

Long Noncoding RNA *ADAMTS9-AS2* Inhibits the Proliferation, Migration, and Invasion in Bladder Tumor Cells

This article was published in the following Dove Press journal:
OncoTargets and Therapy

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Background: Bladder tumor is the fifth most prevalent tumor in men, yet its pathogenesis remains to be fully identified. Albeit a host of long noncoding RNAs (lncRNA) are emerging as new players involved in bladder tumor, the functions of many lncRNAs are still enigmatic. Reports on the deluge of studies on lncRNA *ADAMTS9-AS2* have been convincingly associated with various tumors, but without mention of its roles in bladder tumor. Therefore, the roles of *ADAMTS9-AS2* in bladder tumor cells were explored in our study.

Materials and Methods: Quantitative real-time PCR assays and bioinformatic tools were applied in bladder tumor cells to identify the *ADAMTS9-AS2* and *ADAMTS9* expression. Western blot assays were performed to obtain the protein levels of bladder tumor related key molecules. CCK8, clonogenic assay, scratch wound healing, and transwell assays were separately applied to identify the functional roles of *ADAMTS9-AS2* on proliferation, migration, and invasion in bladder tumor cells.

Results: First, *ADAMTS9-AS2* downregulation in bladder tumor cells was identified. Overexpression and knockdown experiments showed that *ADAMTS9-AS2* expression was positively related to *ADAMTS9*, which is in accordance with the results from GEO database. Second, *ADAMTS9-AS2* contributed to the inhibition of proliferation, migration, and invasion in bladder tumor cells. Third, *ADAMTS9-AS2* was linked with *PI3K/AKT/mTOR* pathway related-molecules, several key autophagy, and apoptotic proteins.

Conclusion: Conjointly, our findings suggested that *ADAMTS9-AS2* might function as a tumor suppressor to restrain the proliferation, migration, and invasion in bladder tumor cells. The potential mechanism of *ADAMTS9-AS2* related to *PI3K/AKT/mTOR* signal pathway was further identified. Of note, we found that *ADAMTS9-AS2* has a significant effect on several key autophagy and apoptotic proteins. Therefore, these observations will provide supportive evidence to *ADAMTS9-AS2* as a potential biomarker in patients with bladder tumor.

Keywords: lncRNA *ADAMTS9-AS2*, proliferation, *PI3K/AKT/mTOR*, bladder tumor

Introduction

Bladder cancer is the fourth most common cancer in male and it develops when abnormal cells grow in an uncontrolled way.¹ Patients are widely categorized as non-muscle-invasive or muscle-invasive bladder tumor.² Common treatment options are recommended, including surgery, chemotherapy, or combined with radiation therapy, but patients often end up with a relatively poor prognosis.³ Moreover, non-muscle-invasive bladder tumor constitutes the majority of patients, more often than not, which

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incurs a high cost and inconvenience due to frequent recurrence.⁴ Therefore, exploring more prognostic biomarkers that participate in the bladder tumor cell growth, migration, and invasion are necessary for the improvement of the patients' prognosis and life quality.

Long noncoding RNAs (lncRNAs) are a kind of non-coding RNA with more than transcripts >200 nt in length.⁵ Ample evidence has indicated their molecular roles in tumor cell growth, metastasis, invasion, and apoptosis.⁶ For example, *TUG1* is involved in cell growth of small cell lung cancer;⁷ knockdown of *GHET1* inhibits cell proliferation and invasion of colorectal cancer;⁸ upregulated *FGF13-AS1* in breast cancer promotes invasion and metastasis.⁹ These studies contribute to a better understanding of the importance of dysregulated lncRNAs in various tumors. Many functions of lncRNAs in tumor biology have been discovered and investigated, but the molecular mechanisms of most lncRNAs in bladder tumors remain largely unknown.

In our study, lncRNA *ADAMTS9-AS2* has drawn our attention due to recent literature reports. *ADAMTS9-AS2* is known to be the antisense transcript of *ADAMTS9*^{10,12} and has been identified as a tumor suppressor gene if mutated.^{13,14} Thus, to get in-depth knowledge of *ADAMTS9-AS2*, extensive researches have been reported regarding its diverse roles in tumors including clear cell renal cell carcinoma,¹⁵ esophageal cancer,¹⁶ and ovarian cancer.¹⁷ Besides, accumulating researches find that *PI3K/AKT* is partially accountable for tumorigenesis and metastasis in bladder cancer.^{18,20} It is well-known that many lncRNAs display abnormal expression in parallel with phosphorylated *AKT1* (p-AKT1) activation of *PI3K/AKT* pathway.^{21,27} Therefore, we proposed a hypothesis that *ADAMTS9-AS2* cooperated with *ADAMTS9* and may participate in the bladder tumor progression by regulating the *PI3K/AKT* signal pathway.

In our study, we concentrated on the potential roles of *ADAMTS9-AS2* by investigating its influence on proliferation, migration, and invasion, which may contribute to the further understanding of bladder tumor and offer new insights for its clinical application in patients.

Materials and Methods

Cell Culture and Treatment

We purchased five bladder tumor cell lines (UC3, 5637, J82, T24, and EJ) and one normal cell line SV-HUC-1 from the American Type Culture Collection (ATCC,

Manassas, VA, USA). Cells were maintained in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (Thermo Fisher Scientific), with a mixture of 100 U/mL of penicillin/streptomycin. Cells were cultivated at 37°C in a humidified atmosphere containing 5% CO₂ in air.

Quantitative PCR (q-PCR) Assays

ADAMTS9-AS2 and *ADAMTS9* expression were detected by q-PCR. Total RNA was first extracted from treated cells using the TRIzol reagent (Thermo Fisher Scientific) based on the manufacturer's instructions. One microgram of the total RNA was purified and used as the template for cDNA synthesis using a PrimeScript RT Reagent Kit with cDNA Eraser (Takara Biotech, Dalian, China). q-PCR was conducted using SYBR Premix Ex Taq (Takara Biotech, Dalian, China) on an ABI 7900 system (Thermo Fisher Scientific). The *GAPDH* was used as an internal control in comparisons of gene expression. Analysis of relative gene expression data was performed using 2^{-ΔΔCt}. The PCR primers are shown as below: *ADAMTS9-AS2*, forward 5'-AAGAAACCCTGATGTCTGGCTGAA-3' and reverse 5'-GTGTTACTTGAGGAGAAAGCGAAA-3'; *ADAMTS9*, forward: 5'-TGGGTTTTCCAGTTTTTCAG-3' and reverse 5'-GTTGATGCTAAAACGACCC-3'; *GAPDH*, forward 5'-ACGGATTTGGTCGTATTGGGCG-3' and reverse 5'-GCTCCTGGAAGATGGTGATGGG-3'.

