MiR-15 suppressed the progression of bladder cancer by targeting BMI1 oncogene via PI3K/AKT signaling pathway

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Abstract. – OBJECTIVE: To investigate the role of microRNA-15 (miR-15) in the progression of bladder cancer (BC) cell and its underlying mechanism.

PATIENTS AND METHODS: Human BC specimens were collected from BC patients during operations. BC cell lines (T24, BIU87, and HT1376) and normal uroepithelial cell lines SV-HUV-1 were cultured. The abilities of cell proliferation and invasion were detected by Methyl thiazolyl tetrazolium (MTT) and transwell assay, respectively. Additionally, the relevant mRNA and protein expressions were measured by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), Western blot and immunohistochemistry, respectively. Furthermore, the luciferase reporter assay was used to verify the target gene of miR-15. Besides, Xenograft tumor formation assay was performed to confirm the effect of miR-15 on tumor growth.

RESULTS: A low expression of miR-15 was detected by qRT-PCR, whereas the high expression of B cell-specific Moloney murine leukemia virus integration site 1 (BMI1) was detected by immunocytochemical assay in BC tissues. Moreover, miR-15 expression and BMI1 expression were significantly associated with the overall survival of BC patients. MTT and transwell assay results stated that the up-regulation of miR-15 inhibited BC cell proliferation, migration, and invasion. BMI-1 was verified as a direct target of miR-15 in BC using Luciferase reporter assay. Besides, miR-15 regulated epithelial-mesenchymal transition (EMT)-related markers, protein kinase B (AKT), and the phosphorylation of AKT protein levels in BC using the Western blot assay. Xenograft tumor formation assay indicated that the over-expression of miR-15 inhibited the tumor growth.

CONCLUSIONS: We stated that miR-15 suppressed BC cell progression by targeting BMI1 through the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway, which provided a potential target for BC treatment.

Key Words: MiR-15, Bladder cancer, Progression, BMI1, PI3K/AKT.

Introduction

Bladder cancer (BC) is one of the most common malignant tumors in the urinary system and causes more than 130,000 deaths a year1-3. There are two types of BC: muscle-invasive BC and non-muscle invasive BC. The survival rate of muscle-invasive BC patients in the last 5 years is about 60%, while that of non-muscle-invasive BC patients is 90%4. The causes of BC are complex and two of the most significant risk factors are poisoning and smoking. Although several treatments, including radiotherapy, surgery, and chemotherapy, are available, the annual incidence and mortality rate of BC disease has been increasing5. Therefore, it is necessary to explore the molecular mechanism in the development and progression of BC. At present, increasing evidence displayed that microRNAs (miRNA) play important roles in the pathogenesis of BC, thus providing a new opportunity to treat BC.

MiRNAs, a kind of endogenous 19 to 22-nucleotide non-coding RNA, were found in eukaryotic organisms. They regulated the protein expression by degrading or weakening the target mRNAs translation. MiRNA was reported to regulate tumors development, cell proliferation, migration, invasion or apoptosis via different molecular mechanisms in various cancers6-8, including miR-15. MiR-15 was down-regulated in prostate cancer and inhibited cell invasion by inhibiting transforming growth factor-β (TGF-β) signaling pathways9. Wang et al10 provided evidence that miR-15 suppressed BC cell progression by targeting BMI1 via PI3K/AKT signaling pathway, which provided a potential target for BC treatment.

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inhibited cell growth of endometrial cancer via Wnt signaling pathway by targeting Wnt3a. Moreover, it was reported by Mou et al\textsuperscript{11} that up-regulation of miR-15 inhibited laryngeal cancer cell proliferation and enhanced cell apoptosis via bcl-2 and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT). Furthermore, low expression of miR-15 in BC was founded by Pekarsky et al\textsuperscript{12}. However, the details of miR-15 role on BC cell progression and its potential mechanism has not been clarified to date. It is well known that the role of epithelial-mesenchymal transition (EMT) in cancer cells metastasis is very important and it is a critical biological progress for the ability to obtain migration and invasion of malignant tumor cells\textsuperscript{13,14}. PI3K/AKT signaling was reported to participate in tumor cell growth, proliferation, and survival\textsuperscript{15}. Thus, it is very important to understand how miR-15 regulates BC cell progression.

