MiR-155 affects proliferation and apoptosis of bladder cancer cells by regulating GSK-3β/β-catenin pathway


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Abstract. – OBJECTIVE: GSK-3β negatively regulates Wnt/β-catenin signaling pathway. The abnormal miR-155 expression is associated with bladder cancer. Bioinformatics analysis revealed a complementary binding site between miR-155 and GSK-3β mRNA. This study investigated the role of miR-155 in the proliferation and apoptosis of bladder cancer cells.

PATIENTS AND METHODS: The dual luciferase reporter gene assay validated the targeted regulation between miR-155 and GSK-3β. Tumor tissues and adjacent tissues were collected from bladder cancer patients and the expression of miR-155 and GSK-3β mRNA was detected by RT-PCR. Bladder cancer cell line BIU-87 cells were cultured in vitro and divided into miR-NC group and miR-155 inhibitor group. The expressions of miR-155, GSK-3β and β-catenin were compared, cell apoptosis was detected by flow cytometry, and cell proliferation was detected by EdU staining.

RESULTS: Compared with adjacent tissues, miR-155 expression was significantly increased in bladder cancer tissues, and GSK-3β mRNA expression was significantly decreased. There was a targeted regulatory relationship between miR-155 and GSK-3β. Compared with SV-HUC-1 cells, miR-155 expression in bladder cancer BIU-87 and 5637 cells was significantly increased, and GSK-3β expression was significantly decreased. Transfection of miR-155 inhibitor significantly increased GSK-3β expression in BIU-87 and 5637 cells, decreased β-catenin expression, increased cell apoptosis, and decreased cell proliferation.

CONCLUSIONS: The increased expression of miR-155 plays a role in reducing the expression of GSK-3β and in promoting the pathogenesis of bladder cancer. Inhibition of miR-155 can up-regulate the expression of GSK-3β, inhibit the activity of Wnt/β-catenin pathway, attenuate proliferation and promote apoptosis of bladder cancer cells.

Key Words: MiR-155, GSK-3β, Wnt/β-catenin, Bladder cancer.

Introduction

Bladder cancer (BC) is one of the common malignant tumors in the genitourinary system. It is one of the top ten malignant tumors in the world, and its incidence rate ranks 9th in systemic malignant tumors.

Glycogen synthase kinase-3β (GSK-3β) is an important negative regulator in Wnt/β-catenin signaling pathway. GSK-3β can phosphorylate β-catenin protein and degrade it, leading to its decreased expression in the cytoplasm, thereby blocking the activation of the Wnt/β-catenin pathway and playing an important role in tumor suppression in the development and progression of various tumors. Studies have shown that the decrease in GSK-3β expression is associated with the occurrence and progression of various tumors such as colorectal cancer and breast cancer, but there are few studies on its role in bladder cancer.

MicroRNAs are a class of endogenous non-coding single-stranded small RNAs with a length of approximately 22-25 nucleotides. They are important epigenetic regulatory molecules that can bind to the 3'-untranslated region (3'-UTR) region of target gene mRNA, leading to degradation of mRNA or inhibition of its translation, and is involved in the regulation of various biological effects such as cell proliferation, differentiation, cycle, and apoptosis. The abnormal expression of miR-155 plays an important role in the occurrence, progression, drug resistance and drug resistance of various tumors such as gastric cancer, breast cancer,
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Bioinformatics analysis revealed a complementary binding site between miR-155 and the 3’-UTR of GSK-3β mRNA. This study compared the expression of miR-155 and GSK-3β in tumor tissues and adjacent tissues of bladder cancer patients, and intervened miR-155 expression in bladder cancer cells cultured in vitro to explore whether miR-155 regulates GSK-3β expression, Wnt/β-catenin pathway activity, affects proliferation and apoptosis of bladder cancer cells.

**Patients and Methods**

**Patients**

Forty patients with bladder cancer who were treated in our hospital from April 2018 to November 2018 were enrolled. The specimens of the tumor tissue removed during the operation were collected, and the adjacent tissues at least 2 cm away from the bladder cancer tissue were collected as controls. The collection of clinical specimens was reviewed and approved by the Hospital Ethics Committee and informed consent was obtained from the patients.

