

miR-451 suppresses bladder cancer cell migration and invasion via directly targeting c-Myc

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Abstract. MicroRNA (miRNA) expression is shown dysregulated in tumors. It has been reported that miR-451 alters gene expression and regulates tumorigenesis in various cancer tissues. However, its underlying biological significance in bladder cancer remains to be clarified. In the present study, we investigated the function and molecular mechanism of miR-451 involved in bladder cancer cell migration and invasion. Our results showed that miR-451 was downregulated in clinical bladder carcinoma tissues compared with adjacent bladder tissues. Overexpression of miR-451 significantly retarded the proliferation, migration and invasion of bladder cancer T24 and 5637 cells *in vitro*. Moreover, the attenuated cell migration and invasion by miR-451 was correlated with increased apoptosis. However, our dual-luciferase reporter assay validated that c-Myc, an oncogene in many tumors, was a direct target gene of miR-451 in bladder cancer. The expression of c-Myc was repressed by miR-451 in bladder cancer cells, and knockdown of c-Myc mimicked the effects of miR-451 overexpression. This discovery suggested that miR-451 is a tumor suppressor modulating bladder cancer cell migration and invasion by directly targeting c-Myc. In addition, apoptosis promoted by miR-451 may participates in this biological behavior. Therefore, target miR-451 may be a novel therapeutic intervention for bladder cancer.

Introduction

Bladder carcinoma is one of the most common malignancies and the second leading cause of death among men in urologic cancer (1). Approximately 75% of the patients have superficial bladder cancer, but they recur and progress to

muscle-invasive cancer after surgical intervention. The 5-year survival rate is only ~60% for patients with muscle-invasive bladder cancer (2,3). Therefore, exploring and understanding the potential molecular mechanism underlying bladder cancer is crucial for the development of novel therapies.

MicroRNAs (miRNAs) are a class of endogenous 19-25 nucleotides small non-coding RNA molecules, which lead to target mRNA silencing or translational repression at the post-transcriptional level through complementary binding to the 3'-untranslated region (3'-UTR) of target genes (4). Dysregulation of miRNAs is a general feature of many tumors, including bladder cancer (5,6). Aberrant expression of miRNAs appear to play crucial roles in various physiological processes of cancer (7). It has been reported that miRNAs could serve as biomarkers for bladder cancer (8). In our preliminary investigation on the changes of miRNA expression profiles in chemotherapy of bladder tumor by miRNA microarray analysis, we observed differentially expressed miRNAs after arsenic trioxide treatment in 5637 and T24 cells, particularly miR-451 showed significantly altered expression. miR-451 is a special miRNA since it has a unique structure and synthetic pathway, and its maturity is mediated by Ago2. Frequent dysregulation of miR-451 was reported in several types of cancers in recent research (9). Intriguingly, expression of miR-451 is not consistent in different tumors. Compared with normal tissues, miR-451 is downregulated in hypopharyngeal squamous cell carcinoma (10), lung (11) and liver cancer (12), and osteosarcoma (13). On the contrary, the expression of miR-451 is upregulated in glioma (14) and colorectal cancer (15). This indicated that distinction of miR-451 expression in tumors may be determined based on the type of tumor. However, the role of miR-451 involved in bladder cancer development and progress is still remains largely unraveled. Therefore, it is necessary to investigate the expression and potential function of miR-451 in bladder cancer.

Our purpose was to analyze miR-451 differential expression between bladder cancer and normal bladder tissues. We used *in vitro* approach to study the role of miR-451 in regulating the biological behavior of bladder cancer cells. Furthermore, we predicted a direct downstream target gene of miR-451 and verified their correlation to deepened our understanding of the role of miR-451 in bladder cancer.

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Table I. Clinicopathological information of the patients with bladder cancer.

Patient no.	Gender	Age (years)	Stage	Grade
1	Male	56	II	Low
2	Male	60	I	Low
3	Male	62	III	High
4	Male	53	III	High
5	Male	61	I	Low
6	Male	59	III	High
7	Female	63	III	High
8	Male	55	II	High
9	Male	59	II	Low
10	Female	45	I	Low
11	Male	55	II	High
12	Male	50	II	Low
13	Male	65	I	Low
14	Female	68	III	Low
15	Female	64	II	Low
16	Male	58	II	Low
17	Male	74	III	High
18	Male	62	II	Low
19	Female	66	II	Low
20	Male	57	III	High

Materials and methods

Human tissue samples. Bladder cancer and adjacent normal tissues were obtained from patients who received partial resection or radical cystectomy at Department of Urological Surgery, the First Affiliated Hospital of Harbin Medical University. None of the patients received any preoperative therapy. After resection, fresh tissues were immediately frozen in liquid nitrogen and then stored at -80°C until RNA extraction. The use of the tissue samples was approved by Institute Ethics Committee. The patients signed informed consent forms. The characteristics of the patients are described in Table I. Bladder tumors were divided into low grade group ($n=12$) and high grade ($n=8$) according to the World Health Organization pathology classification standard (16).

Cell culture and transfection. Human bladder cancer cell lines T24 and 5637 were obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; PAA, USA) under a humid atmosphere with 5% CO_2 at 37°C . The miR-451 mimics and negative control miRNA (miR-NC), siRNA-c-Myc and negative control siRNA (siR-NC) were synthesized by RioBio (Guangzhou, China). Cells were plated to 50-60% confluency in medium and then transfected with oligofectamine reagent (Guangzhou, China) based on the manufacturer's protocol. The miRNAs were used at a final concentration of 100 nM and transfection efficiency detected by real-time RT-PCR.

Table II. Primer sequences.

Name	Primes (5'-3')
miR-451	F AAACCGTTACCATTACTGAGTT
	R GCGAGCACAGAATTAATACGAC
U6	F CTCGCTTCGGCAGCACATATACT
	R ACGCTTCACGAATTTGCGTGTC
c-Myc	F TGCTAAGAAGATTGGTGCTGTA
	R GCGAAGGGCTGAGACATTTAC
GAPDH	F GAAATCCCATCACCATCTTC
	R GAGCCCCAGCCTTCTCCATG

F, forward; R, reverse.

