

SNHG1 promotes proliferation, migration and invasion of bladder cancer cells via the PI3K/AKT signaling pathway

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Abstract. Long non-coding RNA (lncRNA) small nucleolar RNA host gene 1 (SNHG1) has been previously reported to mediate a number of functions during the progression of cancer. However, its involvement in bladder cancer remain unclear. The aim of the present study was to investigate the expression of SNHG1 in bladder cancer and to identify its potential mechanisms. SNHG1 expression was firstly detected in cancer tissues and cells. The effects of SNHG1 on the malignant phenotypes were then investigated. Furthermore, the influence of SNHG1 on the PI3K/AKT signaling pathway was examined. It was demonstrated that SNHG1 expression was significantly upregulated in bladder cancer tissues and cells. Moreover, the loss-of-function experimental results suggested that knockdown of SNHG1 inhibited bladder cancer cell proliferation, migration and invasion, but increased apoptosis; however, SNHG1 overexpression promoted these processes. Mechanistically, rescue assays identified that SNHG1 activated the PI3K/AKT signaling pathway. Therefore, it was speculated that SNHG1 functioned as a carcinogenic lncRNA in bladder cancer via activation of PI3K/AKT.

Introduction

Bladder cancer is the most common malignancy of the urinary system worldwide, with >76,960 new cases and 16,390 mortalities estimated in 2016 (1). Bladder cancer is divided into non-muscle-invasive bladder cancer, characterized by a high recurrence rate (70%), and muscle-invasive bladder cancer, which has a <50% of 5-year overall survival according to biological characteristics (2). Thus, it is important to identify the molecular mechanism of cellular proliferation

in bladder cancer to facilitate the development of a novel and more effective therapy.

As a class of non-coding RNA without the capacity of coding proteins, long non-coding RNAs (lncRNA) are >200 nucleotides in length (3,4). Previous studies have reported that lncRNA are involved in epigenetic, transcriptional and post-transcriptional modulation in various biological processes, including proliferation, differentiation, migration and apoptosis (5,6). lncRNA small nucleolar RNA host gene 1 (SNHG1) located at 11q12.3 locus, functions as an oncogene in a number of cancer types. For instance, Cui *et al* (7) revealed that high SNHG1 expression in non-small cell lung cancer (NSCLC) was significantly correlated with larger tumor size, advanced TNM stage, lymph node metastasis and poor overall survival. Moreover, Hu *et al* (8) reported that knockdown of SNHG1 suppressed gastric cancer cell proliferation both *in vitro* and *in vivo*. Liu *et al* (9) also found that SNHG1 inhibition significantly inhibited cervical cancer cell proliferation, migration and invasion. However, the relationship between SNHG1 expression and bladder cancer, as well as the underlying molecular mechanisms of the oncogenic functions of SNHG1, remains unknown and require further investigation. Furthermore, identifying the downstream targets of SNHG1 will elucidate its critical role in bladder cancer progression.

The PI3K/AKT signaling pathway regulates multiple biological processes, including cell proliferation and apoptosis (10). In most cases, inappropriate activation of PI3K/AKT is speculated to induce tumor formation (11). Increased SNHG1 expression has also been reported to promote the activity of the PI3K/AKT signaling pathway (12). However, the molecular mechanisms underlying this phenomenon are not fully understood.

The aim of the present study was to elucidate the key functions of SNHG1 in the proliferation, apoptosis, migration and invasion of bladder cancer cells *in vitro*, in addition to investigating the signaling pathways that are possibly implicated in this process.

Materials and methods

Tissues. Tumor tissues and homologous adjacent healthy tissues were donated by 60 patients with bladder cancer, who underwent surgery between July 2016 and January 2018

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during their hospitalization in Shaanxi Provincial People's Hospital (Xi'an, China), and were stored at -80°C prior to RNA isolation. The inclusion criteria were as follows: i) Patients diagnosed by pathological biopsies; ii) patients at stage I or II based on the TNM staging system (13); iii) patients with complete medical record; and iv) patients and their families who were willing to participate. The exclusion criteria were as follows: i) Patients who were treated within 3 months before admission; ii) patients who had other diseases, such as chronic diseases and metabolic diseases; and iii) patients who were not willing to donate plasma samples. The 60 patients with bladder cancer included 34 males and 26 females (age range, 27-67 years; mean age, 46.4 ± 5.1 years). Informed consent was obtained and all experimental procedures were approved by the Human Ethics Committee of Shaanxi Provincial People's Hospital.

Cell culture, transfection and treatment. In total, four human bladder cancer cell lines (T24, SW780, J82 and RT4) and normal bladder epithelial HCV-29 cells were purchased from the American Type Culture Collection (ATCC). Cells were incubated in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (all from Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified atmosphere with 5% CO_2 at 37°C .

