Biomedical Research 2018; 29 (3): 530-535 *ISSN 0970-938X*
www.biomedres.info

Significant role of *miR-663b* in bladder cancer and its potential mechanism.

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Abstract

Objective: The present study aimed to explore the role and underlying molecular mechanism of *miR-663b* in the progression of bladder cancer.
Materials and methods: The *miR-663b* expression in human bladder cancer tissues and cell lines was measured by qRT-PCR. We used TargetScan to predict the potential targets of *miR-663b*, and dual-luciferase reporter assay was applied to reveal our prediction. CCK-8 was used for cell proliferation detection, and cell apoptosis was analysed by flow cytometry. In addition, western blotting was carried out to detect the related protein expression.
Results: The findings suggested that *miR-663b* was up-regulated in both bladder cancer tissues and cell lines. *TUSC2* is a direct target of *miR-663b*, and is negatively regulated by *miR-663b*. *MiR-663b* down-regulation significantly reduced the proliferation ability of T24 cells, and T24 cell apoptosis was markedly induced. In addition, *miR-663b* down-regulation enhanced the expression level of p53 and p21 in T24 cells. Moreover, we found that the changes caused by *miR-663b* inhibitor in T24 cells were eliminated by *TUSC2* gene silencing.
Conclusions: Down-regulation of *miR-663b* prevented proliferation and induced apoptosis in bladder cancer cells by directly targeting *TUSC2*. Therefore, we provide a novel promising therapeutic target for bladder cancer treatment.

Keywords: Bladder cancer, *miR-663b*, *TUSC2*, Proliferation, Apoptosis.

Accepted on November 8, 2017

Introduction

Bladder cancer is one of the most common urological tumors. The incidence of bladder cancer ranks fourth in malignant tumors in the world, and a large number of new cases were diagnosed every year [1,2]. In China, bladder cancer is the most common malignant tumor of the urinary system, and more than 90% bladder cancer is transitional cell carcinoma with a high recurrence rate and invasive ability [3]. In recent years, the incidence of bladder cancer in some cities in China showed a steady upward trend. At present, the treatment methods of bladder cancer are mainly surgical resection, radiotherapy and chemotherapy, but the higher recurrence rate and tumor progression rate are still great impacts on the prognosis of bladder cancer patients. The radical cystectomy can effectively reduce the recurrence rate of bladder cancer patients, improve their long-term survival rate, is the standard treatment method of invasive bladder cancer [4]. However, due to postoperative permanent urinary diversion, lifelong wearing urine collection bag, and the presence of abdominal wall stoma, resulting in a series of changes in the body image, mental, psychological and social function of the patients, seriously affect the patients' quality of life [5,6]. Therefore, it is necessary to provide quality transitional care service [7] to ensure the smooth transition of bladder cancer patients from hospital to families, to improve the lack of professional guidance for home rehabilitation, to meet the needs of patients with health care, and to improve the quality of life and prognosis of the patients. At present, the transitional care service of China is still in its infancy, bladder cancer patients with lack of continuity and coordination of nursing activities. Therefore, the search for new and effective strategies for the treatment of bladder cancer is very urgent.

MicroRNAs (miRNAs), a family of small (20-22 nucleotides in length) non-coding single-stranded RNAs, play a critical role in post-transcriptionally regulating gene expression by binding to the 3' untranslated region of the target genes [8-10]. Studies have shown that miRNA abnormal expression is associated with the occurrence of many tumors, including bladder cancer [11-13]. At the same time, miRNAs can play as oncogene or tumor suppressor, and the correct intervention of abnormal expression of miRNA can inhibit tumorigenesis and progression [14]. With the status and role of miRNA in tumor research attracted more and more attention, research on miRNA and bladder cancer has also been developed and deepened.

Previous study found *miR-663b* was highly expressed in the plasma of bladder cancer patients, and *miR-663b* could be a promising novel circulating bio-markers in clinical detection of bladder cancer [15]. However, the expression of *miR-663b* in the tissues of bladder cancer patients and its specific role remain unclear. Therefore, the purpose of this study is to study the expression and specific role of *miR-663b* in bladder cancer, and further to explore the related mechanisms. We hope to provide new and effective targets and more theoretical basis for the treatment of bladder cancer.

