

# Suppressing cell growth and inducing apoptosis by inhibiting miR-23a-5p in human bladder cancer

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**Abstract.** MicroRNAs (miRNAs) are a class of non-coding RNAs, which are important in the initiation and progression of cancer. miRNA-23a-5p (miR-23a-5p) is a novel miRNA, which promotes cell growth and survival in several types of cancer. The aim of the present study was to investigate the expression and functional significance of miR-23a-5p in bladder cancer. Microchip assays have shown that miR-23a-5p is significantly upregulated in human bladder cancer. In the present study, it was found that miR-23a-5p was upregulated in bladder cancer tissues, compared with paired normal urothelial tissues by reverse transcription-quantitative polymerase chain reaction. The impact of miR-23a-5p on cell proliferation, cell migration and apoptosis was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, wound scratch assay and flow cytometry. The inhibition of cell proliferation and induction of apoptosis were also observed in miR-23a-5p inhibitor-transfected T24 and SW780 human bladder cancer cells. These data suggested that miR-23a-5p has an oncogenic role and may be a therapeutic target for bladder cancer.

## Introduction

Bladder cancer is the most common type of cancer of the urinary system, and is associated with high morbidity and mortality rates (1,2). It was estimated that ~430,000 cases of bladder cancer were diagnosed and there were 165,000 cases of bladder cancer-associated mortality worldwide in 2012 (3). Although mortality rates of bladder cancer are decreasing, the

trend is moderate, and the genetic factors contributing to the tumorigenesis and progression of bladder cancer remain to be fully elucidated (4,5).

MicroRNAs (miRNAs), which are ~20 nucleotides in length, are a class of non-coding RNA. Accumulating evidence suggests that miRNAs are central to several pathological processes by regulating ~50% of human protein-coding genes at the post-transcriptional level (6). Several studies have shown that miR-23a-5p, which is suppressed by c-Myc and promyelocytic leukemia protein-retinoic acid receptor- $\alpha$  fusion protein in prostate cancer and acute promyelocytic leukemia, respectively, functions as a tumor suppressor (7). By contrast, miR-23a-5p has been reported to be significantly upregulated in bladder cancer via microchip assays (8,9). Therefore, the expression and function of miR-23a-5p in bladder cancer requires further investigation.

In the present study, it was found that miR-23a-5p was upregulated in bladder cancer tissues, and that the inhibition of miR-23a-5p suppressed the growth and migration abilities of cells, and induced apoptosis.

## Materials and methods

**Patient samples.** A total of 44 patients pathologically diagnosed with urothelial carcinoma of the bladder were included in the present study. All paired bladder cancer tissues and adjacent normal bladder tissues (located at least 2.0 cm outside of visible bladder cancer lesions) were immediately immersed in RNAlater<sup>®</sup> RNA Stabilization agent (Qiagen GmbH, Hilden, Germany) following surgical resection, and were snap-frozen in liquid nitrogen and stored in a cryo freezer at -80°C for further use. All samples were collected at Department of Urology, Peking University Shenzhen Hospital from September 2012 to December 2015. The present study was approved by the Ethics Committee of Peking University Shenzhen Hospital (Shenzhen, China). Written informed consent was obtained from all the patients involved. The clinical parameters of patients are listed in Table I.

**Cell culture.** The T24 and SW780 human bladder cancer cell lines were purchased from the Institute of Cell Biology,

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Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 1% antibiotics (100  $\mu$ /ml penicillin and 100 mg/ml streptomycin sulfates) and 1% glutamate (Gibco; Thermo Fisher Scientific, Inc.), at 37°C in a 5% CO<sub>2</sub> atmosphere (10).