B cell-specific Moloney murine leukemia virus integration site 1 (BMI1), the polycomb ring finger protein, is up-regulated in most cancers and associated with poor prognosis\textsuperscript{16-18}. So far, there have been limited researches on the role of BMI1 in BC. Qin et al\textsuperscript{19} concluded that in addition to endometrial cancer, prostate cancer, and breast cancer, BMI1 was also up-regulated in bladder cancer. For instance, BMI1 acted as a target of miR-139 in regulating BC cell proliferation and self-renewal\textsuperscript{20}. MiR-218 and miR-200c, who was shown to target BMI1, inhibited BC cell progression\textsuperscript{21,22}. Moreover, the down-regulation of BMI1 inhibited breast cancer cell proliferation and invasion as a target of miR-15\textsuperscript{23}. Nevertheless, whether BMI1 is a target of miR-15 in regulating the progression of BC has not been reported.

We investigated miR-15\textsuperscript{*} role in BC cell proliferation, invasion, and metastasis and its molecular mechanism in BC. Our results showed that miR-15 played an inhibitory effect on cell proliferation, invasion, and migration. Moreover, BMI1 was a specific target of miR-15 and miR-15 inhibited BC cell proliferation, invasion, and migration via regulating BMI1 through the PI3K/AKT signaling pathway. These results indicated that miR-15/BMI1/PI3K/AKT signaling pathway might provide a critical target for BC therapy.

**Patients and Methods**

**Tissue Samples**

Human BC specimens were collected from BC patients during operations. All patients recruited in this study were untreated before surgery and should sign informed consent before sample collection. This research was approved by the Ethics Committee of The Second Hospital of Shandong University. Specimens were instantly put into liquid nitrogen when the tumor tissues were removed and then stored in –80°C refrigerator for mRNA and protein detection.

**Cell Culture and Cell Transfection**

T24, BIU87, HT1376 BC cell lines, and normal uroepithelial cell lines SV-HUV-1 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, Rockville, MD, USA) with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL) (Solarbio, Beijing, China), which was incubated at 37°C under 5% CO\textsubscript{2} atmosphere. The Lipofectamine 2000™ reagent (Invitrogen, Carlsbad, CA, USA) was applied for transfecting miR-15 mimic or inhibitor into BC cells. The transfection procedures were performed with the help of the manufacturer’s instructions.

**Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)**

QRT-PCR was used for detecting mRNA expression. Total RNAs were extracted from tissues or cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. Bulge-Loop miRNA-specific RT primers (Ribobio, Guangzhou, China) or random primers with M-MLV reverse transcriptase (Promega, Madison, WI, USA) were used for reverse transcribed for miR-15 or BMI1. ABI 7900 sequence detection system (Thermo Fisher Scientific, Waltham, MA, USA) was used to perform qRT-PCR reactions. All reactions were performed three times. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as an internal control. The primers were designed as follows: miR-15: 5’-TAGCACACATAATGGTTGTG-3’ (forward) and 5’-GTCGTATCCAGTGCAGGGTCCGAGGT-3’ (reverse); BMI1: 5’-TGGACTGACAAATGCTGGAGA-3’ (forward) and 5’-GAAGATTGGTTGGTTCCGGCTG-3’ (reverse); U6: 5’-CTCGCTTCGGCAGCACATATACT-3’ (forward) and 5’-ACGCTTACGAATTGCGTGTC-3’ (reverse); GAPDH: 5’-ATGGGGGAAGGTGAAGGTCG-3’ (forward) and 5’-GGGGTCATTGATGGCAAATA-3’ (reverse). The expression level was analyzed using the 2-ΔΔCq method.
Western Blot Assay
Radioimmunoprecipitation assay (RIPA) lysis buffer was used to extract total protein from BC cells. Bicinchoninic acid (BCA) reagent kit (Beyotime, Shanghai, China) was used to measure protein concentration. 50 μg total proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to NC membrane (Millipore, Billerica, MA, USA). After blocking with skim milk (5-10%) for 2 h at room temperature, the membranes were incubated with the primary antibodies (BMI1, 1:1000, Abcam; GADPH, 1:2000, Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight, subsequently the secondary antibodies for 2 h at room temperature. Finally, the enhanced chemiluminescence kit (ECL, Millipore, Billerica, MA, USA) was used to detect the signals. GAPDH served as a loading control.