**Main Reagents and Materials**

Normal human bladder epithelial SV-HUC-1 was purchased from Shanghai Sixin Bio; human bladder cancer cell lines BIU-87 and 5637 were purchased from Shanghai Fuheng Biological; DMEM, Opti-MEM, FBS, and streptomycin culture medium were purchased from Gibco (Rockville, MD, USA); Fluorescence Quantitative PCR Kit TransScript Green One-Step qRT-PCR SuperMix purchased from Beijing full-form gold bio; TRIZol, transfection reagent Lip 2000 purchased from Invitrogen (Carlsbad, CA, USA); miR-NC, miR-155 mimic, miR-155 inhibitor purchased in Guangzhou Ruibo Bio; EdU cell proliferation assay reagent was purchased from Sigma-Aldrich (St. Louis, MO, USA); rabbit anti-human GSK-3β, β-catenin, β-actin antibody was purchased from Abcam (Cambridge, MA, USA); HRP-conjugated secondary antibody was purchased from Wuhan Boster Bio; pMIR plasmid purchased in Changsha Youbao Bio; Dual-Luciferase Reporter Assay System purchased from Promega (Madison, WI, USA); BCA protein quantification kit was purchased from Jiangsu Biyuntian; FITC Annexin V/PI apoptosis detection reagent was purchased from Beijing Suo Labao Bio.

**Cell Culture**

SV-HUC-1, BIU-87, and 5637 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) medium containing 10% fetal bovine serum (FBS) and 1% streptomycin in a cell culture incubator containing 5% CO₂ at 37°C until cells reached a confluence of 85%. After that, cells were collected using 0.25% trypsin digestion, and sub-cultured at a ratio of 1:4. The experiment was performed when the cells were in a logarithmic growth phase.

**Dual Luciferase Reporter Gene Assay**

HEK293 cells were inoculated with 24-well plates, and after 24 h adherence, pMIR-GSK-3β-WT (or pMIR-GSK-3β-MUT) and miR-155 mimic (or miR-NC) were co-transfected with Lipofectamine 2000. In HEK293 cells, after 6 h, Opti-MEM was replaced with DMEM medium containing 10% fetal bovine serum (FBS), and culture was continued for 48 h. The activity of double luciferase was detected according to the kit instructions.

**Cell Transfection and Grouping**

BIU-87 cells were cultured in vitro and divided into two transfection groups: miR-NC transfection group and miR-155 inhibitor transfection group. The transfection step was as follows: 100 μL of Opti-MEM, 10 μL of Lip 2000 and 10 μL of the plasmid were gently mixed, and then incubated at room temperature for 20 min. When the density of the cells was close to 60%, the original culture solution was discarded, and the PBS was washed twice and added. The mixture was transfected. After 6 hours, the original medium was discarded and replaced with DMEM medium containing 10% FBS and 1% streptomycin. After 72 hours, the cells were collected for various tests.

**qRT-PCR Detection of Gene Expression**

Total RNA was extracted using TRIZol reagent, and the relative expression of the gene was detected by one-step qRT-PCR using TransScript Green One-Step qRT-PCR SuperMix in the 20 μL reaction system including: 1 μg of template RNA, 0.2 μM of pre-primer, 0.2 μM of post-primer, 10 μL of 2×TransStart Tip Green qPCR SuperMix, 0.4 μL of One-Step RT Enzyme Mix, 0.4 μL of Passive Reference Dye II, RNase-free water. The qRT-PCR reaction conditions were: 45°C, 5 min; 94°C, 30 s; (94°C,
5 s; 60°C, 30 s) × 40 cycles, and gene expression was detected on an ABI 7500 Real-time PCR instrument. Primer information was GSK-3β-F: 5'-GGCAGCATGAAATGTCAGA-3'; GSK-3β-R: 5'-GGCAGCATGAAATGTCAGA-3'; β-actin-F: 5'-CATGTACGTTGCTATCCAGGC-3', β-actin-R: 5'-CTCCTTAATGTCAAGCGACGAT-3'.