Plasmid Construction and Cell Transfection

The pcDNA3.1 vector was purchased from Invitrogen (Thermo Fisher Scientific) and small interfering RNAs (siRNAs) were obtained from Ribobio (Guangzhou, China). pcDNA 3.1-*ADAMTS9-AS2* (pcDNA 3.1-AS2) was constructed by cloning the fragment of *ADAMTS9-AS2* into a pcDNA3.1 vector at the BamHI-EcoRI sites. *si-ADAMTS9-AS2-1* and *si-ADAMTS9-AS2-2* (*si-AS2-1* and *si-AS2-2*) and the negative control siRNA (*si-NC*) were provided by Ribobio (Guangzhou, China). Cells were plated to the 12-well plates at a density of 2×10³ cells per well and cultured at least 24 h before transfection. Cells were transfected with pcDNA3.1 or pcDNA3.1-AS2, and *si-NC*, *si-AS2-1* or *si-AS2-2* by using LipofectamineTM 2000 (Thermo Fisher Scientific) following the manufacturer's instructions. Cells were incubated for 24 h and then collected for further assays after transfection. The target sequences were listed as

follows: pcDNA3.1-AS2, forward 5'-GGGGTACCAA CTTGACGTACACACG-3' and reverse 5'-AGCCGGAA TTCTTTTCTGTTTTTATAATGTAC-3'; si-AS2-1 5'-GCATGACGCAACTTTGCTA-3' (sense), si-AS2-2 5'-CCTGTCTACAGGCTGATAT-3' (sense).

Cell Proliferation and Clonogenic Assays

The CCK8 assays (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) and clonogenic assays were conducted to evaluate cell proliferation ability. As for the CCK8 assays, cells were cultured at a density of 4×10^4 cells/well in 96-well culture plates for 18 h prior to transfection. Given the transfection efficiency of si-AS2-2 was higher than si-AS2-1, si-AS2-2 was selected in the experiments below. Cells were transfected with pcDNA 3.1 or pcDNA 3.1-AS2, and si-NC or si-AS2-2. Cells were treated with 10 mL/well of CCK8 solution during the last four hours of culture. The OD value was plotted using the 450 nm absorbance by Multiskan FC (Thermo Fisher Scientific) at each time point. In terms of clonogenic assays, in brief, cells at a density of 500 cells/well were cultured in six-well plates and cultured for two weeks under 37°C containing 5% CO₂. The colonies were fixed with 4% paraformaldehyde for 10 min, stained with 0.1% crystal violet for 15 min and washed twice with PBS. The number of clones of each well were calculated with ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). The clone formation rate of each well was counted. All experiments were performed in triplicate.

Cell Migration Assays

The scratch wound healing assay was applied to observe cell migration ability. Cells were cultured at a density of 1×10^6 cells/well in six-well culture plates for 18 h. Uniform wounds were generated prior to transfection (pcDNA 3.1 or pcDNA 3.1-AS2; si-NC or si-AS2-2). Microphotographs were taken at 0, 24, 48, and 72 h after transfection, and images were obtained under a magnification of 40× for each group. All experiments were performed in triplicate.

Cell Invasion Assays

The invasive ability of transfected cells was determined by transwell assay. Cells were cultured in 100 μL serum-free DMEM after transfection for 48 h. Transwell insert chamber coated with Matrigel (BD Biosciences, San Jose, CA, USA) was used according to manufacturer's instruction. By contrast, the migrated cells on the bottom side were

fixed with 4% polyoxymethylene and stained with 0.1% crystal violet for 20 min at 4°C. The invading cells at 100× magnification were counted using the Adobe Photoshop CS5. All experiments were performed in triplicate.

Western Blot Assays

Total cell lysates were prepared with sample buffer and boiled at 95°C for five minutes. Protein samples were transferred to 10% polyacrylamide gel electrophoresis (SDS-PAGE) for three hours, and then the samples were moved to polyvinylidene fluoride (PVDF). After incubation with primary antibodies (1:10,000) for *ADAMTS9*, *AKT*, p-AKT1, *PIK3CB*, mTOR, p-mTOR, *LC3*, *BECNI*, *SQSTM1*, Bax and Bcl-2 at 4°C overnight. All these antibodies were purchased from ABclonal (China). Peroxidase-conjugated goat anti-mouse (1:10,000) was used as a secondary antibody for *GAPDH* and *BECNI* at 37°C for one hour. Peroxidase-conjugated goat anti-rabbit IgG (1:10,000) were used as a secondary antibody for other proteins at 37°C for one hour. The PVDF membrane was developed using ECL chemiluminescent reagent (EMD Millipore, Billerica, MA, USA). *GAPDH* was used as an internal control. The images were obtained using Bio-Rad Gel Doc XR⁺ system (Bio-Rad Laboratories Inc., Hercules, CA, USA). Each sample was examined in triplicate.

Gene Expression Omnibus (GEO) Data Extraction

Using GEO database (<https://www.ncbi.nlm.nih.gov/geo/>), the clinical samples of bladder cancer were obtained. First, the gene expression data sets of bladder cancer and adjacent tissues were processed by R language. Second, the matrix of gene expressions was generated and uploaded to Gene Set Enrichment Analysis (GSEA) platform (<http://software.broadinstitute.org/gsea/index.jsp>) for enrichment analysis. Further, the expression profiles, pathway enrichment and the gene enrichment maps were obtained, separately. Finally, the correlation coefficient map between of *ADAMTS9-AS2* and *ADAMTS9* was calculated by Cor function in the R-language.

Statistical Analysis

All data were analyzed with GraphPad Prism 7.0 software (GraphPad Software Inc., La Jolla, CA, USA) and the data were expressed as mean ±SD. Student's *t*-test was used to analyze differences between two groups. All experiments

were performed in triplicate. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Downregulation of *ADAMTS9-AS2* in Bladder Tumor Cells

ADAMTS9-AS2 expression was determined in one normal cell line SV-HUC-1 and five bladder tumor cell lines (UC3, 5637, J82, T24, and EJ) using qPCR. Compared to the normal cell line SV-HUC-1, *ADAMTS9-AS2* expression was downregulated in UC3, 5637, J82, T24, and EJ cells, apparently in UC3 and 5637 (Figure 1, $P < 0.001$). Thus, bladder tumor cell lines UC3 and 5637 were chosen for the functional experiments. Besides, the *ADAMTS9* expression was also identified using qPCR. It suggested that *ADAMTS9* was downregulated in UC3, 5637, J82, T24, and EJ cell lines (Figure S1). The results suggested that *ADAMTS9-AS2* and *ADAMTS9* were downregulated in bladder tumor cells.