RNA extraction and quantitative real-time PCR (qPCR). Total RNAs were extracted from human tissues and cell lines with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the protocol as previously described (10). RNA purity and concentrations were assessed by measuring the absorbance at 260 and 280 nm using a NanoDrop[®] ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Samples at optical density (OD) 260/280 nm ratios between 1.80-2.00 were used for further analysis. Total RNA was reverse-transcribed into cDNA with One Step PrimeScript miRNA cDNA Synthesis kit (Takara, Otsu, Japan). qRT-PCR reactions were conducted using the ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA), followed by 95°C for 30 sec and 40 thermal cycles of 95°C for 5 sec and 60°C for 30 sec. The U6 or GAPDH were used as the internal endogenous controls for miR-451 or siR-c-Myc and the $2^{-\Delta\Delta\text{Ct}}$ method was used to calculate the relative expression levels. The sequences of the primers are shown in Table II.

Cell proliferation assay. Cell proliferation was measured using Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Cells were seeded into 96-well plates at 5,000 cells/well. After 24 h cultivation, cells were transfected with miR-451 mimics, miR-NC, si-c-Myc and siR-NC. CCK-8 reagent 10 μl was added into each plate after cells were transfected 0, 24, 48, 72 and 96 h. The cells were cultured for another 1 h and the absorbance at 450 nm was measured with a spectrophotometric plate reader (Bio-Rad, Richmond, CA, USA).

Cell Transwell assay. Cell Transwell assay was performed in 24-well plates with Transwell chamber (8.0- μm pore size polycarbonate filter) as previously described (11). Briefly, cells (5×10^4) in 100 μl serum-free medium were placed into the upper chamber with or without Matrigel (BD Biosciences, Bedford, MA, USA). RPMI-1640 medium (1 ml) containing 10% FBS was added in the lower chamber. After cultured for 48 h, cells on the upper chamber were washed, fixed and stained with methanol and crystal violet, then counted by inverted microscopy at a magnification of $\times 100$ (Sony, Tokyo, Japan).

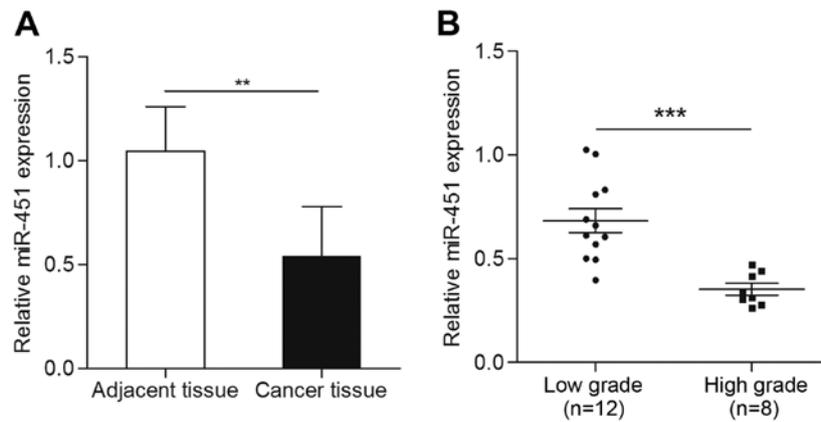


Figure 1. Expression of miR-451 in bladder tissue. (A) Expression level of miR-451 in bladder cancer tissues by RT-PCR. (B) Comparison of expression level of miR-451 in a group of low grade with a group of high grade.

Dual-luciferase reporter assay. The luciferase reporter assay was carried out as previously described (12). In brief, the recombinant vectors (HaoGe, Shanghai, China) were constructed with wild-type (WT)-c-Myc sequences or mutant (MUT)-c-Myc sequences inserted between the hRluc and the hLuc gene. Cells (2×10^4) were seeded into 96-well plates, after growth to 70-80% confluence, and cotransfected with miR-NC or miR-451 mimics and the WT c-Myc 3'-UTR vector and the MUT c-Myc 3'-UTR vector using Lipofectamine 2000 (Invitrogen). After transfecting for 24 h, the firefly and *Renilla* luciferase activities were measured consecutively through a Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA). *Renilla* luciferase intensity (*Renilla*/firefly) was used as normalized data.

Western blotting. Total proteins were extracted from cells by PARIS kit (Ambion, Carlsbad, CA, USA), and protein concentration was detected by BCA protein assay kit (Beyotime, China). Then, proteins were separated using 10% SDS-PAGE gels, and transferred to polyvinylidene fluoride (PVDF) membranes. Next, the membranes were blocked with 5% non-fat dried milk for 2 h and incubated with rabbit anti-MMP-7 (1:1,000; Cell Signaling Technology, Danvers, MA, USA), anti-PARP1 antibody (1:1,000; ProteinTech Group, Inc., Chicago, IL, USA), anti-c-Myc antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-GAPDH antibody (ProteinTech) at a 1:2,000 dilution overnight at 4°C. Membranes were washed three times in PBST, and incubated with a goat anti-rabbit antibody at a 1:2,000 dilution for 2 h. Blots were detected by an enhanced chemiluminescence (ECL) system (Pierce Biotechnology Inc., Rockford, IL, USA).

Cell apoptosis analysis. The transfected cells were harvested and washed twice by ice cold PBS. Cells, were then resuspended at a density of 1×10^6 cells/ml in 1X binding buffer, and stained with an Annexin V-FITC apoptosis detection kit (Clontech, Beijing, China). Cell apoptosis was detected using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Statistical analysis. Data are expressed as means \pm standard deviation (SD). Statistical significance was evaluated by

Student's t-test or one-way analysis of variance (ANOVA). Differences were considered statistically significant at $p < 0.05$. All experiments were repeated three times.

