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Original Article

miR-451 inhibits invasion and proliferation of bladder cancer by regulating EMT

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Abstract: MicroRNAs (miRNAs) have recently been reported play a crucial role in some tumors. In order to investigate the association of miR-451 with bladder cancer, we investigate the expression of miR-451 in bladder cancer tissues and its role in biological behavior of T24, 5637 and J28 bladder cancer cell lines. Quantitative RT-PCR results showed miR-451 was significantly down-regulated in bladder cancer tissues and paracancerous tissues compared with normal bladder tissues. miR-451 expression was significantly associated with histological differentiation degree and TNM stage. Over-expression of miR-451 was established by transfecting miR-451 mimics into T24, 5637 and J28 cells, and its effects on the biological behavior of bladder cancer were studied using transwell assay, migration assay, adhesion assay, MTT and flow cytometry. Results indicated over-expression of miR-451 significantly inhibited cell proliferation, migration, invasion and induced apoptosis of the bladder cancer cells. Furthermore, we investigated the expression level of EMT related proteins in transfected 5637 cells by western blot. Results shown E-cadherin was up-regulated more significantly than N-cadherin, vimentin and Snail. N-cadherin and vimentin were up-regulated significantly when miR-451 was inhibited in miR-451 inhibitor group, however, no significant changes in mimics group. In conclusion, miR451 should be a tumor-suppressing gene in bladder cancer. miR-451 could maintain the bladder tumor cells in epithelial phenotype, inhibit EMT process, thereby reducing the invasion and migration of tumor cells.

Keywords: miR-451, bladder cancer, EMT

Introduction

Bladder cancer is the 7th most common cancer worldwide, and the 4th most common cancer among males in economically developed countries [1]. Although most of the cases (70-75%) are diagnosed as non-muscle-invasive disease during initial treatment, approximately 70% of them still recur; moreover, approximately 20% of the cases present with muscle invasion associated with a strong propensity toward deadly metastases [2]. Novel approaches to inhibit the invasion of tumors are valuable for the treatment of bladder cancer.

MiRNAs are an abundant class of small non-coding RNAs that modulate gene expression in a posttranscriptional manner through se quence-specific binding to target mRNA [3]. Abnormal expression of miRNAs is linked with various human disorders, especially cancer development and progression [4], where miRNAs can act as oncogenes (e.g., miR-21 [5, 6], miR-638 [7] and miR-200 family [8]) or tumor suppressors (e.g., miR-29b [9], MiR-133a [10], miR-137 [11], miR-145 [12], MiR-146b [13]). MiR451 is one of these miRNAs, which has been reported can inhibit the proliferation of several tumor cells, including cervical cancer [14], gastrointestinal cancer [15], glioblastoma stem cells [16].

Epithelial mesenchymal transition (EMT) is closely related to the transformation and infiltration of tumor cell. Much research on the relationship between miRNAs and tumor development and metastasis is focused on the expression of EMT related proteins. In this

study, we evaluated miR-145 expressions in clinical bladder cancer specimens and cell lines, analyzes the relation between miR451 and bladder carcinoma and its clinical pathological characteristics. Furthermore, we inspect the biological characteristics and the expression of EMT associated protein in transfected cell of miR451 to explore the proliferation and metastasis mechanism of bladder cancer cell.

Materials and methods

Tissue specimens

A total of 40 tissue specimens were obtained from our Hospital. All patients gave informed consent prior to collection of specimens according to institutional guidelines. Tumor specimens, adjacent tissues (2.5 cm away from the tumor edge) and normal tissues were all collected from bladder cancer at the time of radical cystectomy, and snap-frozen in the operating room immediately after surgery. The normal tissues (non-tumor tissues) were confirmed to be those surrounding tumor tissues and free of cancer cells by pathologic examination. The histopathology of the disease was determined by two pathologists according to the criteria of the World Health Organisation. Clinical staging was done according to the Union for International Cancer Control classification.

Cell culture and transfection

Human bladder tumor cell line T24, 5637 and J82 were purchased from China Academia Sinica Cell Repository (Shanghai, China), in which T24 and 5637 cells were cultured in RPMI 1640 (Gibco, USA) supplemented with 10% fetal calf serum (Gibco, USA), J82 cells were maintained in Opti-MEM® I medium (Gibco, USA) with 10% fetal calf serum. All cells were cultured in a humidified incubator at 37°C in 5% CO_2.