Methyl Thiazolyl Tetrazolium (MTT) Assay
Cell proliferation was detected by MTT (Sigma-Aldrich, St. Louis, MO, USA) assay. The cells (5×10³/well) were added into 96-well plates and cultured for 48 h at 37°C with 5% CO₂. Then, we added the MTT medium (20 μL) to each well. After incubation at 37°C for another 4 h, the MTT medium was sucked out and 100 μL of dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was then added and incubated for additional 10 min, the plates were then read at a wavelength of 490 nm at 24, 48, 72, and 96 h to measure the absorbance of each well.

Transwell Assay
Cell invasion and migration were examined by transwell assay. For migration, the upper and lower chambers were divided by the transwell chamber with the 8 μm pore size polycarbonate inserted. 2×10⁴ BC cells were grown in the upper chambers with serum-free DMEM and the lower chambers were filled with DMEM containing 10% FBS. Once incubated at 37°C for 48 h, the cells in the upper chambers were migrated to the lower side. The non-migrated cells were removed by a cotton swab. Then, the cells were fixed and stained. The microscope was applied for photographing the migrated cells. For invasion assay, the Matrigel chambers were created by coating with Matrigel. Other experimental steps were similar to the migration assay.

Dual Luciferase Reporter Assay
The recombinant pMIR-reporter luciferase vector was applied for luciferase assay. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was carried out to con-transfected miR-15 mimic with pMIR-reporter luciferase vector containing the 3’-untranslated region (3’-UTR) of wild or mutant BMI1 into T24 cells. Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) was then used to measure the luciferase activity.

Immunohistochemistry
Immunohistochemistry was applied for measuring BMI1 protein density. Briefly, 4% paraformaldehyde was used to fix the BC tissues for 12 h. Then, paraffin section sections (8 μm) were incubated with 3% H₂O₂ in phosphate-buffered saline (PBS). After blocking with 5% goat serum at temperature for 2 h, the primary antibody anti-BMI1 (Cell Signaling Technology, Danvers, MA, USA) was added and incubated for 24 h at 4°C. Next, the sections were incubated with biotinylated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at 37°C. Subsequently, the sections were stained using the diaminobenzidine (DAB) mixture (Solarbio, Beijing, China), followed by dehydrated using a graded alcohol series, clear using xylene and cover slipped using neutral balsam. Finally, the protein density of per section was determined by the Image Pros Plus 5.0 software (Silver Springs, MD, USA).

Xenograft Tumor Formation Assay
The nude mice (3-5 weeks old) were purchased from Shanghai Lab Animal Research Center (Shanghai, China). The animal experiments were approved by the Animal Ethics Committee of Shandong University and conducted with the help of the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. T24 cells (1×10⁶) treated with pre-miR-15 plasmid were injected into the right flank of nude mice. The tumor volume was observed every 3 days. After 4 weeks, CO₂ asphyxiation was used to sacrifice the mice and the tumors were used for further study.

Statistical Analysis
All experiments were repeated in triplicate. Statistical Product and Service Solutions (SPSS) v.19.0 software (IBM, Armonk, NY, USA) was used to perform statistical analyses and GraphPad Prism 5.02 Software (La Jolla, CA, USA).
was used to complete graph presentations. Results are represented as the mean ± SD (standard deviation), and the differences between two groups were evaluated using Student’s $t$-test in SPSS. Comparison between multiple groups was done using One-way analysis of variance (ANOVA) test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ were considered as statistically significant.

