

Effects of targeted *Notch1* silencing on the biological processes of the T24 and 5637 cells *in vitro*

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Abstract. The present study aimed to investigate the roles of *Notch1* in the biological processes of bladder cancer cells (BCCs) *in vitro*. Short hairpin (sh)RNA targeting *Notch1* was designed and constructed, and the T24 and 5637 BCCs were selected for transfection. The cells were classified into two groups: shRNA negative control (NC) and *Notch1* shRNA. MTT and Transwell assays, and flow cytometry were performed to examine the changes in cell proliferation, invasiveness, and apoptosis, respectively. In addition, reverse transcription-quantitative PCR and western blot analysis was used to detect the mRNA and protein expression levels of apoptosis-related proteins (Bax, Bid and Bcl2) and epithelial-mesenchymal transition factors (vimentin and E- and N-cadherin). Compared with that in the shRNA NC group, the *Notch1* shRNA group showed significantly decreased cell proliferation rate and invasiveness; increased apoptotic rate; elevated mRNA expression levels of Bax, Bid and E-cadherin; and reduced mRNA expression levels of Bcl2, N-cadherin and vimentin. The trends for protein expression levels were the same as those for mRNA levels. *Notch1* silencing inhibited invasion and promoted apoptosis of BCCs.

Introduction

Bladder cancer is a prevalent urinary system malignancy, that seriously threatens human health and life. According to the study ~430,000 new cases of bladder cancer were reported worldwide in 2012, of which 165,000 were fatal (1). Bladder cancer is divided into non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC) (2). In total, ~75% of patients with bladder cancer are diagnosed with NMIBC (3). Transurethral resection of bladder tumors is the major form of treatment for NMIBC. Regular intravesical chemotherapy is administered following surgery to prevent recurrence (4). However, ~50-70% of patients with NMIBC suffer from disease recurrences, and 30-40% of these patients suffer from disease progression (3,5,6). Data obtained from studies published between 2006 and 2011 showed the 4-year survival rate of patients with MIBC after progression from high-risk NMIBC was 35% (7). Pelvic lymph node dissection is not only a treatment method, but also provides important information on prognosis. Studies have shown that the risk of lymphatic metastasis in muscular invasive bladder cancer was over 24% and was associated with the depth of tumor invasion (pT2a 9-18%, pT2b 22-41%, pT3 41-50%, pT4 41-63%). Therefore, pelvic lymph node dissection is an important part of radical cystectomy. Due to the high incidence and recurrence rates, it is necessary to investigate the relevant genetic pathways underlying the genesis and development of bladder cancer.

Notch1 is involved in the proliferation and differentiation of various cells, such as human dental pulp stem cells (8,9) and neural stem cells (8). Studies have demonstrated that *Notch1* expression in bladder cancer cells (BCCs) was associated with their malignancy (10,11). *Notch1* silencing, using shRNA, could inhibit the proliferation of 5637 BCCs (12). However, the specific mechanism of action of *Notch1* in BCCs remains unclear.

Therefore, in the present study, *Notch1* was silenced in human T24 and 5637 BCCs to observe its effects on the invasion potential, apoptosis, and epithelial-mesenchymal transition (EMT) of these cells.

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Materials and methods

Experimental materials. Human T24 and 5637 BCCs were purchased from Procell Life Science & Technology Co., Ltd. The BCA protein assay kit was purchased from Beyotime Institute of Biotechnology, while the PLVX-shRNA2 vector was purchased from Addgene, Inc. TRIzol[®] reagent and restriction endonucleases (*Exo*RI and *Bam*HI) were purchased from Thermo Fisher Scientific, Inc. Reverse transcription (RT) and SYBR-Green PCR kits were purchased from Takara Biotechnology, Co., Ltd. Rabbit monoclonal antibodies against E-cadherin, N-cadherin, vimentin, and Bcl2 were purchased from Cell Signaling Technology, Inc. Rabbit polyclonal antibodies against Bad and Bid were purchased from ProteinTech Group, Inc. and Abcam, respectively. The Transwell chambers were purchased from BD Biosciences, while the apoptosis detection and cell cycle detection kits were purchased from Nanjing KeyGen Biotech Co., Ltd.

Cell culture. The T24 and 5637 cell lines were removed from liquid nitrogen and thawed in a water bath at 37°C for up to 1 min. The cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/l) and 10% FBS, and maintained at 37°C in a humidified incubator with 5% CO₂. The cells were passaged when they reached ~80% confluence.

Plasmid construction and transfection. The Notch1 scrambled negative control short hairpin (sh) RNA (shRNA NC) and Notch1 interference (Notch1 shRNA) sequences used were biosynthesized by Shanghai GenePharma Co., Ltd., and designed according to GeneID, 4851 (Table I). After annealing to form double strands, the shRNA NC or Notch1 shRNA sequences were cloned into the PLVX-shRNA2 vector pre-digested with *Exo*RI and *Bam*HI. After being transfected into competent *Escherichia coli* DH5 α cells, the plasmids were extracted from one single colony and Sanger sequencing was performed to verify the presence of the cloned plasmids. For shRNA (2 μ g) and PLVX vector transfection, the T24 and 5637 cell lines during the logarithmic growth stage were seeded into 24-well plates to adjust the cell density to 4x10⁵/well, which corresponded to 70-80% confluency. In accordance with the manufacturer's instructions, Lipofectamine[®] 2000 transfection (Invitrogen; Thermo Fisher Scientific, Inc.) reagent was used to transfect the plasmids into the T24 and 5637 cell lines. After 8 h of transfection, at 37°C with 5% CO₂, the RPMI 1640-medium containing 1% ampicillin, used upon transfection (30 μ g/ml) was replaced with normal medium. The cells were cultured in a constant-temperature incubator at 37°C with 5% CO₂. After 48 h, the cells were collected for subsequent experiments. Notch1 mRNA expression levels in the T24 and 5637 transfected cells were detected using RT-quantitative PCR.