Has-miR-451 mimics, negative control RNA duplex (NC) and miR-451 inhibitor were synthesized by Ribo Bio, Guangzhou, China. siRNA duplexes with non-specific sequences were used as siRNA negative control (NC). Transfections were performed using Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer's instructions. The final concentration of small RNAs was 50 nM. Blank control was performed without any transfection.

RNA isolation, reverse transcription and QRT-PCR

To quantify miR-451 expression in tumor tissues, adjacent tissues, normal tissues and different bladder tumor cell line (T24, 5637 and J82), total RNA was extracted respectively using Trizol reagent (Invitrogen) according to the manufacturer's protocol and determined by spectrophotometry and spectrophotometric (NanoDrop 2000, USA). Then RNA was reverse transcribed into cDNA using a PrimeScripts RT reagent Kit with gDNA Eraser (Takara, China). Real time PCR (qPCR) was performed using a SYBR premix Ex TaqTM kit (Takara, China) on the Applied Biosystems 7500 Real-time PCR system (Life Technologies, USA). Each sample was analyzed in triplicate. U6 was used as an endogenous control. All samples were normalized to internal controls and fold changes were calculated through relative quantification ($2^{-\Delta\Delta CT}$).

Scratch wound migration assay

7×10^5 T24, 5637 and J82 cells were seeded in 6-well plates respectively and grown to 60% confluence in complete medium. Then the cells were transfected with 50 nM miR-451 mimic, non-specific control miRNA mimic was used as the control group. Continue cell culture until the plates were covered, vertical scratches were then made using a 200 μl plastic filter tip to create a 'wound' of approximately 200 μm in diameter. The cells were then rinsed with PBS three times and incubated at 37°C. The average distance of migrating cells was determined under an inverted microscope at 0, 6, 12 and 24 h. Migration distance was calculated using image-prolog processing software. Formula: migration distance = total area/height. The experiment was performed in triplicate.

Cell adhesion assay

A 96-well micro plate was coated with 2 μg matrigel per well and left to air-dry. Wash away the excess glue using serum-free medium. Then add transfected cell suspension into 96-well plate (4.0×10^5 cells/well) and incubated for 2 h at 37°C. Removing non-adherent cells by washing with PBS. The adherent cells were then reacted with 5 mg/mL MTT solution medium at 37°C for 4 h. Removing the liquid, add 150 μl DMSO and incubated for 10 min in

concussion incubator. The optical density was measured at 490 nm. Results were expressed as the percentage of total cells assuming that the adhesion of cells in the control was 100%. All experiments were carried out in triplicate, and three independent assays were conducted.

Matrigel invasion assays

Transwell filters (8 μm; Millipore, USA) were coated with 100 μL Matrigel (BD Biosciences, CA) at 1:8 dilutions in serum-free media and incubated overnight. Discard excess solution and added 50 μl serum-free medium to hydrate Matrigel glue. 200 μl transfected cell suspension (2.5×10^5 cells/ml) was added to the upper chamber and 750 μL media with 30% FBS were added to the bottom wells. After 16 h culture, cells were fixed with 3.7% formaldehyde for 2 h, stained with 1% crystal violet. Then counted and photographed under the inverted microscope.

Cell proliferation assay

T24, 5637 and J82 cells were plated in 96-well plates and transfected with miR-451 mimics or non-specific control miRNA mimic as described above. After 48 h transfection, 20 μl MTT (5 g/L) (Ameresco, USA) was added to each well and incubated at 37°C for 4 h. Then cell proliferation was assessed by measuring the absorbance at 490 nm using Multiskan MS (Labsystems, Finland). Five replicate wells were set up in each group and repeated three independent.

Flow cytometry analysis

T24 and 5637 cells were seeded in 6-well plates and incubated overnight till 50-70% confluence. Then transfected with miR-451 mimics or miRNA inhibitor as described above and harvested at 48 h. Cells in each group were trypsinized and resuspended in ice-cold PBS, then the cell density was adjusted to $1\text{-}5\times10^5$ cells/mL with ice-cold PBS. Added 500 μL Binding Buffer and 5 μL Annexin V-FITC. After fully mixed, 5 μL Propidium Iodide (50 mg/ml) was added and the system was kept in dark at room temperature for 5-15 min. Finally, cells were analyzed for cell apoptosis using a Becton Dickinson FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA). The percentage of apoptotic cells was quantified using Cell Quest software, respectively. This experiment was performed in triplicate.

