Target inhibition on GSK-3β by miR-9 to modulate proliferation and apoptosis of bladder cancer cells

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Abstract. – OBJECTIVE: Glycogen synthase kinase-3β (GSK-3β) can negatively regulate Wnt/β-catenin signaling pathway via degrading β-catenin protein, and plays suppressing roles in various tumors. Its role in bladder cancer pathogenesis is still unclear. In bladder cancer tissues, expression of microRNA-9 (miR-9) is significantly elevated. This study investigated the effect of miR-9 in modulating GSK-3β expression, Wnt/β-catenin pathway activity, and proliferation or apoptosis of bladder cancer cells.

PATIENTS AND METHODS: Dual luciferase reporter gene assay confirmed targeted regulation between miR-9 and GSK-3β. Bladder cancer tissues were collected to measure expression of miR-9, GSK-3β mRNA using adjacent tissues as the control. Expression of miR-9 and GSK-3β was also measured in HBEC, RT4 and TCCSUP cells. Cultured RT4 and TCCSUP cells were transfected with miR-9 inhibitor or pSicoR-GSK-3β. The expression of miR-9, GSK-3β and β-catenin was compared, followed by using flow cytometry assay for cell apoptosis and EdU staining for cell proliferation.

RESULTS: Comparing to adjacent tissues, bladder cancer tissues illustrated significantly elevated miR-9 expression and lower GSK-3β mRNA. Bioinformatics analysis revealed complementary binding sites between miR-9 and 3’-UTR of GSK-3β mRNA, indicating targeted regulation between miR-9 and GSK-3β. Comparing to HBEC cells, RT4 and TCCSUP cells had significantly elevated miR-9 expression and lower GSK-3β expression, with enhanced proliferation. Transfection of miR-9 inhibitor or pSicoR-GSK-3β significantly elevated GSK-3β expression and suppressed β-catenin expression, promoted cell apoptosis and inhibited proliferation.

CONCLUSIONS: MiR-9 up-regulation plays a role in suppressing GSK-3β expression and facilitating bladder cancer pathogenesis. Inhibition of miR-9 could potentiate GSK-3β expression, suppress proliferation of bladder cancer, and facilitate apoptosis.

Key Words: miR-9, GSK-3β, Wnt/β-catenin, Prostate cancer, Proliferation, Apoptosis.

Introduction

Bladder cancer (BC) is a commonly occurred malignant tumor in urinary-reproductive system. It is one of the ten most popular cancers worldwide and locates on the ninth incidence of system malignant tumors¹. BC has typical malignant features including predisposition towards invasion and metastasis, and has higher post-operation recurrent rate (60-70%), leading to higher treatment difficulty, and worse prognosis. Therefore, BC has become a major challenge in urinary-reproductive tumors². Therefore, the investigation of pathogenesis mechanism of BC is of critical importance for unrevealing BC pathogenesis, and for improving treatment efficiency, decreasing recurrent or mortality rate. As a critical protein in canonical Wnt/β-catenin signal pathway, β-catenin is closely related with activation of this pathway. The Wnt/β-catenin abnormally potentiates the expression of various down-stream target genes, therefore, closely related with occurrence, progression and metastasis of multiple genes³,⁴. Glycogen synthase kinase-3β (GSK-3β) is a critical negative regulator in Wnt/β-catenin signaling pathway. It can phosphorylate β-catenin protein for its further degradation, suppressing its cytoplasmic expression and impeding the activation of Wnt/β-catenin pathway. Therefore, the GSK-3β plays important tumor suppressing functions in occurrence and progression of multiple tumors⁵-⁷. The previous researches showed that GSK-3β down-regulation or dysfunction was closely correlated with
occurrence, progression, metastasis and drug resistance of various tumors including colon cancer and breast cancer. However, little has been known about its correlation with BC pathogenesis. MicroRNA (miR) is a group of endogenous non-coding small single stranded RNA with about 22-25 nucleotides length. As an important epigenetic regulator, miR can bind with 3’-untranslated region (3’-UTR) of target gene mRNA to degrade mRNA or suppress its translation. Therefore, miR is correlated with modulation of various biological effects of cell proliferation, differentiation, cell cycle and apoptosis. MiR-9 is a widely studied miR molecule, and plays oncogene-like or tumor suppressor gene role in tumor pathogenesis or progression due to variation of target genes. Multiple researches showed significantly elevated miR-9 expression in BC tumor tissues, indicating its potential tumor-facilitating role. Therefore, this study investigated the role of miR-9 in modulating GSK-3β expression, activity of Wnt/β-catenin signal pathway, and proliferation or apoptosis of BC cells.