MTT assay. The cells in the logarithmic growth phase were cultured overnight in an incubator at 37°C with 5% CO₂. Then, 100 μ l/well cell suspension (5x10⁴ cells/ml) were seeded in a 96-well plate and cultured at 37°C with 5% CO₂ for 4 h. After incubation with MTT (Nanjing SenBeiJia Biological Technology Co., Ltd.), 0.15 ml DMSO (Beijing Solarbio Science and Technology Co., Ltd.) was added and the suspension was shaken for 10 min. Optical density (OD) at 568 nm

was measured using a microplate reader. The experiments were performed independently 3 times.

Transwell assay. After 48 h of successful transfection, the cells in each group were digested with 0.25% trypsin, collected and centrifuged at 300 x g, at 4°C. The cells were then washed twice with pre-cooled PBS. Cells were suspended in a serum-free RPMI-1640 medium and counted using the plate count method, by visually observing the number of cells. Next, 0.8 ml medium with 10% FBS was transferred into a 24-well plate, which was then placed in a Transwell chamber. Then, 1 mg/ml Matrigel (100 μ l) was added vertically to the bottom of the upper Transwell chamber. After the Matrigel solidified, 200 μ l cell suspension (1x10⁷) was added to the upper Transwell chamber and cultured at 37°C in a humidified incubator with 5% CO₂ for 24 h. The Transwell was then removed, the chamber was washed with PBS and the cells were fixed in 10% methanol for 30 min at 4°C. Then, the membrane was removed and the cells were stained with crystal violet (0.5%) at room temperature for 20 min, followed by a wash with PBS. Images were acquired and the cell numbers calculated using a counting slide, under a light microscope (magnification, x20; Olympus Corporation). The experiments were performed independently 3 times.

Flow cytometry for apoptosis. After 2 days in culture, the cells were subjected to trypsin digestion (0.25%) and then collected in a flow cytometry tube. A total of ~1x10⁵ suspended cells were centrifuged at 300 x g, at 4°C. Detection was conducted following the instructions of the Annexin V-APC/7-AAD detection kit. The cells were resuspended in binding buffer (0.05 ml; 5x10⁵/ml), followed by the addition of the 7-AAD solution (5 μ l), and incubated for 15 min at room temperature. Finally, 0.45 ml binding buffer and 1 μ l Annexin V-APC were added at room temperature in the dark for 15 min. The cell cycle distribution was evaluated using a FACSVerse[™] flow cytometer (Beckman Coulter, Inc.) and the data were analyzed using FlowJo v10 software (FlowJo LLC). The experiments were performed independently 3 times.

Cell cycle analysis. The cells were cultured for 48 h following transfection, then digested with 0.25% non-EDTA trypsin, followed by centrifugation at 300 x g, at room temperature for 5 min to harvest the cells. The cells were then resuspended in 100 ml PBS, and fixed by slowly adding 700 ml pre-cooled 80% ethanol to reach a final concentration of 70%, following incubation at 4°C for 4 h before centrifugation at 300 x g for 5 min, at 4°C. Then, RNase (100 ml; 50 μ g/ml) was added, and the cells were placed in a water bath at 37°C for 30 min to permeabilize the cells. Finally, PI solution (400 μ l; 50 μ g/ml) was added, and the cells were stained at 4°C for 30 min in the dark. The cell cycle distribution was evaluated using FACSVerse[™] flow cytometer (Beckman Coulter, Inc.) and the data were analyzed using FlowJo v10 software (FlowJo LLC). The experiments were performed independently 3 times.

RT-qPCR. After the T24 and 5637 cells were transfected and cultured for 48 h, total RNA was isolated using TRIzol[®] following the manufacturer's instructions. RT was performed to synthesize cDNA using a Prime Script RT kit (Takara, cat.no.RR037A; Takara Bio, Inc.) at 65°C for 10 min [RNA, oligo(dT), random primer and

Table I. shRNA sequence list.

Name	Orientation	Sequence (5'-3')
Notch1-shRNA	Forward	GATCCCCAACATCCAGGACAACATTTCAAGAGAAT GTTGTCTCGGATGTTGGTTTTTCTCGAGG
	Reverse	AATTCCTCGAGAAAAAACCAACATCCAGGACAA CATTCTCTTGAAATGTTGTCTCGGATGTTGGG
shRNA-NC	Forward	GATCCTTCTCCGAACGTGTACAGTTTCAAGAGAACGT GACACGTCTCGAGAAATTTTTCTCGAGG
	Reverse	AATTCCTCGAGAAAAAATTCTCCGAACGTGT CACGTTCTCTTGAAACGTGACACGTCTCGAGAAAG

shRNA, short hairpin RNA; NC, negative control.

Table II. Primer sequences for E-cadherin, N-cadherin, vimentin, Bcl-2, Bad, Bid and GAPDH.