Results

Expression of miR-451 is downregulated in bladder cancer tissues. The expression of miR-451 was detected by qRT-PCR in paired 20 bladder cancer tissues and matched adjacent non-cancer tissues in the present study. The results showed that expression of miR-451 in bladder cancer tissues was significantly decreased when compared with adjacent non-cancerous tissues ($p < 0.01$) (Fig. 1A). In order to further determine the relation of miR-451 and bladder cancer, we analyzed miR-451 expression disparity in different grade bladder cancer tissues. The expression of miR-451 in low grade bladder cancer tissues was found noticeable higher than that of the high grade tissues ($p < 0.001$) (Fig. 1B). These results suggested that miR-451 may be associated with bladder cancer.

miR-451 overexpression suppresses the proliferation, migration and invasion of bladder cancer cells. To study the tumor suppressive role of miR-451 in bladder cancer, we examined the proliferation, migration and invasion of bladder cancer cells after the different transfections. qRT-PCR was carried out for miR-451 expression evaluation after transfections. The results showed the expression of miR-451 was increased in miR-451 transfected cells compared with mock or miR-NC ($p < 0.001$) (Fig. 2A). Next, we performed CCK-8 cell proliferation assay. The results indicated that bladder cancer cells transfected with miR-451 showed significantly low proliferation rate than controls (Fig. 2B). Cell migration and invasion are key steps during tumor progression and metastasis (17). We detected the migration and invasion capability of bladder cancer cells after transfection with miR-451 by Transwell assay. Fig. 2C showed that overexpression of miR-451 significantly induced the migration ability of bladder cancer cells compared with the controls by Transwell migration assay ($p < 0.001$). Similarly results were observed in Transwell invasion assay, cells transfected with miR-451 showed attenuated invasiveness compared to the controls ($p < 0.001$) (Fig. 2D). These results suggest that overexpression

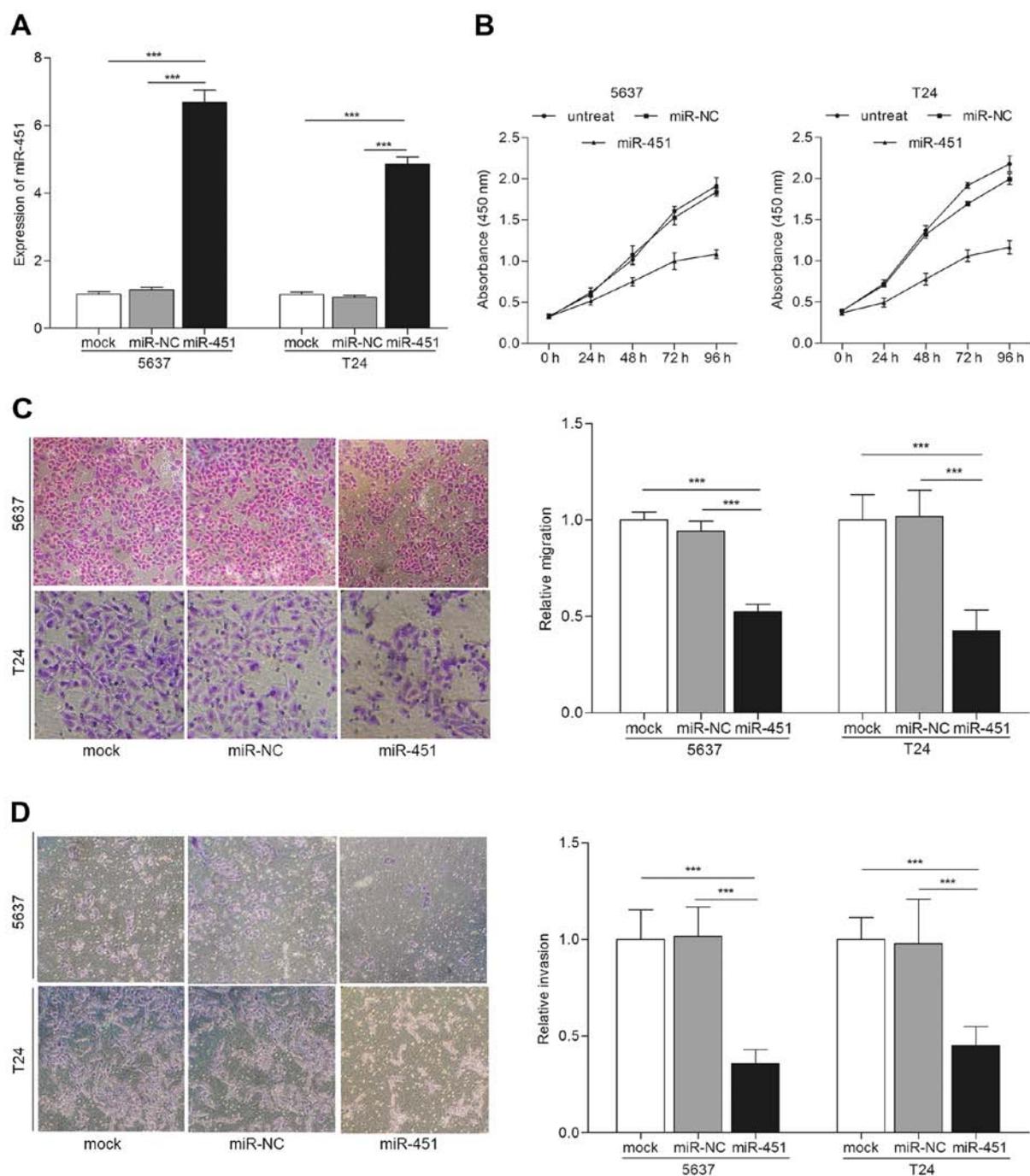


Figure 2. miR-451 regulates proliferation, migration and invasion of bladder cancer cell lines. (A) The miR-451 expression was evaluated in 5637 and T24 cells after transfection by real-time RT-PCR. (B) Cell proliferation assays in bladder cancer cells 5637 and T24. (C) Cell Transwell migration assay showed migration ability of cells transfected with miR-451. (D) Cell Transwell invasion assay in bladder cancer cells.