For knockdown of SNHG1 in SW780 cells, the sequences of short hairpin (sh)RNA targeting for SNHG1 (5'-CAGCAG TTGAGGGTTTGCTGTGTAT-3') were designed by Shanghai GenePharma Co., Ltd. For overexpression, the full-length SNHG1 was cloned into a pcDNA3.1 vector (Shanghai GenePharma Co., Ltd.) to overexpress SNHG1 in RT4 cells, and the primer sequences were as follows: SNHG1 forward, 5'-GGGGTACCGTTCTCATTTTTCTACTGCTCGTG-3' and reverse, 5'-CGGGATCCATGTAATCAATCATTTTATTAT TTTCATC-3'. The empty vector and scrambled shRNA for SNHG1 were used as negative controls. Cell transfections were conducted using shRNAs and plasmids, both at a final concentration of 100 nM, using Lipofectamine[®] 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Cells were treated with the PI3K agonist 740Y-P (50 μM ; Sigma-Aldrich; Merck KGaA) or the PI3K inhibitor LY294002 (50 μM ; Sigma-Aldrich; Merck KGaA) for 24 h at 37°C , 48 h after transfection.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from tissues or T24, SW780, J82, RT4, and HCV-29 cells using the TRIzol[®] Reagent (Thermo Fisher Scientific, Inc.) and then converted into cDNA by reverse transcription using PrimeScript[™] RT Master Mix (cat. no. RR036Q; Takara Biotechnology Co., Ltd.), according to the manufacturer's protocol. qPCR was performed using Path-ID[™] Multiplex One-Step RT-PCR Kit (cat. no. 4442136; Applied Biosystems; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol in an ABI 7500 RT PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) to determine the relative quantification of SNHG1 expression. A total of 1 μg total RNA was reversely transcribed using oligo(dT) primer at 42°C for 1 h and 2 μl the reverse transcription reaction mix was amplified by PCR with denaturation at 95°C for 2 min, followed by 50 cycles of 95°C for 30 sec, 55°C for

30 sec and 72°C for 1 min. The thermocycling conditions were as follows: Initial denaturation at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. Relative expression was calculated with the $2^{-\Delta\Delta\text{C}_q}$ method as previously described (14), where GAPDH was used as the internal control. The primer sequences were as follows: SNHG1 forward, 5'-AGGCTGAAGTTACAGGTC-3' and reverse, 5'-TTGGCT CCCAGTGTCTTA-3'; and GAPDH forward, 5'-GTCAAC GGATTTGGTCTGTATT-3' and reverse, 5'-AGTCTTCTG GGTGGCAGTGAT-3'.

Cell Counting Kit-8 (CCK-8) assay. Cell proliferation was assessed using a CCK-8 assay (Beyotime Institute of Biotechnology). At 0, 24, 48, 72 and 96 h post-transfection, cells were re-seeded into culture medium on 96-well plates at a density of 5×10^3 cells/well and incubated at 37°C overnight. CCK-8 solution (Beyotime Institute of Biotechnology) with 10 $\mu\text{l}/\text{well}$ was then added and incubated at 37°C for another 2 h according to the manufacturer's instruction. Absorbance was measured with a microplate reader at a wavelength of 450 nm.

Colony formation assay. After 24 h transfection, SW780 and RT4 cells were re-seeded onto 6-well plates at a density of 500 cells per well and cultured for 3 days. After washing with PBS, cells were fixed with 4% paraformaldehyde solution at 37°C for 15 min and stained with 0.1% crystal violet solutions at room temperature for 10 min. The number of colonies containing >50 cells was analyzed manually using light microscope (magnification, x200). The experiments were performed in triplicate.

Apoptosis detection. For the detection of the percentage of early apoptotic cells, SW780 and RT4 cells were seeded in 24-well plates (2×10^5 cells/well) for 48 h, and stained using Annexin V-FITC apoptosis assay (5 μl ; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 20 min. The stained cells were analyzed using BD FACSCalibur[™] flow cytometer (Beckman Coulter, Inc.) and FlowJo software (version X; FlowJo LLC). Living cells were in the lower left quadrant. The upper right quadrant represented necrotic and late apoptotic cells, whilst the lower right quadrant represented early apoptotic cells, which were quantified.

Migration assay. Wound-healing assay was performed to measure the cell migration capacity of SW780 and RT4 cells. Transfected cells after 48 h transfection were subsequently cultured in DMEM in a six-well culture plate at a density of 5×10^5 cells/well. When the confluence reached 95%, the cells were washed and the culture medium was replaced with serum-free DMEM. A scratch was made through the single cell layer using a 10 μl pipette tip and the cells were washed again with warmed PBS. After 24 h incubation in serum-free medium, images of the migrating cells were captured using a light microscope (magnification, x200; Nikon Corporation). Representative images after wounding were captured with a light microscope. The percent closure of the cells into the wound field was measured using ImageJ software (version 1.46r; National Institutes of Health). The rate of wound healing was calculated as: Wound healing