Name	Primer	Sequence	Size, bp
E-cadherin	Forward	5'-CGTAGCAGTGACGAATGTGG-3'	175
	Reverse	5'-CTGGGCAGTGTAGGATGTGA-3'	
N-cadherin	Forward	5'-CTTGCCAGAAAACCTCCAGGG-3'	213
	Reverse	5'-TGTGCCCTCAAATGAAACCG-3'	
Vimentin	Forward	5'-CGCCAACTACATCGACAAGG-3'	166
	Reverse	5'-GGCTTTGTCTGTTGGTTAGCT-3'	
Bcl-2	Forward	5'-GCCTTCTTTGAGTTCGGTGG-3'	192
	Reverse	5'-GAAATCAAACAGAGGCCGCA-3'	
Bad	Forward	5'-GGGACGGAGGACGACG-3'	272
	Reverse	5'-CACTGGGAGGGGGCGG-3'	
Bid	Forward	5'-GGGAAGAATAGAGGCAGA-3'	304
	Reverse	5'-GACATCACGGAGCAAGGA-3'	
GAPDH	Forward	5'-TCAAGAAGGTGGTGAAGCAGG-3'	115
	Reverse	5'-TCAAAGGTGGAGGAGTGGGT-3'	

water], then on ice for 5 min. The samples were then incubated at 25°C for 10 min, with the addition of dNTPs, RNaseA, buffer, MTV reverse transcriptase. Next, the samples were incubated at 37°C for 60 min, then heated to 70°C for 10 min. Subsequently, qPCR was subsequently performed using the SYBR-Green PCR kit, according to the manufacturer's instructions (cat. no. RR430S; Takara Bio, Inc.), to analyze the mRNA expression levels of vimentin, Bcl2, Bad, Bid, and E- and N-cadherin (primer sequences are listed in Table II). The following thermocycling conditions were used for the qPCR: Initial denaturation at 95°C for 30 sec, then 40 cycles of 95°C for 30 sec and 60°C for 30 sec. The 2^{-ΔΔC_q} method was used to measure the relative mRNA expression levels (13); GAPDH was used as the internal reference gene. The experiments were performed independently 3 times.

Western blot analysis. The T24 or 5637 cells were lysed with lysis buffer (50 mM Tris-HCl, 0.5 mM EDTA, 1% SDS and 1 mM DTT) on ice. The concentration of the extracted proteins from the cells were determined using a BCA assay kit (cat. no. DEM183-1000T; Beijing BioDee Biotechnology Co., Ltd.).

A total of 20 μg protein was mixed with 4 μl 2X SDS sample buffer. After denaturation, the samples were separated using 10% SDS-PAGE, transferred to PVDF membranes and blocked with 5% skimmed milk. Then, the membranes were incubated with the following primary antibodies: Bad (cat. no. ab32445; 1:1,000), Bid (cat. no. ab32060; 1:1,000), vimentin (cat. no. ab92547; 1:1,000), E-cadherin (cat. no. ab40772; 1:1,000), N-cadherin (cat. no. ab98952; 1:1,000), Bcl2 (cat. no. ab182858; 1:1,000) and GAPDH (cat. no. ab8245; 1:1,000) (all from Abcam) overnight at 4°C. The membranes were then washed with PBS and horseradish peroxidase-labeled sheep anti-rabbit IgG secondary antibody (1:50,000) was added and incubated at room temperature for 60 min. Enhanced chemiluminescence assay (cat. no. WBKLS0500; EMD Millipore) was used to detect the densitometry of the target proteins, then the OD was measured using ImageJ software (v1.8.0.112; National Institutes of Health). The experiments were performed independently 3 times.

Statistical analysis. Statistical analyses were performed using SPSS (version 22.0; IBM Corp.). Data were presented

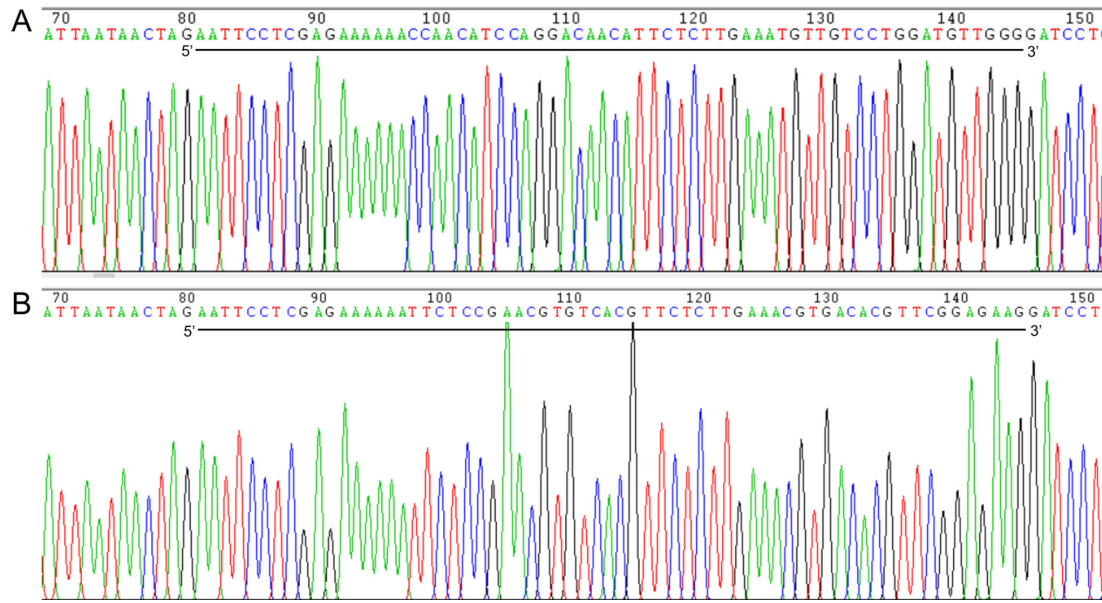


Figure 1. Sequencing and identification of *Notch1* (A) shRNA and (B) NC vector plasmid. The underlined bases correspond to the inserted *Notch1* shRNA or the NC sequence, and the non-underlined nucleotides correspond to the PLVX-shRNA2 vector backbone.