TGF-β inducing analysis

The untransfected cells (T24, 5637, J82) were cultured for 24 h, then added TGF-β1 (10 ng/ml) and cultured with untreated reference samples for another 24 h. After rinsing the cells twice with PBS and fixed with cold acetone/methanol mixture (1:1 vol.%), the cells were observed and photographed under the inverted microscope. The cell line with the most obvious epithelial mesenchymal transition will be selected for the expression analysis of related proteins using western blot.

Western blot assay

48 h after transfection, total protein lysates were extracted using radioim-munoprecipitation assay (RIPA) lysis buffer (Sigma-Aldrich) and quantified with a Bicin Choninic Acid (BCA) protein assay kit (Thermo). Four groups were performed: miR-451 mimics, miR-451 inhibitor, NC and blank control. Proteins from each group were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes (PVDF; Bio-Rad). The membranes were blocked and then probed with antibodies against E-cadherin, vimentin (Cell Signaling Technology, Danvers, MA), N-cadherin, snail (Chemicon, Billerica, USA), and β-actin (Sigma). After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence detection kit (Millipore).

Statistical analysis

All the above experiments were independently repeated three times. The results were given as means ± standard deviations (SDs). All statistical analyses were performed using SPSS 19.0 statistics software. Group comparisons were analyzed with one-way analysis of variance (ANOVA) with $P < 0.05$ as statistically significant difference.

Results

Down-regulation of miR-451 in bladder cancer tissues

To testify the correlation between miR-451 and bladder cancer, the expression level of miR-451 was measured by RT-PCR in bladder can-