ADAMTS9-AS2 was Positively Related to *ADAMTS9* in 5637 and UC3 Cells

Using the GEO database, a weak positive correlation with 0.42 has been observed between *ADAMTS9-AS2* and *ADAMTS9* (Figure S2). Importantly, to pinpoint the relationship between *ADAMTS9-AS2* and *ADAMTS9*,

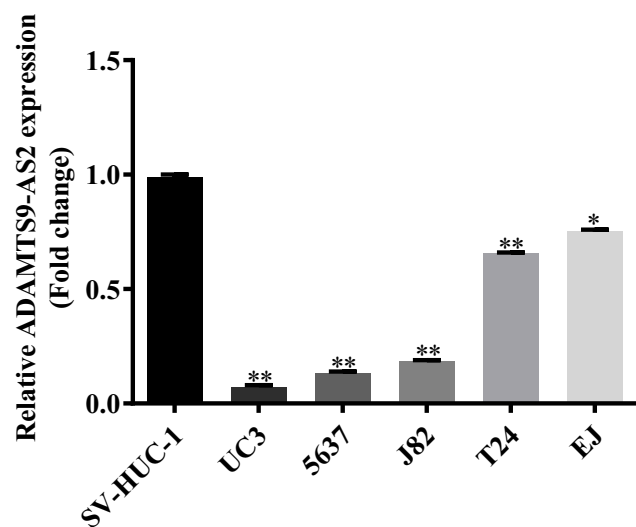


Figure 1 Downregulation of *ADAMTS9-AS2* in bladder tumor cells.

Notes: Based on the qPCR assays, *ADAMTS9-AS2* expression in bladder tumor lines was determined in one normal cell line SV-HUC-1 and five bladder tumor cell lines (UC3, 5637, J82, T24, and EJ). Compared to the normal cell line SV-HUC-1, the expression of *ADAMTS9-AS2* was downregulated in UC3, 5637, J82, T24, and EJ cell lines, apparently in UC3 and 5637 cells. * $P < 0.01$; ** $P < 0.001$.

Abbreviations: q-PCR, quantitative PCR 12 13.

ADAMTS9-AS2 knockdown and overexpression experiments were conducted separately. *ADAMTS9-AS2* overexpression experiments were conducted in the first place. After pcDNA3.1 control and pcDNA3.1-*AS2* were transfected into 5637 and UC3 cell lines, respectively, *ADAMTS9-AS2* expression was rising in parallel with increasing *ADAMTS9* expression (Figure 2A and B; $P < 0.01$). For *ADAMTS9-AS2* knockdown experiments, si-NC, si-*AS2*-1 and si-*AS2*-2 were transfected into 5637 and UC3 cell lines, respectively, which showed that downregulated *ADAMTS9-AS2* resulted in the reduction of *ADAMTS9* expression and no significant changes in UC3 cells transfected with si-*AS2*-1 group (Figure 2C and D; $P < 0.05$). The results suggested that *ADAMTS9-AS2* was positively related to *ADAMTS9* in bladder tumor cells.

ADAMTS9-AS2 Inhibits the Proliferation Ability of 5637 and UC3 Cells

To further explore the effect of *ADAMTS9-AS2* on proliferation ability, *ADAMTS9-AS2* overexpression and knockdown experiments in 5637 and UC3 cell lines were performed. The CCK8 and clonogenic assays demonstrated that in cells transfected with pcDNA3.1-*AS2* group, the proliferation ability was curbed compared with those transfected with pcDNA3.1 control group (Figure 3A–D; $P < 0.05$), while it was promoted in cells transfected with si-*AS2*-2 as compared to si-NC group (Figure 3A–D; $P < 0.05$). The results suggested that *ADAMTS9-AS2* inhibits the proliferation ability of bladder tumor cells.

ADAMTS9-AS2 Suppresses the Migration Ability of 5637 and UC3 Cells

The scratch wound healing assays showed that the migration ability in 5637 and UC3 cells transfected with the pcDNA 3.1-*AS2* group was restrained in comparison with those in pcDNA 3.1 group (Figure 4A–D). On the flip side, cells were transfected with si-NC or si-*AS2*-2 to further confirm the influence of *ADAMTS9-AS2*, which showed that cell migration ability in the si-*AS2*-2 group was strengthened compared to si-NC (Figure 4A–D). Concisely, *ADAMTS9-AS2* suppresses the migration ability of bladder tumor cells.

ADAMTS9-AS2 Restrains the Invasion Ability of 5637 and UC3 Cells

The transwell assays found that the invasion ability of 5637 and UC3 in cells transfected with the pcDNA

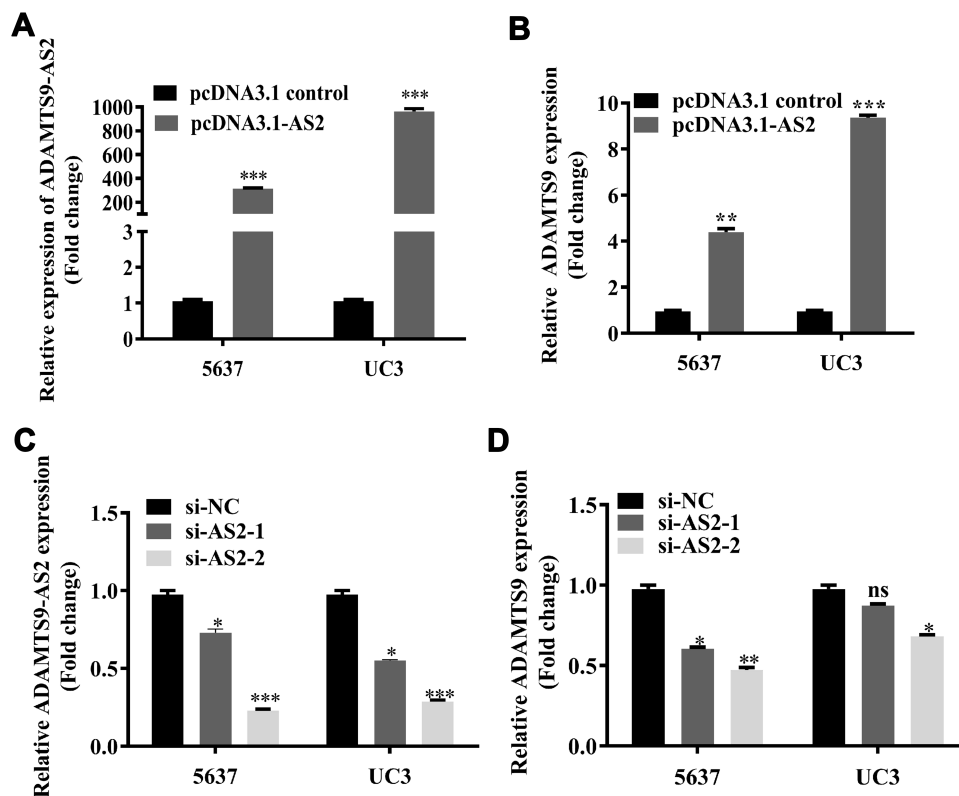


Figure 2 ADAMTS9-AS2 was positively related to ADAMTS9 in 5637 and UC3 cells.