of miR-451 suppresses biological behavior of bladder cancer *in vitro*.

miR-451 reduces cell migration and invasion by increasing apoptosis. Previous studies implied that apoptosis represents one of the important processes in cancer metastasis (18). Thus, we investigated the possible mechanism of miR-451 involved in inhibiting bladder cancer cell migration and invasion. Significantly increased apoptotic proportions were observed in bladder cancer cells transfected with miR-451 ($p < 0.05$) (Fig. 3A). We also detected the expression of key

apoptosis related protein, PARP (19). The results showed that cleaved PARP1 protein expression was apparently increased in miR-451 transfected cells compared with the controls by western blot assay ($p < 0.001$) (Fig. 3B). A previous study showed that MMPs served as metastasis-related factors in malignancy mediated tumor cell apoptosis (18). Then, expression of MMP-7 was detected by western blotting. We observed that miR-451 overexpression led to lower expression level of MMP-7 protein (Fig. 3B). These results suggest that apoptosis may mediate bladder cancer cell migration and invasion.

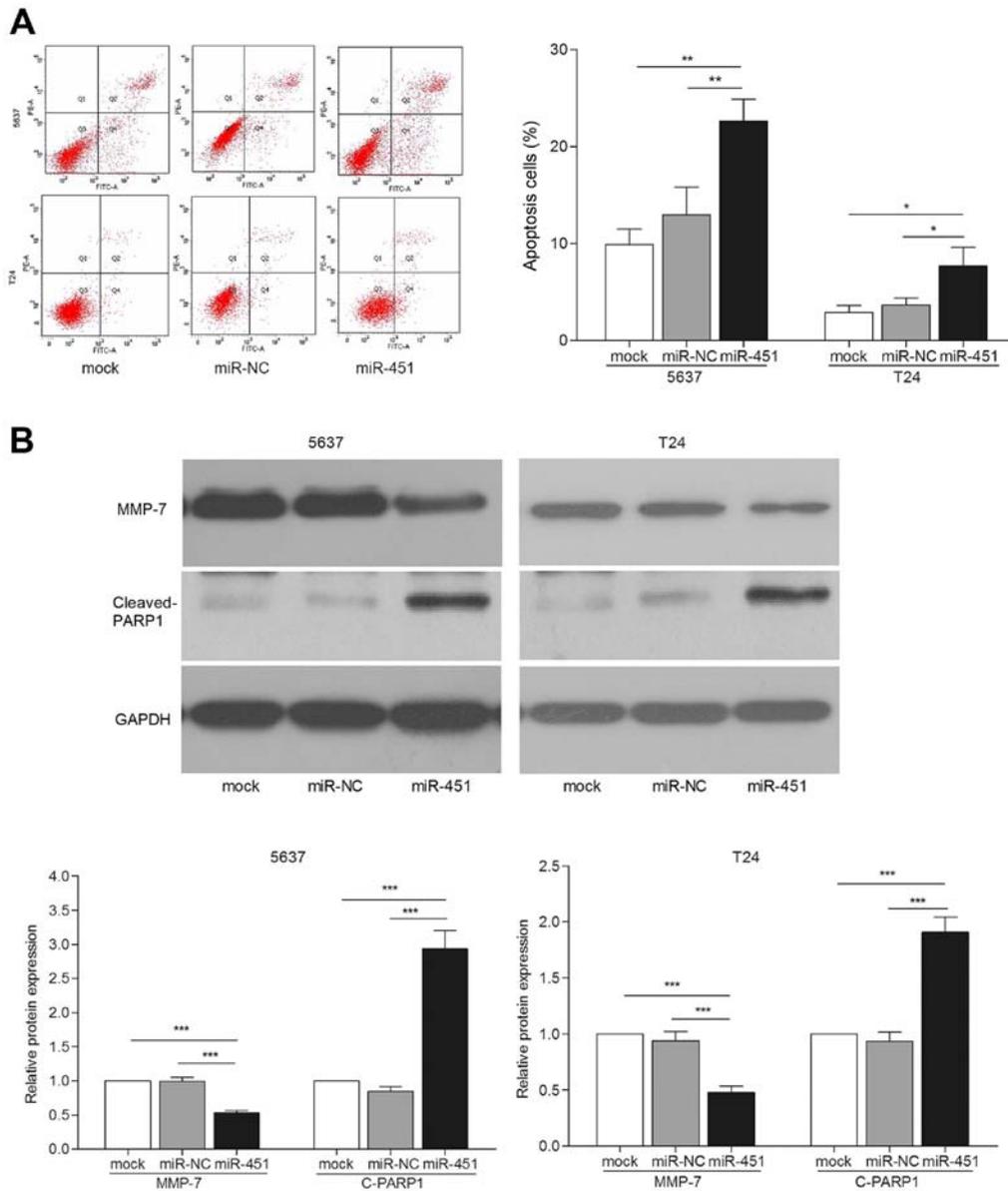


Figure 3. miR-451 mediates bladder cancer cells 5637 and T24 apoptosis. (A) Flow cytometry analysis showed apoptosis in bladder cancer cells. (B) Protein expression level of MMP-7 and cleaved PARP1 (C-PARP1) in 5637 and T24 cells by western blot assay.

miR-451 regulates c-Myc expression by directly targeting its 3'-UTR. To understand the details of antitumorigenic mechanism of miR-451 in bladder cancer, we explored target gene of miR-451 by multi-bioinformatics analysis, such as TargetScan, MicroRNA and PicTar. After analysis, we found that 3'-UTR of c-Myc mRNA contained one conserved complementary binding site with miR-451 (Fig. 4A). This indicated that c-Myc may be a putative target gene of miR-451. In order to validate whether c-Myc was directly regulated by miR-451 with its 3'-UTR, we co-transfected the luciferase plasmid harboring the wild- or mutant-type 3'-UTR of c-Myc, and then carried out luciferase reporter assay. The results confirmed that luciferase activity of c-Myc 3'-UTR containing wild-type was suppressed in miR-451 re-expressed cells, but not containing mutant type ($p < 0.01$) (Fig. 4B). Next, we detected expression levels of c-Myc in 5637 and T24 cell lines by western blot assay. The results showed that c-Myc protein levels are markedly reduced in cells transfected with miR-451 compared

with that in control cells ($p < 0.01$) (Fig. 4C). Moreover, we observed an inverse correlation between miR-451 and c-Myc protein level in bladder cancer tissues (Fig. 4D). The above demonstrates that c-Myc is a direct downstream target gene of miR-451 in bladder cancer cells.

