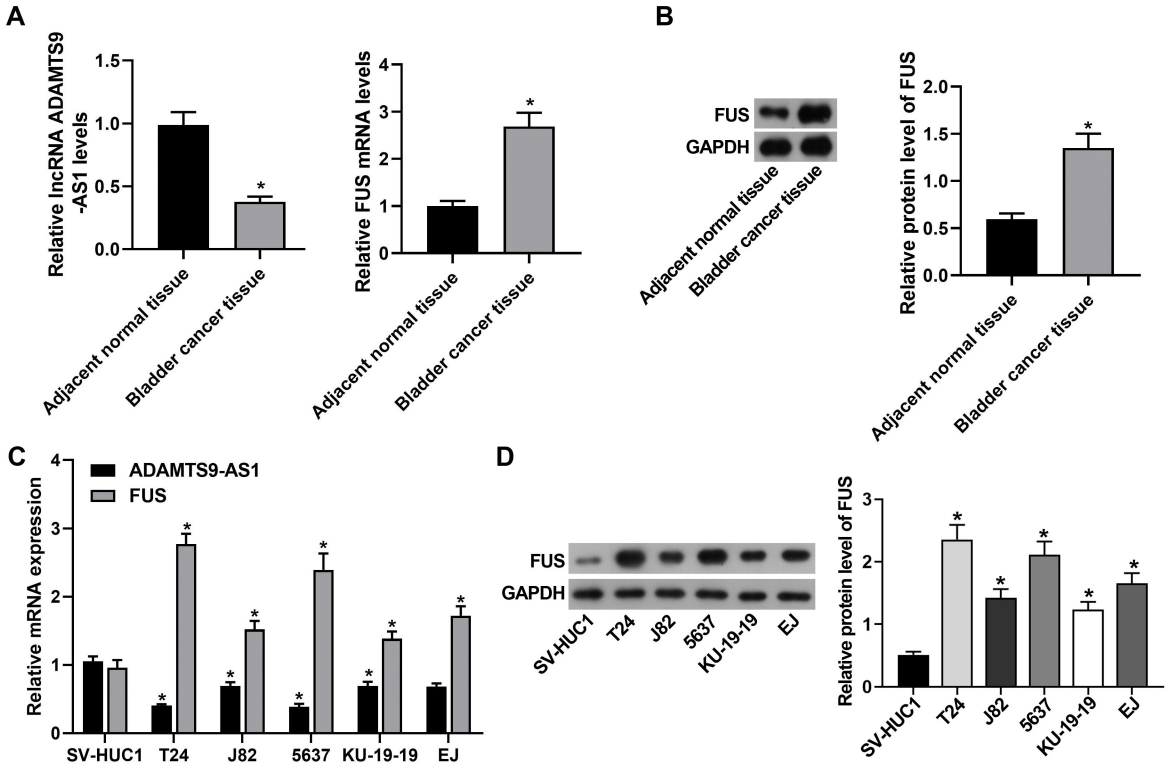

Fig. 1. Expression levels of long non-coding RNA (lncRNA) *ADAMTS9-AS1* and fused in sarcoma (*FUS*) in bladder cancer (BC) tissues and BC cell lines. (A) Expression levels of *ADAMTS9-AS1* and *FUS* mRNA levels in BC tissues and peritumoral tissues were detected by reverse-transcription quantitative polymerase chain reaction (RT-qPCR). (B) Expression of FUS protein in BC tissues and peritumoral tissues as detected by Western blotting. (C) Expression levels of *ADAMTS9-AS1* and *FUS* mRNA in SV-HUC1, T24, J82, 5637, KU-19-19, and EJ cells as detected by RT-qPCR. (D) Western blots of FUS protein in SV-HUC1, T24, J82, 5637, KU-19-19, and EJ cells, and the corresponding quantitative expression level in each cell line. Each experiment was performed in triplicate. $^{*}p < 0.05$ vs Adjacent normal tissue or SV-HUC1 cell. ADAMTS9-AS1, ADAM Metallopeptidase with Thrombospondin Type 1 Motif 9 Antisense RNA 1.