Patients and Methods

Patients
A total of 38 BC patients (20 males and 18 females, aging between 51 and 73 years, average age = 62.7 years) who received treatment in our hospital between September 2016 and March 2017, were recruited in this study. All patients were primarily diagnosed and treated, without any anti-tumor therapy prior to admission. Tumor tissue samples were collected during surgery, and tumor adjacent tissues were collected from bladder mucosal tissues from at least 5 cm from cancer edge. This study was approved by the Ethical Committee of Lanzhou University Second Hospital (Lanzhou, China). All of the patients signed the consents form for this study.

Inclusive criteria: 1- signed informed consents; post-op diagnosis as BC by pathology.

Exclusive criteria: 1- patients with severe liver/ kidney dysfunction; 2- complicated with other tumors or malignant blood disease; 3 non-primary diagnosis, or received radio-, chemo-, immune-therapy or Chinese medicine beforehand.

Major Reagents and Materials

Human bladder epithelial cells (HBEC), human BC cell line RT4 and TCCSUP were purchased from Huiying Biotech. Co. Ltd. (Xiamen, China). Dulbecco’s modified eagle medium (DMEM), optional minimal essential medium (Opti-MEM), fetal bovine serum (FBS) and streptomycin-penicillin culture medium were purchased from Gibco (Grand Island, NY, USA). RNA extraction kit EasyPure RNA Kit, fluorescent quantitative PCR kit TransScript Green One-Step qRT-PCR SuperMix were purchased from Transgen Biotech. Co. Ltd. (Beijing, China). Transfection reagent Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). MiR-normal control (miR-NC), miR-9 mimic and miR-9 inhibitors were purchased from RiboBio (Shanghai, China). EdU cell proliferation test kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse anti-human GSK-3β was purchased from Abcam Biotech. (Cambridge, MA, USA). Rabbit anti-human β-catenin and β-actin were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Horseradish-peroxidase (HRP) conjugated secondary antibody was purchased from Sango Biotech. (Shanghai, China). pGRE-luc luciferase reporter plasmid was purchased from Biovector Science Lab., Inc., (Beijing, China). Dual-Luciferase reporter assay system was purchased from Promega (Madison, WI, USA). Over-expression plasmid pSicoR was purchased from Youbao Bio (Shanghai, China). Radioimmunoprecipitation assay (RIPA) lysis buffer, bicinchoninic acid (BCA) protein quantification kit and fluorescein isothiocyanate (FITC)-Annexin V/propidium iodide (PI) cell apoptosis kit were purchased from Beyotime Biotech. (Shanghai, China).
6 h, Opti-MEM was switched to DMEM medium containing 10% FBS for 48 h further incubation. In dual luciferase activity assay, the culture medium was discarded, and cells were rinsed twice in PBS. Each well was added with 100 μl Passive Lysis Buffer for 15 min incubation, followed by 1000 r/min centrifugation for 5 min. A total of 50 μl supernatant of lysate were incubated with 50 μl luciferase substrate, and the luciferase activity was immediately quantified. The reaction was quenched by 50 μl Stop & Glo buffer, and Renilla luciferase activity was immediately measured. The ratio between luciferase activity and Renilla luciferase activity was calculated to reflect relative luciferase activity.

**Construction of GSK-3β**
CDS fragment of GSK-3β gene was amplified, and the target fragment length was determined by gel electrophoresis. Via dual enzymatic digestion, the fragment was ligated into pSicoR over-expression plasmid. After transforming competent bacteria, positive clones were selected for amplification and further extraction of recombinant plasmid containing targeted fragment. Sequencing was performed to confirm correct insertion of GSK-3β gene fragment, and the plasmid was named as pSicoR-GSK-3β. Empty plasmid pSicoR-blank was used as the control.