To further confirm that the role of miR-451 on the biological behavior of bladder cancer cells was through targeting c-Myc, we knocked down the expression of c-Myc in bladder cancer cells. We transfected cells with c-Myc siRNA. Both the expression levels of c-Myc mRNA and protein were reduced compared with those in the controls (Fig. 5A and B). Using cell proliferation technique, we observed the significantly reduced cell proliferation in si-c-Myc transfected bladder cancer cells (Fig. 5C). Both Transwell migration and invasion assays indicated that c-Myc knockdown played an inhibitory effect on the bladder cancer cell migration and invasion compared with control groups ($p < 0.001$) (Fig. 6A and B). In addition, protein levels of MMP-7 were significantly repressed in cells transfected

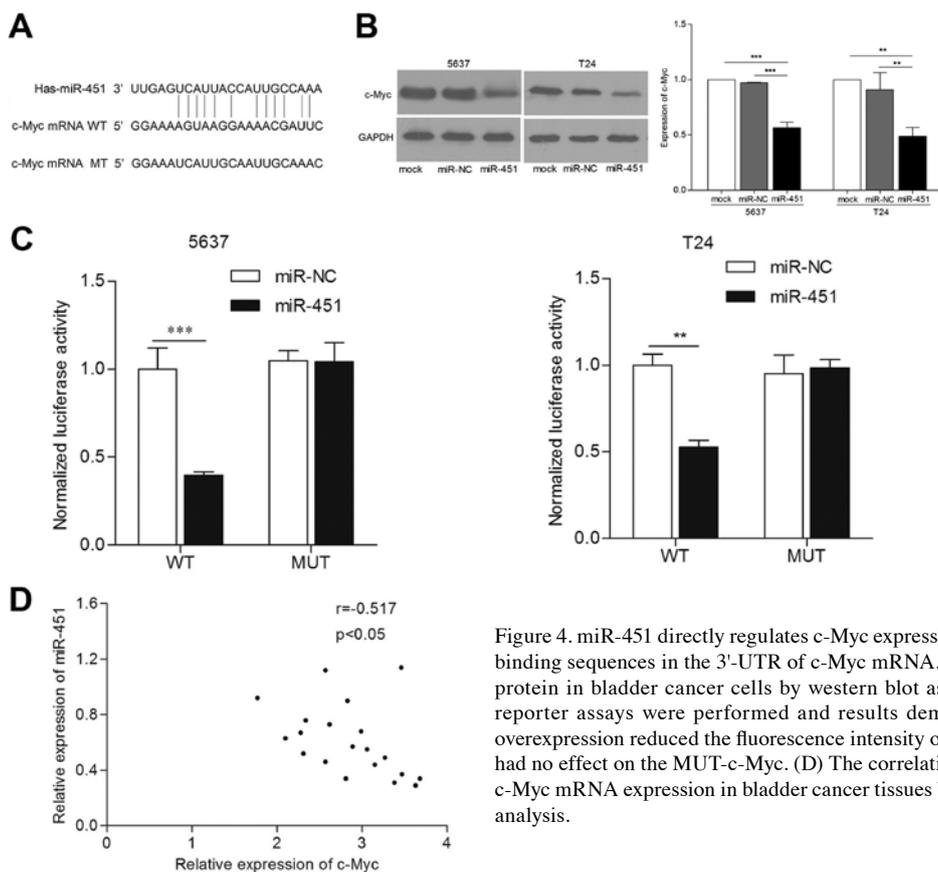


Figure 4. miR-451 directly regulates c-Myc expression. (A) Putative miR-451 binding sequences in the 3'-UTR of c-Myc mRNA. (B) Expression of c-Myc protein in bladder cancer cells by western blot assay. (C) Dual luciferase reporter assays were performed and results demonstrated that miR-451 overexpression reduced the fluorescence intensity on the WT-c-Myc, while it had no effect on the MUT-c-Myc. (D) The correlation between miR-451 and c-Myc mRNA expression in bladder cancer tissues by spearman's correlation analysis.