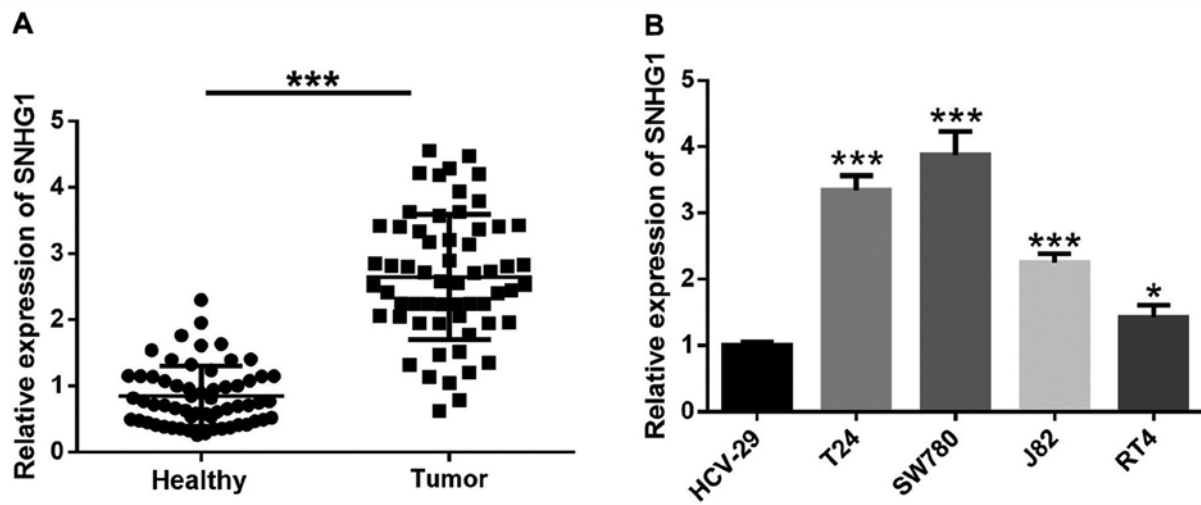


Figure 1. lncRNA SNHG1 expression is significantly upregulated in bladder cancer tissues and cells. (A) SNHG1 expression in 60 paired bladder cancer tissues and adjacent healthy tissues. (B) SNHG1 expression in four bladder cancer cell lines, T24, SW780, J82 and RT4 and human immortalized bladder epithelial HCV-29 cells using reverse transcription-quantitative PCR. * $P < 0.05$, *** $P < 0.001$ vs. HCV-29 cells or adjacent healthy tissues. SNHG1, small nucleolar RNA host gene 1; lncRNA, long non-coding RNA.

rate = (wound width at 0 h - wound width at 24 h) / wound width at 0 h $\times 100\%$.

Transwell assay. Cell invasion was analyzed with Transwell chambers (8.0- μm pore size with polycarbonate membrane; BD Biosciences) that were pre-coated with Matrigel (BD Biosciences) for 6 h at 37°C. Cells resuspended in serum-free medium at a density of 1×10^4 cells/well were added to the upper chamber, while 500 μl DMEM with 10% FBS was added to the bottom chamber. After 24 h, cells that failed to migrate were removed from the upper part of the filters by scrubbing with a cotton swab, and the membrane was fixed with 4% formaldehyde at room temperature for 5 min and stained with 0.5% crystal violet at room temperature for 10 min. The invasive cells were counted using light microscope at $\times 200$ magnification from 10 different fields of each filter. The invaded cell rates were calculated using the following formula: Mean test group invaded cell number / mean blank control invaded cell number $\times 100\%$.

Western blotting. Total protein from cultured cells were extracted using RIPA buffer (Beyotime Institute of Biotechnology). Protein concentration was quantified using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of protein (40 μg) were electrophoresed in a 10% SDS-PAGE and then electro-blotted onto a PVDF membrane (EMD Millipore). The membranes were incubated with primary antibodies against GAPDH (cat. no. 5174; 1:1,000 dilution; Cell Signaling Technology, Inc.), PI3K (cat. no. 17366; 1:1,000 dilution; Cell Signaling Technology, Inc.), phosphorylated (p)-AKT (cat. no. 4060; 1:1,000 dilution; Cell Signaling Technology, Inc.), AKT (cat. no. 10176-2-AP; 1:1,000 dilution; ProteinTech Group, Inc.), proliferating cell nuclear antigen (PCNA; cat. no. 13110; 1:1,000 dilution; Cell Signaling Technology, Inc.), Bcl-2 (cat. no. 3498; 1:1,000 dilution; Cell Signaling Technology, Inc.), Bax (cat. no. 5023; 1:1,000 dilution; Cell Signaling

Technology, Inc.), N-Cadherin (cat. no. 22018-1-AP; 1:3,000 dilution; ProteinTech Group, Inc.) and E-Cadherin (cat. no. 20874-1-AP; 1:5,000 dilution; ProteinTech Group, Inc.) at 4°C overnight. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. sc-2004; 1:5,000 dilution; Santa Cruz Biotechnology) for 1 h at room temperature and visualized using the enhanced chemiluminescence method (Cytiva). GAPDH was used as an internal control. Image-Pro Plus 7.0 software (Media Cybernetics, Inc.) was used to perform the densitometric analysis.