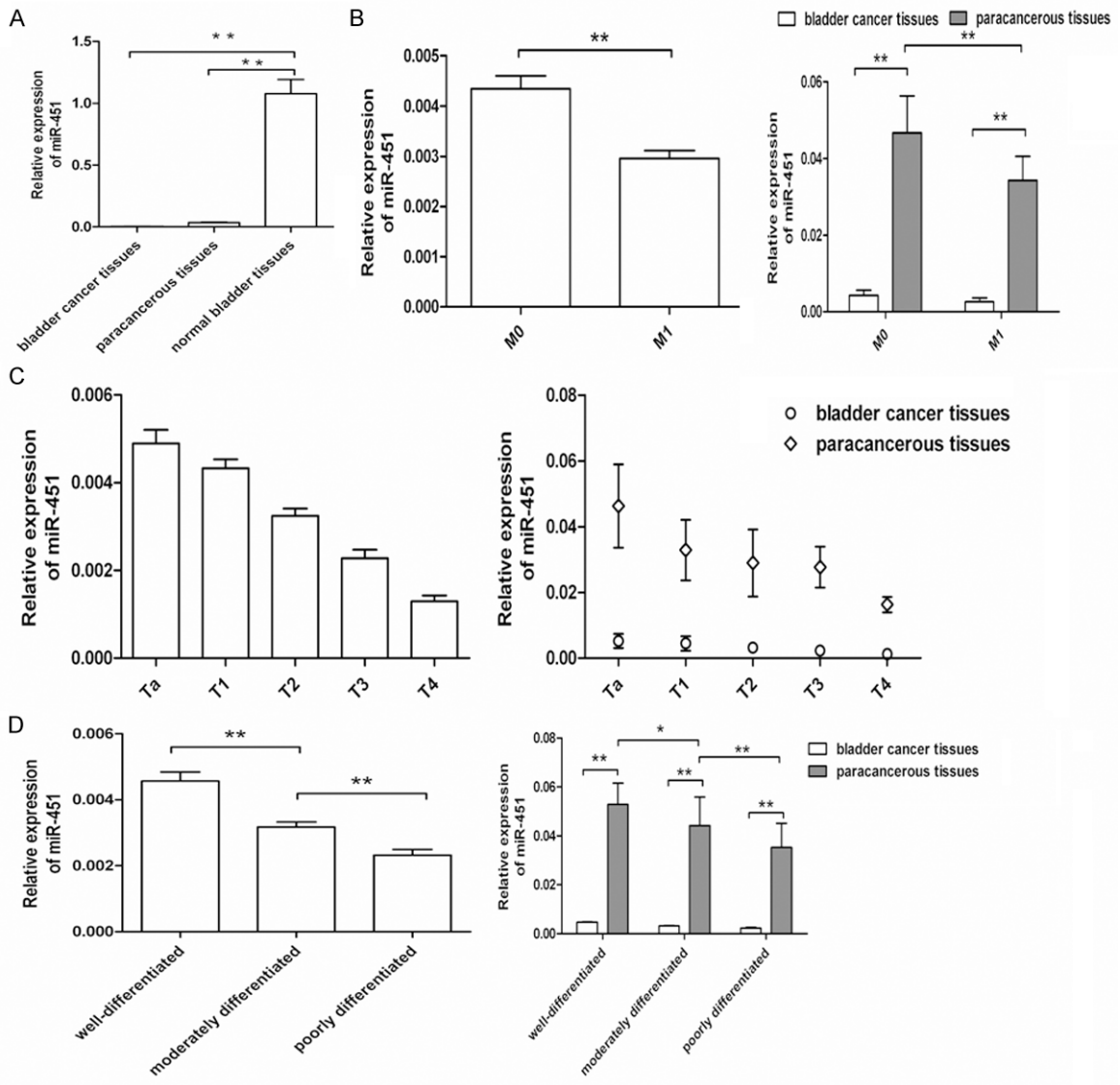


Figure 1. miR-451 expression in bladder cancer tissues and matched adjacent and normal tissues. A. Comparison of miR-451 expression in bladder cancer tissues, paracancerous tissues and normal bladder tissues. B. The expression level of miR-451 in distant migration tumor cells and no distant migration tumor cells. C. The expression level of miR-451 in Ta-T4 group. D. The expression level of miR-451 in bladder cancer tissues and corresponding paracancerous tissues of different differentiation. The term $2^{-\Delta\Delta CT}$ was used to describe the expression level of miR-451. Small nuclear RNA U6 served as internal normalized references for miR-451. X ± SD is represented in the images. miR-451 expression in normal bladder tissues was normalized and calculated as 1. $^{**}P < 0.01$.

cer tissues, paracancerous tissues and normal bladder tissues. We found a significantly higher level of miR-451 expression in the normal samples relative to the levels in the tumor or paracancerous tissues ($P < 0.01$) (**Figure 1A**). Furthermore, analyze the relationship between clinicopathological grading and miR-451 expression, we found that miR-451 expression was down-regulated with the development of bladder cancer (from Ta to T4) ($P < 0.05$) (**Figure 1C**); in no distant migration tumor cells, the expression of miR-451 was significantly higher than that of distant migration tumor cells ($P < 0.01$) (**Figure 1B**); miR-451 expression of high differentiation tumor cells was higher than that of low differentiated tumor cells ($P < 0.01$) (**Figure 1D**). The corresponding paracancerous tissues also conformed to these trends.

Up-regulation of miR-451 in low metastasis potential cell lines

T24, 5637 and J82 cell lines are bladder cancer cells, which were used commonly in bladder