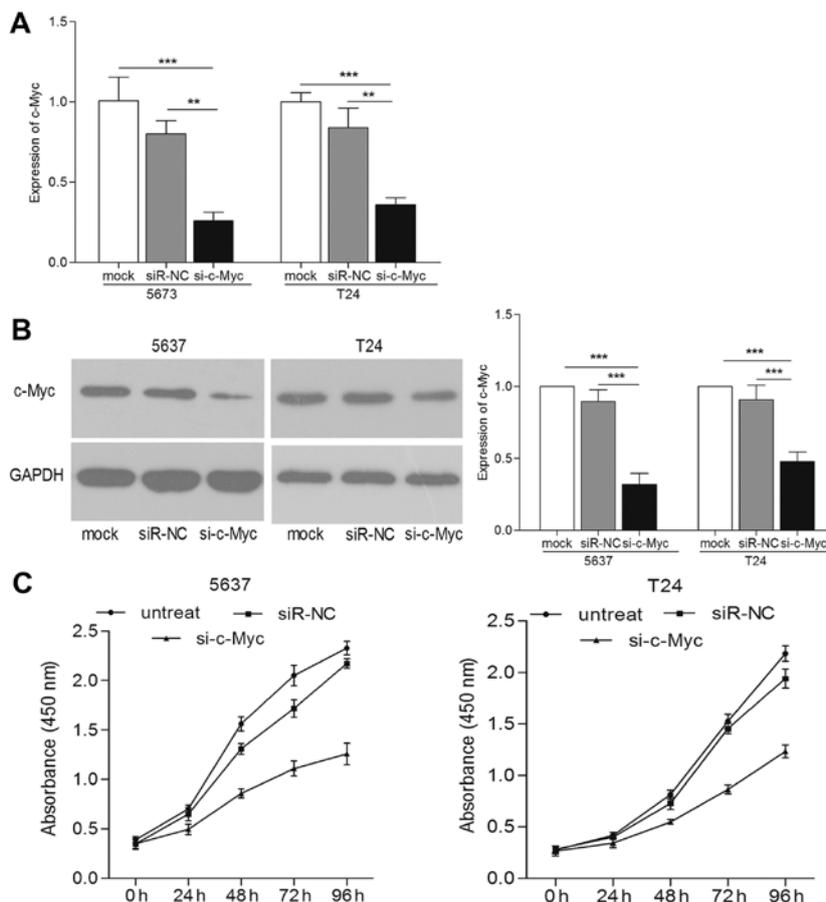


Figure 5. c-Myc knockdown regulates bladder cancer cell proliferation. Expression level of c-Myc mRNA (A) and protein (B) was conducted by RT-PCR and western blot assays. (C) Cell proliferation assay was demonstrated in si-c-Myc transfected and control cells.

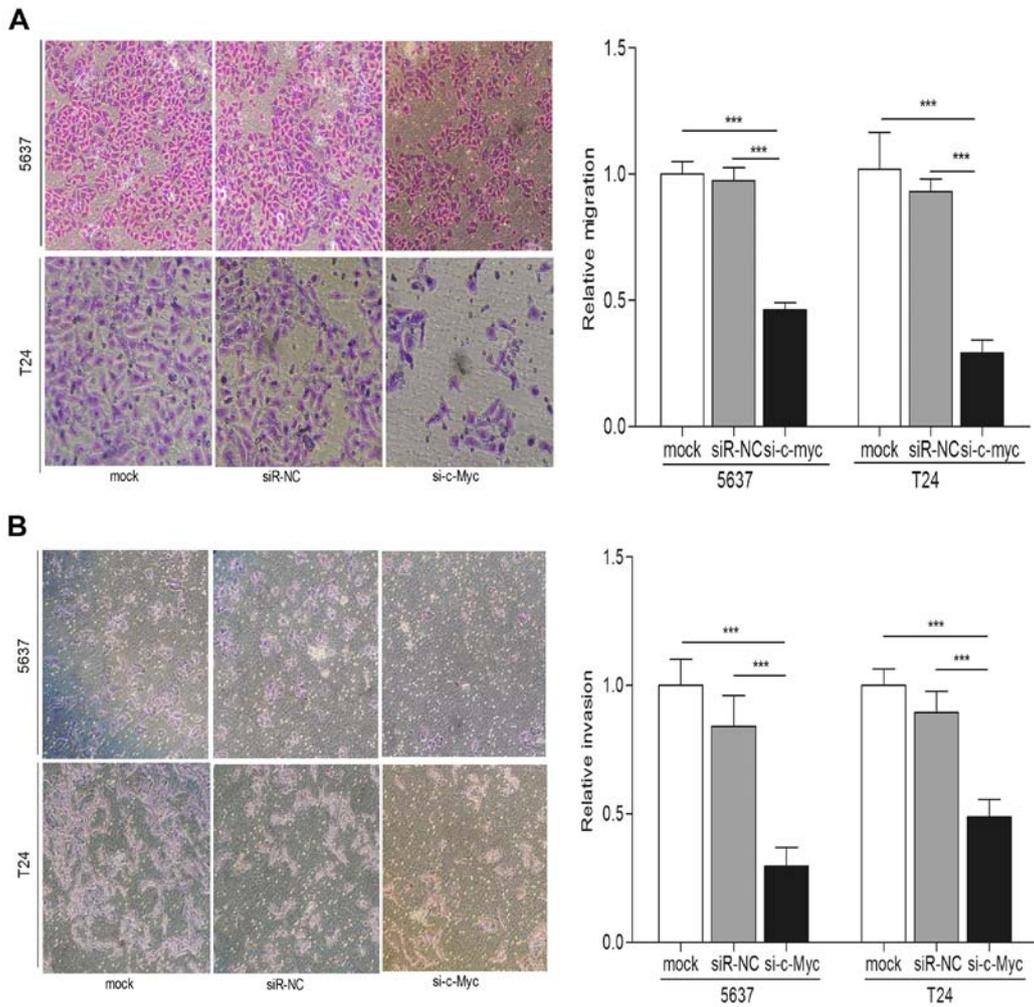


Figure 6. c-Myc knockdown inhibits cell migration and invasion. (A) Cell migration was detected using the Transwell migration assay. (B) Cell invasion ability was tested by Transwell invasion assay.

