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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 lu reporter gene assay confirmed targeted r a-tion between miR-9 and GSK-3β. Bladder ca tissues were collected to measure expres of miR-9, GSK-3β mRNA using adjacent tissu as the control. Expression of m d GSK-3β was also measured in HBEC CCSUP cells. Cultured RT4 and SUP s were transfected with miR-9 in or or p GSK-3β. The expression of m SK- enin was compared, low g flow cytometry assay for c poptos d EdU staining for cell prolife n.

RESULTS: C g to adjac ssues, bladder canc s illustrated significantly elevated m expr and lower GSK-3β mRNA. Bi formatics a revealed complementa nding sites be miR-9 and 3'-UTR of -3β mRNA, indicat g targeted regulatio etwee -9 and GSK-3β. Comparing to l cells 4 and CCSUP cells had signific ed mi expression and lower GSK ressio th enhanced proliferation. Tran n c R-9 inhibitor or pSicoR-3 sign elevated GSK-3β expression and suppre d β-catenin expression, promot ell apoptosis and inhibited proliferation.

S: MiR-9 up-regulation plays a n ssing GSK-3β expression and facilitating bladder cancer pathogenesis. Inhibition of miR-9 could potentiate GSK-3β expression to suppress proliferation of bladder cancer, and facilitate apoptosis.

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

roduction

der can BC) is a commonly occurred mal in urinary-reproductive system. It is one of the ten most popular cancers worldwide and locates on the ninth incidence malignant tumors[1]. 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 tumors[2]. 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 genes[3,4]. 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 tumors[5-7]. The previous researches showed that GSK-3β down-regulation or dysfunction was closely correlated with

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occurrence, progression, metastasis and drug resistance of various tumors including colon cancer[8] and breast cancer[9]. 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'-untranlsated 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[10]. 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[11-13]. Multiple researches[14-16] 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 75 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. 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 consent form for this study.

Inclusive criteria: Signed informed consents; Non-drop diagnosis of 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), BC cell line RT4 and TCCSUP were purchased from Huiying Biotech. Co. Ltd. (Changsha, China). Dulbecco's modified eagle medium (DMEM), optional minimal essential medium (α-MEM), fetal bovine serum (FBS) and streptomycin-penicillin culture medium were purchased from Gibco (Grand Island, NY, USA). RNA extraction 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, 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 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).

Cell Culture

HBEC, RT4 and TCCSUP cells were all incubated in DMEM medium containing 10% FBS and 1% streptomycin-penicillin, and were cultured in a 37°C chamber with 5% CO_2. Cells were passed at 1:4 ratio, and those cells at log-growth phase with satisfactory status were used for further experiments.

Dual Luciferase Reporter Gene Assay

HEK293 cells were inoculated into 24-well plate. After 24 h attaching growth, 100 ng pGRE-GSK-3β-wt-3'-UTR (or pGRE-GSK-3β-mut-3'-UTR), 50 pmol miR-9 mimic (miR-NC or miR-9 inhibitor), 10 ng pRL-TK were co-transfected into HEK293 cells using Lipo 2000 reagent. After

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-9 mimic transfection group (50 pmol), miR-9 inhibitor transfection group (50 pmol), pSicoR-blank transfection group (100 ng), pSicoR-GSK-3β group (100 ng). After transfection, assays were performed. In transfection, 100 µl Opti-MEM, 1 µl Lipofectamine 2000 and 100 ng plasmid were gently mixed for 20 min in room temperature. When cell density reached 50%-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 cells collected after 72 h incubation.

qRT-PCR Assay for Gene Expression

TransScript Green One-Step qRT-PCR SuperMix was used to perform one-step qRT-PCR using total 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$_F$: 5'-CGCGC GTTGGTT ATCTA G-3', miR-9P$_R$: 5'-GTGCA GGGTC CGAGG T-3'; U6P$_F$: 5'-CTCGC TTCGGCAGC ACATA TACTG AG-3'; U6P$_R$: 5'-GAAC GCTTC ACG AATTTG CGTGTC AT-3'; GSK-3βP$_F$: 5'-TGGTC GCCAT CAAGA AAC G-3'; GSK-3βP$_R$: 5'-CTTT GGCT GACTA T-3'; β-actinP$_F$: 5'-GA CTAAG GCCAA C-3'; β-actinP$_R$: 5'-TGTCA AC GATTT CC-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 was added into each 5×10^6 cells. After 20 min iced incubation, the supernatant was transferred into new pre-cold Eppendorf (EP) tubes. 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 placed 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