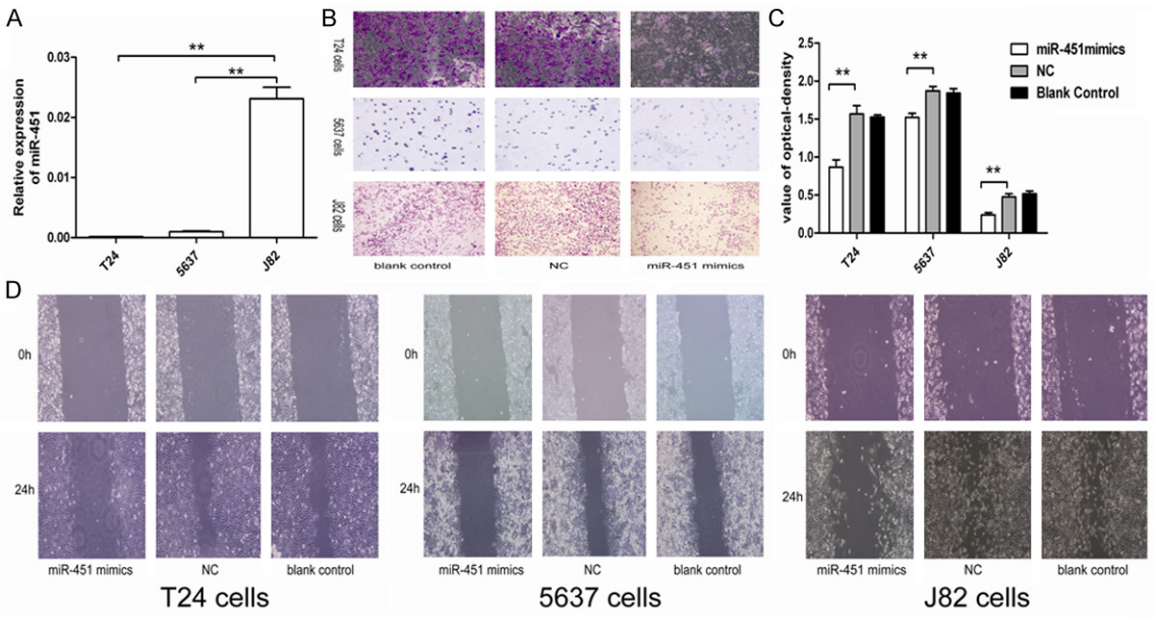


Figure 2. A. miR-451 expression in T24, 5637 and J82 cell lines. B. miR-451 over-expression in T24, 5637 and J82 cell lines inhibit cell invasion compared with the control group and NC group. C. miR-451 over-expression in T24, 5637 and J82 cell lines weakened cell adhesion ability compared with the control group and NC group. D. miR-451 over-expression in T24, 5637 and J82 cell lines inhibits cell migration compared with the control group and NC group. $^{**}P < 0.01$.

cancer research. Their metastasis potential is discrepant, the order is: T24>5637>J82. Before transfection, the expression level of miR-451 in the cells was measured by RT-PCR. The results showed that (**Figure 2A**), the expression of miR-451 was negatively correlated with the metastatic potential. miR-451 was highly expressed in low metastatic potential cell lines (J82), and low expressed in highly metastatic potential cell lines (T24) ($P < 0.01$).

Cell migration and invasion capability in bladder cancer cells decreased by miR451

Transfection efficiency was measured by observing under fluorescence microscope at the 5th hour after transfected. The transfection efficiency reached 90% and meets the qualification of further experiments. The expression level of miR-451 in transfected cells were detected after 48 h using qRT-PCR. Results showed the expression of miR-451 in miR-451mimics transfection group was significantly higher than NC and blank control group ($P < 0.05$). No significant difference between NC and blank control group ($P < 0.05$).

The migration and invasion capability of T24, 5637 and J82 cell lines were examined at 0, 6, 12 and 24 h by scratch wound migration assay and transwell invasion assay after transfection. The results showed the migration distance of miR-451 mimics group was obviously shorter than NC and blank control group (**Figure 2D**). The difference was statistically significant ($P < 0.05$). And there was no significant difference between NC and blank control group ($P > 0.05$). In parallel, the results of transwell invasion assay showed the invasion ability of miR-451 mimics group was obviously lower than NC and blank control group (**Figure 2B**). The difference was statistically significant ($P < 0.05$). And there was no significant difference between NC and blank control group ($P > 0.05$). This indicated that up-regulation of miR-451 inhibited the migration and invasion of T24, 5637 and J82 cells.

Cell adhesion capability in bladder cancer cells decreased by miR451

To further determine the effect of miR-451 on cell adhesion, adhesion assay was performed. The results showed the optical density of miR-451 mimics group was obviously lower than NC and blank control group (**Figure 2C**). The difference was statistically significant ($P < 0.05$). And there was no significant difference between NC