Notes: (A, B) ADAMTS9-AS2 overexpression experiments were first conducted. pcDNA3.1 control and pcDNA3.1-AS2 were transfected into 5637 and UC3 cell lines, respectively. It suggested that ADAMTS9-AS2 expression was rising in parallel with increasing ADAMTS9 expression. (C, D) For ADAMTS9-AS2 knockdown experiments, si-NC, si-AS2-1 and si-AS2-2 were transfected into 5637 and UC3 cell lines, correspondingly, which showed that downregulated ADAMTS9-AS2 resulted in the reduction of ADAMTS9 expression and only no significant changes in UC3 cells transfected with si-AS2-1 group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Abbreviations: siRNA, small interfering RNA; ns, no significance; NC, negative control.

3.1-AS2 group had abated compared to the corresponding NC group (Figure 5A–D; $P < 0.01$). Cells were transfected with si-NC or si-AS2-2 to further identify the roles of ADAMTS9-AS2. The knockdown experiments in 5637 and UC3 cell lines suggested that cell invasion ability in si-AS2-2 groups were enhanced compared to their corresponding si-NC group (Figure 5A–D; $P < 0.01$). Collectively, these findings suggested that ADAMTS9-AS2 inhibits the proliferation, migration, and invasion ability of bladder tumor cells.

ADAMTS9-AS2 Blocked PI3K/AKT/mTOR Signal Pathway in 5637 and UC3 Cells

As mentioned earlier, the activation of PI3K/AKT was closely related to abnormal expression of a large number of lncRNAs.^{21,27} To investigate the potential mechanism of ADAMTS9-AS2 in bladder cancer, therefore, the associations between ADAMTS9-AS2 and PI3K/AKT/mTOR pathway related markers were analyzed. Western blot assays were first conducted to analyze the expression of

p-AKT, PIK3CB and p-mTOR at phosphorylation level. ADAMTS9-AS2 overexpression experiment or knockdown experiment were conducted in 5637 and UC3 cell lines. It showed that the expressions of p-AKT, PIK3CB and p-mTOR were inhibited in cells transfected with pcDNA 3.1-AS2 group compared to pcDNA 3.1 group (Figure 6A and B). ADAMTS9-AS2 knockdown experiment suggested that the expression of p-AKT, PIK3CB and p-mTOR was enhanced in the si-AS2-2 group compared to si-NC group (Figure 6A and B). ADAMTS9-AS2 overexpression or knockdown had no significant effects on AKT1 and mTOR (Figure 6A and B). These results suggested that ADAMTS9-AS2 might block the PI3K/AKT/mTOR signal pathway in bladder cancer cells.

ADAMTS9-AS2 was Essential to Autophagy and Apoptosis in 5637 and UC3 Cells

The PI3K/AKT/mTOR pathway has been widely explored to reflect the apoptosis process.^{28,30} In fact, the mechanistic target of rapamycin (mTOR) serves as a central

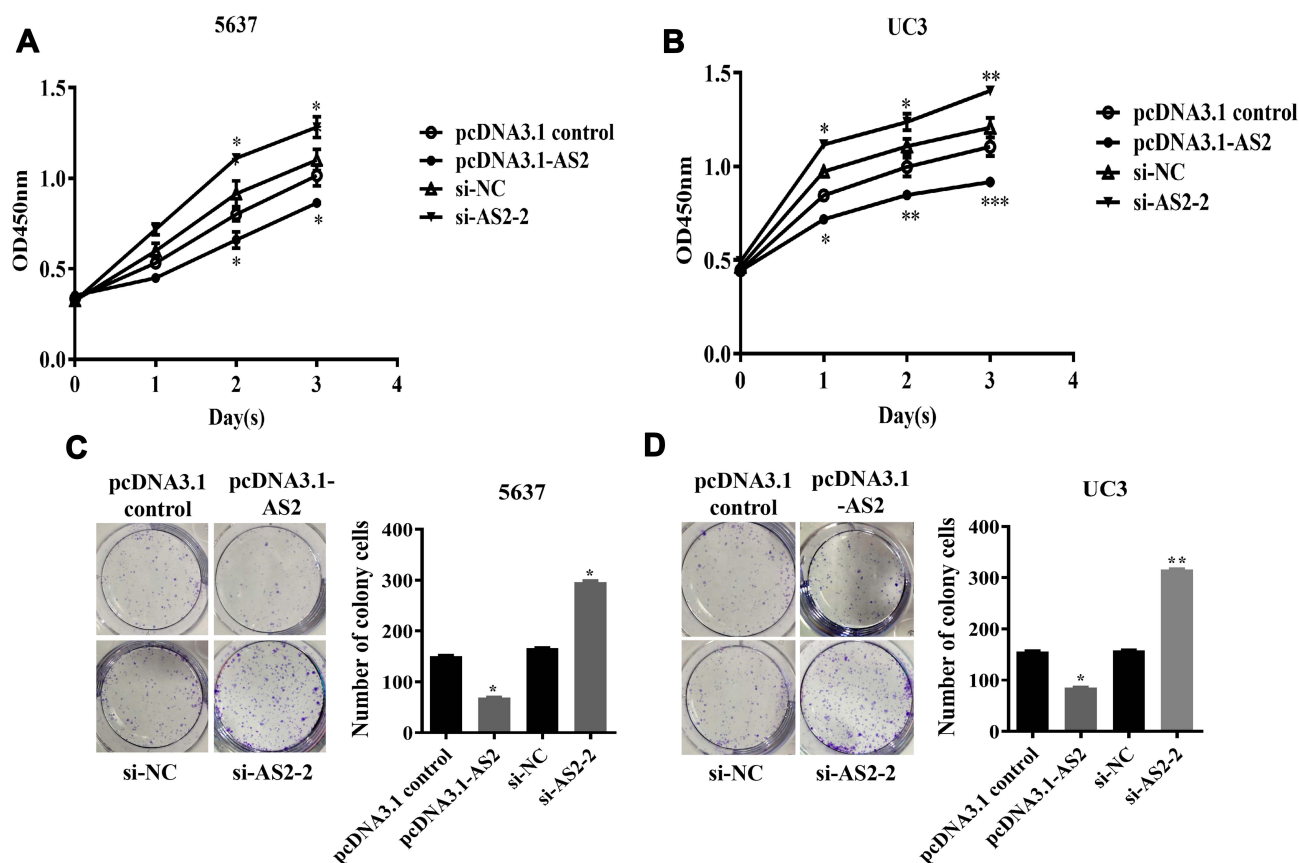


Figure 3 ADAMTS9-AS2 inhibits the proliferation ability of 5637 and UC3 cells.