Results

Expression Levels of LncRNA ADAMTS9-AS1 and FUS in BC Tissues and BC Cell Lines

To assess the expression of lncRNA *ADAMTS9-AS1* and *FUS* in BC tissues and cell lines, we performed RT-qPCR and Western blotting analyses. BC tissues showed a significant reduction in *ADAMTS9-AS1* levels compared to peritumoral tissues (Fig. 1A, $p < 0.05$), along with a marked increase in FUS mRNA and protein levels (Fig. 1A,B, $p < 0.05$). We cultured normal bladder epithelial cell line SV-HUC1 and five other BC cell lines *in vitro*, namely T24, J82, 5637, KU-19-19, and EJ, to detect and verify their expression levels of *ADAMTS9-AS1* and *FUS*. It was found that *ADAMTS9-AS1* levels significantly decreased and *FUS* mRNA levels significantly increased in all of the tested BC cell lines when compared with SV-HUC1 (Fig. 1C, $p < 0.05$). In addition, FUS protein expressions also increased to varying degrees across the five BC cell lines (Fig. 1D, $p < 0.05$). Among them, T24 and 5637 cells featured the most prominent increase in *FUS* expression. Consequently, these two cell lines were chosen for further experiments to investigate their biological behavior and sensitivity to cisplatin.

Upregulation of ADAMTS9-AS1 Inhibited the Proliferation and Migration of BC Cells

To explore the effects of *ADAMTS9-AS1* on the biological behavior of T24 and 5637 cells, we examined the proliferation and migration of these cells following their transfection with pcDNA3.1-ADAMTS9-AS1 to upregulate the level of *ADAMTS9-AS1*. Compared with the T24+pcDNA3.1 group and the 5637+pcDNA3.1 group, *ADAMTS9-AS1* levels in the T24+pcDNA3.1-ADAMTS9-AS1 group and the 5637+pcDNA3.1-ADAMTS9-AS1 group were significantly increased (Fig. 2A, $p < 0.05$). Through the colony formation assay results, we found that the clone formation efficiency in the T24+pcDNA3.1-ADAMTS9-AS1 group and the 5637+pcDNA3.1-ADAMTS9-AS1 group were significantly lower than that in the T24+pcDNA3.1 group and the 5637+pcDNA3.1 group, respectively (Fig. 2B, $p <$