AbnormalExpression of miR-9 and GSK-3β

qRT-PCR assay showed t pared to tumor adjacent tissues, B tissu showed significantly elevated iR-9 ression (Mann-Whitney U = < 0 1A). Comparing to tumor adjacent tissues, BC tissues showed remarkably decreas mRNA expression (Mann-Whitn = 6 < 0.001, Figure 1B).

Up-regulation of miR-9 and Down-Regulation of G -3β in ells

Flow cytometry resu owed sig higher EdU positive n RT4 nd TC cells comparing to H C cells icating more potent proliferati ity BC c lines than HBEC cell Fig qRT-P results showed that, pared to C c BC cell lines RT4 a CSUP show icantly elevated mi sion, whilst K-3β mRNA expressi was antly decreased (Figure 2B). Western blot r showed significantly low -3β protein ssion in RT4 and T UP cells than HBEC cells, whilst β-catenin protein expressi n level was remarkably higher than HBEC s (Figure 2C). Online gene diction by m RNA.org showed the existe f target complementary binding sites betw nd 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-

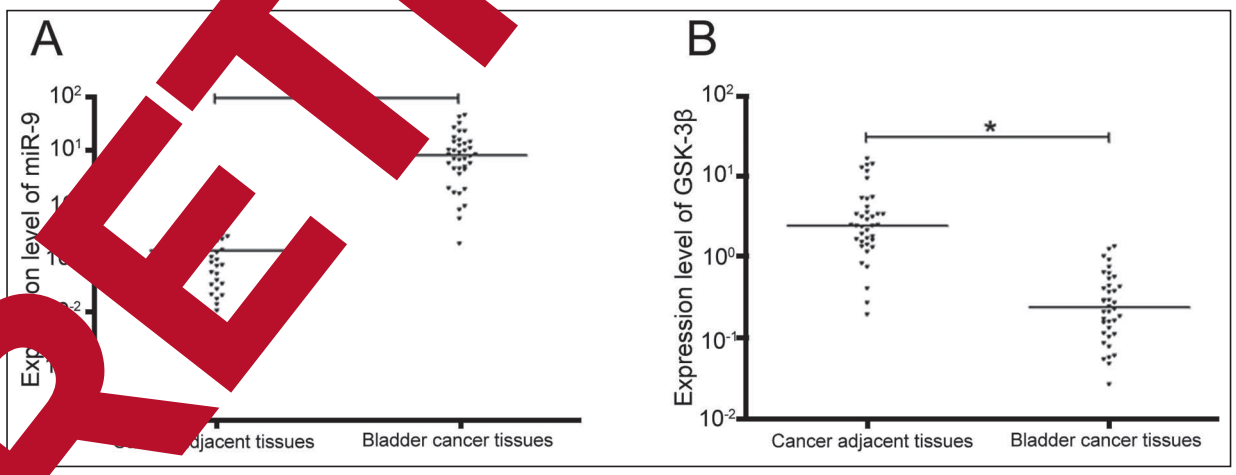
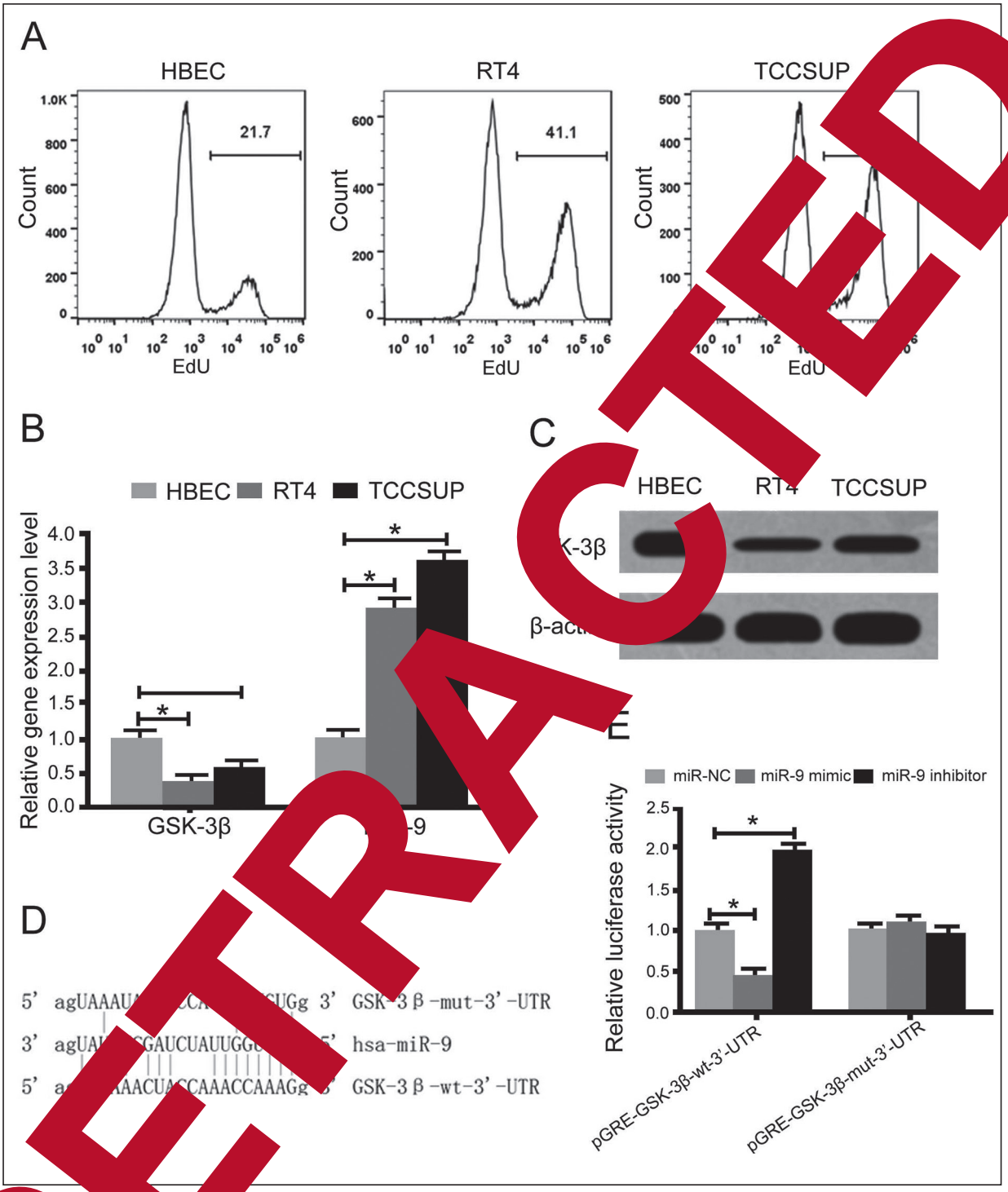


Figure 1. Abnormal expression pattern of miR-9 and GSK-3β. ***(A)*** qRT-PCR for miR-9 expression in BC tissues and tumor adjacent tissues. ***(B)*** qRT-PCR for measuring GSK-3β mRNA expression in BC or adjacent tissues. *$p < 0.001$ compared to cancer adjacent tissues.



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.

tivity of HEK293 cells transfected with pGRE-GSK-3β-mut-3'-UTR (Figure 2E), confirming targeted regulation between miR-9 and 3'-UTR of GSK-3β mRNA.