Materials and Methods

Clinical samples

A total of 25 paired bladder cancer tissues and the adjacent normal tissues were obtained from the Beijing Luhe Hospital. Informed consent was obtained from every patient, and this study was approved by the Ethics Committee of Beijing Luhe Hospital.

Cell culture and cell transfection

Bladder cancer cell lines T24, 5637, J82, UMUC3, and a normal bladder cell line (SV-HUC-1) were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplement with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA) and 1% streptomycin-penicillin mix solution, and then incubated at 37°C with 5% CO_2.

The *miR-663b* inhibitor, its negative control (NC) was obtained from the GenScript (Nanjing). T24 cells were transfected with *miR-663b* inhibitor, its Negative Control (NC) or *miR-663b* inhibitor+*TUSC2*-siRNA by using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instruction. Cells without any treatment were used as the control. 48 h after cell transfection, following analysis were performed.

Dual luciferase reporter assay

We used bioinformatics software (TargetScan) to predict the potential targets of *miR-663b*, and *TUSC2* was found to be interacted with *miR-663b* at 3'UTR. To confirm our prediction, dual luciferase reporter assay was performed. Wild-type and mutant 3'-UTRs of *TUSC2* were amplified and then cloned into the psiCHECK-2 reporter. *MiR-663b* and *miR-663b-TUSC2*-WT 3'UTR or *miR-663b-TUSC2*-MUT 3'UTR vector were co-transfected into T24 cells by using lipofectamine 2000 per as the instructions supplied by the manufacturer. 48 h after the transfection, the luciferase activity was analysed by performing the dual-luciferase reporter assay system (Promega, USA).

Cell proliferation assay

48 h after transfection, cell proliferation assay was performed to detect cell proliferation ability. In brief, T24 cells were collected, re-suspended, and then re-plated into 96-well culture plates (Corning Costar, Corning, NY, USA). The cells were incubated for 24 h at 37°C with 5% CO_2. Subsequently, CCK-8 solution (10 μg/ml) was added to each well, and then incubated at 37°C for 2 h. Finally, the OD value at 450 nm was determined using a micro-plate reader (Thermo Labsystems, Waltham, MA, USA). Every test was repeated at least 3 times.

Apoptosis analysis assay

48 h after transfection, cell apoptosis analysis assay was carried out to analyse cell apoptosis. The transfected T24 cells were washed with PBS, fixed with 70% ethanol, labeled with annexin V-FITC and Propidium Iodide (PI), and then incubated at room temperature for 30 min. At the end of the test, flow cytometry (Becton Dickinson, New Jersey, USA) was applied to analyse cell apoptosis, and the rate of apoptotic cells was calculated. Tests were repeated at least three times.

Western blot analysis

48 h after cell transfection, western blotting was used to detect protein expression. RIPA buffer (Auragene, Changsha, China) was used to extract the total cellular proteins. The BCA protein quantitative kit (Thermo, USA) was carried out to detect protein concentration. Protein samples were separated by using 11% SDS-PAGE and then transferred to Polyvinylidene Fluoride (PVDF) membranes. After blocking with 5% skim milk at room temperature for 1 h, the membranes were then incubated with a primary antibody (anti-*TUSC2* 1:1000; anti-*p53* 1:1000; anti-*p21* 1:1000; anti-*β-actin* 1:1000; CST, MA, USA) at 4°C overnight. Subsequently, the membranes were washed with TBST solution for 3 times. At last, the membranes were incubated with a HRP-conjugated secondary antibody (Anti-rabbit IgG, HRP-linked Antibody; 1:5,000) at room temperature for 4 h. The protein bands were visualized using an ECL kit (Applygen, Beijing, China) per as the manufacturer's protocol.