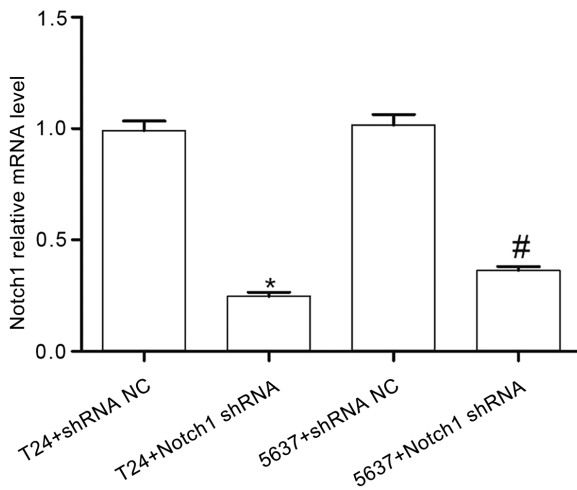


Figure 2. Changes in *Notch1* mRNA expression following transfection with shRNA were detected using reverse transcription-quantitative PCR. * $P < 0.05$ vs. T24+shRNA NC; # $P < 0.05$ vs. 5637+shRNA NC. shRNA, short hairpin RNA; NC, negative control.

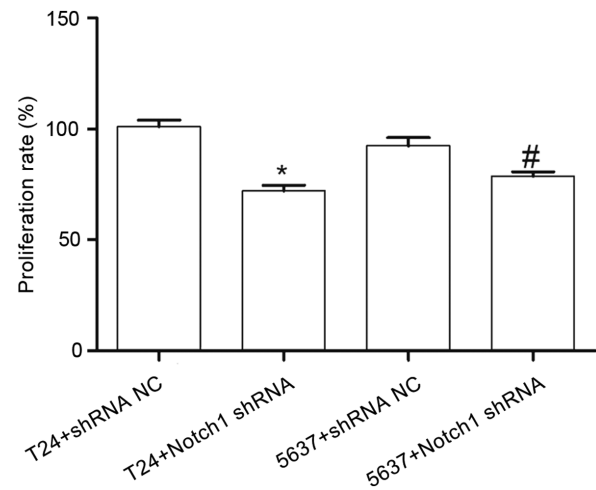


Figure 3. Changes in cell proliferation rate in the T24 and 5637 bladder cancer cells following transfection with *Notch1* shRNA were detected using a MTT assay. * $P < 0.05$ vs. T24+shRNA NC; # $P < 0.05$ vs. 5637+shRNA NC. shRNA, short hairpin RNA; NC, negative control.

as mean \pm SD, and independent t-test was used to investigate intergroup distinctions. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Target gene *Notch1* was successfully constructed. As shown in Fig. 1, the constructed *Notch1* shRNA vector was sequenced, and the inserted sequence and locus were correct. The interfering vector plasmid of the target gene *Notch1* was successfully constructed.

***Notch1* mRNA expression level in BCCs following *Notch1* shRNA.** To investigate the role of *Notch1* in the BCCs, plasmid transfection was used to suppress *Notch1* expression in the T24 and 5637 cell lines. *Notch1* expression was detected

using RT-qPCR. Compared with that in the T24+shRNA NC group, the expression level of *Notch1* in the T24+*Notch1* shRNA group was significantly decreased ($t = 16.310$; $P < 0.001$). Compared with that in the 5637+shRNA NC group, the expression level of *Notch1* in the 5637+*Notch1* shRNA group was also significantly decreased ($t = 13.28$; $P < 0.001$) (Fig. 2). These results indicated that the *Notch1* gene was successfully silenced and inhibited the expression of *Notch1* mRNA.

Effect of *Notch1* shRNA on the proliferation of the T24 and 5637 cell lines. Compared with that in the T24+shRNA NC group, the cell proliferation rate in the T24+*Notch1* shRNA group was significantly decreased ($t = 7.583$; $P < 0.05$). Similarly, compared with that in the 5637+shRNA NC group, the cell proliferation rate in the 5637+*Notch1* shRNA group was also significantly decreased ($t = 3.283$; $P < 0.05$) (Fig. 3). These results