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 pa... inside mammalian cells, and it is inv... embryonic development[17,18], tissue/orga...r-phogenesis[19,20], and process of cell prolifera... apoptosis, migration and invasion[21,22]. Simil... to β-catenin, GSK-3β is an import... compone... of Wnt/β-catenin signal pathy... contrast... with positive regulator β-c...in, G...3β is a negative regulator. Withi...t/β-cat... signal pathway, GSK-3β can ...oryla... sine residue at N-ter...us ...in protein, and forms a comple...th axin ...denomatous polyposis coli (A... thus degrad... catenin protein, loweri...tosolic expres...n level, and inhibitin... clea...location or function, eventually blocking activ... of Wnt/β-catenin pathway ... suppressing i...bition on cell apopto... facilitating on ce... proliferation[5-7]. MiR... a wid...tudied microRNA. Previous stu...owe... onco... role in occurrence of pr...er[12] a...arian cancer[13], whilst i...gastri...er it ...ys a tumor suppressor ...Multi...s[14-16] showed significantly elev... miR-9 ...BC tumor tissues, indicating ...oncogene-like role in BC pathogenesis. In..., dual luciferase gene reporter ...y found targeted regulation between miR-9 ...GSK-3β mRNA, without further knowledge a... its regulatory relationship in BC. We thus ...mpared miR-9 and GSK-3β expression between BC tumor tissues and adjacent tissues, and among normal bladder mucosa... cell line and BC cell lines RT4 ...CCSU... The results showed significantly ...ated miR-9 expression in tumor tissues, w... GSK-3β expression was remarkably suppres...e above results suggested that abn...al up-r...on of miR-9 may be an impo...t pathogen... suppressing GSK-3β e...ession ...d facili...s BC pathogenesis. I...further...rk regarding miR-9 and BC, Pig...all...owed t...com-pared to normal ...dde..., musc...vasive bladder cance...MIBC) p...p...ted significantly el...d miR-9 exp...Even when comparin...mor tissues ...m non-muscle inva... bla...ncer (NMIBC) tissues, miR-9 expression w...ormally elevated by 7.61... Moreover, c...ring to those with r...ely lower miR-9 expression, patients with ...her miR-9 ex...ession presented significantly ...er recurrenc...e survival (RFS) or overall ...ival (OS), s...esting that miR-9 up-regula...as an ...vorable factor for prognosis. Wan...and that compared to normal bladder mucosal tissues, BC tumor tissues had ...ignificantly elevated miR-9 expression. Xie et ...showed abnormally elevated miR-9 ...ver ...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[15] 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[15]. Xie et al[16] found that transfection of pre-miR-9 in