**Cell Transfection and Grouping**
Cultured RT4 and TCCSUP cells were divided into four groups: miR-NC transfection group (50 pmol), miR-9 inhibitor transfection group (50 pmol), pSicoR-blank transfection group (100 ng), pSicoR-GSK-3β group (100 ng). 72 h after transfection, assays were performed. In transfection, 100 μl Opti-MEM, 10 μl Lipo 2000 and 10 μl plasmid were gently mixed for 20 min room temperature. When cell density reached nearly 60%, original culture medium was discarded, and cells were rinsed twice in PBS. Transfection mixture was added, and original medium was discarded after 6 h. DMEM medium containing 10% FBS and 1% penicillin-streptomycin was switched. Further assays were performed on cells collected after 72 h incubation.

**qRT-PCR Assay for Gene Expression**
Transcript Green One-Step qRT-PCR SuperMix was used to perform one-step qRT-PCR using RNA extracted by EasyPure RNA Kit as the template, to measure relative expression level of genes. Within 20 μl reaction system, 1 μg template RNA, 0.2 μM forward primer, 0.2 μM reverse primer, 10 μl 2XTransStart Tip Green qPCR SuperMix, 0.4 μl One-Step RT Enzyme Mix, 0.4 μl Passive Reference Dye II and RNase-free water filled up to 20 μl, were added. qRT-PCR conditions were: 45°C for 5 min, 94°C 30 s, followed by 40 cycles each consisting of 94°C 5 s and 60°C 30 s. On ABI 7500 Real-time fluorescent quantitative PCR, gene expression was measured. Primer sequences were: miR-9P: 5'-CGGCC GTCTT TGGTT ATCTA GC-3'; miR-9P: 5'-GTGCA GGTC CGAGG T-3'; U6P: 5'-TTTGA AAC-GA TACAG AGAAG ATT-3'; U6P: 5'-GGAAC GCTTC ACGAA TTTG-3'; GSK-3βP: 5'-TG-GTC GCCAT CAAGA AAGTA TTG-3'; GSK-3βP: 5'-GGCAG TGTTT GGCTC GACTA T-3'; β-actinP: 5'-AGTTA CTCCG AAGCA TTC-3'; β-actinP: 5'-GGTCA CGCAG GCCAC GTATG C-3';

**Western Blot**
Cells from all transfection groups were digested by trypsin and were collected. The lysate mixture was centrifuged at 300 ×g for 5 min and the supernatant was collected. 100 μl RIPA lysis buffer were added into each 5×10^6 cells. After 20 min iced incubation, the supernatant was transferred into new pre-cold Eppendorf (EP) tubes. After quantifying protein quality and concentration by bicinchoninic acid (BCA) approach, 40 μg proteins were separated in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After transferring to the membrane, blocking was performed using Phosphate-buffered solution and Tween-20 (PBST-20) containing 5% defatted milk powder. Primary antibody (GSK-3β at 1:2000, β-catenin at 1: 2000, β-actin at 1:8000) was added for 4°C overnight incubation. After three times of PBST rinsing, HRP conjugated secondary antibody (1:20000) was added for 60 min room temperature incubation. With three times of PBST rinsing, enhanced chemiluminescence (ECL) chromogenic substrate was added for 2-3 min room temperature incubation. The membrane was exposed in dark room for development and imaging. The film was scanned for collecting data.

**Flow Cytometry for Cell Apoptosis**
Following the manual instruction, cells were re-suspended in 100 μl Annexin V Binding Buffer. 5 μl FITC Annexin V were added, followed by 10 μl PI solution. After 15 min room temperature incubation, 400 μl Annexin V Binding Buffer were added, and cell apoptosis was measured on EPICS XL-MCL flow cytometry.
Flow Cytometry for Cell Proliferation

EdU Flow Cytometry Kit was used for testing cell proliferation. In brief, cells were plated in culture medium containing 10 μM EdU culture medium at 37°C for 2 h incubation, and were inoculated into 60 mm culture dish. After 72 h incubation, cells were digested by trypsin and were collected. After fixation in paraformaldehyde and permeabilization in saponin, 500 μl testing buffer were added for 30 min of dark incubation at room temperature, and 300×g centrifugation was performed. Cells were then re-suspended in 500 μl wash reagent, and loaded onto FC500 MCL flow cytometry for measuring cell proliferation.

Statistical Analysis

SPSS 18.0 was used for statistical analysis (SPSS Inc., Chicago, IL, USA). Measurement data were presented as mean ± standard deviation (SD), and comparison between groups was performed by Student t-test or Mann-Whitney U test. A statistical significance was defined when p < 0.05.