**Western Blot**

The cells of each transfaction group were collected by trypsinization, centrifuged at 300 g for 5 min, the supernatant was collect followed by addition of 100 μL RIPA lysate, and the protein supernatant was transferred to a new pre-cooled Eppendorf (EP) tube after 15 min lysis on ice. After quantification of the concentration by BCA method, 40 μg was separated by electrophoresis on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene difluoride (PVDF) membrane, blocked with 5% skim milk powder in PBST for 60 min at room temperature, and incubated with the primary antibodies (GSK-3β, β-catenin and β-actin with a dilution of 1:2000, 1:1000, 1:8000, respectively) overnight at 4°C. After washing the membrane 3 times with PBST, the membrane was incubated with HRP-conjugated secondary antibody (1:8000, 60 min) at room temperature followed by washing with PBST 3 times and subsequent addition of ECL chemiluminescence solution for 2-3 min incubation at room temperature. After that, the membrane was exposed and developed.

**Flow Cytometry Detection of Cell Apoptosis**

Follow the instructions, cells were resuspended in 100 μL Annexin V Binding Buffer, followed by addition of 5 μL FITC Annexin V and 10 μL PI for 15 min incubation at room temperature. After that, 400 μL of Annexin V Binding Buffer was added and cell apoptosis was measured by EPICS XL-MCL flow cytometry.

**Flow Cytometry Detection of Cell Proliferation**

The above miR-NC and miR-155 inhibitor transfected cells were collected by trypsinization and incubated in medium containing 10 μM EdU for 2 h at 37°C, re-inoculated into 60 mm culture dishes, and returned to the incubator for 72 h. After collecting cells by trypsin digestion, the cells were fixed at room temperature by paraformaldehyde, and saponin was permeabilized. 500 μL of the test solution was added, incubated at room temperature for 30 min in the dark, centrifuged once at 300 g, and resuspended in 500 μL of wash reagent followed by analysis of cell proliferation by FC500 MCL flow cytometry.

**Statistical Analysis**

Statistical analysis was performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were expressed as mean ± standard deviation (SD). The comparison between the measurement data of the groups was performed by Student t-test or Mann-Whitney U test. p<0.05 was considered statistically significant.

**Results**

**Abnormal Expression of MiR-155 and GSK-3β in Bladder Cancer**

The results of qRT-PCR showed that the expression of miR-155 was significantly increased in tumor tissues of bladder cancer patients compared with adjacent tissues (Figure 1A). The expression of GSK-3β mRNA in bladder cancer tissues was significantly lower than that in adjacent tissues (Figure 1B).

**Targeted Regulatory Relationship Between MiR-155 and GSK-3β mRNA**

Bioinformatics analysis revealed a complementary binding site between miR-155 and the 3'-UTR of GSK-3β mRNA (Figure 2A). Dual luciferase gene reporter assays showed that transfection of miR-155 mimic significantly reduced the relative luciferase activity in pMIR-GSK-3β-WT transfected HEK293T cells, but miR-NC or miR-155 mimic did not affect the relative luciferase activity in the HEK293T cells transfected with pMIR-GSK-3β-MUT (Figure 2B), indicating that miR-155 has a targeted regulatory relationship with the 3'-UTR region of GSK-3β mRNA.

**Increased MiR-155 and Decreased GSK-3β Expression in Bladder Cancer Cells**

The results of qRT-PCR showed that the expression of miR-155 was significantly increased in bladder cancer BIU-87 and 5637 cells compared with that in normal bladder epithelial SV-HUC-1 cells (Figure 3A), while GSK-3β mRNA expression was significantly reduced (Figure 3B). Western blot analysis showed that the expression of GSK-3β protein in bladder cancer BIU-87 and 5637 cells was significantly lower than that in SV-
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HUC-1 cells (Figure 3C).

**Inhibition of MiR-155 Expression Inhibits Bladder Cancer Cell Proliferation and Promotes Apoptosis**

The results of qRT-PCR showed that the expression of miR-155 in BIU-87 cells was significantly decreased in miR-155 inhibitor transfected group compared with miR-NC transfected group (Figure 4A), while the expression of GSK-3β mRNA was increased (Figure 4B). Western blot analysis showed that compared with miR-NC transfection group, the expression of GSK-3β protein in BIU-87 cells was significantly increased in miR-155 inhibitor transfection group, while the expression of β-catenin protein was significantly decreased (Figure 4C). Flow cytometry analysis showed that transfection of miR-155 inhibitor significantly increased apoptosis (Figure 4D) and inhibited proliferation (Figure 4E) of in BIU-87 cells.