QRT-PCR

Total RNA from T24 cells was abstracted by using TRIZOL reagent (Takara, Japan) following the manufacturer's instructions. A260/A280 ratio was calculated to assess the integrity and quality of the RNA. U6 (for miRNA) and GAPDH (for mRNA) were used as the internal control. *MiR-663b* was reversely transcribed into cDNA by using the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies, USA) according to the manufacturer's instructions. Real-time PCR was performed by using SYBR Premix Ex Taq (Takara, Japan) according to the manufacturer's instructions. Primer sequences for PCR were listed as following:

GAPDH-forward: 5'CTTTGGTATCGTGGAAGGACTC3'

GAPDH-reverse: 5'GTAGAGGCAGGGATGATGTTCT3'

U6-forward: 5'GCTTCGGCAGCACATATACTAAAAT3'

U6-reverse: 5'CGCTTCACGAATTTGCGTGTCAT3'

TUSC2-forward: 5'GGAGACAATCGTCACCAAGAAC3'

TUSC2-reverse: 5'TCACACCTCATAGAGGATCACAG3'

p53-forward: 5'CTGCCCTCAACAAGATGTTTTG3'

p53-reverse: 5'CTATCTGAGCAGCGCTCATGG3'

p21-forward: 5'ATGAAATTCACCCCCTTTCC3'

p21-reverse: 5'CCCTAGGCTGTGCTCACTTC3'

miR-663b-forward: 5'CATAATAAATAGGCGGGGCG3'

miR-663b-reverse: 5'CAGAGCAGGGTCCGAGGTA3'

The relative gene expression was determined by the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

SPSS 16.0 statistical software (SPSS, Chicago, IL, United States) was used for statistical analyses. Data are present as the mean ± SD. Student's t-test or ANOVA was performed to compare differences between groups. A value of $p<0.05$ was considered as a significant difference.

Results

MiR-663b is up-regulated in bladder cancer

To detect the level of *miR-663b* in bladder cancer tissues, bladder cancer cell lines (T24, 5637, J82, UMUC3), and the normal bladder cell line (SV-HUC-1), qRT-PCR was performed. We found that compared with the control, the level of *miR-663b* was significantly increased in bladder cancer tissues (Figure 1A). Meanwhile, the bladder cancer cell lines (T24, 5637, J82, UMUC3) showed a higher expression of *miR-663b* compared to the normal bladder cell line (SV-HUC-1). T24 cells showed a more obvious up-regulation (Figure 1B). These data indicated that *miR-663b* was up-regulated in bladder cancer. T24 cells were used for further analysis.

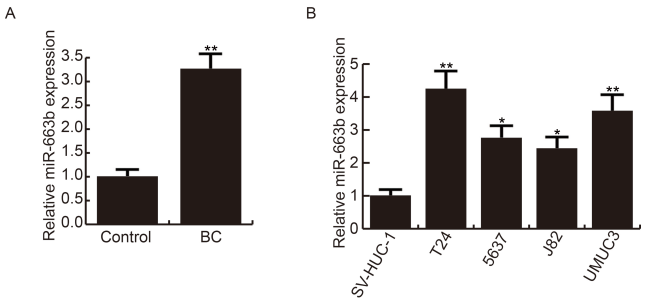

Figure 1. *miR-663b is up-regulated in bladder cancer. QRT-PCR was used to determine the miR-663b expression. A: relative miR-663b expression in bladder cancer tissues. BC: Bladder Cancer tissues; Control: the adjacent normal tissues. B: relative miR-663b expression in bladder cancer cell lines. SV-HUC-1: the normal bladder cell line; T24, 5637, J82, UMUC3: human bladder cancer cell lines. *, **p<0.05, 0.01 vs. control. Experiments were repeated 3 times.*