Results

Abnormal Expression of miR-9 and GSK-3β

qRT-PCR assay showed that, compared to tumor adjacent tissues, BC tissues showed significantly elevated miR-9 expression (Mann-Whitney U = 53, p < 0.001, Figure 1A). Comparing to tumor adjacent tissues, BC tissues showed remarkably decreased GSK-3β mRNA expression (Mann-Whitney U = 63, p < 0.001, Figure 1B).

Up-regulation of miR-9 and Down-Regulation of GSK-3β in BC Cells

Flow cytometry results showed significantly higher EdU positive rate in RT4 and TCCSUP cells comparing to HBEC cells, indicating more potent proliferation ability of BC cell lines than HBEC cells (Figure 2A). qRT-PCR results showed that, compared to HBEC cells, BC cell lines RT4 and TCCSUP showed significantly elevated miR-9 expression, whilst GSK-3β mRNA expression was significantly decreased (Figure 2B). Western blot results showed significantly lower GSK-3β protein expression in RT4 and TCCSUP cells than HBEC cells, whilst β-catenin protein expression level was remarkably higher than HBEC cells (Figure 2C). Online gene prediction by microRNA.org showed the existence of targeted complementary binding sites between miR-9 and 3'-UTR of GSK-3β mRNA (Figure 2D). Dual luciferase reporter gene assay showed that transfection of miR-9 mimic significantly decreased relative luciferase activity inside HEK293 cells transfected with pGRE-GSK-3β-wt-3'UTR, whilst miR-9 inhibitor significantly elevated relative luciferase activity of HEK293 cells transfected with pGRE-GSK-3β-wt-3'UTR (Figure 2E). The transfection of miR-9 mimic or miR-9 inhibitor, however, had no significant effect on relative luciferase ac-

Figure 1. Abnormal expression pattern of miR-9 and GSK-3β. (A) qRT-PCR for miR-9 expression in BC tissues and tumor adjacent tissues. (B) qRT-PCR for measuring GSK-3β mRNA expression in BC or adjacent tissues. *p < 0.001 compared to cancer adjacent tissues.
Figure 2. Up-regulation of miR-9 and down-regulation of GSK-3β in BC cells. (A) Flow cytometry for cell proliferation. (B) qRT-PCR for gene expression. (C) Western blot for protein expression. (D) Binding sites between miR-9 and 3'-UTR of GSK-3β mRNA. (E) Dual luciferase reporter gene assay. *p < 0.05 comparing between two groups.
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MiR-9 Inhibition Up-Regulated GSK-3β Expression, Suppressed RT4 or TCCSUP Cell Proliferation and Induced Cell Apoptosis

Transfection of miR-9 inhibitor or pSicoR-GSK-3β significantly up-regulated GSK-3β expression in RT4 (Figure 3A) and TCCSUP cells (Figure 3B), significantly suppressing β-catenin expression level in RT4 and TCCSUP cells. Flow cytometry results showed that, after transfecting miR-9 inhibitor or pSicoR-GSK-3β, proliferation potency of RT4 and TCCSUP cells was significantly compromised (Figure 3C), and cell apoptosis was significantly potentiated (Figure 3D).