**Discussion**

Bladder cancer is characterized by easy invasion, easy metastasis, and high recurrence rate. It is difficult to treat and its prognosis is very poor. Therefore, to study the pathogenesis of bladder cancer, explore the abnormal changes in the pathogenesis of bladder cancer signal molecules is of great significance to improve the diagnosis, treatment effect, as well as prognosis.

Wnt/β-catenin is a widely existing signaling pathway in mammals involved in the regulation of embryonic development, tissue and organogenesis, cell proliferation, apoptosis, migration and invasion. Elevated level of β-catenin protein and accumulation in the cytoplasm of the nucleus can lead to abnormal activation of the Wnt/β-catenin signaling pathway, which is closely related to the occurrence, progression and metastasis of tumors.

**Figure 1.** Abnormal expression of miR-155 and GSK-3β in bladder cancer. (A) qRT-PCR detection of miR-155 expression in bladder cancer tissues; (B) qRT-PCR detection of GSK-3β mRNA expression in bladder cancer tissues. * Represents $p<0.05$ compared with adjacent tissues.

**Figure 2.** Targeted regulatory relationship between miR-155 and GSK-3β mRNA. (A) Schematic diagram of the interaction site between miR-155 and the 3’-UTR of GSK-3β mRNA; (B) Dual luciferase gene reporter assay. *Represents $p<0.05$ compared to miR-NC.
Unlike β-catenin, a positive regulator in the Wnt/β-catenin signaling pathway, GSK-3β is a negative regulator, and GSK-3β phosphorylates the serine/threonine at the amino terminus of β-catenin. The residue forms a complex with axin and adenomatous polyposis coli (APC), which degrade the β-catenin protein, thereby reducing the expression of β-catenin in the cytoplasm, thereby blocking the activation of the Wnt/β-catenin pathway and attenuating the inhibition of apoptosis by the Wnt/β-catenin pathway and promoting cell proliferation.2-4

Multiple studies8,12,13 have shown that abnormal changes in miR-155 expression are associated with the development, progression, and metastasis of various tumors21,22. Unlike β-catenin, a positive regulator in the Wnt/β-catenin signaling pathway, GSK-3β is a negative regulator, and GSK-3β phosphorylates the serine/threonine at the amino terminus of β-catenin. The residue forms a complex with axin and adenomatous polyposis coli (APC), which degrade the β-catenin protein, thereby reducing the expression of β-catenin in the cytoplasm, thereby blocking the activation of the Wnt/β-catenin pathway and attenuating the inhibition of apoptosis by the Wnt/β-catenin pathway and promoting cell proliferation.2-4

Multiple studies8,12,13 have shown that abnormal changes in miR-155 expression are associated with the development, progression, and metastasis of bladder cancer. Bioinformatics analysis revealed a complementary binding site between miR-155 and the 3’-UTR of GSK-3β mRNA. This study compared the expression of miR-155 and GSK-3β in tumor tissues and adjacent tissues of bladder cancer patients, and intervened miR-155 expression in bladder cancer cells cultured in vitro to explore whether miR-155 regulates GSK-3β expression, Wnt/β-catenin pathway activity, play a role in the proliferation and apoptosis of bladder cancer cells.