### Results

**The Effect of MiR-15 on the Survival of BC Patients**

To explore the relationship between miR-15 and the survival rate of BC patients, we first used RT-PCR to detect miR-15 expression in BC tissues. As shown in Figure 1A, miR-15 mRNA expression in BC tissues was significantly lower than normal tissues. Moreover, we also found that the down-regulation of miR-15 was correlated with tumor size ($p=0.037$) and clinical stage ($p=0.049$) as shown in Table I. Furthermore, Kaplan-Meier survival curve stated that the lower the miR-15 expression, the lower the survival rate of BC patients, whereas the higher the miR-15 expression, the higher the survival rate of BC patients ($p=0.0128$, Figure 1B). Thus, we concluded that miR-15 might predict the prognosis of BC patient.

**The Inhibitory Effect of MiR-15 on BC Cell Progression**

To examine the role of miR-15 in the proliferation, invasion, and migration of BC, we first

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Statistical analyses were performed by the $\chi^2$-test. *$p < 0.05$ was considered significant.
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measured miR-15 expression in three BC cell lines and normal SV-HUV-1 cells. As Figure 2A shown, miR-15 expresional level was decreased in both BC cell lines compared with normal cells. We then detected miR-15 expression in T24 cells after transfection with miR-15 mimic or inhibitor and the results showed that the transfection was very successful (Figure 2B). MTT results stated that miR-15 mimic inhibited BC cell proliferation, while miR-15 inhibitor enhanced BC cell proliferation (Figures 2C-2D). Transwell assay showed that increasing the expresional level of miR-15 suppressed the migration and invasion of BC cells, whereas decreasing miR-15 promoted BC cell migration and invasion (Figures 2E-2F). The results concluded that the effect of miR-15 on BC cell progression was suppressive.

**Figure 2.** The suppression effect of miR-15 examined on BC cell proliferation, migration and invasion. A, MiR-15 expression was tested in BC cell lines by qRT-PCR. B, MiR-15 expression was examined in T24 cells after treated with miR-15 mimic or inhibitor by qRT-PCR. C-D, BC cells viability was measured after treated with miR-15 mimic or inhibitor by MTT assay. E-F, BC cell migration and invasion were measured after treated with miR-15 mimic or inhibitor by transwell assay (magnification × 40). **p<0.01.

**The Effect of MiR-15 on Tumor Growth In Vivo**

To explore the miR-15 effect on tumor growth, the nude mice were injected with miR-15 plasmid or miR-NC treated T24 cells subcutaneously. We found that re-expression of miR-15 significantly declined the tumor volume contrast to the control group (Figure 3A). Moreover, the tumors with miR-15 plasmid grew more slowly than that with miR-NC (Figure 3B). These findings suggested that miR-15 suppressed BC tumor growth in vivo.

**BMI1, the Target of MiR-15 in BC Cells**

To inquire into the target of miR-15 in BC cells, TargetScanHuman was firstly applied for predicting the candidate gene of miR-15. Figure 4A shows that miR-15 and BMI1 have the binding sites. Luciferase reporter assay was then used to
verify this prediction in T24 cells. The luciferase activity in miR-15 mimic group was significantly reduced contrast to control group in wild-type as expected, while there was no significant difference in mut-type (Figure 4B). Subsequently, BMI1 expression level in T24 cells after re-expression or knockdown of miR-15 was examined by RT-PCR. As shown in Figure 4C, results showed that BMI1 expression was markedly decreased in miR-15 mimic group, and miR-15 inhibitor enhanced BMI1 expression significantly (Figure 4C). Finally, we detected the relationship between miR-15 and BMI1. Results showed that miR-15 and BMI1 were negatively correlated (Figure 4D).