**Cell transfection.** For the suppression of miR-23a-5p, chemically synthesized miR-23a-5p inhibitor and negative control (NC) were purchased from GenePharma (Shanghai, China). The sequences were as follows: Sense, 5'-AAAUCCCAUCCCCAGGAACCCC-3' and antisense, 5'-CAGUACUUUUGUGUAGUACAA-3', respectively. Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was mixed with the 100 pmol miR-23a-5p inhibitor or NC for transfection, according to the manufacturer's protocol.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis.** Total RNA was extracted from the tissue samples or the transfected cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The RNA samples with 260/280 ratios of 1.8-2.0 were used for further experiments. Total RNA was converted into cDNA using the miScript II RT kit (Qiagen GmbH). The primer sequences were as follows: miR-23a-5p, forward 5'-GGGGUUCUGGGGAUGGGAUUU-3' and reverse: Universal primer, provided with the miScript SYBR®-Green PCR kit (Qiagen GmbH); U6 forward, 5'-CTCGCTTCGGCAGCACACA-3' and reverse, 5'-ACGCTTCACGAATTTGCGT-3'. The qPCR procedure was performed to determine the expression levels of miR-23a-5p using the miScript SYBR®-Green PCR kit (Qiagen GmbH), according to the manufacturer's protocol. The thermocycling conditions for RT-qPCR were as follows: 95°C for 15 min, followed by 40 cycles of 94°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec. The reactions were performed in triplicate using the LightCycler 480 real-time PCR system (Roche Applied Science, Mannheim, Germany). U6 was selected as the internal control. The expression level was determined as the fold difference relative to U6, which was based on the following equation: Relative expression =  $2^{-\Delta\Delta C_q}$ ,  $\Delta\Delta C_q = (\text{mean}C_{q_{\text{cancer}}} - \text{mean}C_{q_{\text{U6}}}) - (\text{mean}C_{q_{\text{normal}}} - \text{mean}C_{q_{\text{U6}}})$  (11).

**3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.** Cell proliferation was analyzed using an MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) assay. The T24 and SW780 cells, at ~5,000 cells per-well, were plated into 96-well plates, with five replicate wells for each condition. After 24 h, each well was transfected with 5 pmol miR-23a-5p inhibitor or NC. Cell growth was assessed at 0, 24, 48 and 72 h post-transfection. The MTT (20  $\mu$ l) was added into each well and incubated at 37°C for 6 h. The MTT medium was then discarded and 12  $\mu$ l dimethyl sulphoxide (Sigma-Aldrich; Merck KGaA) was added. Following agitation for 30 min at room temperature, the optical density (OD) value of each sample was measured with an enzyme immunoassay instrument (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a wavelength of 490 nm (with 630 nm as the reference wavelength).

Table I. Clinicopathological features of patients with bladder cancer.

Characteristic	Cases (n)
Median age (range), years	67 (38-85)
Sex	
Male/female	36/8
Histological type	
Transitional cell carcinoma	44
Grade	
Low/high	18/26
Stage	
I/II/III/IV	18/21/5/0

Grade and stage according to the 2009 American Joint Committee on Cancer staging system (20).

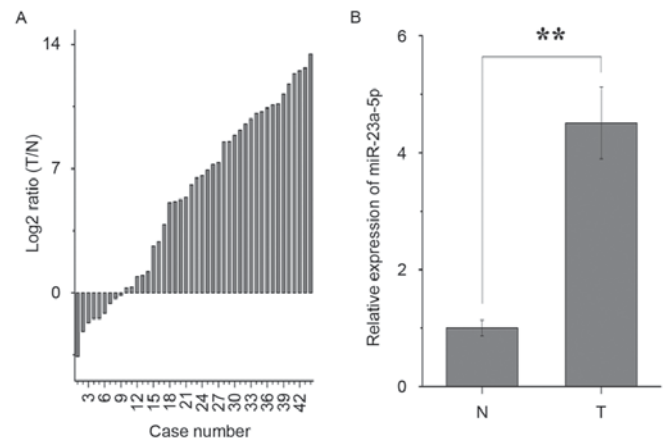


Figure 1. Expression of miR-23a-5p in 44 paired bladder cancer tissues and adjacent normal tissues. (A) Log 2 ratios of the expression of miR-23a-5p in 44 paired bladder cancer tissues to adjacent normal tissues. (B) Relative expression of miR-23a-5p in bladder cancer tissues and adjacent normal tissues. \*\*P<0.01. miR, microRNA; T, bladder cancer tissues; N, adjacent normal tissues.

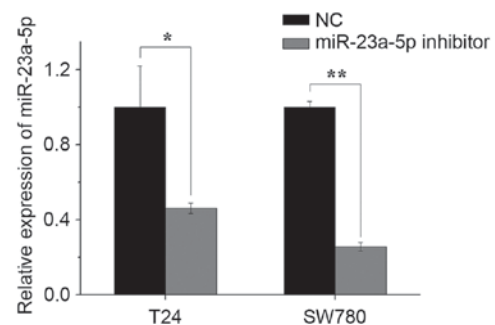


Figure 2. Validation of cell transfection efficiency. The relative expression of miR-23a-5p in T24 and SW780 cells transfected with miR-23a-5p inhibitor or negative control. \*P<0.05; \*\*P<0.01. miR, microRNA; NC, negative control.