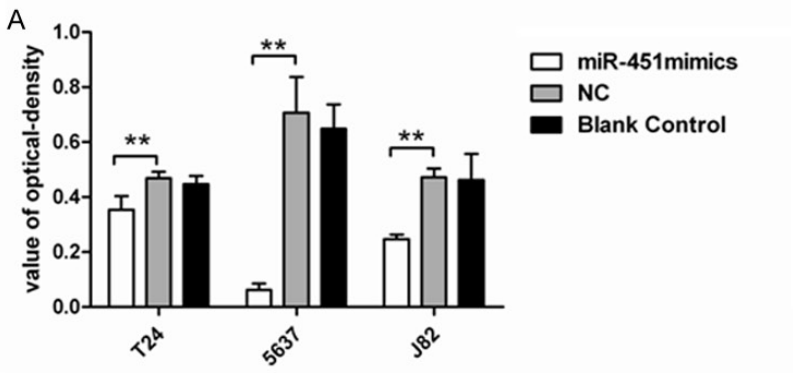
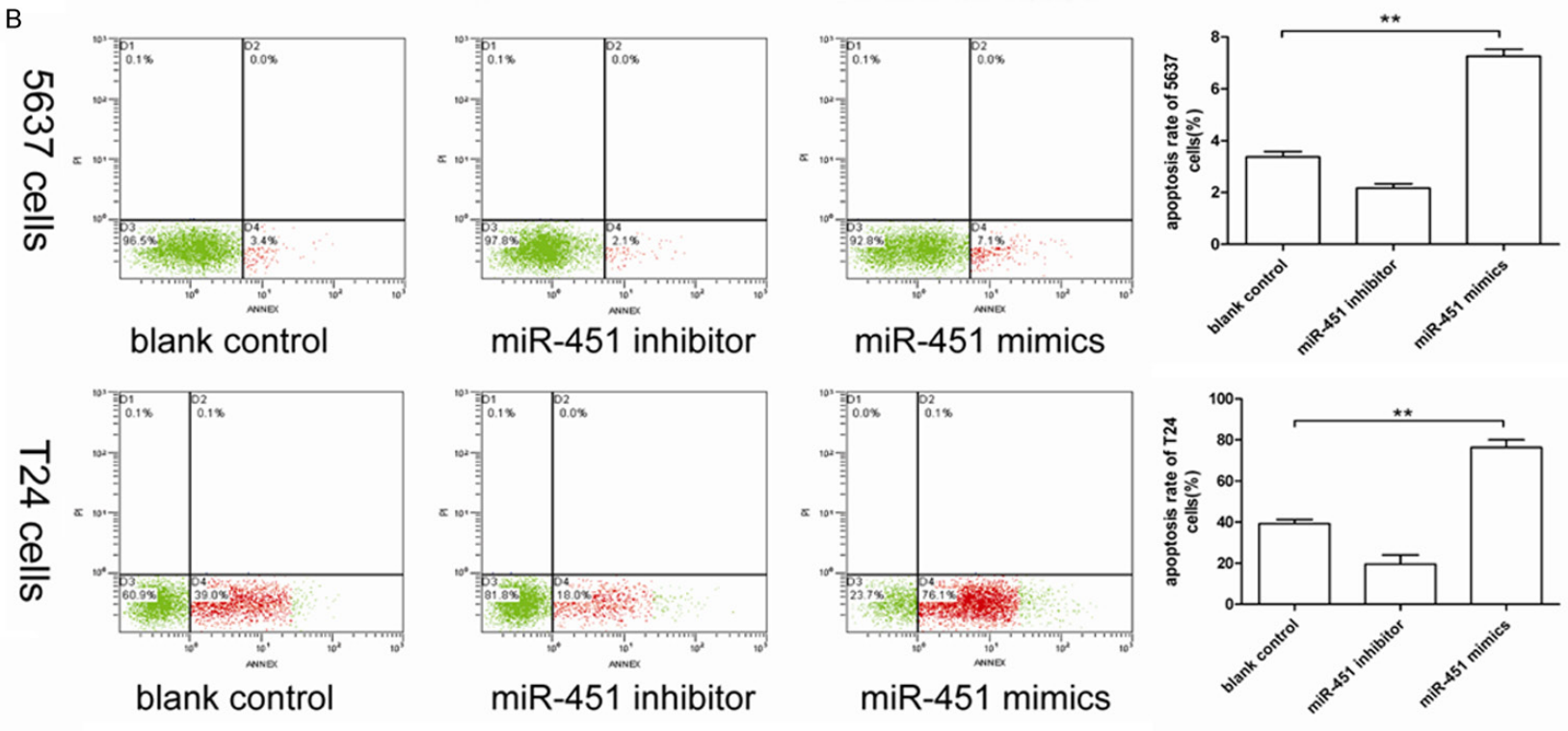


Figure 3. miR-451 reduced cell proliferation and induced apoptosis in bladder cancer cells. A. miR-451 over-expression significantly reduced cell proliferation in T24, 5637 and J82 cell lines. B. miR-451 over-expression significantly induced apoptosis in T24 and 5637 cell lines. $**P < 0.01$.



and blank control group ($P > 0.05$). This indicated that up-regulation of miR-451 weakened the adhesion ability of T24, 5637 and J82 cells.