Notes: (A and C) In 5637 cells, CCK8 assays and clonogenic assays demonstrated that cells in transfected with the pcDNA 3.1-AS2 group, the proliferation ability was curbed compared with those transfected with pcDNA3.1 control, while it was promoted in cells transfected with si-AS2-2 as compared to si-NC group. (B and D) In UC3 cells, similar results obtained from CCK8 and clonogenic assays, the proliferation ability was inhibited in the ADAMTS9-AS2 overexpression group but enhanced in the ADAMTS9-AS2 knockdown group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Abbreviations: siRNA, small interfering RNA; NC, negative control.

controller of cell growth signaling to autophagy.³¹ To further reveal the functional roles of *ADAMTS9-AS2*, its effect on autophagy and apoptosis was investigated. Similarly, *ADAMTS9-AS2* overexpression and knockdown experiments were conducted in 5637 and UC3 cell lines. Expressions of several key autophagy (LC3-I, LC3-II, *BECN1*, and *SQSTM1*) and apoptotic proteins (Bax and Bcl-2) were observed by Western blot assays.

For *ADAMTS9-AS2* overexpression experiment, cells were transfected with pcDNA 3.1 or pcDNA 3.1-AS2. We found that *ADAMTS9*, LC3-II, *BECN1*, and proapoptotic Bax expression were increased, but *SQSTM1* and antiapoptotic Bcl-2 expression were decreased in pcDNA 3.1-AS2 group compared to their corresponding pcDNA 3.1 group (Figure 7A and B). For the *ADAMTS9-AS2* knockdown experiment, we noticed that *ADAMTS9*, LC3-II, *BECN1*, and proapoptotic Bax expression were reduced in cells transfected with si-AS2-2 group compared with si-NC, while *SQSTM1* and antiapoptotic Bcl-2 expression were elevated

in si-AS2-2 group compared to si-NC group (Figure 7A and B). We did not detect obvious impact on LC3-I in *ADAMTS9-AS2* overexpression or knockdown experiment (Figure 7A and B). All these results indicate a potential regulatory mechanism of *ADAMTS9-AS2* involved in autophagy and apoptosis in bladder tumor cells.

Discussion

It has been well-recognized that a host of lncRNAs were closely related to the pathogenesis of bladder cancer.³² Nevertheless, the roles of *ADAMTS9-AS2* in the bladder tumor remain largely unknown. It is well known that *ADAMTS9-AS2* is an antisense transcript of *ADAMTS9*.^{11,33} A growing number of research has shown that a pair of sense and antisense transcripts commonly plays a pivotal role in the tumors, like *SPINT1-AS1* and *SPINT1* in colorectal cancer,³⁴ *FOXF1-AS1* and *FOXF1* in osteosarcoma cells;³⁵ *AFAP1-AS1* and *AFAP1* in breast cancer;³⁶ *MAPT-AS1* and *MAPT* in breast cancer.³⁷ From the GEO analysis results, red

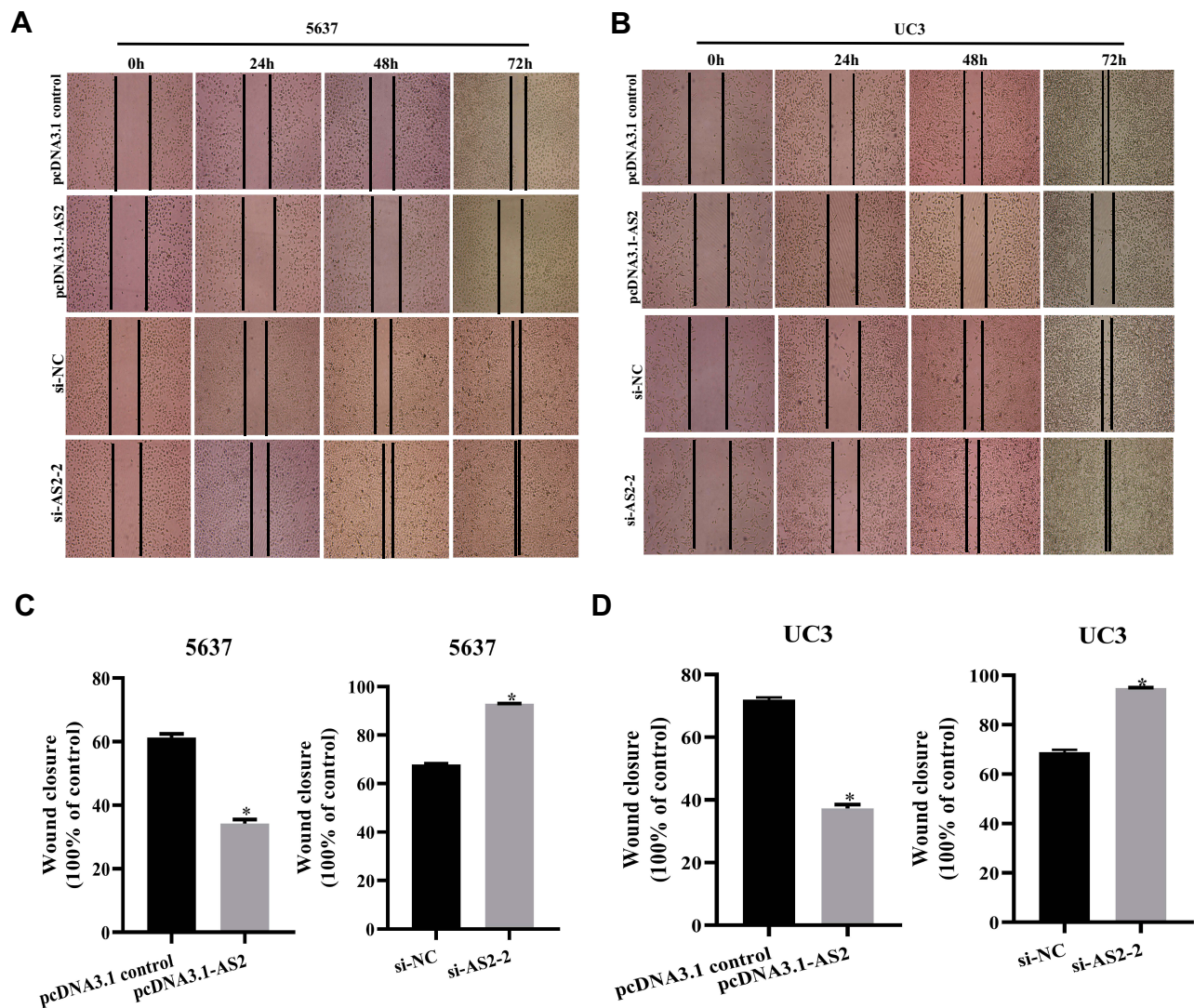


Figure 4 ADAMTS9-AS2 suppresses the migration ability of 5637 and UC3 cells.