Discussion

Wnt/β-catenin is a widely signaling pathway inside mammalian cells, and it is involved in embryonic development, tissue/organ morphogenesis, and process of cell proliferation, apoptosis, migration and invasion. Similarly to β-catenin, GSK-3β is an important component of Wnt/β-catenin signal pathway, but in contrast with positive regulator β-catenin, GSK-3β is a negative regulator. Within Wnt/β-catenin signal pathway, GSK-3β can phosphorylate serine/lysine residue at N-terminus of β-catenin protein, and forms a complex with axin and adenomatous polyposis coli (APC), thus degrading β-catenin protein, lowering its cytosolic expression level, and inhibiting nuclear translocation or function, eventually blocking activation of Wnt/β-catenin pathway and suppressing its inhibition on cell apoptosis or facilitating on cell proliferation. MiR-9 is a widely studied microRNA. Previous study showed its oncogene role in occurrence of prostate cancer and ovarian cancer, whilst in gastric cancer it plays a tumor suppressor role. Multiple studies showed significantly elevated miR-9 in BC tumor tissues, indicating its oncogene-like role in BC pathogenesis. In this research, dual luciferase gene reporter assay found targeted regulation between miR-9 and GSK-3β mRNA, without further knowledge about its regulatory relationship in BC. We thus compared miR-9 and GSK-3β expression between BC tumor tissues and adjacent tissues, and among normal bladder mucosal epithelial cell line and BC cell lines RT4 or TCCSUP. The results showed significantly elevated miR-9 expression in tumor tissues, whilst GSK-3β expression was remarkably suppressed. The above results suggested that abnormal up-regulation of miR-9 may be an important pathogenic factor suppressing GSK-3β expression and facilitating BC pathogenesis. In a further work regarding miR-9 and BC, Pignot et al showed that compared to normal bladder tissues, muscle invasive bladder cancer (MIBC) patients presented significantly elevated miR-9 expression. Even when comparing with tumor tissues from non-muscle invasive bladder cancer (NMIBC) tissues, miR-9 expression was abnormally elevated by 7.61 folds. Moreover, comparing to those with relatively lower miR-9 expression, patients with higher miR-9 expression presented significantly lower recurrence free survival (RFS) or overall survival (OS), suggesting that miR-9 up-regulation was an unfavorable factor for prognosis. Wang et al found that compared to normal bladder mucosal tissues, BC tumor tissues had significantly elevated miR-9 expression. Xie et al also showed abnormally elevated miR-9 level in BC tumor tissues compared to that in normal bladder epithelium. Moreover, miR-9 expression in infiltrative BC tissues also showed higher miR-9 expression than superficial BC tissues. All these studies indicated that abnormally elevated miR-9 expression might form on oncogenic factor of BC, similarly with abnormal miR-9 expression pattern observed in BC tissues and cell lines from this study. Our further assays showed that transfection of miR-9 inhibitor and/or pSico-GSK-3β significantly elevated GSK-3β expression in T24 and 5637 cells, decreased β-catenin expression, remarkably elevated cell apoptosis, and inhibited proliferation. Wang et al transfected BC cell lines T24 and 5637 cells with miR-9 mimic to up-regulate its expression, and found that over-expression of miR-9 could increase the expression of cyclin D1, MMP9, Bcl-2 and survivin in T24 and 5637 cells. Meanwhile, over-expression of miR-9 also decreased E-cadherin expression, therefore, facilitating cell proliferation, cycle progression, invasion and chemotherapy drug resistance. Moreover, suppression of miR-9 expression weakened malignant biological properties of BC cells via inhibiting target gene LASS2 expression. Xie et al found that transfection of pre-miR-9 in...
Figure 3. Inhibition of miR-9 up-regulated GSK-3β expression, suppressed proliferation and induced apoptosis of RT4 and TCCSUP cells. (A) Western blot for protein expression of RT4 cells. (B) Western blot for protein expression inside TCCSUP cells. (C) EdU staining for cell proliferation. (D) Flow cytometry for cell apoptosis.
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BC T24 cells remarkably potentiated miR-9 expression and suppressed the level of target gene CBX7. However, the up-regulation of CBX7 significantly inhibited invasion of T24 cells, supporting our observation that up-regulation of miR-9 could weaken malignant biological features of BC cells. In a research about the relationship between GSK-3β and BC, Shahjee et al used anti-proliferative factor (APF) to suppress proliferation of BC cells and observed significantly decreased expression of phosphorylated and inactivated form of GSK-3β. These results indirectly suggested that the involvement of GSK-3β inhibition in suppressed proliferation of BC cells. Fan et al found that during the suppression of GSK-3β activation in suppressed proliferation results indirectly suggested that the involvement of GSK-3β and BC cells, both of which showed that suppression of miR-9 expression in facilitating BC pathogenesis. Moreover, we showed that suppression of miR-9 expression could elevate GSK-3β expression and weaken malignant property of BC cells, whilst over-expression of GSK-3β had tumor suppressor effects on BC cells, similarly to Shahjee et al. Fan et al and Pan et al, showing modulatory effects of GSK-3β on biological property of BC cells. This study connected miR-9 with GSK-3β, and revealed the role of miR-9 up-regulation in suppressing GSK-3β expression and in facilitating BC pathogenesis. Moreover, we showed that suppression of miR-9 expression could elevate GSK-3β expression and weaken malignant property of BC cells, both of which had not been reported previously and thus are novelty of this work.

Conclusions

We showed that miR-9 up-regulation plays a role in suppressing GSK-3β expression and in facilitating BC pathogenesis. Suppressing of miR-9 could enhance GSK-3β expression, inhibit BC cell proliferation, and potentiate cell apoptosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References