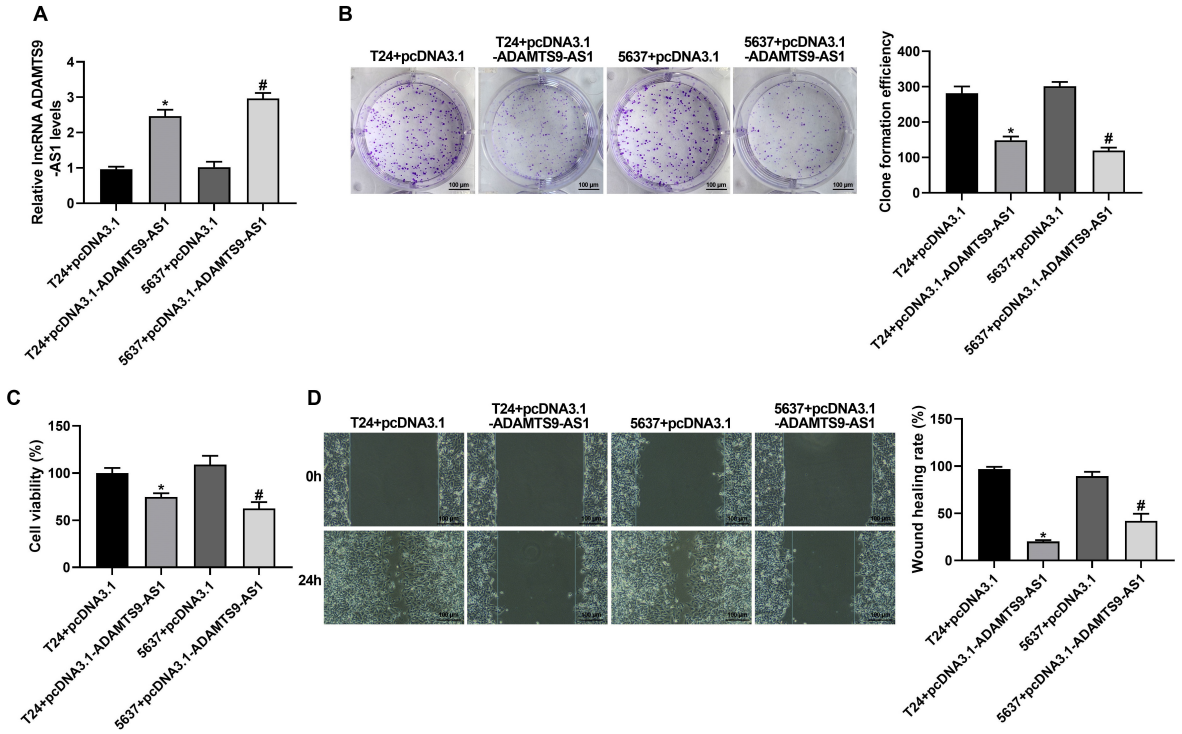

Fig. 2. Upregulation of *ADAMTS9-AS1* inhibited the proliferation and migration of BC cells. (A) Detection of *ADAMTS9-AS1* expression levels in different permutations of BC cell lines and vector usage. (B,C) Detection of BC cell proliferation by means of clone formation assay and Cell Counting Kit-8 (CCK-8) assay. (D) Determination of BC cell migration capacity by wound healing assay. Each experiment was performed in triplicate. $^{*}p < 0.05$ vs T24+pcDNA3.1; $^{\#}p < 0.05$ vs 5637+pcDNA3.1.

0.05). Similarly, compared with the T24+pcDNA3.1 group and the 5637+pcDNA3.1 group, cell viability and wound healing rate in the T24+pcDNA3.1-ADAMTS9-AS1 group and the 5637+pcDNA3.1-ADAMTS9-AS1 group were significantly reduced, which effectively inhibited the migration and proliferation ability of T24 and 5637 cells (Fig. 2C,D, $p < 0.05$).

Upregulation of ADAMTS9-AS1 Promoted Apoptosis and Cisplatin Sensitivity of BC Cells

Compared with the T24+pcDNA3.1 group and the 5637+pcDNA3.1 group, the apoptosis ratio of BC cells elevated significantly after upregulation of T24+pcDNA3.1-ADAMTS9-AS1 and 5637+pcDNA3.1-ADAMTS9-AS1 (Fig. 3A, $p < 0.05$). Besides, the expression of pro-apoptotic proteins cleaved caspase3 and cleaved-PARP was also elevated (Fig. 3B, $p < 0.05$). Our experimental findings also showed that the transfection of pcDNA3.1-ADAMTS9-AS1 remarkably decreased the IC50 values of cisplatin in both T24+pcDNA3.1-ADAMTS9-AS1 and 5637+pcDNA3.1-ADAMTS9-AS1 cells (Fig. 3C, $p < 0.05$), a clear sign that an *ADAMTS9-AS*1 upregulation could effectively improve the sensitivity of BC cells to cisplatin.

ADAMTS9-AS1 Directly Targeted FUS

According to the results predicted by the online database StarBase v2.0 database (http://starbase.sysu.edu.cn/), *FUS* is the downstream target protein of *ADAMTS9-AS1*, and *FUS* and *ADAMTS9-AS1* were pinpointed to the complementary binding site (Fig. 4A). To verify their relationship, dual luciferase reporter assay was used. The results revealed that the relative activities of luciferase of T24+pcDNA3.1-ADAMTS9-AS1 and the 5637+pcDNA3.1-ADAMTS9-AS1 were significantly weakened in the FUS-WT group (Fig. 4B, $p < 0.05$), proving that *ADAMTS9-AS1* can directly target *FUS*.