Notes: (A and C) In 5637 cells, transwell assays found that invasion ability of cells transfected with the pcDNA 3.1-AS2 group had abated compared to the corresponding pcDNA 3.1 control group, while cell invasion ability in si-AS2-2 groups was enhanced compared to their corresponding si-NC group. (B and D) In UC3 cells, ADAMTS9-AS2 overexpression also restrained the migration ability, and its knockdown promoted the migration ability of cells. * $P < 0.05$

Abbreviations: siRNA, small interfering RNA; NC, negative control.

represents positive correlation, which indicated that *ADAMTS9-AS2* and *ADAMTS9* had a weak positive correlation. Therefore, the potential association of *ADAMTS9-AS2* and *ADAMTS9* gave rise to the insights into the investigation of bladder cancer. Using the qPCR assays, *ADAMTS9-AS2* expressions in one normal cell line SV-HUC-1 and five bladder cancer cell lines (UC3, 5637, J82, T24, and EJ) were first identified. Because *ADAMTS9-AS2* was apparently downregulated in the 5637 and UC3 cell lines, the two cell lines were applied in following assays. From the *ADAMTS9-AS2* overexpression and knockdown experiments, the association between *ADAMTS9-AS2* and *ADAMTS9* were

identified. We found that *ADAMTS9-AS2* was positively related to *ADAMTS9* in 5637 and UC3 cells, which is in line with the previous study.³⁸ Albeit the diverse roles of *ADAMTS9-AS2* has been reported in a variety of tumors,^{15,17} it still remains largely unclear whether *ADAMTS9-AS2* could bring out the effects on bladder tumor cells. To identify the functional roles of *ADAMTS9-AS2* in bladder tumor cells, CCK8, clonogenic assay, scratch wound healing, and transwell assay were performed, respectively. We found that *ADAMTS9-AS2* overexpression inhibits the proliferation, migration, and invasion ability in bladder cancer lines 5637 and UC3. Conversely, its knockdown had positive results.

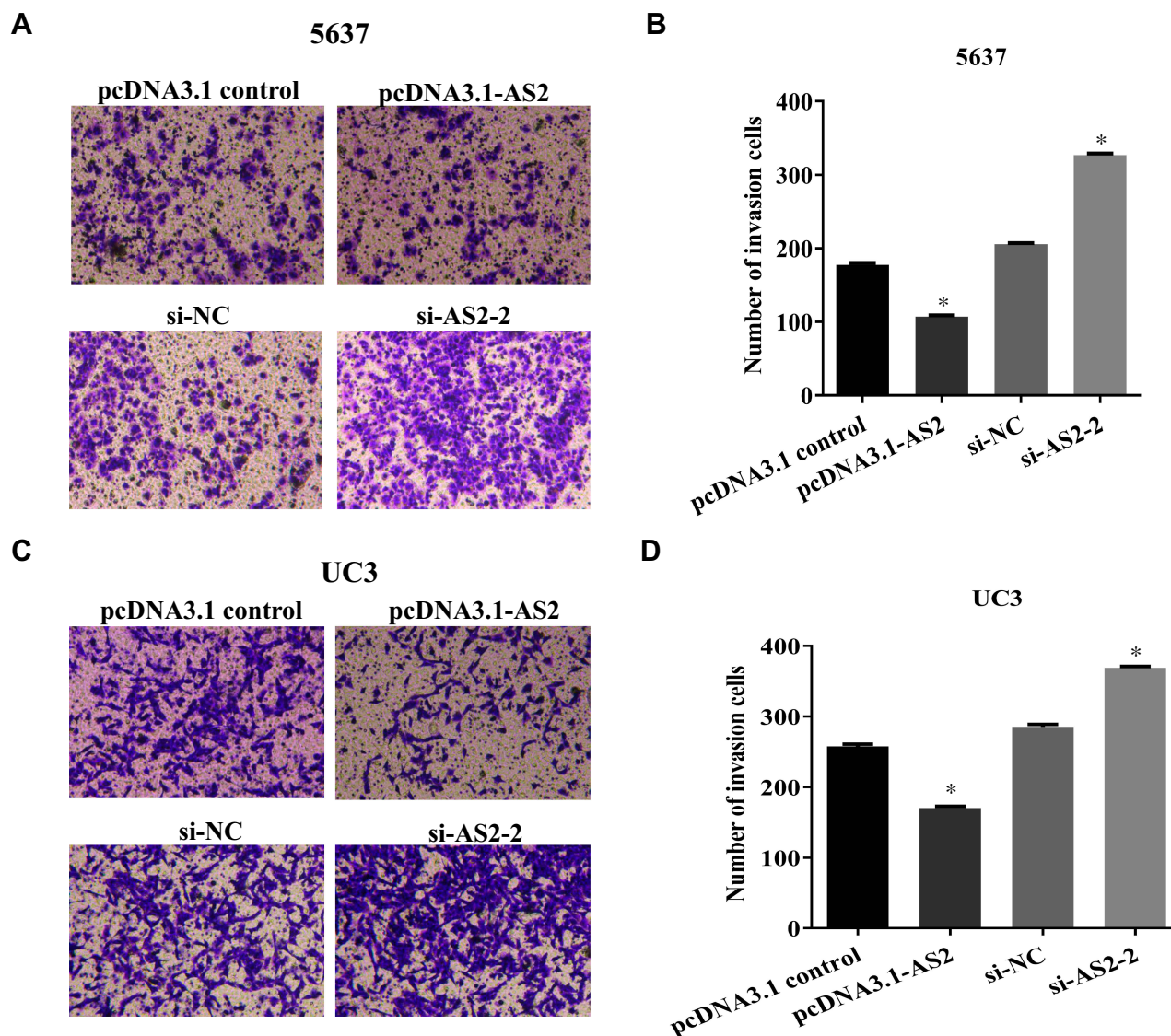


Figure 5 *ADAMTS9-AS2* restrains the invasion ability of 5637 and UC3 cells.