**Wound-healing assay.** Cell migration was determined with a wound-healing assay. Briefly, ~3x10<sup>5</sup> cells were seeded into

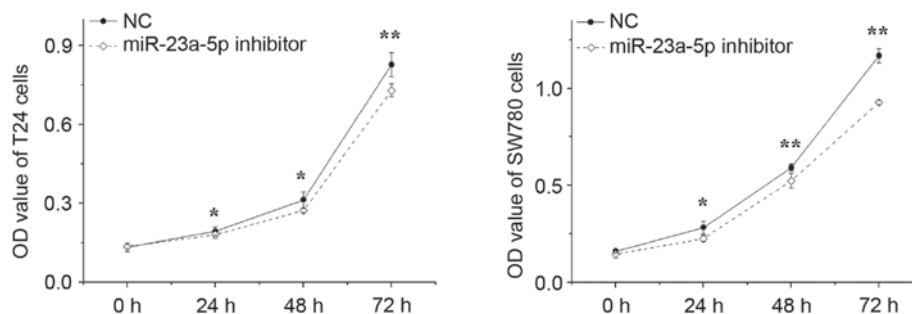


Figure 3. Proliferation abilities of T24 and SW780 cells. Cell proliferation was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay at 0, 24, 48 and 72 h, respectively. \* $P < 0.05$ ; \*\* $P < 0.01$ . NC, negative control; miR, microRNA; OD, optical density.

12-well plates at equal density and grown to 80-90% confluence. Artificial gaps/wounds were generated using a 200- $\mu$ l sterile pipette tip following transfection. The regions of the wounds were marked and images were captured using a digital camera system (Olympus Corporation, Tokyo, Japan). The cell migration distance (mm) was calculated using the HMIAS-2000 (v1.0; Champion Image Co., Ltd., Wuhan, China) software program. Each experiment was repeated three times.

**Flow cytometry.** Flow cytometry was performed to determine the early apoptotic rate. The cells were seeded ( $\sim 3 \times 10^5$  cells per well) into 6-well plates at equal density. At 48 h post-transfection, the cells in each well, including floating cells, were collected and stained with 3  $\mu$ l propidium iodide (PI) and 5  $\mu$ l Annexin V-fluorescein isothiocyanate (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Within 30 min, each sample was measured and analyzed on a flow cytometer (Coulter Epic XL-4; Beckman Coulter, Inc., Brea, CA, USA) using EXPO32 ADC software V1.2 (Beckman Coulter).

**Statistical analysis.** All data are expressed as the mean  $\pm$  standard deviation. The significance of differences were determined using SPSS version 19.0 software (IBM Corps., Armonk, NY, USA). The differences in the expression of miR-23a-5p between bladder cancer tissues and adjacent normal tissues were analyzed using a paired-sample t-test. The independent-samples t-test was used to analyze other data.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**miR-23a-5p is upregulated in bladder cancer.** The results of the RT-qPCR analysis showed that miR-23a-5p was significantly upregulated in 35/44 patients with bladder cancer (Fig. 1A). As shown in Fig. 1B, miR-23a-5p was upregulated in bladder cancer tissues, compared the adjacent normal tissues, with an average of a 4.509-fold increase in expression, in accordance with previous studies (8,9). These results indicated that miR-23a-5p may have an oncogenic role in bladder cancer. The clinical parameters of patients are listed in Table I.

**miR-23a-5p inhibitor downregulates the expression of miR-23a-5p.** The silencing efficiency of the miR-23a-5p inhibitor was validated using RT-qPCR analysis 48 h post-transfection. As shown in Fig. 2, the expression levels of