Overexpression of FUS Reversed the Effects of ADAMTS9-AS1 on Proliferation and Migration

In the previous experiments, we showed that *ADAMTS9-AS1* directly targeted *FUS*, and *ADAMTS9-AS1* upregulation inhibited the proliferation and migration of BC cells. After further transfection with *FUS* overexpression plasmid, we found that FUS mRNA and protein expression levels were increased in T24+pcDNA3.1-ADAMTS9-AS1+FUS overexpression group and 5637+pcDNA3.1-ADAMTS9-AS1+FUS overexpression group when compared with the T24+pcDNA3.1-ADAMTS9-AS1 and 5637+pcDNA3.1-ADAMTS9-AS1 up-regulation groups (Fig. 5A,B, $p < 0.05$). Meanwhile, the cell viability and

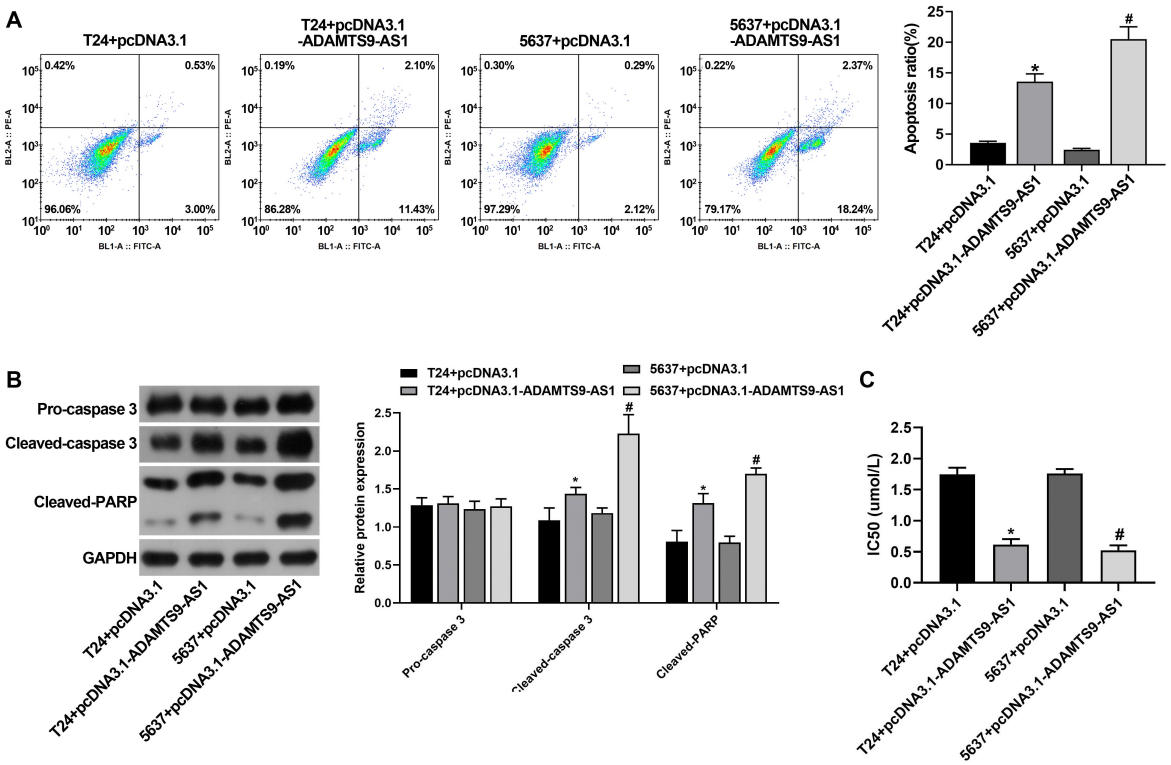

Fig. 3. Upregulation of ADAMTS9-AS1 promoted apoptosis and cisplatin sensitivity of BC cells. (A) Flow-cytometric determination of apoptosis in different permutations of BC cell lines and vector usage. (B) Western blots of pro-caspase3, cleaved caspase3, and cleaved-poly-ADP-ribose polymerases (PARP) in different permutations of BC cell lines and vector usage, and the corresponding quantitative expression level in each group. (C) Measurement of cisplatin half-effective concentration (IC50) values of cisplatin in different permutations of BC cell lines and vector usage. Each experiment was performed in triplicate. $^{*}p < 0.05$ vs T24+pcDNA3.1; $^{\#}p < 0.05$ vs 5637+pcDNA3.1.