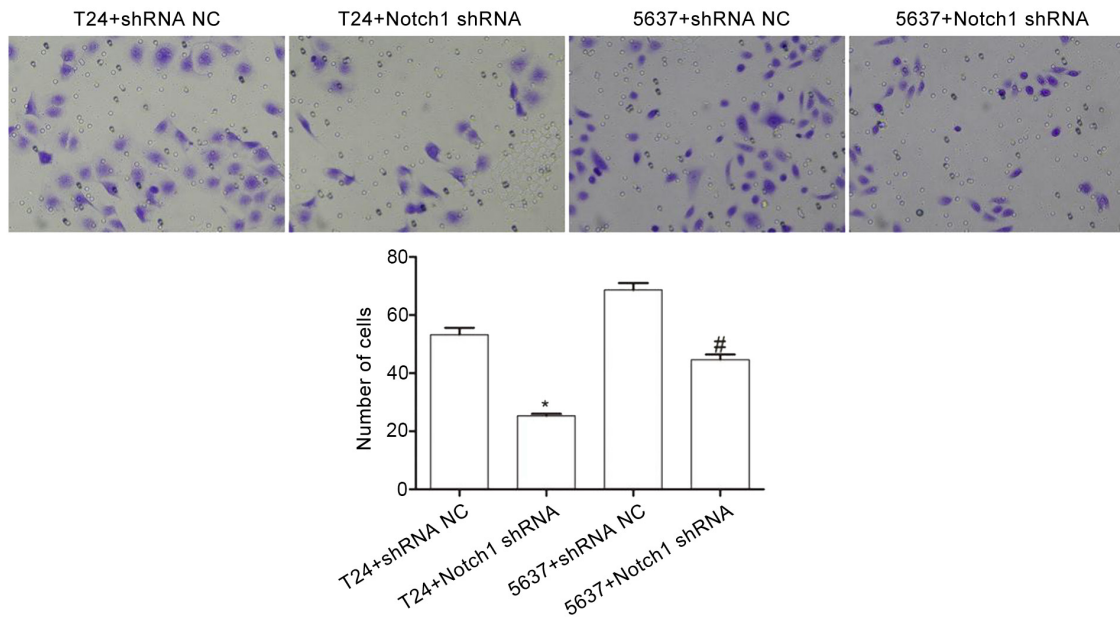


Figure 4. Changes in the number of invasive cells in the T24 and 5637 bladder cancer cell lines following transfection with *Notch1* shRNA were detected using a Transwell assay. Scale, 200 μ m. * P <0.05 vs. T24+shRNA NC; # P <0.05 vs. 5637+shRNA NC. shRNA, short hairpin RNA; NC, negative control.

indicated that by silencing the *Notch1* gene, the proliferation of the T24 and 5637 cells was decreased.

Effect of *Notch1* shRNA on the invasive ability of the T24 and 5637 cell lines. Compared with that in the T24+shRNA NC group, the number of invasive cells in the T24+Notch1 shRNA group was significantly decreased ($t=11.538$; $P<0.001$). Similarly, compared with that in the 5637+shRNA NC group, the number of invasive cells in the 5637+Notch1 shRNA group was significantly decreased ($t=8.205$; $P<0.05$) (Fig. 4). These results indicated that by silencing the *Notch1* gene, the invasive ability of the T24 and 5637 cells was decreased.

Effect of *Notch1* shRNA on apoptosis and the cell cycle in the T24 and 5637 cell lines. To investigate the effect of Notch1 on BCC apoptosis, flow cytometry was used to detect T24 and 5637 cell apoptosis upon Notch1 silencing. Compared with that in the T24+shRNA NC group, the apoptosis rate in the T24+Notch1 shRNA group was significantly increased ($t=-34.083$; $P<0.001$). Compared with that in the 5637+shRNA NC group, the apoptosis rate in the 5637+Notch1 shRNA group was also significantly increased ($t=-14.316$; $P<0.001$) (Fig. 5A). These results indicated that by silencing the *Notch1* gene, the rate of apoptosis of the T24 and 5637 cells was increased.

Flow cytometry was also used to investigate the cell cycle. The results revealed that compared with that in the T24+shRNA NC group, the proportion of cells in the G_1 phase was significantly increased ($t=-6.929$; $P<0.05$), but the proportion of cells in the G_2 phase was significantly decreased ($t=5.631$; $P<0.05$) in the T24+Notch1 shRNA group; no obvious differences were noted in the proportion of cells in S phase ($t=0.264$; $P=0.805$). In addition, compared with that in the 5637+shRNA NC group, the proportion of cells in the G_1 phase was significantly increased ($t=-21.302$; $P<0.001$), but the proportion of cells in S phase was significantly decreased ($t=23.462$; $P<0.001$) in the 5637+Notch1 shRNA group; no

obvious differences were noted in the proportion of cells in the G_2 phase ($t=1.072$; $P=0.344$) (Fig. 5B). These results indicated that by silencing the *Notch1* gene, the proportion of the T24 and 5637 cells in the G_1 phase was increased.

Effect of *Notch1* on the mRNA expression levels of *N-cadherin*, *vimentin*, *Bcl2*, *E-cadherin*, *Bad* and *Bid* in the BCCs. Compared with that in the T24+shRNA NC, the mRNA expression level of *E-cadherin* ($t=-8.804$; $P<0.001$), *Bad* ($t=-11.07$; $P<0.001$), and *Bid* ($t=-10.718$; $P<0.001$) was significantly increased, but the mRNA expression level of *N-cadherin* ($t=11.725$; $P<0.001$), *vimentin* ($t=12.597$; $P<0.001$), and *Bcl2* ($t=10.627$; $P<0.001$) was significantly decreased in the T24+Notch1 shRNA group (Fig. 6).

Compared with that in the 5637+shRNA NC, the mRNA expression level of *E-cadherin* ($t=-13.689$; $P<0.001$), *Bad* ($t=-23.999$; $P<0.001$), and *Bid* ($t=-12.805$; $P<0.001$) was significantly increased, but the mRNA expression of *N-cadherin* ($t=0.105$; $P<0.001$), *vimentin* ($t=9.649$; $P<0.001$), and *Bcl2* ($t=7.966$; $P<0.001$) was significantly decreased in the 5637+Notch1 shRNA group (Fig. 6). These results indicated that by silencing the *Notch1* gene, the mRNA expression levels of *N-cadherin*, *vimentin* and *Bcl2* were decreased, while *E-cadherin*, *Bad* and *Bid* were increased in the T24 and 5637 cells.