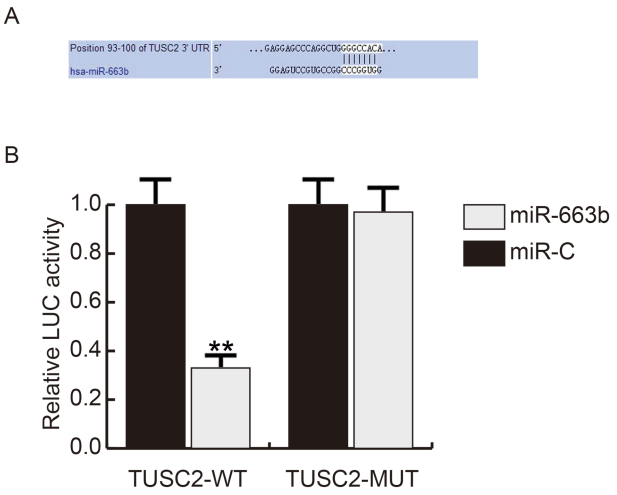

Figure 2. *TUSC2 is a direct target of miR-663b. A: Interaction between miR-663b and 3'UTR of TUSC2 was predicted using TargetScan; B: Luciferase activity of a reporter containing a wild-type TUSC2 3'UTR or a mutant TUSC2 3' UTR are presented. "TUSC2-MUT" indicates the TUSC2 3' UTR with a mutation in the miR-663b binding site. UTR: Untranslated Region. **p<0.01 vs. control. Experiments were repeated 3 times.*

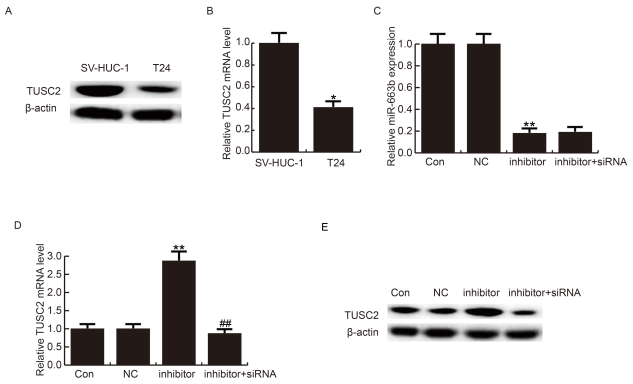

Figure 3. *Expression of TUSC2 in different groups. QRT-PCR was used to determine the miR-663b and mRNA expression, and Western blotting was used for protein level detection. A: protein level of TUSC2 in SV-HUC-1 and T24 cells; B: mRNA level of TUSC2 in SV-HUC-1 and T24 cells; C: relative miR-663b expression in different groups; D: protein level of TUSC2 in different groups; E: mRNA level of TUSC2 in different groups. Control: cells without any treatment; NC: cells transfected with the negative control of miR-663b inhibitor; inhibitor: cells transfected with miR-663b inhibitor; inhibitor +siRNA: cells co-transfected with miR-663b inhibitor and TUSC2-siRNA. All data are presented as the mean ± SD of three independent experiments. *, **p<0.05, 0.01 vs. control; ##p<0.01 vs. inhibitor.*

TUSC2 is a target of miR-663b

To study the exact role of *miR-663b* in bladder cancer, we used bioinformatics software (TargetScan) to predict the potential targets of *miR-663b*, and *TUSC2* was found to be interacted with *miR-663b* at 3'UTR (Figure 2A). The results of luciferase reporter gene assay confirmed our prediction (Figure 2B).

Furthermore, we detected the level of *TUSC2* in T24 cells, and the findings suggested that *TUSC2* was low expressed in T24

cells (Figures 3A and 3B). Then, to analyse the role of *miR-663b* in T24 cells, T24 cells were transfected with *miR-663b* inhibitor, its negative control, or *miR-663b* inhibitor +*TUSC2*-siRNA, and the transfection efficiency was evaluated using qRT-PCR (Figure 3C). As shown in Figure 3D and 3E, *TUSC2* was negatively regulated by *miR-663b* in T24 cells. *MiR-663b* inhibitor markedly enhanced the expression level of *TUSC2*, and this increase was eliminated by *TUSC2*-siRNA.

MiR-663b down-regulation inhibits T24 cell proliferation

To study the effect of *miR-663b* on T24 cell proliferation, cell proliferation ability was detected by using CCK-8 assay. As shown in Figure 4, *miR-663b* inhibitor significantly inhibited T24 cell proliferation, and this decline was eliminated by *TUSC2*-siRNA.