Statistical analysis. SPSS 19.0 software (IBM Corp.) was used to perform statistical analysis. Data are presented as the mean \pm standard deviation from ≤ 3 independent experiments. Data comparisons were performed by either unpaired (or paired for tissue samples) Student's *t*-test (between two groups) or one-way ANOVA followed by Tukey's test (among three groups). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

lncRNA SNHG1 expression is significantly upregulated in bladder cancer tissues and cells. The expression of SNHG1 in bladder cancer tissues was significantly higher compared with the adjacent healthy tissues (Fig. 1A). Moreover, SNHG1 expression was significantly increased in four bladder cancer cell lines, T24, SW780, J82 and RT4 compared with human immortalized bladder epithelial HCV-29 cells (Fig. 1B).

lncRNA SNHG1 promotes bladder cancer cell proliferation. To investigate the association between SNHG1 expression and the proliferative ability of bladder cancer cells, pcDNA-SNHG1, sh-SNHG1 and the empty plasmids were transfected into bladder cancer cells. Transfection with sh-SNHG1 significantly downregulated SNHG1 expression in SW780 cells,

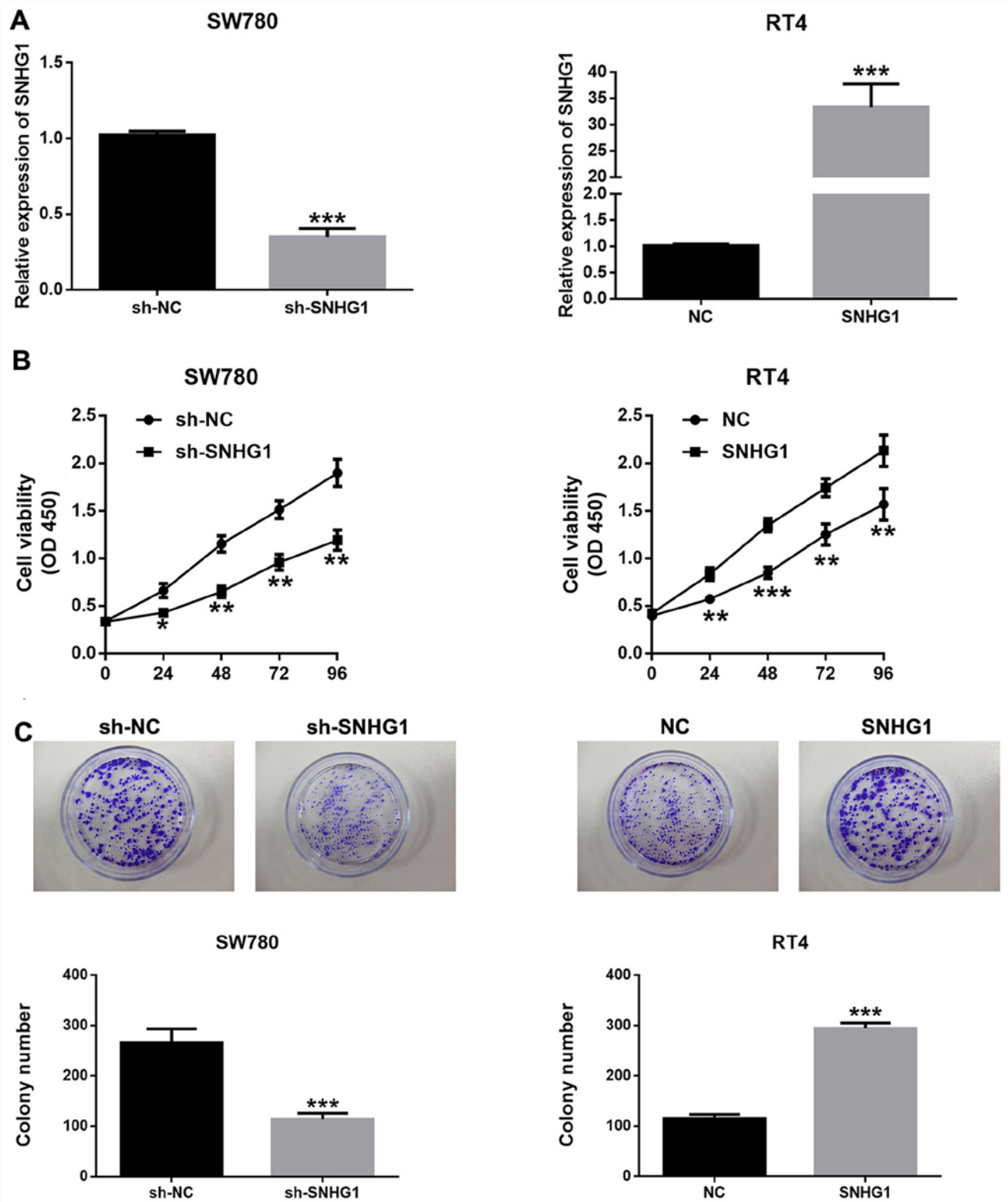


Figure 2. lncRNA SNHG1 promotes bladder cancer cell proliferation. (A) SNHG1 expression, (B) Cell Counting Kit-8 cell viability assay and (C) colony formation assay results in SW780 cells transfected with sh-SNHG1, and in RT4 cells transfected with pcDNA-SNHG1. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. sh-NC or NC. NC, negative control; sh, short hairpin RNA; SNHG1, small nucleolar RNA host gene 1; OD, optical density; lncRNA, long non-coding RNA.

while pcDNA-SNHG1 transfection significantly promoted SNHG1 expression in RT4 cells (Fig. 2A).

The potential biological effects of SNHG1 on the proliferation of bladder cancer cells were detected by CCK-8 assay and colony formation assay. It was demonstrated that

SNHG1 silencing significantly inhibited the proliferation of SW780 cells, while SNHG1 overexpression led to the opposite results (Fig. 2B and C). These findings indicated that SNHG1 facilitated the proliferation and enhanced the viability of bladder cancer cells.