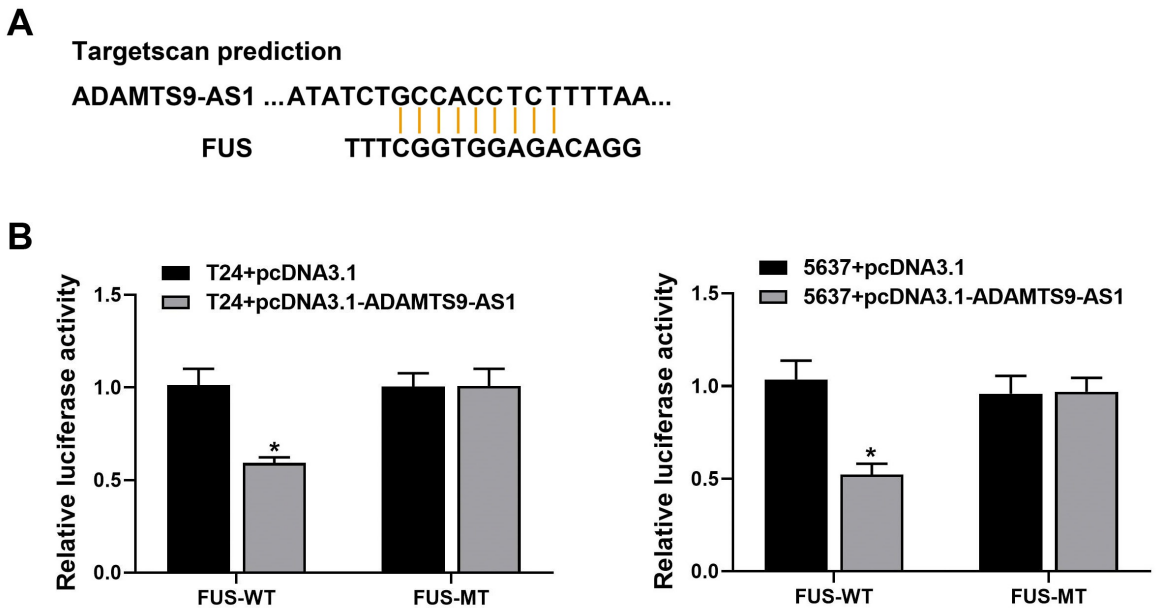

Fig. 4. *ADAMTS9-AS1* directly targeted *FUS*. (A) Prediction of the binding sites of *ADAMTS9-AS1* and *FUS* using StarBase v2.0 database (http://starbase.sysu.edu.cn/). (B) Determination of relative luciferase activity of different groups to verify the relationship between *ADAMTS9-AS1* and *FUS*. Each experiment was performed in triplicate. $^{*}p < 0.05$ vs T24 (5637) +pcDNA3.1.