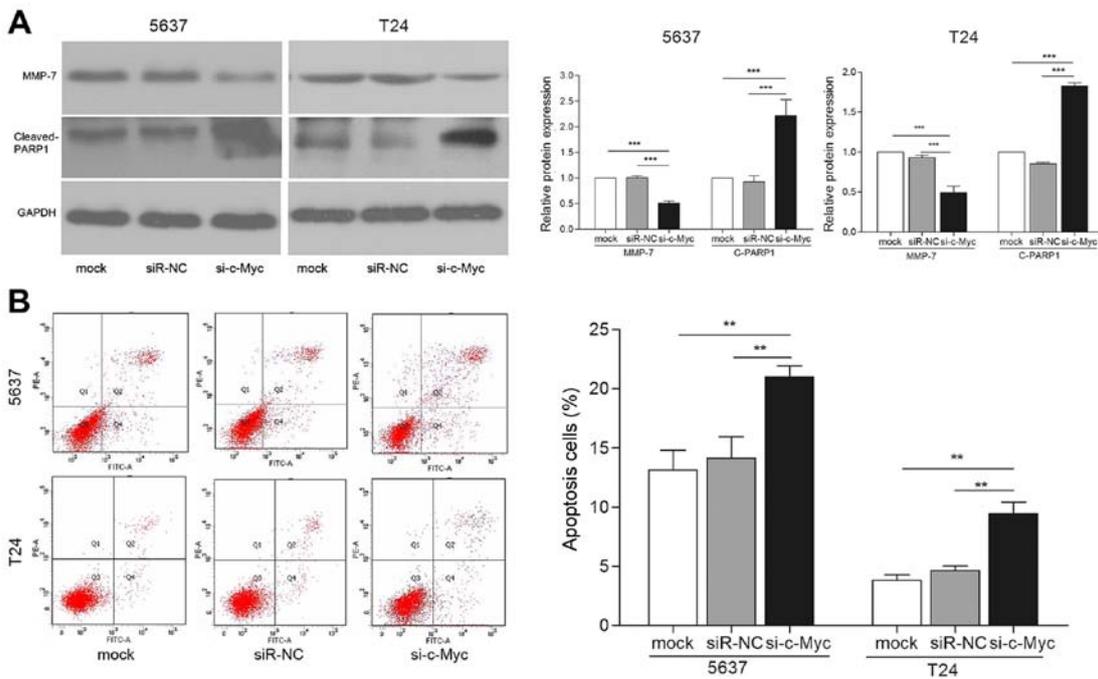


Figure 7. c-Myc knockdown promotes bladder cancer cell apoptosis. (A) Western blot assay detected protein level of MMP-7 and cleaved PARP1 level in bladder cancer cells transfected with si-c-Myc and controls. (B) Flow cytometric assay show percentage of apoptotic cells in 5637 and T24 cells.

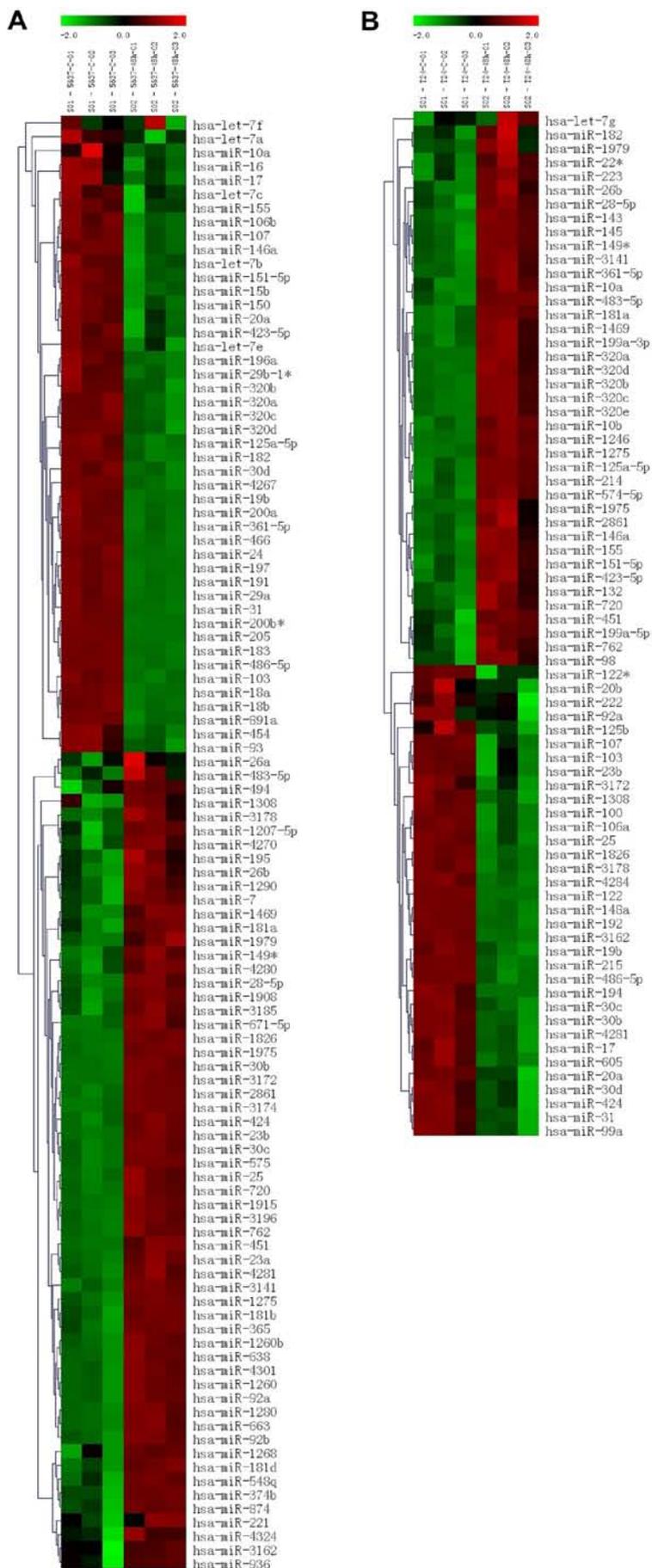


Figure 8. Heatmap illustrating expression of miRNAs in response to arsenic trioxide in bladder cancer cells, miRNAs from each time point were labeled with Cy5. (A and B) Heatmap of 5637 and T24 cells after arsenic trioxide treatment by miRNA microarray analysis, 5637-c-01,02,03 and T24-c-01,02,03 represent result of untreated 5637 cells, 5637-48h-01,02,03 and T24-48h-01,02,03 represent results of arsenic trioxide treated 5637 cells.

with si-c-Myc (Fig. 7A). Furthermore, cells transfected with si-c-Myc showed increased apoptosis (Fig. 7A and B). Taken together, these effects induced by knockdown of c-Myc are consistent with miR-451 overexpression in bladder cancer cells. Thus, we concluded that c-Myc may be a downstream functional regulator of miR-451.