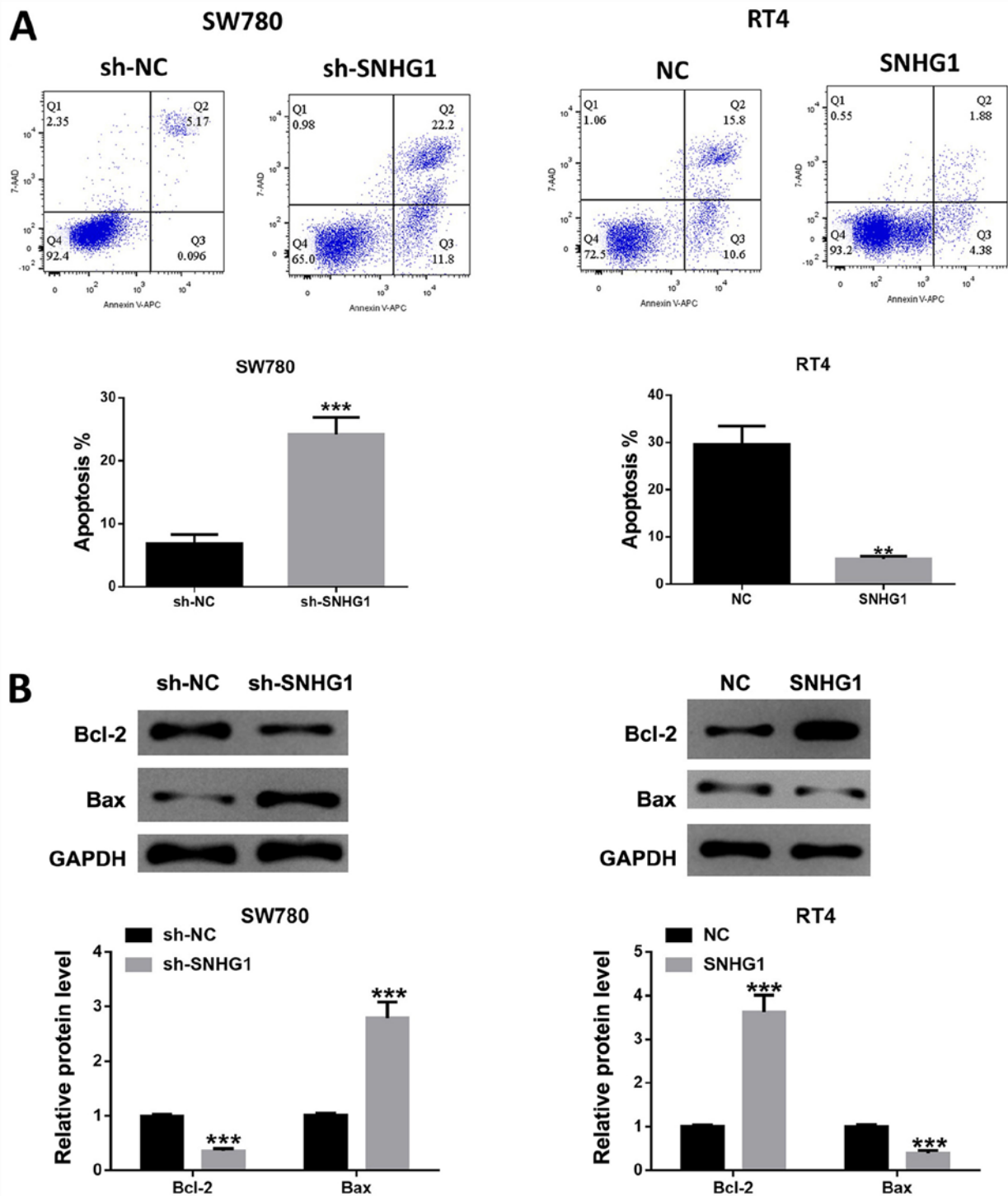


Figure 3. lncRNA SNHG1 inhibits bladder cancer cell apoptosis. SW780 cells were transfected with sh-SNHG1 and in RT4 cells were transfected with pcDNA-SNHG1 (A) Percentage of apoptotic cells using flow cytometry. (B) Protein expression levels of Bcl-2 and Bax were measured using western blotting. ** $P < 0.01$, *** $P < 0.001$ vs. sh-NC or NC. NC, negative control; sh, short hairpin RNA; SNHG1, small nucleolar RNA host gene 1; lncRNA, long non-coding RNA.

lncRNA SNHG1 inhibits bladder cancer cell apoptosis. Cell apoptosis analysis results indicated that the number of apoptosis cells was significantly increased in SW780 cells after SNHG1 silencing, while SNHG1 overexpression repressed the number of apoptotic cells (Fig. 3A). Furthermore, the western blotting results demonstrated that the ratio of Bcl-2/Bax was significantly reduced after SNHG1 knockdown in SW780 cells, while SNHG1 overexpression in RT4 cells led to the opposite

effect (Fig. 3B), which was in line with the aforementioned flow cytometric analysis results.