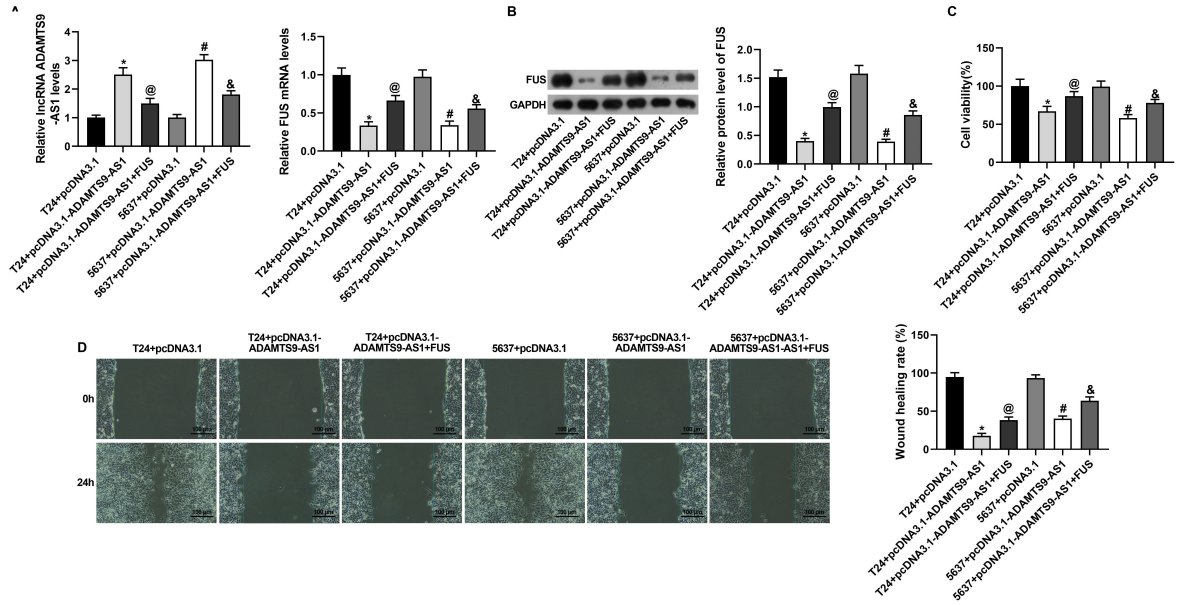

Fig. 5. Overexpression of *FUS* reversed *ADAMTS9-AS1* effects on proliferation and migration. (A) Expression levels of *ADAMTS9-AS1* and *FUS* mRNA as detected by RT-qPCR. (B) Western blot of *FUS* in different permutations of BC cell lines and vector usage, and the corresponding quantitative expression level in each group. (C) Cell viability of BC cells as measured by CCK-8 assay. (D) Wound healing ratio of BC cells in each group as detected by wound healing assay. Each experiment was performed in triplicate. $^{*}p < 0.05$ vs T24+pcDNA3.1; $^{\#}p < 0.05$ vs 5637+pcDNA3.1; $^{@}p < 0.05$ vs T24+pcDNA3.1-ADAMTS9-AS1; $^{\&}p < 0.05$ vs 5637+pcDNA3.1-ADAMTS9-AS1.

wound healing ratio of BC cells were increased (Fig. 5C,D, $p < 0.05$), suggesting that overexpression of *FUS* reversed the effects of *ADAMTS9-AS1* on proliferation and migration.

Overexpression of FUS Reversed the Effects of ADAMTS9-AS1 on Cisplatin Sensitivity and Apoptosis

After co-transfection with pcDNA3.1-ADAMTS9-AS1 and *FUS* overexpression plasmid, the IC50 values of cisplatin in T24 and 5637 cells became remarkably higher than those for the T24+pcDNA3.1-ADAMTS9-AS1 group and the 5637+pcDNA3.1-ADAMTS9-AS1 group (Fig. 6A, $p < 0.05$), indicating that the resistance of BC cells to cisplatin was enhanced. Moreover, there was a reduction in the apoptosis ratio in the T24+pcDNA3.1-ADAMTS9-AS1+FUS overexpression group and 5637+pcDNA3.1-ADAMTS9-AS1+FUS overexpression group, which was accompanied by a decrease of cleaved caspase3 and cleaved-*PARP* expression (Fig. 6B,C, $p < 0.05$).

Discussion

Arising mostly in bladder mucosa, BC is one of the most common malignant tumors, clinching the 10th spot among all tumors when ranked based on their incidence rates [25]. Each year, the world sees an addition of more than 570,000 new patients and 210,000 deaths caused by BC. In China, BC stands as the most common tumor of the urinary system and ranks seventh among all other tumors in men based on the BC incidence rate [26]. In recent years, a collection of factors, such as the mushrooming of smoking habits, long-term exposure to industrial chemicals, and aging, have concertedly contributed to an overall increase in BC incidence, which has sparked widespread concern [27]. Chemotherapy—a therapeutic approach to inhibiting or reversing tumor progression by using synthetic or natural biochemical factors—is employed to suppress or retard the progression of BC as well. Chemotherapy drugs commonly used in BC include interferon, gemcitabine, doxorubicin, and platinum [28]. Among them, the cisplatin-based GC (gemcitabine + cisplatin) regimen and MVAC (methotrexate + vinblastine + adriamycin + cisplatin) regimen are the first-line regimens for metastatic BC treatment [29]. These systemic adjuvant chemotherapy or combined chemotherapy regimens could extend the median survival of BC patients by 7–9 months but are unable to contribute to a satisfactory overall prognosis [30]. Adding to the limitation of the current chemotherapy regimens for BC is cisplatin resistance, which represents a huge obstacle to the clinical efficacy of chemotherapy resistance. Therefore, enhancing the sensitivity of BC cells to cisplatin is a promising therapeutic strategy.