Notes: (A, B) In 5637 cells, the transwell assay found that the invasion ability of cells transfected with the pcDNA 3.1-AS2 group was inhibited compared to the corresponding NC group, whereas the invasion ability was enhanced in the si-AS2-2 group compared to the si-NC group. (C, D) In UC3 cells, similarly, cell invasion ability was inhibited in the *ADAMTS9-AS2* overexpression group, whereas it was enhanced in the *ADAMTS9-AS2* knockdown group compared to their corresponding NC group. * $P < 0.01$.

Abbreviations: siRNA, small interfering RNA; NC, negative control.

Given the information from GEO database and several pairs of sense and antisense related studies,^{34,37} we speculated that *ADAMTS9-AS2* might play a role in bladder tumor by regulating the *ADAMTS9* expression. Besides, *ADAMTS9* functions as a critical player in the process of *AKT/mTOR* signaling pathway as evidenced by Chen et al.^{33,39} Noticeably, the activation of the *PI3K/AKT/mTOR* signaling pathway is widely recognized as an essential part of regulatory mechanism in tumors including bladder cancer.^{40,42} As previously mentioned, *ADAMTS9-AS2* expression was correspondent with *ADAMTS9*. We thus proceeded with deep

investigation concerning the association of *ADAMTS9-AS2* and *PI3K/Akt/mTOR* pathway related markers. Results based on Western blot assays indicated that *ADAMTS9-AS2* inhibited p-AKT, *PIK3CB*, and p-mTOR. Previous study noticed that *PI3K/Akt/mTOR* was closely related to autophagy and apoptosis.^{31,43} The autophagy related several key markers (*LC3-I*, *LC3-II*, *BECN1*, and *SQSTM1*) and apoptotic proteins (*Bax* and *Bcl-2*) were further explored. It suggested that *ADAMTS9-AS2* promoted *LC3-II*, *BECN1*, and proapoptotic *Bax*, but inhibited *SQSTM1* and antiapoptotic *Bcl-2* expression. The data we present might highlight the

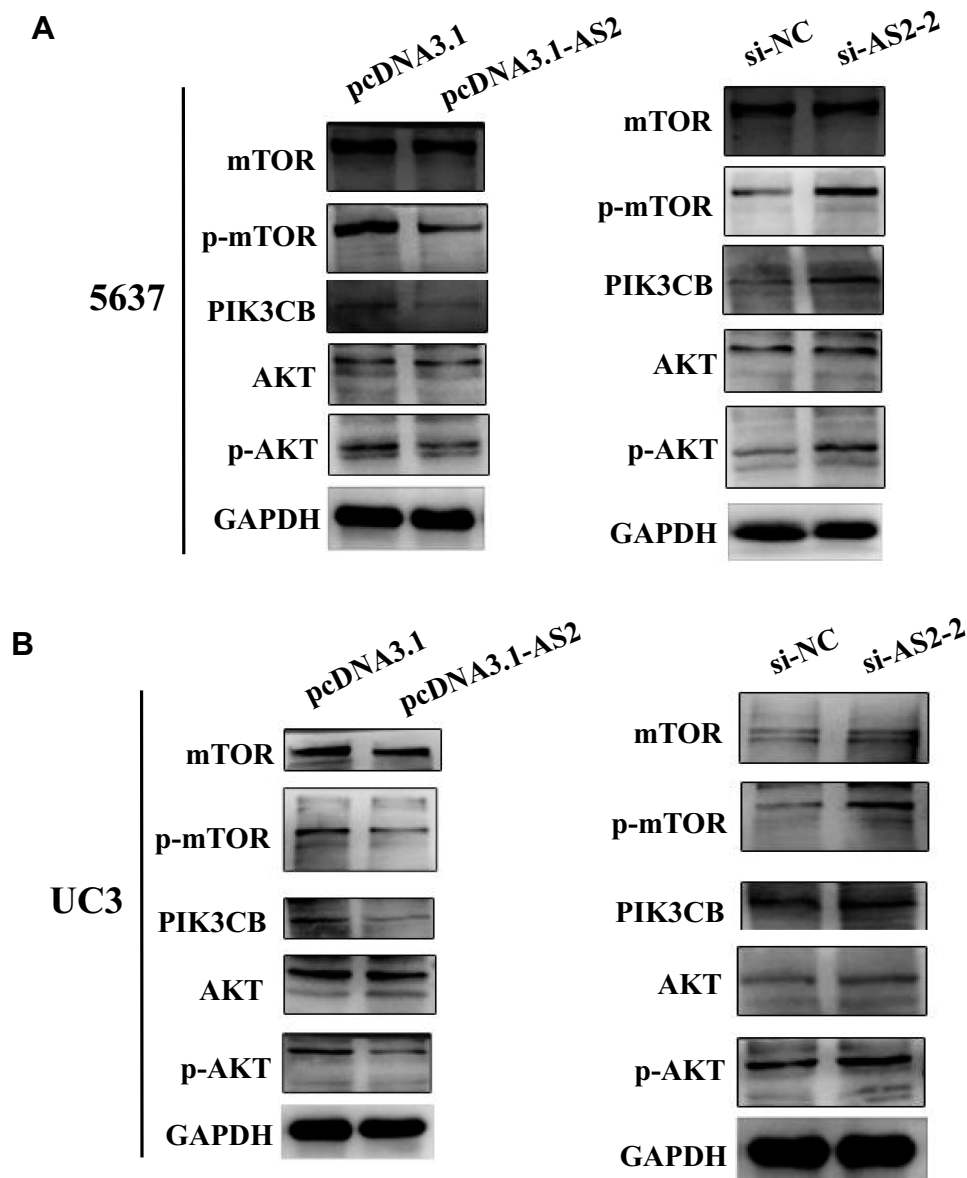


Figure 6 ADAMTS9-AS2 blocked PI3K/AKT/mTOR signal pathway in 5637 and UC3 cells.

Notes: (A) In 5637 cells, from the results of Western blot assays, the expressions of p-AKT, PIK3CB, and p-mTOR were inhibited in cells transfected with the pcDNA 3.1-AS2 group compared to the pcDNA 3.1 group, while p-AKT, PIK3CB, and p-mTOR were enhanced in the si-AS2-2 group compared to si-NC group. ADAMTS9-AS2 overexpression or knockdown had no significant effects on AKT1 and mTOR. (B) In UC3 cells, similarly, Western blot assays also indicated that p-AKT, PIK3CB, and p-mTOR were restrained in the ADAMTS9-AS2 overexpression group, whereas their expressions were enhanced in the ADAMTS9-AS2 knockdown group.