**The Effect of BMI1 on the Survival of BC Patients**

We explored the effect of BMI1 on the survival of BC patients. The results of IHC showed that the positive BMI1 protein was detected in the...
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nucleus of BC tissues (Figure 5A). Moreover, the protein intensity of BMI1 was markedly increased in BC tissues compared with the adjacent normal tissues (Figure 5B). In addition, the Kaplan-Meier survival curve stated that the lower the BMI1 expression, the higher the survival rate of BC patients, whereas the higher the BMI1 expression, the lower the survival rate of BC patients \( (p=0.0285, \text{Figure 5C}) \). Thus, we concluded that BMI1 was associated with the prognosis of BC patients.

**PI3K/AKT Signaling Pathway Was Involved in the Progression of BC Regulated by MiR-15**

To delve into the precise molecular mechanism of miR-15 in BC, we test EMT-related markers \( (\text{N-cadherin, Vimentin, and E-cadherin}) \) in T24 cells. As we expected, re-expression of miR-15 inhibited N-cadherin and Vimentin expression while increased E-cadherin expression. Inversely, silencing miR-15 enhanced N-cadherin and Vimentin expression and suppressed E-cadherin expression (Figure 6A). Therefore, we concluded that miR-15 regulated cell invasion and migration by regulating EMT. In addition, we detected AKT protein expression in T24 cells after treated with miR-15 mimic or inhibitor to further explore the underlying mechanism of miR-15 on cell proliferation. We found that increasing miR-15 repressed the phosphorylation of AKT expression remarkably. On the contrary, decreasing miR-15 enhanced the phosphorylation of AKT expression level (Figure 6B). Taken together, miR-15 was examined to regulate EMT and AKT pathway in BC progression.

![Figure 5](image)

**Figure 5.** Higher expression of BMI1 in BC tissues. A-B, BMI1 protein expression was measured in BC tissues by immunohistochemistry (magnification × 40). C, MiR-15 expression and overall survival (OS) in BC patients were negatively correlated. **\( p<0.01 \).**
Discussion

It is well known that BC is one of the most common cancers in all urological tumors and its incidence is rising every year. Therefore, looking for an accurate mechanism may provide a research idea for the treatment of BC. In our work, we found that miR-15 expression was decreased in BC cells and over-expression of miR-15 inhibited BC cell progression via PI3K/AKT signaling pathway by targeting BMI1. Our study provided an important treatment strategy for BC.

MiR-15 has been shown to play an important role in the development of various tumors. Zidan et al. reported that miR-15 was down-regulated in prostate cancer tissues and acted as a suppressor. Liu et al. suggested that miR-15 was reduced in colon cancer and up-regulation of miR-15 induced cell apoptosis. In our report, we clarified that miR-15 expression was lower in BC tissues and acted as a tumor suppressor in regulating the progression of BC, which is line with the previous studies that miR-15 was reduced in BC cells. We also found that re-expression of miR-15 inhibited BC cell progression while inhibiting miR-15 enhanced the progression of BC. In addition, we first identified BMI1 as a target of miR-15 in BC.

BMI1, widely expressed in different human cancers, played important roles in tumors progression, including breast cancer, colorectal cancer, and lung cancer. It has been stated that it acted as a prognostic marker in oral cancer and osteosarcoma. In our work, we stated that BMI1 was up-regulated in BC tissues and it up-regulation was related to the poor prognosis of BC patients, which in accordance with the previous study that BMI1 expression was up-regulated in bladder cancer. However, it is the first time that we found BMI1 acted as a specific target of miR-15 in regulating the development of BC. We also stated that increasing miR-15 inhibited EMT and PI3K/AKT pathway in regulating BC progression.

Conclusions

We demonstrated for the first time that miR-15 targeted BMI1 to inhibit BC tumorigenesis via PI3K/AKT signaling. MiR-15, therefore, represented a novel therapeutically relevant cellular target for the treatment of BC patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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