Effect of *Notch1* on the protein expression levels of *vimentin*, *Bcl2*, *N-cadherin*, *Bad*, *Bid* and *E-cadherin* in the BCCs. The protein expression levels of apoptosis-related proteins and EMT factors in the T24 and 5637 cells were examined. Compared with that in the NC groups, the protein expression levels of *E-cadherin* (T24 cells, $t=-6.39$; $P<0.05$; and 5637 cells, $t=-4.95$; $P<0.05$), *Bad* (T24 cells, $t=-11.372$; $P<0.001$; and 5637 cells, $t=-6.988$; $P<0.05$), and *Bid* (T24 cells, $t=-13.85$; $P<0.001$; and 5637 cells, $t=-15.383$; $P<0.001$) were significantly increased, but the protein expression levels of *N-cadherin* (T24 cells, $t=9.957$; $P<0.05$; and 5637 cells, $t=11.624$; $P<0.001$), *vimentin*

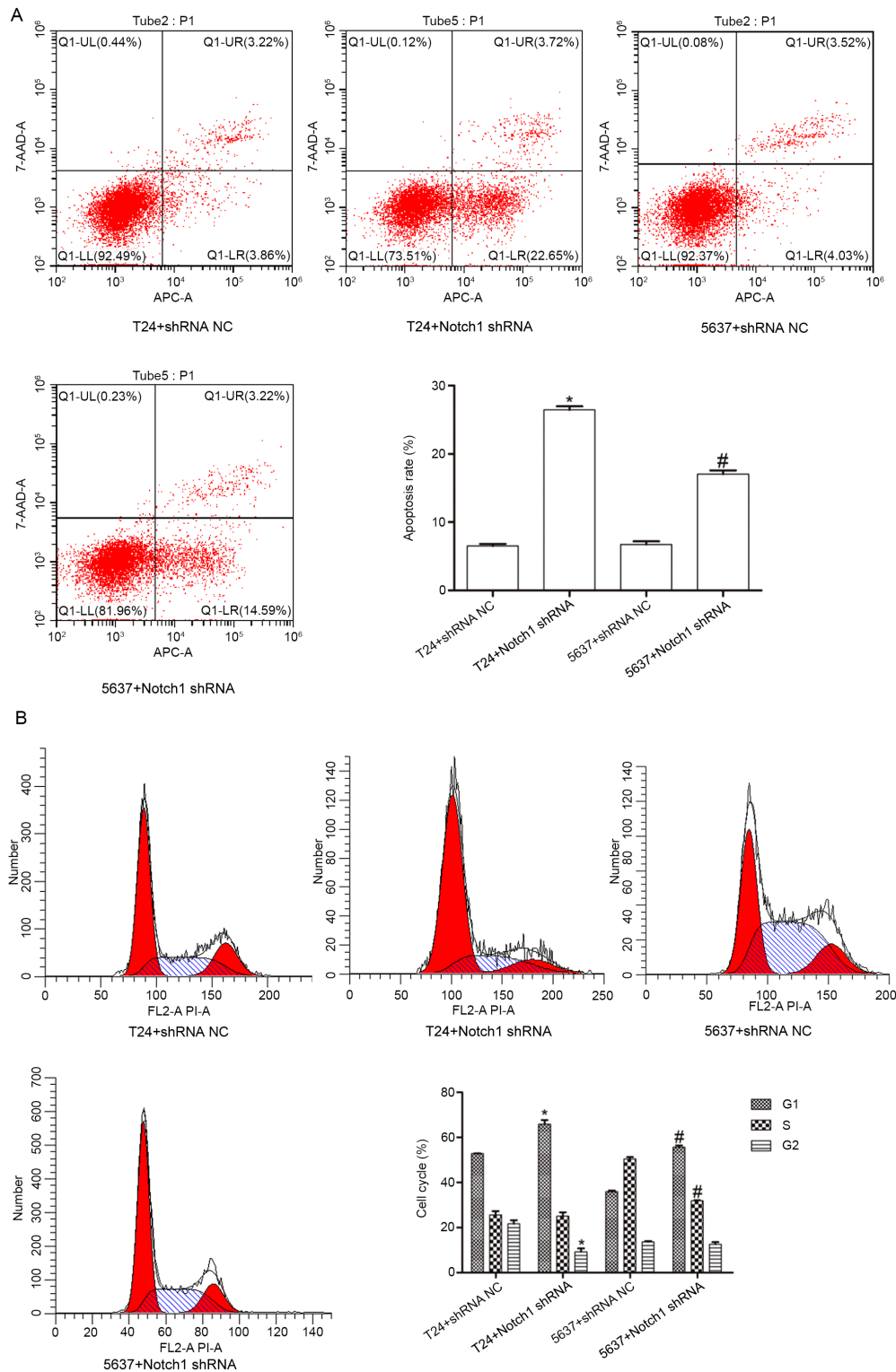


Figure 5. Changes in the apoptotic rate and cell cycle in the BCCs following transfection with *Notch1* shRNA were detected using flow cytometry. (A) Apoptotic rate of the BCCs in each group. (B) Changes in the cell cycle of the BCCs in each group. * $P < 0.05$ vs. T24+shRNA NC; # $P < 0.05$ vs. 5637+shRNA NC. BCCs, bladder cancer cells; shRNA, short hairpin RNA; NC, negative control.

(T24 cells, $t=6.502$; $P < 0.05$; and 5637 cells, $t=4.681$; $P < 0.05$), and Bcl2 (T24 cells, $t=12.576$; $P < 0.001$; and 5637 cells, $t=8.061$; $P < 0.05$) were significantly decreased in the *Notch1* shRNA group (Fig. 7). These results indicated that by silencing the *Notch1* gene, the protein expression levels of N-cadherin, vimentin, and Bcl2 were decreased, while E-cadherin, Bad and Bid were increased in the T24 and 5637 cells.