The mechanisms of cisplatin resistance have been well understood in recent years, mainly involving the increased frequency of DNA repair, the stimulation of drug efflux pumps, the activation of pro-survival pathways, the de-

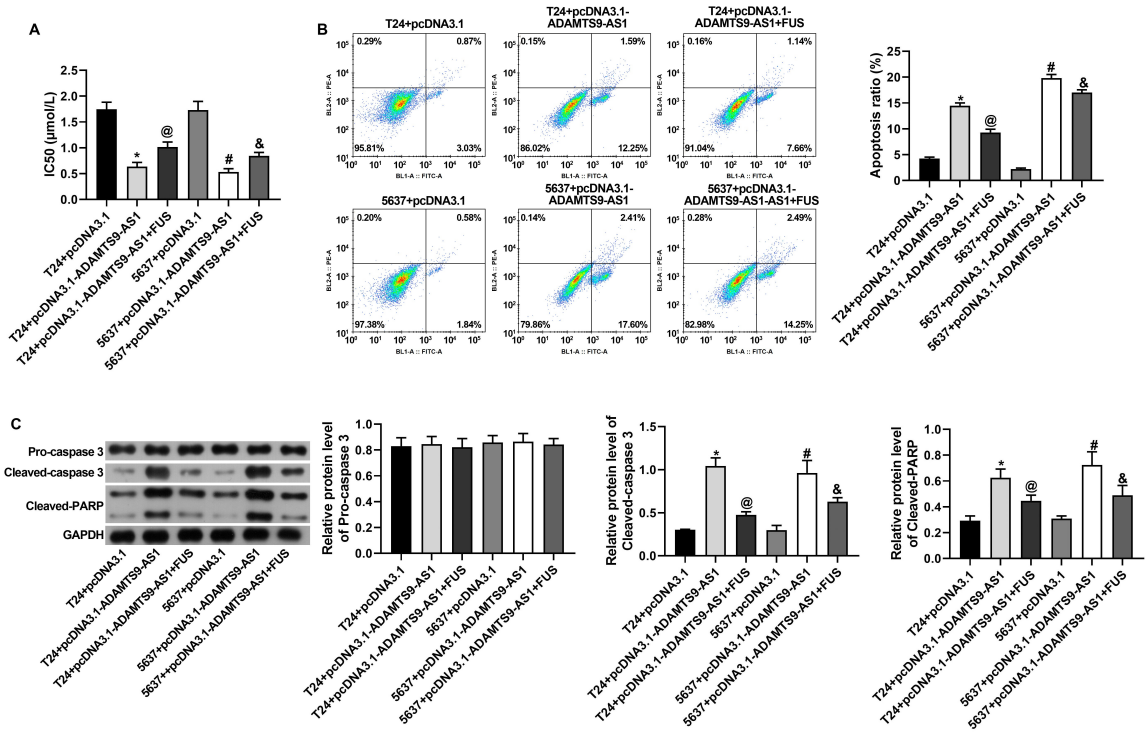

Fig. 6. Overexpression of FUS reversed the effects of ADAMTS9-AS1 on cisplatin sensitivity and apoptosis. (A) Measurement of IC50 values of cisplatin. (B) Flow-cytometric determination of BC cell apoptosis. (C) Western blots of pro-caspase3, cleaved caspase3, and cleaved-PARP in different permutations of BC cell lines and vector usage, and the corresponding quantitative expression level in each group. Each experiment was performed in triplicate. $^{*}p < 0.05$ vs T24+pcDNA3.1; $^{\#}p < 0.05$ vs 5637+pcDNA3.1; $^{@}p < 0.05$ vs T24+pcDNA3.1-ADAMTS9-AS1; $^{\&}p < 0.05$ vs 5637+pcDNA3.1-ADAMTS9-AS1.