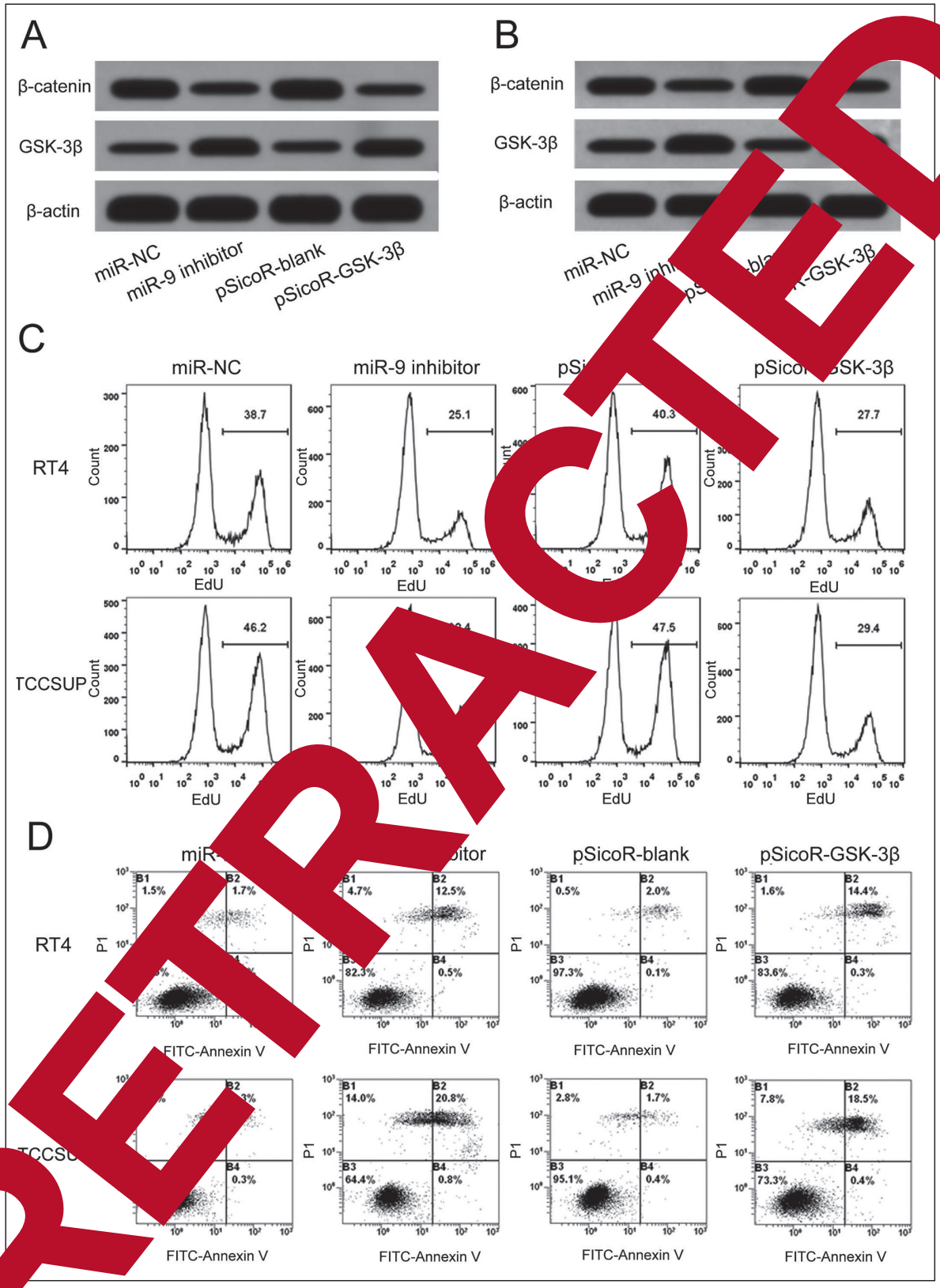


Figure 3. Inhibition of miR-9 up-regulated GSK-3β expression, suppressed proliferation and induced apoptosis of RT4 and TCCSUP cells. ***(A)*** Western blot for protein expression of RT4 cells. ***(B)*** Western blot for protein expression inside TCCSUP cells. ***(C)*** EdU staining for cell proliferation. ***(D)*** Flow cytometry for cell apoptosis.

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[16]. In a research about the relationship between GSK-3β and BC, Shahjee et al[23] 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 activation in suppressed proliferation of BC cells. Fan et al[24] found that during the suppression of BC cell T24 proliferation by miR-29c down-regulation, phosphorylated level of GSK-3β was significantly decreased, indicating that hyper-activity of GSK-3β could inhibit BC cell proliferation. Pan et al[25] found that fibronectin could inactivate GSK-3β, up-regulate cyclin D1 expression to antagonize mitomycin C (MMC) induced G0/G1 phase arrest of BC cell line T24. Furthermore, fibronectin could also accelerate the cell cycle progression and proliferation, indicating the role of GSK-3β in weakening cycle and proliferation[25]. We found abnormally decreased GSK-3β expression in BC cells, while over-expression of GSK-3β had tumor suppressor effects on BC cells, similarly to Shahjee et al[23], Fan et al[24] and Pan et al[25], suggesting modulatory effects of GSK-3β on biological property of BC cells. This study detected the miR-9 with GSK-3β, and revealed the role of miR-9 regulation in suppressing GSK-3β expression and in facilitating BC tumorigenesis. 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 invasion, and potentiate cell apoptosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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