Discussion

Previous studies have confirmed that the Notch signaling pathway and EMT play key roles in the occurrence, development, and metastasis of human tumors, such as lung, breast, prostate and colorectal cancers (14,15). However, in bladder cancer, the association between the Notch signaling pathway

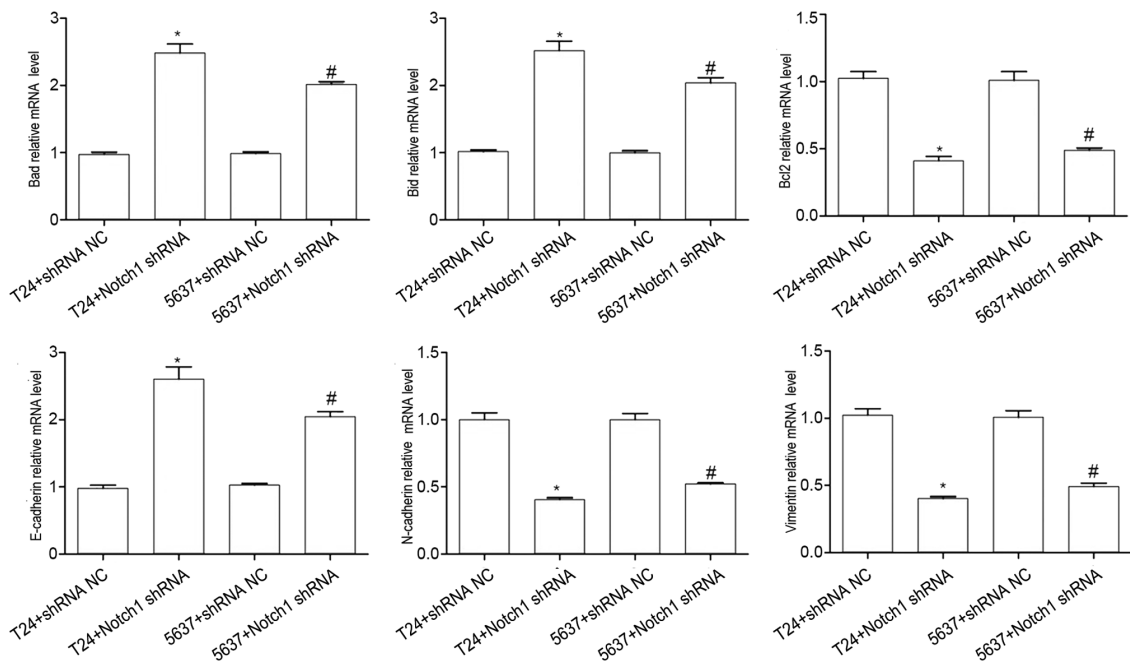


Figure 6. Changes in vimentin, Bcl2, Bad, Bid, and E- and N-cadherin mRNA expression levels in the T24 and 5637 bladder cancer cells following transfection with *Notch1* shRNA were detected using reverse transcription-quantitative PCR. *P<0.05 vs. T24+shRNA NC; #P<0.05 vs. 5637+shRNA NC. shRNA, short hairpin RNA; NC, negative control.