Discussion

It is well known that miRNAs are pivotal players in the highly complex world of gene regulation; they regulate over 30% of protein-coding genes, play a critical role in normal cellular processes such as proliferation, apoptosis, migration, invasion and metabolism (20). In recent years, numerous studies found that dysregulation of miRNAs serve as modulator and lead to the development of human cancers such as bladder cancer (21). Various miRNAs (e.g., miR-137, miR-424 and miR-195/497) present differential expression in bladder cancer. In addition, they also functionally regulate biological behavior of bladder cancer (6,22,23). Altered expression of miR-451 has been reported in some types of cancers and involved in cancer progression. For example, the expression of miR-451 in glioblastoma tissues is higher than that in matched normal adjacent brain tissue, and miR-451 suppressed the migration of glioblastoma cells by CAB39 (14). Using a T cell acute lymphoblastic leukemia xenograft model, it was observed that tumor development was delayed in miR-451-treated mice (24). Kovalchuk *et al* showed that increased miR-451 expression level enhanced sensitivity of resistant MCF-7 cells to doxorubicin (25). These results suggest that miR-451 may be a critical regulator in neoplasm formation and progress. Our preliminary study found that miR-451 expression was significantly upregulated in arsenic trioxide treatment in bladder cancer cells (Fig. 8). Therefore, the relation between miR-451 with development and progress of bladder cancer was evaluated. In the present study, we found miR-451 was downregulated in bladder cancer tissues compared with adjacent non-tumor tissues. This result is consistent with the result of Zeng *et al* (26). Furthermore, miR-451 was proved negatively correlated with cancer grade, miR-451 in high grade of bladder cancer was markedly lower. Then, we focused on and explored potential function of miR-451 by restored expression of miR-451 in bladder cancer cell lines. The results revealed that overexpressed miR-451 markedly inhibited proliferation, migration and invasion of bladder cancer cells. These results predicted that miR-451 may act as an anti-oncogene and regulate bladder cancer formation and progression.

Apoptosis is the common form of programmed cell death, as an important mechanism that negatively regulates cancer development. Mehlen *et al* found that apoptosis plays a key role in inhibiting process of cancer metastasis (18). MMPs are a class of critical protein usually involved in tumor cell invasion and migration (27). However, evidence also showed that MMPs play a role in the regulation of cell apoptosis. The ability of tumor cells to resist apoptosis was induced by MMP-7 in early tumor development though cleaving CD95 (28). Abraham *et al* found that MMP-15 knockdown led to an increase of the cancer cell apoptosis (29). Based on this, we detected whether miR-451 attenuate bladder cancer migra-

tion and invasion through activation of apoptosis. Our results showed that miR-451 overexpression induced cells apoptosis, increased expression of cleaved PARP and decreased the level of MMP-7, which are associated with tumor apoptosis and metastasis. These results indicated that overexpression of miR-451 promoted apoptosis, which may represent a way for inhibiting migration and invasion of bladder cancer.

The region of 5'-end of miRNA can perfectly or near-perfectly bind complementary sequences with 3'-UTR of mRNA. Such binding induced target mRNA silencing or translation-repressed at post-transcriptional level. A dysfunctional miRNA-guided mRNA silencing pathway would lead to altered expression of target genes involved in cellular processes of tumorigenesis (30). Every miRNA has a large amount of downstream target genes, each target gene is also modulated by numerous miRNAs. Arduous function of miRNAs in cancer not only correlated with the tumor type, but also associated with diverse target genes. Therefore, exploration of more target genes of miRNAs may help us further understand the mechanisms of miRNA in cancer development. Several target genes of miR-451 have been found in certain cancers in previous research, such as ESDN/DCBLD2 (10), PSMB8 (11) and CAB39 (14). In the present study, c-Myc was predicted as a direct target gene of miR-451 by bioinformatics analysis, and then identified in bladder cancer cells using RT-PCR, western blot and luciferase reporter assays.

c-Myc, a central gene in mammalian cells, is a member of the family of Myc genes involved in diverse physiological processes, embryonic development, cell cycle, cell proliferation, apoptosis and protein synthesis (31). In normal cells, expression and function of c-Myc are regulated by developmental or mutagenic signals. Thus, the mRNA and protein levels of c-Myc are low, c-Myc is one of the most highly amplified oncogenes among many different human cancers such as colon cancer, T-cell leukemia, lung and bladder cancer (32). It has been reported that altered expression of c-Myc is induced by upstream signal pathways (33). In addition, various studies found miRNAs may be upstream regulators of c-Myc by targeting its mRNA, then inhibiting its translation (34,35). In addition, miRNA has been proved to be able to regulate chemoresistance of cancer cells by inhibiting c-Myc (36,37). The present study confirmed that c-Myc was directly targeted by miR-451 in bladder cancer. Overexpression of miR-451 inhibited mRNA and protein of c-Myc. The results of luciferase reporter assay demonstrated that c-Myc had a binding site with miR-451 at its 3'-UTR. The expression of c-Myc is reduced in miR-451 overexpressed cells. Further study showed that c-Myc knockdown could repress bladder cancer cell proliferation, migration and invasion indicating that the reduced c-Myc expression exert similar effects of miR-451 overexpression in bladder cancer cells. Taken together, these findings may suggest that miR-451 suppresses the biologic activity of bladder cancer by negatively regulating c-Myc.

In conclusion, our findings provide evidence that miR-451 is downregulated in bladder cancer tissues and is involved in multiple cellular biological behaviors. We also confirmed a mechanism that miR-451 reduced migration and invasion of bladder cancer cells probably by inducing apoptosis. Furthermore, miR-451 is a tumor-suppressor by directly inhibiting c-Myc expression in bladder cancer. These data

enrich our knowledge of miR-451 that plays a significant role in bladder cancer formation and progression. It may provide novel therapeutic targets for bladder cancer at gene level.

Acknowledgements

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