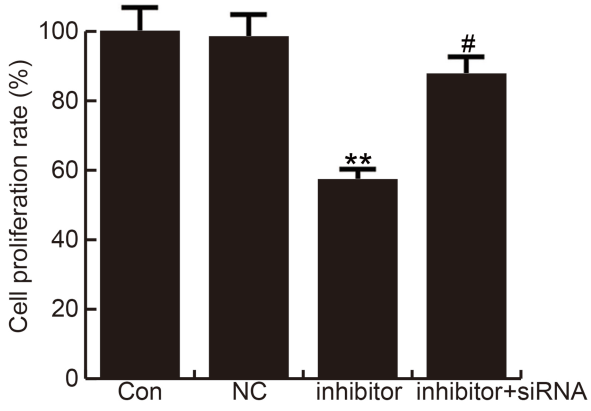

Figure 4. *MiR-663b down-regulation inhibits the proliferation of T24 cells. Cell proliferation was analysed by using CCK8 assay in the T24 cells. Control: cells without any treatment; NC: cells transfected with the negative control of miR-663b inhibitor; inhibitor: cells transfected with miR-663b inhibitor; inhibitor+siRNA: cells co-transfected with miR-663b inhibitor and TUSC2-siRNA. All data are presented as the mean ± SD of three independent experiments. $^{**}p<0.01$ vs. control; $^{\#}p<0.05$ vs. inhibitor.*

MiR-663b down-regulation induces T24 cell apoptosis

Effect of *miR-663b* on T24 cell apoptosis was analysed by using flow cytometry. The findings suggested that compared with the control, *miR-663b* inhibition significantly induced T24 cell apoptosis, and this increase was reversed by *TUSC2*-siRNA (Figure 5A). The cell apoptosis rate was calculated and presented (Figure 5B).

MiR-663b down-regulation enhances the expression of p53 and p21

To explore the extract underlying molecular mechanism of the role of *miR-663b* played in T24 cells, *p53* and *p21* expression was detected by using qRT-PCR and Western blotting respectively. As shown in Figure 6, compared with the control, the protein level of *p53* and *p21* was notably enhanced by *miR-663b* down-regulation, and the effect was eliminated by *TUSC2*-siRNA (Figure 6A). The same trend results were obtained from qRT-PCR (Figures 6B and 6C).

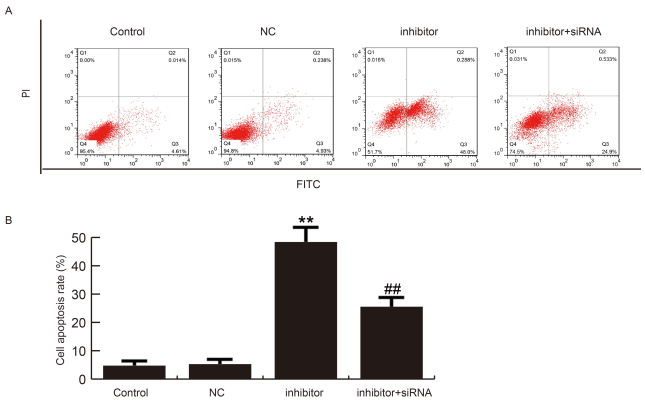

Figure 5. *MiR-663b down-regulation induces the apoptosis of T24 cells. Flow cytometry was used to detect the cell apoptosis (A), and data was analysed (B). Q1: dead cells; Q2: late apoptosis cells; Q3: early apoptosis cells; Q4: survival cells. Cell apoptosis=early apoptosis+late apoptosis. Control: cells without any treatment; NC: cells transfected with the negative control of miR-663b inhibitor; inhibitor: cells transfected with miR-663b inhibitor; inhibitor +siRNA: cells co-transfected with miR-663b inhibitor and TUSC2-siRNA. All data are presented as the mean ± SD of three independent experiments. $^{**}p<0.01$ vs. control; $^{\#\#}p<0.01$ vs. inhibitor.*