fects of apoptotic pathways, and the production of cisplatin-isolating proteins [31]. However, multidrug resistance mechanisms may be implicated in tumor progression, enhancing the capability of cancer cells to repair cisplatin-induced DNA damage, causing abnormal proliferation and migration, and inhibiting apoptosis [32]. Biomarkers from genomic screening can inform the formulation of more targeted therapy for patients. For BC, the currently known lncRNA biomarkers for cisplatin resistance include macrophage stimulating 1 pseudogene 2 (*MST1P2*) [33], metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) [34], taurine-upregulated gene 1 (*TUG1*) [35], maternally expressed gene 3 (*MEG3*) [36], and so on.

As demonstrated in prior bioinformatic analyses, the expression of lncRNA *ADAMTS9-AS1* is decreased in BC and bladder urothelial carcinoma, relating to good prognosis for patients [19,37,38], but such association is unconfirmed due to a lack of experimental evidence. Therefore, in the current study, we examined the expression level of *ADAMTS9-AS1* in the cancer tissues and peritumoral tissues acquired from BC patients. The results demonstrated that *ADAMTS9-AS1* levels were downregulated in BC cancer tissues when compared to normal peritumoral tissues, aligning well with the bioinformatics results mentioned above. Interestingly, we found that the *ADAMTS9-AS1* levels were also reduced in a variety of BC cell lines, including T24, J82, 5637, KU-19-19, and EJ, with the most significant downregulation noted in T24 and 5637 cells. Although *ADAMTS9-AS1* exerts tumor suppressive effect on various tumors, such as breast cancer [39], prostate cancer [40], and ovarian cancers [41], *ADAMTS9-AS1* effects on the biological behavior and cisplatin sensitivity of BC cells have not been reported in the literature. Thus, this study was designed to investigate these aspects by upregulating the *ADAMTS9-AS1* level in T24 and 5637 cells. We found that *ADAMTS9-AS1* upregulation led to a decline in colony number, cell viability, and wound healing, together with an increase in apoptosis as well as expression of cleaved caspase3 and cleaved-*PARP*. Furthermore, increasing the *ADAMTS9-AS1* level could elevate the sensitivity of BC cells to cisplatin, as evidenced by a reduction of the IC50 value of cisplatin. We demonstrated the targeting relationship between *FUS* and *ADAMTS9-AS1* by dual luciferase reporter assay. In BC cells, the upregulation of *ADAMTS9-AS1* decreased the protein and mRNA expression of *FUS*, suggesting that *ADAMTS9-AS1* negatively regulated *FUS* expression. And the upregulation of *FUS* decreased the *ADAMTS9-AS1* level, which also suggested that there was a possible negative regulatory mechanism between the *FUS* and *ADAMTS9-AS1*. Meanwhile, it was found that the over-

expression of *FUS* could reverse the effects of *ADAMTS9-AS1* on the biological behavior and cisplatin sensitivity of BC cells.

Conclusions

In conclusion, the upregulation of lncRNA *ADAMTS9-AS1* can inhibit the proliferation and migration but promote apoptosis and cisplatin sensitivity of BC cells by regulating *FUS*, thus providing a theoretical basis for *ADAMTS9-AS1* as a potential therapeutic target in BC treatment.

Availability of Data and Materials

The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

ZY and GXT designed the research study. CYY performed the research. YMC and HJZ made data acquisition. ZY analyzed the data and GXT wrote the manuscript. WFX helped perform the analysis with constructive discussions. MZY helped perform the research with supervision. All authors have involved in the drafting and critical revision of the manuscript. All authors have reviewed and approved the final manuscript. All authors have agreed to be accountable for all aspects of their work.

Ethics Approval and Consent to Participate

This paper is a retrospective study and The Ethics Committee of The 908th Hospital of Joint Logistic Support Force of PLA has granted an exemption. The informed consent was obtained from the participants. Research procedures were complied with the Helsinki Declaration.

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Conflict of Interest

The authors declare no conflict of interest.

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