lncRNA SNHG1 increases bladder cancer cell migration and invasion. The suppression of SNHG1 expression decreased the migratory capacity of SW780 cells, while the migratory capacity of RT4 cells was increased in SNHG1-overexpressing cells (Fig. 4A). In addition, the Transwell invasion assay

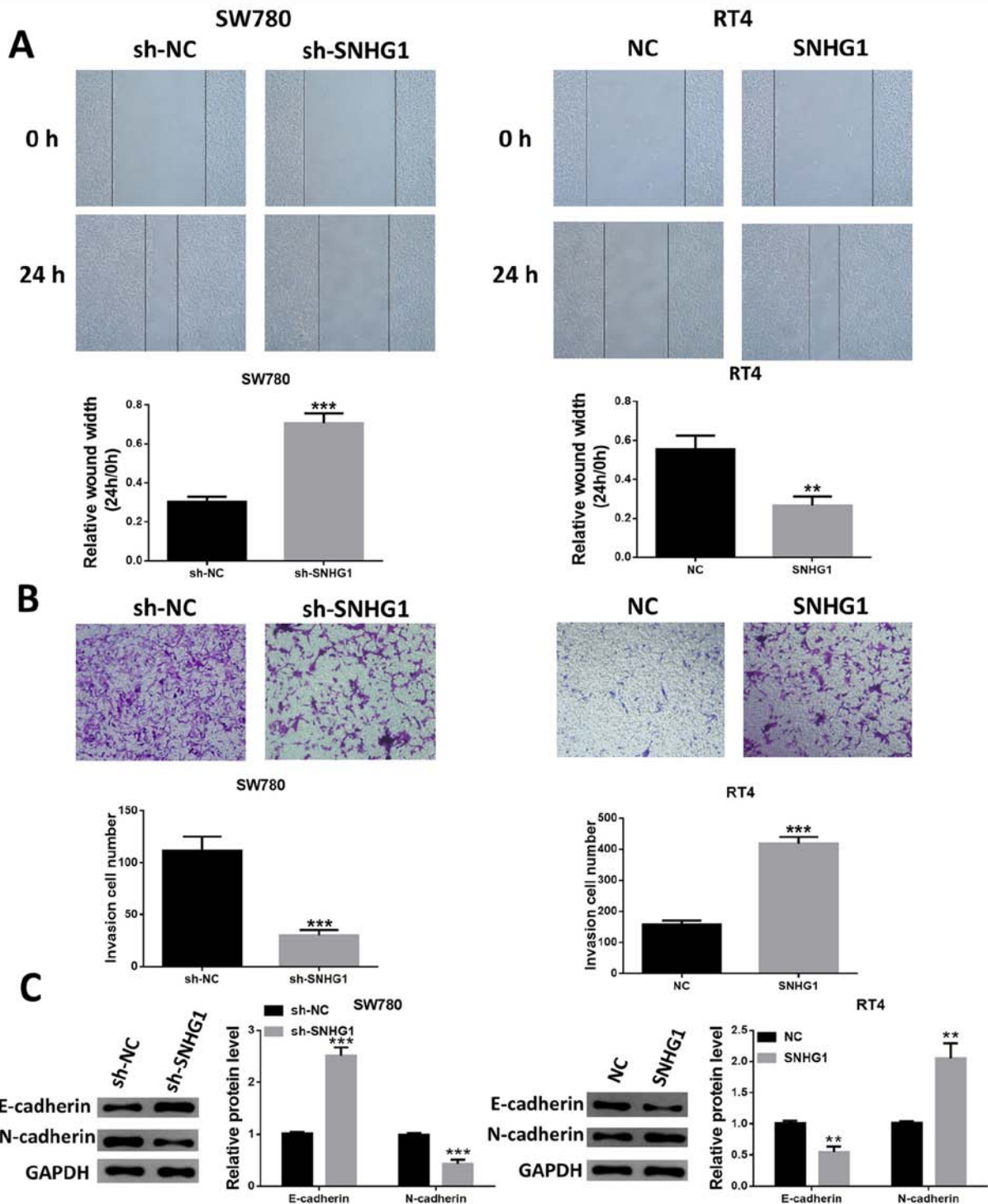


Figure 4. IncRNA SNHG1 elevates bladder cancer cell migration and invasion. SW780 cells were transfected with sh-SNHG1 and RT4 cells were transfected with pcDNA-SNHG1. Cell migration and invasion capacities determined by (A) wound healing and (B) Transwell assay. Magnification, x200. (C) Protein expression levels of E-cadherin and N-cadherin. ** $P < 0.01$, *** $P < 0.001$ vs. sh-NC or NC. NC, negative control; sh, short hairpin RNA; SNHG1, small nucleolar RNA host gene 1; IncRNA, long non-coding RNA.

identified similar results, indicating that SW780 cells with SNHG1 knockdown had reduced cell invasion, while RT4 cells with SNHG1 overexpression exhibited enhanced invasive ability (Fig. 4B).

The expression level changes of the epithelial-mesenchymal transition markers E-cadherin and N-cadherin were also examined. Silencing of SNHG1 upregulated E-cadherin expression, but resulted in N-cadherin downregulation.