Abbreviations: siRNA, small interfering RNA; NC, negative control.

biological functions of *ADAMTS9-AS2* in bladder tumor. In addition, we further explored the reasonable mechanism that *ADAMTS9-AS2* inhibited the proliferation, migration, and invasion via *PI3K/Akt/mTOR* signaling pathway. Nevertheless, our study exists some limitations, such as, functional experiments were performed only in two cell lines; the precise molecular mechanism underlying the *ADAMTS9-AS2* and *ADAMTS9* require further investigation. Therefore, further studies will be considered to reveal the specific mechanisms of *ADAMTS9-AS2* in bladder tumor.

Conclusion

Our results found the functional roles and potential mechanisms of *ADAMTS9-AS2* in bladder tumor. It suggested that *ADAMTS9-AS2* could inhibit cell proliferation, migration, and invasion ability in bladder tumor. Our work might provide valuable insights into a possible mechanism that *ADAMTS9-AS2* is a tumor suppressor involved in the *PI3K/AKT/mTOR* signal pathway causing cell autophagy and apoptosis in bladder tumor.

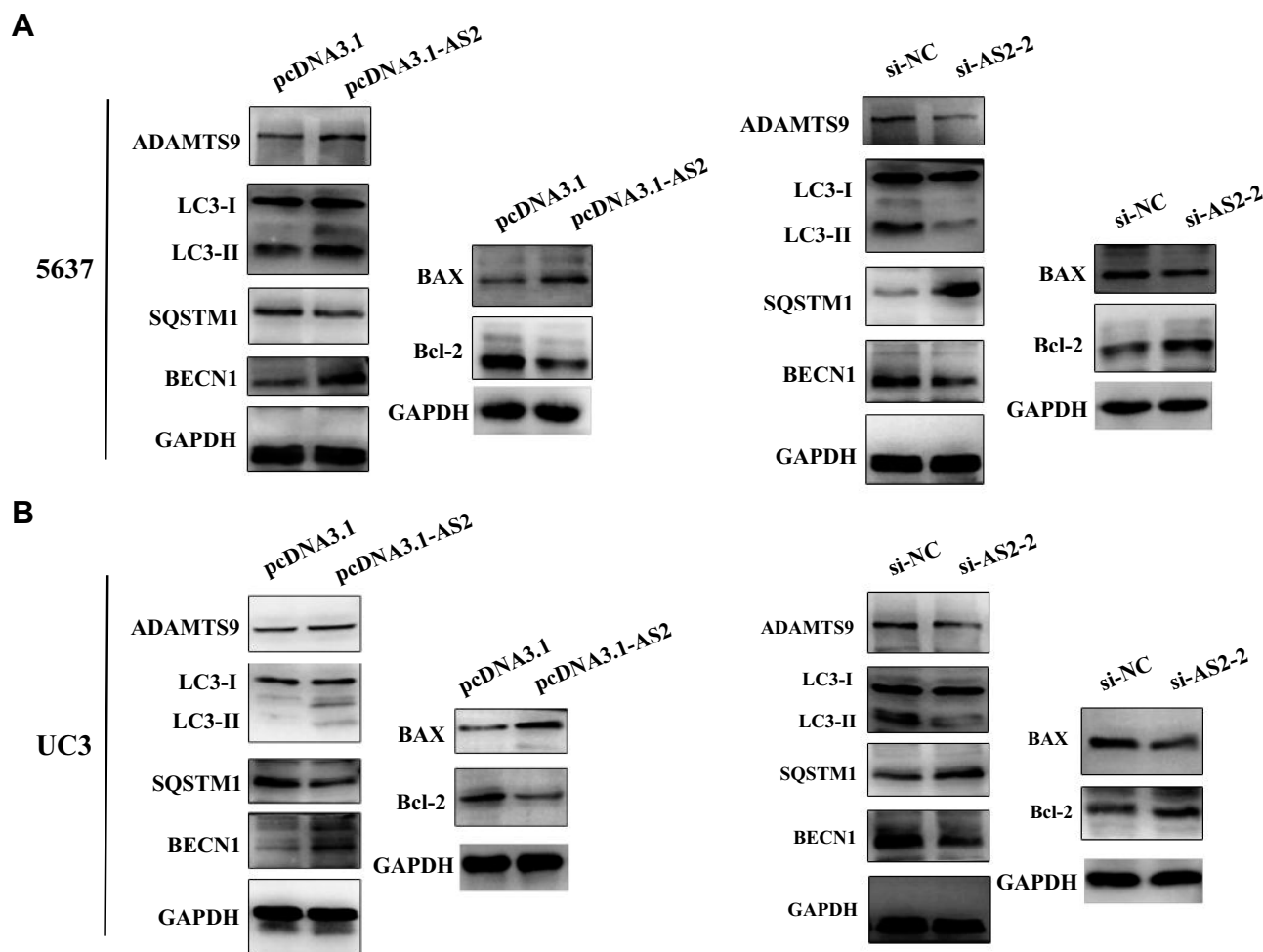


Figure 7 ADAMTS9-AS2 was essential to autophagy and apoptosis in 5637 and UC3 cells.

Notes: (A) In 5637 cells, from the results of Western blot assays, ADAMTS9, LC3-II, BECN1, and proapoptotic Bax expression were increased in pcDNA 3.1-AS2 group, but reduced in cells transfected with si-AS2-2 group compared with corresponding NC group. In addition, SQSTM1 and antiapoptotic Bcl-2 expression were decreased in the pcDNA 3.1-AS2 group, whereas they were elevated in the si-AS2-2 group compared to the NC group. (B) In UC3 cells, similar results were obtained from Western blot assays. ADAMTS9, LC3-II, BECN1, and proapoptotic Bax expressions were rising in the ADAMTS9-AS2 overexpression group, while they were reduced in the ADAMTS9-AS2 knockdown group; SQSTM1 and antiapoptotic Bcl-2 expressions were decreased as ADAMTS9-AS2 overexpression, while they were abated as ADAMTS9-AS2 knockdown. **Abbreviations:** siRNA, small interfering RNA; NC, negative control.

Abbreviations

lncRNA, long non-coding RNA; siRNA, small interfering RNA; q-PCR, quantitative PCR.

Funding

The support in this work was obtained from the Scientific Research Project of Hubei Provincial Health Committee (WJ2019M194), Xisike-Hengrui Cancer Research Fund (Y-HR2018-328), and Xisike-BMS Cancer Immunotherapy Research Fund (Y-BMS2019-003).

Disclosure

The authors confirmed no conflicts of interest in this work.

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