The results of clinical samples showed that the expression of miR-155 was abnormally increased in tumor tissues of patients with bladder cancer compared with adjacent tissues, and the expres-
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Figure 4. Inhibition of miR-155 expression can significantly inhibit bladder cancer cell proliferation and promote apoptosis. (A) qRT-PCR detection of intracellular miR-155 expression; (B) qRT-PCR detection of intracellular GSK-3β mRNA expression; (C) Western blot detection of intracellular protein expression; (D) Flow detection of apoptosis; (E) EdU staining for cell proliferation. * Represents $p < 0.05$ compared to miR-NC.
sion of GSK-3β was significantly decreased, suggesting that the increased expression of miR-155 may play a role in reducing the expression of GSK-3β and promoting the pathogenesis of bladder cancer. Dual luciferase activity assay showed that transfection of miR-155 mimic significantly reduced relative luciferase activity in pMIR-GSK-3β-WT transfected HEK293T cells, but miR-NC or miR-155 mimic did not affect the relative luciferase activity of HEK293T cells transfected with pMIR-GSK-3β-MUT, confirming the targeted regulation relationship between miR-155 and GSK-3β mRNA. The results of cell culture in vitro showed that compared with normal bladder epithelial SV-HUC-1 cells, the expression of miR-155 in bladder cancer BIU-87 and 5637 cells was significantly increased, while the expression of GSK-3β was significantly increased, further suggesting that miR-155 and GSK-3β abnormalities are associated with bladder cancer. In the study of the relationship between miR-155 and bladder cancer, Peng et al.12 showed that the expression of miR-155 was significantly increased in tumor tissues of bladder cancer patients compared with adjacent tissues. The expression level of its targeted tumor suppressor gene DMTF1 was significantly reduced. Wang et al.23 indicated that the expression of miR-155 in urine of patients with bladder cancer was abnormally higher than that of the control group, suggesting that miR-155 may play a role in promoting cancer in bladder cancer. Wang et al.13 detected that the expression of miR-155 was significantly increased in tumor tissues of bladder cancer patients compared with adjacent tissues. Compared with patients with lower miR-155 expression, the survival and prognosis of patients with higher expression of miR-155 were significantly worse. Xie et al.24 observed that the expression of miR-155 was abnormally elevated in tumor tissues of bladder cancer patients, and the increased expression of miR-155 was an indicator for poor prognosis. Zhang et al.25 and others showed that compared with healthy controls and cystitis patients, the expression of miR-155 in urine of patients with bladder cancer was significantly increased, and the expression of miR-155 was related to the bladder cancer stage, indicating that detection of miR-155 expression can be used as a predictor of prognosis in patients with bladder cancer. In this study, the expression of miR-155 in bladder cancer tumor tissues and bladder cancer cell lines was abnormally elevated, and miR-155 may be a cancer-promoting factor in the pathogenesis of bladder cancer, which was consistent with Peng et al.12 and Wang et al.23. The further results of this study showed that transfection of miR-155 inhibitor significantly increased the expression of GSK-3β in BIU-87 cells, decreased the expression of β-catenin, increased apoptosis, and decreased cell proliferation. The results showed that miR-155 can promote the cancer-promoting effect of bladder cancer by inhibition of GSK-3β, while down-regulating the expression of miR-155 can increase the expression of tumor suppressor GSK-3β and inhibit Wnt/β-catenin. The activity of the pathway plays a role in weakening the proliferation ability of bladder cancer cells and inducing cell apoptosis. In the study of the relationship between miR-155 and the biological effects of bladder cancer, Pan et al.26 found that in the bladder cancer cells, the expression of miR-155 was increased, which can promote cancer, and administration also of several alternative drugs. The active constituents of Hedyotis diffusa and Scutellaria barbata L. can significantly down-regulate the expression of miR-155 and promote the apoptosis of bladder cancer cells. Overexpression of miR-155 can significantly activate the activity of Akt pathway and up-regulate the expression of anti-apoptotic factor Bel-2 and Mcl-1, which antagonizes the apoptosis-inducing effect of Hedyotis diffusa and Scutellaria barbata L. on bladder cancer cells and plays a role as a cancer-promoting gene. Peng et al.12 showed that overexpression of miR-155 in bladder cancer um-uc-3 and T24 cells down-regulated the expression of DMTF1, promoted cell proliferation, and enhanced cell growth and tumor formation in nude mice. Similar to overexpression of miR-155, siRNA interferes with the expression of DMTF1 can promote the cancer-promoting effect, promote the proliferation and cycle progression of bladder cancer cells, and enhance the ability of cell cloning. This study links miR-155 and GSK-3β and reveals that elevated expression of miR-155 plays a role in down-regulating GSK-3β expression and promoting bladder cancer, while inhibition of miR-155 expression increases GSK-3β expression, attenuating the malignant characteristics of bladder cancer cells. However, whether miR-155 regulates GSK-3β affects the biological effects of bladder cancer cells in vivo is unclear, and requires further research in animal studies.

Conclusions

The increased expression of miR-155 plays a role in reducing the expression of GSK-3β and
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promoting the pathogenesis of bladder cancer. Inhibition of miR-155 can up-regulate the expression of GSK-3β, inhibit the activity of Wnt/β-catenin pathway, attenuate proliferation and promote apoptosis of bladder cancer cells.

Conflict of interest
The authors declare no conflicts of interest.

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References