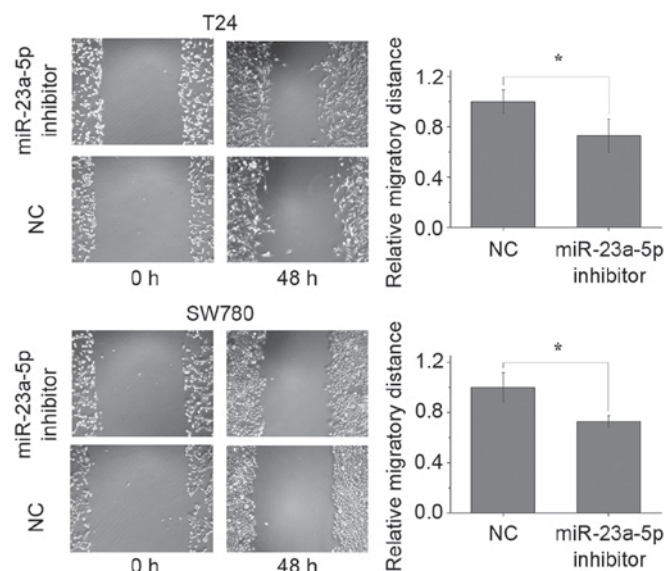


Figure 4. Wound-healing assay examination of the migration of T24 and SW780 cells. Cell migration was determined following transfection with the miR-23a-5p inhibitor or negative control at 0 and 48 h. \* $P < 0.05$ . miR, microRNA; NC, negative control.

miR-23a-5p in the miR-23a-5p inhibitor group were decreased by 53.7% in T24 cells and 74.3% in the SW780 cells, compared with those in the NC group.

**Silencing miR-23a-5p suppresses cell growth.** The effect of miR-23a-5p inhibitor on the growth of bladder cancer cells was determined using MTT assays. The outcomes revealed that, following silencing of miR-23a-5p, the proliferation of T24 cells decreased by 6.23% ( $P < 0.05$ ), 12.71% ( $P < 0.05$ ) and 11.72% ( $P < 0.01$ ), and the proliferation of SW780 cells decreased by 19.91% ( $P < 0.05$ ), 11.24% ( $P < 0.05$ ) and 20.55% ( $P < 0.05$ ) at 24, 48 and 72 h, respectively, compared with proliferation in the NC group (Fig. 3). These results indicated that the downregulation of miR-23a-5p significantly decreased the proliferation of bladder cancer cells.

**Silencing miR-23a-5p attenuates cell migration.** The present study performed wound-healing assays to observe the effect of miR-23a-5p on cell migratory ability. The results showed that the migratory distances of the miR-23a-5p inhibitor group were significantly decreased by 27.2% ( $P < 0.05$ ) and 27.3% ( $P < 0.05$ ) for the T24 cells and SW780 cells, at 48 h