Over expression of miR-451 decreased cell proliferation and induced apoptosis of bladder cancer cells

The effect of miR-451 on proliferation of bladder cancer cells were analyzed at the 48th after transfection. The results demonstrated that the T24, 5637 and J82 cells transfected with miR-451 mimics exhibited a significant decrease of cell proliferation compared with NC and blank control group ($P < 0.01$), while there was no apparent difference between NC and blank control group ($P > 0.05$) (**Figure 3A**). To further determine the effect of miR-451 on cell apoptosis, our data showed that miR-451 mimic significantly induced apoptosis in 5637 and T24 cells ($P < 0.01$) compared with the control group by flow cytometry analysis, respectively (**Figure 3B**). Apoptosis rate increased from 3.4% to 7.1% in 5637 cells and 39.0% to 76.1% in T24 cells after transfected.

miR-451 affects the expression of EMT related proteins

EMT has been identified as a key role in the invasion of various cancer cells by the transformation of polarized and adherent epithelial cells into motile and invasive mesenchymal cells. EMT can be induced by various growth factors, transforming growth factor-β (TGF-β) is one important inducer of EMT [17]. Experiment of TGF-β induction showed cell morphology changed from the initial compact polygons into loose spindle, especially 5637 cell line (**Figure 4A**). Furthermore, to explore which protein was regulated by miR-451 in the EMT process, we investigated the expression of four EMT related proteins, E-cadherin, N-cadherin, Vimentin and Snail by Western blot. 5637 cell lines, cell morphology changed the most obviously after TGF-β induction, were transfected with NC, miR-451 mimics, miR-451 inhibitor. Results indicated the expression of E-cadherin was increased in miR-451 mimics group compared with NC and untransfected groups (**Figure 4B**, **4C**). Moreover, E-cadherin expression was highest than the other three proteins in miR-451 mimics group. N-cadherin was up-regulated significantly when miR-451 was inhibited in miR-451 inhibitor group. This indicated miR-451 represses the expression of N-cadherin, while promoting the induction of E-cadherin.

Discussion

Several studies have demonstrated that miR-451 can inhibit the proliferation of cervical cancer [14], gastrointestinal cancer [15] and glioblastoma stem cells [16]. Detection revealed miR-451 was significantly down-regulated in bladder cancer tissues and paracancerous tissues compared with normal bladder tissues. Furthermore, results show miR-451 expression was significantly associated with histological differentiation degree and TNM stage, which suggests that miR-451 might be a tumor suppressor gene in bladder cancer.

T24, 5637 and J82 cells were used as experimental material in vitro. Detection of miR-451 expression indicated the transfer ability of the three cell lines were negatively related to the expression level of miR-451. After transfection of miR-451 mimics, our results showed that miR-451 over-expression significantly inhibited cell proliferation and induced apoptosis of the bladder cancer cells. And migration and invasion of the three cell lines were inhibited significantly too. The adhesion ability of T24 cell line was weakened more obviously than 5637 and J82 cell lines, the reason might be T24 cells had the least miR-451 before transfection, so the effect of miR-451 over-expression was more obviously. Furthermore, we induced EMT by TGF-β and found cell morphology changed the most obviously in 5637 cell line after TGF-β induction. Then we investigated the expression level of EMT related proteins in transfected 5637 cells to explore which protein was regulated by miR-451 in the EMT process. Results shown E-cadherin was up-regulated more significantly than N-cadherin, Vimentin and Snail. N-cadherin was up-regulated significantly when miR-451 was inhibited in miR-451 inhibitor group. This indicated miR-451 represses the expression of N-cadherin, while promoting the induction of E-cadherin. E-cadherin has been identified as the epithelial state (E state) marker in EMT process, while N-cadherin as the mesenchymal state (M state) marker [18]. This result means miR-451 could maintain the bladder tumor cells in epithelial phenotype, inhibit EMT process, thereby reducing the invasion and migration of tumor cells.