Furthermore, the expression of E-cadherin was significantly reduced, while the expression of N-cadherin was upregulated following the overexpression of SNHG1 in RT4 cells (Fig. 4C).

PI3K/AKT axis affects the carcinogenesis of SNHG1. The addition of 740Y-P, a PI3K activator, reversed the down-regulation of PI3K and AKT phosphorylation levels and the

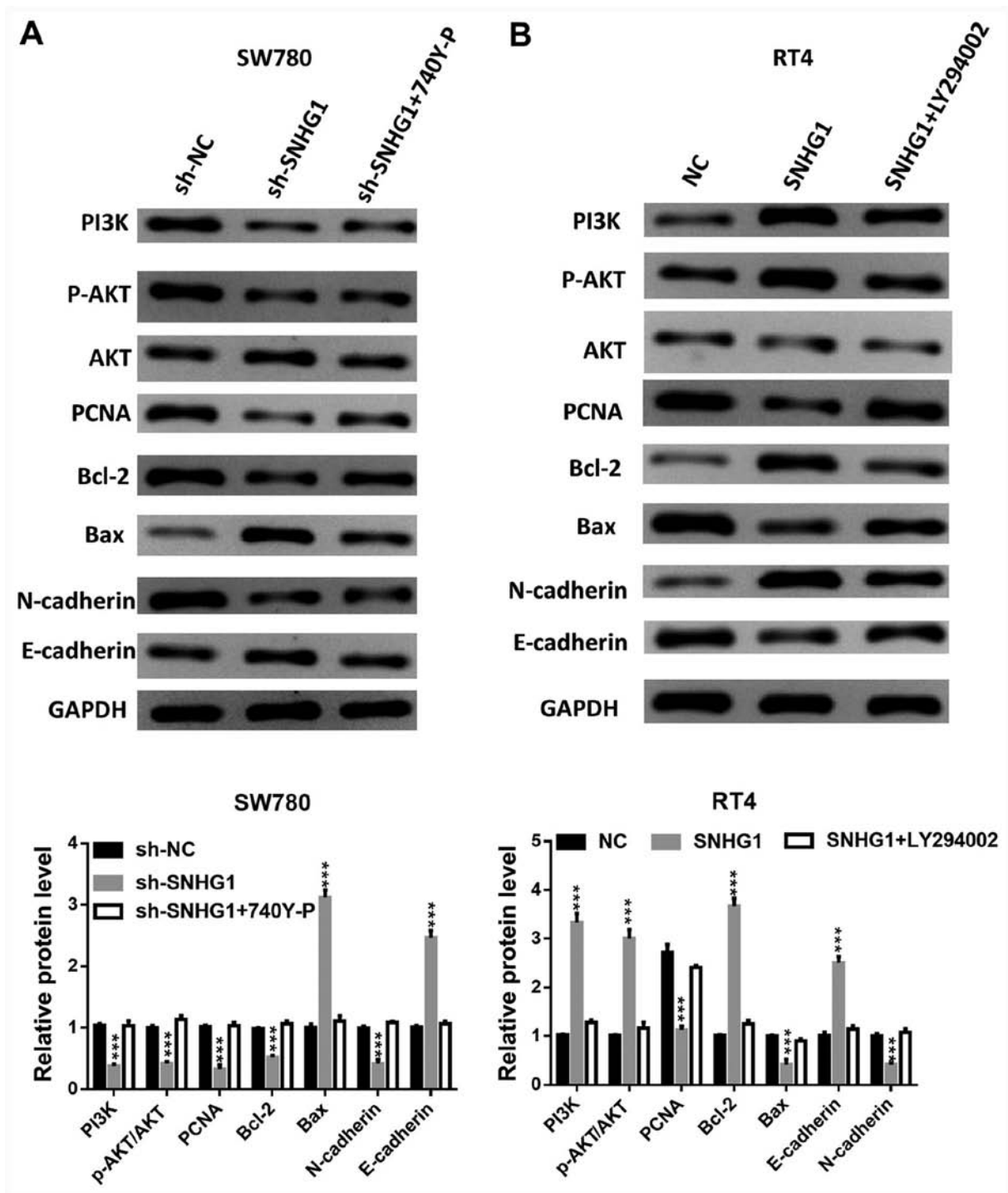


Figure 5. PI3K/AKT axis affects the carcinogenesis of SNHG1. The phosphorylation levels of PI3K and AKT, the protein expression levels of AKT, PCNA, Bcl-2, Bax, N-cadherin and E-cadherin in (A) SW780 cells transfected with sh-SNHG1 + PI3K activator 740Y-P, and (B) in RT4 cells transfected with pcDNA-SNHG1 + PI3K inhibitor LY294002. *** $P < 0.001$ vs. sh-NC or NC. NC, negative control; sh, short hairpin RNA; SNHG1, small nucleolar RNA host gene 1; p-, phosphorylated; PCNA, proliferating cell nuclear antigen.

expression levels of PCNA, Bcl-2 and N-cadherin, which were caused by silenced SNHG1. However, 740Y-P reduced the enhanced Bax and E-cadherin protein expression levels induced by silenced SNHG1 (Fig. 5A).