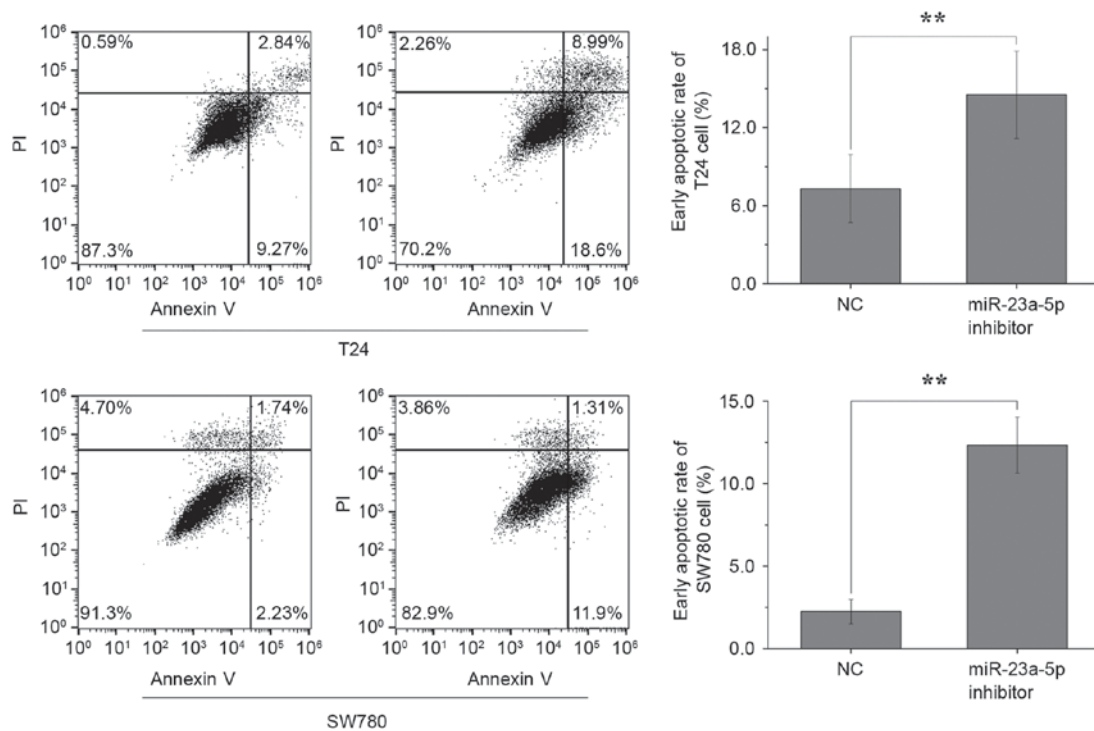


Figure 5. Early apoptotic rates (%) of T24 and SW780 cells were measured using flow cytometry. \*\* $P < 0.01$ . NC, negative control; miR, microRNA; PI, propidium iodide.

post-transfection, compared with distances in the NC group (Fig. 4). This suggested that the silencing of miR-23a-5p attenuated the migratory abilities of the bladder cancer cells.

**Silencing miR-23a-5p induces apoptosis.** Finally, flow cytometric analysis was performed to determine whether the miR-23a-5p inhibitor had an effect on bladder cancer cell apoptosis. The results demonstrated that the average early apoptotic rates of the T24 cells transfected with miR-23a-5p inhibitor and NC, were 7.31 and 14.53%, respectively ( $P < 0.01$ ), whereas the average early apoptotic rates of the SW780 cells transfected with miR-23a-5p inhibitor and NC were 2.25 and 12.33%, respectively ( $P < 0.01$ ) (Fig. 5). These data suggested that the silencing of miR-23a-5p induced bladder cell apoptosis.

## Discussion

miRNAs have emerged as key post-transcriptional regulators of gene expression and are involved in the regulation of almost every cellular process (6). Dysregulation of miRNAs are associated with several human pathologies, including tumorigenesis and tumor progression (6). Oncogenic miRNAs targeting key tumor suppressor genes, including the miR-17-92 cluster and miR-21, are upregulated in cancer (12). miR-17 and miR-20a, of the miR-17-92 cluster, target E2F transcription factor 1, a cell cycle regulator involved in cell division and apoptosis (13). miR-21 in breast cancer and colon cancer leads to increased tumor growth by downregulating programmed cell death 4, a protein involved in the promotion of cellular apoptosis (14,15). The miR-200 family is among the most downregulated tumor suppressive miRNAs in cancer (1). ETS proto-oncogene 1 (ETS1) is one target of miR-200, and the loss of the miR-200-mediated repression of ETS1 results in angiogenic responses in cancer cells (16).

miR-23a-5p has been found to be dysregulated in several types of cancer (7). As a functional downstream target of cAMP responsive element binding protein 1, miR-23a-5p represses the tumor suppressor gene phosphatase and tensin homolog and promotes cell growth and survival in glioma (17). The overexpression of miR-23a-5p in pancreatic ductal adenocarcinoma is involved in increasing the transformation of epithelial mesenchymal transition-like cell shape and its integration into mesothelial monolayers through altering the expression of E-cadherin,  $\beta$ -catenin and Wnt-related genes (18). Interleukin 6 receptor (IL6R), an evolutionarily conserved antiproliferative protein, has been confirmed as a direct target gene for miR-23a in gastric adenocarcinoma (19).

To the best of our knowledge, the present study is the first to confirm the upregulation of miR-23a-5p in bladder cancer using RT-qPCR analysis, and to correlate miR-23a-5p with the development of bladder cancer. In the present study, the relative expression of miR-23a-5p was quantified in 44 paired bladder cancer tissues and adjacent normal tissues. The data suggested that miR-23a-5p was significantly upregulated in the bladder cancer tissues and that miR-23a-5p may have a functional role in bladder cancer. To further investigate the role of miR-23a-5p in bladder cancer, a wound-healing assay, MTT assays and flow cytometric analysis were performed in bladder cancer-related cells. Cell proliferation, the suppression of migration and the induction of apoptosis were observed in the miR-23a-5p inhibitor-transfected T24 and SW780 cells. The resulting data suggested that oncogenic miR-23a-5p may have a fundamental role in the tumorigenesis and tumor progression of bladder cancer.

In conclusion, the present study indicated that miR-23a-5p functions as an oncogene in bladder cancer by affecting cell proliferation, migration and apoptosis. Further investigations are



required to predict and confirm the upstream and downstream genes, understand the molecular mechanisms, and examine the clinical application of miR-23a-5p in bladder cancer.

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