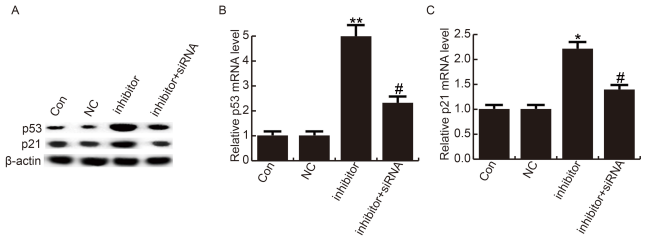

Figure 6. *p53 and p21 expression before and after miR-663b down-regulation. A: protein levels of p53 and p21 in T24 cells; B and C: relative mRNA levels of p53 and p21 in T24 cells. Control: cells without any treatment; NC: cells transfected with the negative control of miR-663b inhibitor; inhibitor: cells transfected with miR-663b inhibitor; inhibitor+siRNA: cells co-transfected with miR-663b inhibitor and TUSC2-siRNA. All data are presented as the mean ± SD of three independent experiments. *, **p<0.05, 0.01 vs. control; $^{\#}p<0.051$ vs. inhibitor.*

Discussion

Bladder cancer is one of the most common human malignant tumors, and its pathogenesis is complex, involving a lot of genes' expression, aberrant functions and changes in multiple signaling pathways. At present, the molecular genetics pf the development and progression of bladder cancer is still unclear. In recent years, the pathological relevance and significance of miRNAs in bladder cancer have attracted more and more attention. In the present study, we studied the expression, biological function and molecular mechanisms of *miR-663b* and its target gene in the pathogenesis of bladder cancer.

We found that *miR-663b* inhibition played an important role in repressing bladder cancer cell proliferation and inducing cell apoptosis. *MiR-663b* directly targeted *TUSC2* and negatively

regulated the expression of *TUSC2*. *MiR-663b* low-expression thus indirectly promoted the expression of *p53* and *p21*, which may contribute to the prevention of T24 cell proliferation and the induction of T24 cell apoptosis. First, we proved the higher expression of *miR-663b* in bladder cancer cell lines T24, 5637, J82, and UMUC3 compared with the normal bladder cell line (SV-HUC-1). And T24 cells showed a more obvious up-regulation. Therefore, in this study, T24 cell line was used for the investigation of bladder cancer *in vitro*. We next revealed a novel functional link between *miR-663b* and *TUSC2* in the progress of bladder cancer. Our results also found that *miR-663b* inhibitor significantly prevented the proliferation and increased apoptosis of T24 cells *in vitro*. *MiR-663b* inhibition significantly enhanced the expression level of *p53* and *p21*. Moreover, we found that the effects of *miR-663b* inhibitor on T24 cells could be eliminated by *TUSC2* siRNA. These results indicated that *miR-663b* served as an oncogene in the development of bladder cancer, and *miR-663b* inhibitor played a critical role in tumor prevention, thus, *miR-663b* may serve as a promising therapeutic target in bladder cancer treatment.

Various studies suggested that miRNAs play critical roles in the diagnosis, therapy and prognosis of a variety of cancers *via* participating in tumorigenesis, tumor growth and tumor metastasis [16,17]. Therefore, miRNAs may be promising treatment targets for cancer. Increasing evidence has indicated that many miRNAs are involved in bladder cancer cell proliferation, migration and invasion [18-22]. To date, the high expression of *miR-663b* in the plasma of bladder cancer patients has been revealed, however, to the best of our knowledge, the expression of *miR-663b* in the tissues of bladder cancer patients and its specific role remain unclear. Here, our results strongly demonstrated that *miR-663b* was significantly up-regulated in bladder cancer cell lines, and *miR-663b* inhibitor markedly repressed the proliferation, induced apoptosis of T24 cells.

Tumor suppressor candidate 2 (*TUSC2*), also known as *FUS1*, is a recognized tumor suppressor gene [23,24]. A variety of allele losses and genetic alterations are observed in various cancers, including breast cancer, lung cancer and so on [24-26]. However, no interaction between *miR-663b* and *TUSC2* in bladder cancer cells has been reported previously. In the present study, we revealed that *TUSC2* was a direct target of *miR-663b*.

In summary, we found for the first time that *miR-663b* was up-regulated in bladder cancer tissues and cell lines, and down-regulation of *miR-663b* prevented the proliferation of bladder cancer cells and induced cell apoptosis *via* directly targeting *TUSC2*. Therefore, *miR-663b* may serve as a promising therapeutic target for the treatment of bladder cancer.

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