The application of LY294002, a PI3K inhibitor, led to a partial abrogation of SNHG1 overexpression-induced PI3K and AKT phosphorylation, Bcl-2 and E-cadherin upregulation, and PCNA, Bax and N-cadherin downregulation (Fig. 5B).

Discussion

The initiation and development of bladder cancer involves multiple molecular mechanisms, including abnormal expression of growth factors, adhesion molecules and angiogenic factors (15-17). Therefore, it is important to identify novel biomarker that may be useful for tumor prevention and therapy. In the present study, upregulated SNHG1 expression was identified in bladder cancer tissues and cells. Furthermore, SNHG1

knockdown significantly suppressed bladder cancer cell proliferation, migration and invasion, as well as promoted apoptosis. However, the overexpression of SNHG1 led to an opposite effect via activation of the PI3K/AKT signaling pathway.

lncRNAs are associated with tumor growth and metastatic potentials in several types of cancer, including bladder cancer. For example, Pei *et al* (18) reported that lncRNA cancer susceptibility candidate 2 via the inhibition of the Wnt/ β -catenin signaling pathway, inhibited bladder cancer cell proliferation, migration and invasion, but promoted apoptosis. Wang *et al* (19) also showed that high expression of lncRNA OIP5 antisense RNA 1 (OIP5-AS1) was a poor predictor of bladder cancer prognosis, and the knockdown of OIP5-AS1 expression decreased cell viability, as well as promoted cell-cycle arrest and apoptosis in bladder cancer. Moreover, Gao *et al* (20) revealed that lncRNA zinc finger E-box binding homeobox 2 AS1 increased the proliferation, migration and invasion, but reduced the apoptosis of bladder cancer cells as competing endogenous RNA sponges microRNA (miR)-200b to elevate the expression of fascin-1. Previous studies have also reported that SNHG1 serves important roles in the progression of cancer types, including NSCLC (21), glioma (9), pancreatic cancer (22), cholangiocarcinoma (23) and osteosarcoma (24).

Tumor progression is associated with the expression or modulation of several gene products that control apoptosis and proliferation. Apoptosis is an important negative growth regulatory mechanism in tumors (25). In some malignancies, the apoptotic index may reflect the degree of carcinogenicity (26). Bcl-2 is a potent inhibitor of apoptosis and increases proliferation (27). Furthermore, the Bcl-2/Bax ratio is the critical determinant for the induction or inhibition of apoptosis (28). PCNA is present in nuclei throughout the cell cycle and is synthesized in the late G₁ and S phases (29). Cell migration is a process that is essential during embryonic development, throughout adult life and in some pathological conditions (30). Cadherins, specifically the neural cell adhesion molecule N-cadherin, play an important role in migration (31). In cancer, cadherins control the balance between suppression and promotion of invasion (32). For example, E-cadherin functions as an invasion suppressor and is downregulated in most carcinomas, while N-cadherin, as an invasion promoter, is frequently upregulated (33). The present results suggested that lncRNA SNHG1 was significantly upregulated in bladder cancer tissues and cells. Moreover, shRNA-mediated SNHG1 downregulation impaired cell proliferation, migration and invasion, but facilitated cell apoptosis; however, SNHG1 overexpression led to opposite results.

The PI3K/AKT pathway has been reported to exert important roles in regulating cell cycle and the proliferative, antiapoptotic, metastatic and invasive abilities of cancer cells. For example, the overexpression of lncRNA AB073614 significantly improved the proliferation, migration and invasion of colorectal cancer cells, and decreased the rates of apoptosis and G₁ phase cell cycle arrest by targeting the PI3K/AKT signaling pathway (34). It has also been revealed that miR-802 expression inhibited NSCLC tumor growth by deactivating the PI3K/AKT/mTOR pathway by targeting fibroblast growth factor receptor 1 (35). In addition, Zhang *et al* (36) demonstrated that laminin subunit β -3 promoted pancreatic ductal adenocarcinoma (PDAC) cell cycle progression, proliferation, invasion and migration, as well as inhibited apoptosis by upregulating the PI3K/AKT

signaling pathway. A previous study reported also that SNHG1 acted as an oncogenic lncRNA, and promoted tumorigenesis in PDAC via the PI3K/AKT signaling pathway (12). The present study investigated whether SNHG1 modulated the PI3K/AKT pathway in bladder cancer cells. It was demonstrated that SNHG1 silencing reduced the phosphorylation levels of PI3K and AKT, whilst SNHG1 overexpression induced the activation of the PI3K/AKT axis. The PI3K activator 740Y-P and the inhibitor LY294002 reversed the previous effects of SNHG1 knockdown in SW780 cells and SNHG1 overexpression in RT4 cells, respectively. Based on these findings, it was speculated that SNHG1 may promote the tumorigenic process, at least partly via activating the PI3K/AKT pathway in bladder cancer.

However, several limitations should be considered when interpreting the present results. For instance, the number of patients was limited and *in vivo* experiments were not performed. Furthermore, the other molecular mechanisms that may be involved require further investigation.

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Availability of data and materials

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

Authors' contributions

QD conducted the majority of the experiments, wrote the manuscript and analyzed the data. JC designed the study and revised the manuscript. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures were approved by the Human Ethics Committee of Shaanxi Provincial People's Hospital (Xi'an, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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