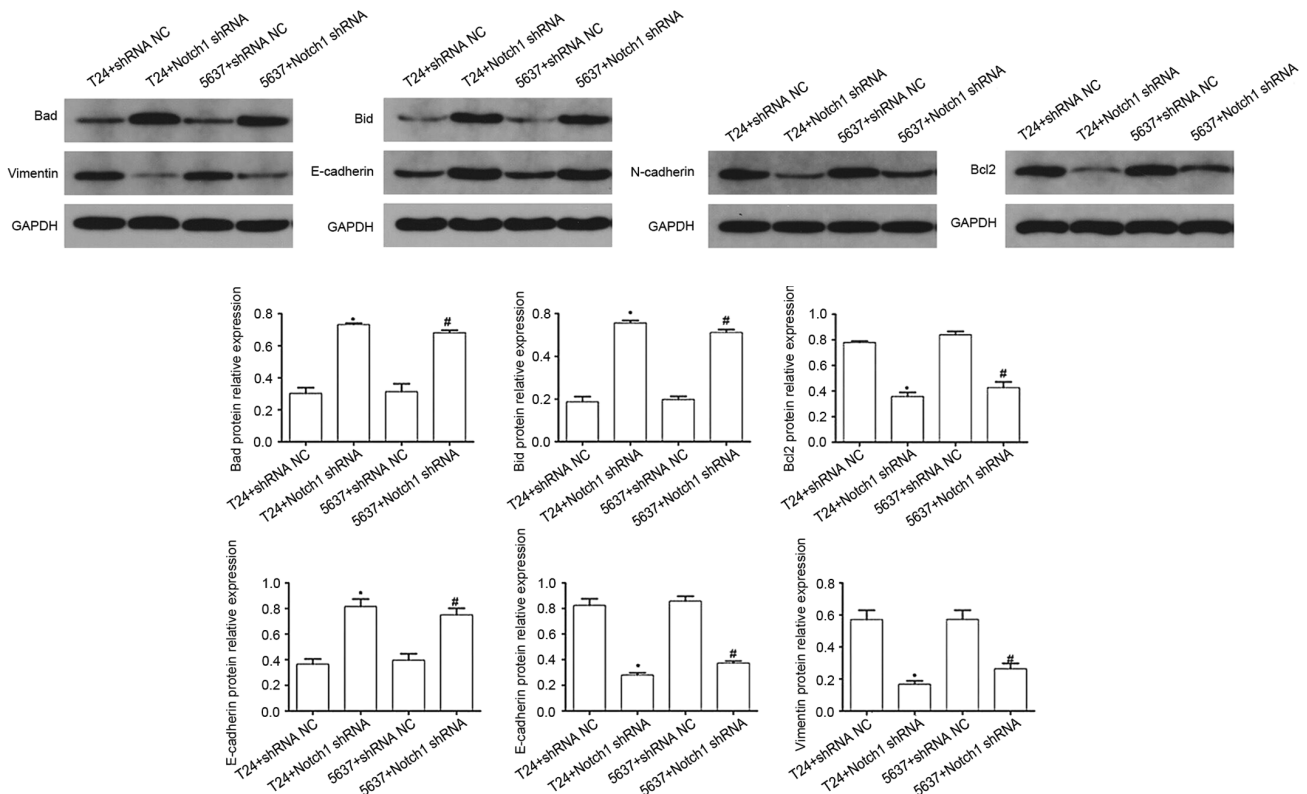


Figure 7. Changes in vimentin, Bcl2, Bad, Bid, E- and N-cadherin protein expression levels in T24 and 5637 bladder cancer cells following transfection with *Notch1* shRNA were detected using western blot analysis. *P<0.05 vs. T24+shRNA NC; #P<0.05 vs. 5637+shRNA NC. shRNA, short hairpin RNA; NC, negative control.

and EMT has not yet been fully elucidated. The Notch signaling pathway is abundant in mammals and is involved in the regulation of numerous life processes, including the maintenance of the dynamic balance of cell differentiation, proliferation,

and apoptosis (16,17). The Notch signaling pathway comprises Notch receptors and ligands, as well as intracellular effector molecules (18). There are four Notch receptors, including Notch1, Notch2, Notch3 and Notch4; the Notch ligands

include protein jagged (JAG)1, JAG2, δ -like protein (DLL)1, DLL3 and DLL4 (17). An increase in the signaling levels in the Notch signaling pathway may induce the development of a variety of tumors, such as lung and breast cancers, and increase Notch1 mRNA expression level is the most frequently observed abnormality in tumor tissues, such as lung and breast (19-23). Notch1 has also been found to participate in the occurrence and development of diverse tumors, such as lung, breast, prostate and colorectal cancers by affecting cell proliferation, differentiation and apoptosis (19-23). *Notch1* shRNA silencing could inhibit cell proliferation and invasion of the SGC-7901 gastric cancer cell line (19). Notch1 suppression, by genetic interference, could also inhibit the proliferation and invasion of breast cancer cells (20). Goriki *et al* (12) reported that increased Notch1 protein expression level in bladder cancer was associated with an increased pathological grade.

In the present study, the proliferation and invasion of the BCCs upon Notch1 shRNA was significantly reduced, whereas cell apoptosis was increased. The mRNA and protein expression level of the apoptosis-related proteins, Bad and Bid in the Notch1 shRNA group was significantly increased, whereas the expression level of the anti-apoptotic protein, Bcl2 was significantly decreased, suggesting that inhibition of *Notch1* promoted apoptosis of the BCCs. These findings suggested that suppression of *Notch1* inhibited T24 and 5637 cell proliferation and invasion, and promoted apoptosis.

EMT refers to the ability of epithelial cells to infiltrate and migrate (by losing polarity and loosening the tight junctions between cells), which is stimulated by certain factors, such as inflammation (24). Under physiological conditions, EMT of epithelial cells can repair injured normal tissues (25). In addition, EMT assumes a critical role in tumor occurrence and progression. EMT is a starting point and an important event in metastasis cascade reactions, where epithelial cancer cells lose polarity and cell-cell contact, and therefore the migratory ability of cancer cells is enhanced (26). The E-cadherin, N-cadherin, and vimentin proteins are related to EMT. A decrease in E-cadherin expression has been considered to be the most significant feature of EMT (27). E-cadherin maintains the tight junctions between epithelial cells and plays an important role in maintaining the morphological stability of cells (28). Increased vimentin and N-cadherin expression ensures that cancer cells acquire mesenchymal characteristics, that promote migration and invasion (27). N-cadherin mainly mediates the adhesion between nerve tissue and fibroblasts, while vimentin is widely distributed in interstitial cells (27). Notch1 can induce EMT by mediating the expression of various EMT-related genes, such as Snail1 and Snail2 (29,30). Natsuzaka *et al* (31) reported that Notch1 protein expression levels were increased in NCI-H2023 squamous cell carcinoma (SCC) cell line and was associated with a poor prognosis for esophageal SCC.

To date, there have been relatively few studies on the role of the Notch1 signaling pathway in BCCs. Therefore, to investigate whether Notch1 shRNA plays a role in bladder cancer by inhibiting EMT of BCCs, the mRNA and protein expression level of EMT-related factors were detected using RT-qPCR and western blot analysis in BCCs after *Notch1* silencing with shRNA. E-cadherin expression level was found to be notably augmented at both the mRNA and protein expression level, whereas vimentin and N-cadherin was reduced upon *Notch1* silencing. E-cadherin was associated with BCC metastasis and invasion, whereas

N-cadherin and vimentin proteins can promote cell adhesion and signal transduction (32). This suggests that the inhibition of Notch1 may inhibit the expression level of EMT-relevant proteins, thereby inhibiting BCC invasion and metastasis.

In conclusion, inhibiting *Notch1* expression was found to inhibit the invasion of human BCCs, promote their apoptosis, and inhibit EMT. However, there are still a number experiments required to validate the results. Future studies should focus on the *Notch1* gene and its association with clinical outcomes of patients with bladder cancer.

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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

KWZ, XH, YS, QW, QM and KSZ conceived and designed the project. KWZ and XH acquired the data. KWZ and XH confirm the authenticity of all the raw data. KWZ, XH, YS, QW, QM and KSZ analyzed and interpreted the data. QM and KSZ wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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