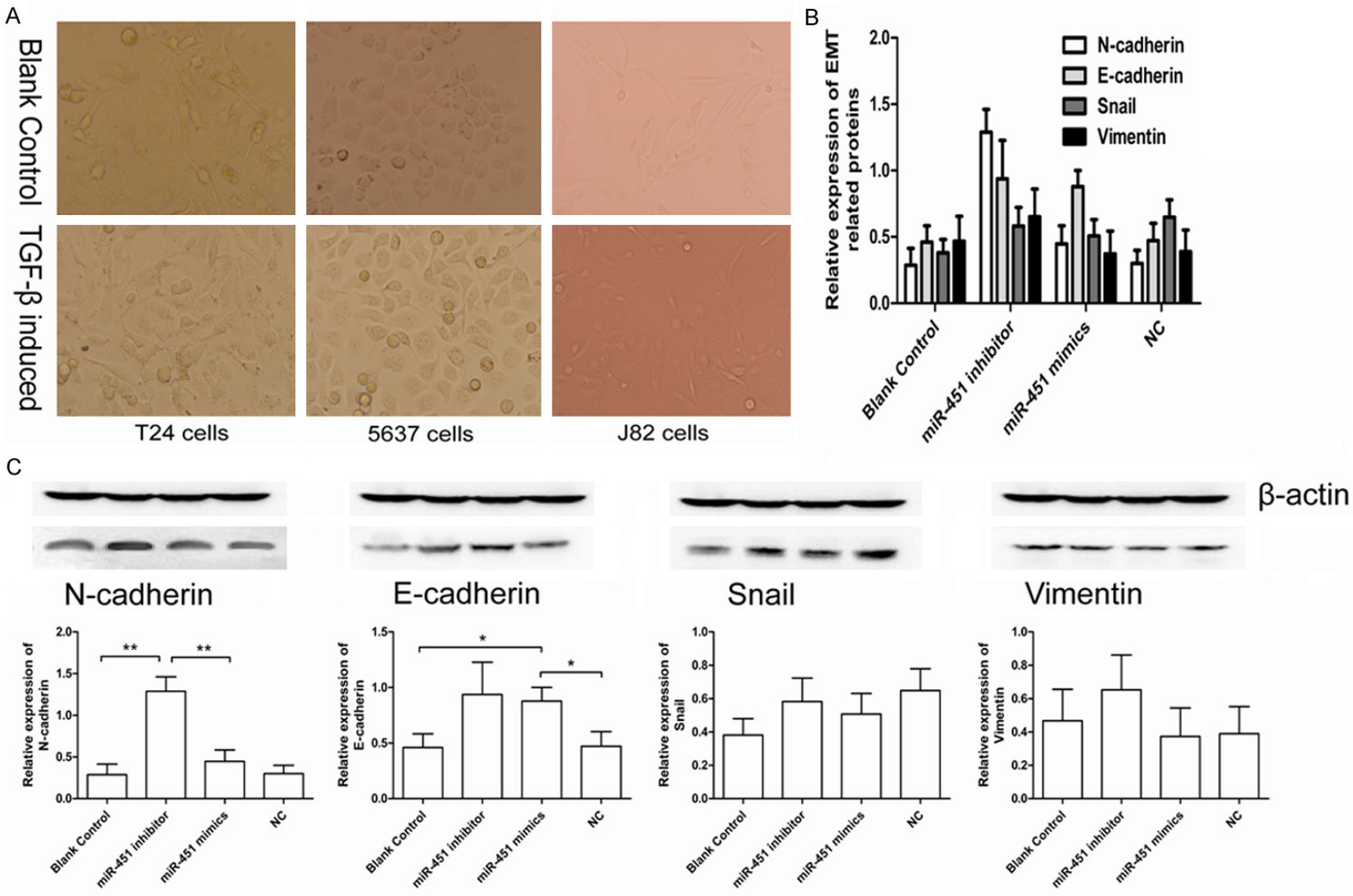


Figure 4. A. TGF-β induced EMT. B, C. Expression of four EMT related proteins in 5637 cells. miR-451 over-expression promotes the induction of E-cadherin, and down-regulated stimulate N-cadherin expression. The expression level of β-actin was used as an internal control. $^{**}P < 0.01$. $^{*}P < 0.05$.

miR-451 is located on chromosome 17q11.2, which region was known to be amplified in various cancers, and is in close proximity to HER2 (17q12) and TRAF4 (TNF receptor-associated factor 4). TRAF4 is a subunit of TNF receptor that interacts with TGF-β1 [19, 20]. Kim's [21] research reported that CAB39 is the target gene of miR451, and miR451 inhibit glioma cell migration and proliferation by regulating the CAB39/AMPK pathway. Liu [14] indicated that IL6R should be the target gene of miR451, which suppresses tumor cell growth by IL6-STAT3-HIF signaling pathway. Gal [16] identified two target sites for SMAD in the upstream promoter region of miR-451 and believe that miR-451 may be activated by the SMAD pathway. Our research shown TGF-β could induce EMT process. Howbeit, there was no significant change of Snail expression, which was identified as crucial transcription factors involved in a TGF-β mediated EMT.

In conclusion, the data presented in this study, demonstrate that miR451 should be a tumor-suppressing gene in bladder cancer. Overexpression of miR-451 inhibited cell proliferation, migration, invasion, adhesion and induced apoptosis of bladder cancer cells. miR-451 could maintain the bladder tumor cells in epithelial phenotype, inhibit EMT process, thereby reducing the invasion and migration of tumor cells. This study indicates that restoration of miR-451 may be a rational therapeutic strategy for the treatment of bladder cancer in the future.

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Disclosure of conflict of interest

None.

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