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MiR-155 affects renal carcinoma cell proliferation, invasion and apoptosis through regulating GSK-3β/β-catenin signaling pathway

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Abstract. – OBJECTIVE: Glycogen Synthase Kinase-3β (GSK-3β) negatively regulates Wnt/β-catenin signaling pathway through degrading β-catenin protein. It plays an inhibitory role in various tumors, while the influence in the pathogenesis of renal carcinoma has not been elucidated. MicroRNA-155 (MiR-155) was found to be upregulated in renal carcinoma tissue. Bioinformatics analysis revealed the complementary binding site between miR-155 and 3'-UTR of GSK-3β. This study investigated the influence of miR-155 in regulating GSK-3β expression, Wnt/β-catenin signaling pathway activity, and renal carcinoma cell proliferation, invasion, and apoptosis.

PATIENTS AND METHODS: The targeted regulatory relationship between miR-155 and GSK-3β were tested by dual luciferase assay. Renal carcinoma tissue and benign renal tissue were collected to detect miR-155, GSK-3β expressions. MiR-155, GSK-3β, and β-catenin levels were compared between 786-O and A-498 cells. Renal carcinoma 786-O cells were cultured in vitro and divided into blank, miR-NC, anti-miR-155, pIRES2-GSK-3β groups. Cell apoptosis was evaluated by flow cytometry. Cell invasion was determined by transwell assay. Cell proliferation was assessed by EdU staining.

RESULTS: MiR-155 targeted and regulated GSK-3β expression. GSK-3β and β-catenin expressions were significantly increased, while GSK-3β level was significantly lowered in renal carcinoma tissue compared with benign renal tissue. MiR-155 and β-catenin expressions were significantly higher, whereas GSK-3β level was significantly downregulated in 786-O cells compared with A-498 cells. Anti-miR-155 or pIRES2-GSK-3β transfection significantly up-regulated GSK-3β expression, attenuated β-catenin level, restrained cell proliferation and invasion, and enhanced cell apoptosis.

CONCLUSIONS: MiR-155 promoted renal carcinoma pathogenesis. Inhibition of miR-155 increased GSK-3β expression, attenuated Wnt/β-catenin signaling pathway, weakened proliferation and invasion, facilitated apoptosis in renal carcinoma cells.

Key words:
miR-155, GSK-3β, Wnt/β-catenin, Renal carcinoma, Proliferation.

Introduction

Renal cell carcinoma (RCC) is a type of malignant tumor derived from uriniferous tubule epithelial system. Its morbidity accounts for the 12th in malignancy and 2nd in urinary tract malignant tumor[1]. RCC is featured as low sensitivity to radiotherapy and chemotherapy, and high postoperative recurrence rate, thus causing severe impact on quality of life and health.

β-catenin is a key protein in Wnt/β-catenin signaling pathway that is closely associated with tumorigenesis, progression, and metastasis[2,3]. Glycogen synthase kinase-3β (GSK-3β) is a negative regulator of Wnt/β-catenin signaling pathway that locates in the upstream of β-catenin. It degrades β-catenin through phosphorylation to maintain it at relative low level, thus suppressing the excessive activation of Wnt/β-catenin signaling pathway. It plays a tumor suppressor role in tumorigenesis by promoting cell apoptosis and restraining cell proliferation[4-6]. It was showed that GSK-3β expression and downregulation mediated Wnt/β-catenin signaling pathway enhancement was related to the occurrence, progression, metastasis, and poor prognosis of thyroid cancer[7], intestinal cancer[8], prostate cancer[9], and breast cancer[10]. However, its role in RCC has not been clarified. MicroRNA (miRNA) is a

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type of endogenous single stranded non-coding RNA at the length of 22-25 nt. It plays a degrading or inhibiting role on mRNA by binding with the 3'-UTR, thus participating in multiple biological processes, such as cell proliferation, differentiation, tissue and embryonic development, angiogenesis, and immune response[11]. The role of miRNA abnormity in tumorigenesis and development bring more and more attention[12,13]. It was reported that miR-155 markedly increased in RCC tumor tissue, indicating that RCC may be treated as an oncogene of RCC[14,15]. Bioinformatics analysis revealed the complementary binding site between miR-155 and 3'-UTR of GSK-3β. This study investigated the influence of miR-155 in regulating GSK-3β expression, Wnt/β-catenin signaling pathway activity, and renal carcinoma cell proliferation, invasion, and apoptosis.

Patients and Methods

Clinical Information

A total of 36 cases of RCC patients rece[illegible]ved surgery in our hospital between June 20[illegible] December 2016 were enrolled, including 20 [illegible]s and 16 females with mean age at 56.7 ± 11.9 y[illegible] old. No patients received radiotherapy or chem[illegible] therapy before surgery. RCC tu[illegible]ue wa[illegible] stored at -80°C. The specimens [illegible]e di[illegible]d in to 10 in G1, 15 in G2, and 11 in [illegible]accordi[illegible]to cell differentiation degree. There [illegible]22 [illegible] and 14 in stage III-IV ac[illegible]ding [illegible]M staging. Another 22 cases of [illegible]l tissue w[illegible]obtained from benign renal le[illegible]cluding 11 [illegible]es and 11 females with a[illegible]ge a[illegible]57.3 ± 13.1 years old. No statisti[illegible]difference[illegible]ge and gender were observe[illegible]etween two grou[illegible]

Main Re[illegible]nts [illegible]d Ma[illegible]erials

Human R[illegible]-O a[illegible]ormal renal proximal [illegible] epit[illegible]l c[illegible]HK-2 were purchased fr[illegible]Sh[illegible]hai B[illegible]otechnology Co., Ltd. (Sha[illegible]hina) Roswell Park Memorial Inste[illegible]0 and Dulbecco Minimum Es[illegible]Medium (DMEM) mediums were obtaine[illegible]m Lonza (Allendale, NJ, USA). Penicillin-st[illegible]omycin was derived from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was got from Gemini Bio Products (West Sacramento, CA, USA). Total RNA extraction reagent GenElute™ Total RNA Purification Kit was bought from Sigma-Aldrich (St. Louis, MO, USA). Real-time PCR reagent TransScript Green One-Step qRT-PCR SuperMix was obtained from TransGen (Beijing, China). Transfection kit FuGENE6 was purchased from Roche Pharma (Basel, Switzerland). MiR-NC, miR-155 mimic, and anti-miR-15[illegible]re purchased from RiboBio (Guangzh[illegible]EdU flow cytometry reagent was [illegible]rom Sig[illegible]ldrich (St. Louis, MO, USA[illegible]bbit anti-h[illegible]n GSK-3β and β-catenin antibod[illegible]ere pro[illegible]ed by Cell Signaling Te[illegible]ology ([illegible]rb[illegible]MA, USA). Mouse anti-h[illegible]an Survivin [illegible]-actin antibodies were [illegible]ained[illegible]m Activ[illegible] motif (Carlsbad, CA, U[illegible]H[illegible] radi[illegible]peroxidase (HRP) conju[illegible]d se[illegible]ary a[illegible]ody was got from Jacks[illegible]Immuno[illegible](West Grove, PA, USA[illegible]luciferase[illegible]porter gene vector pM[illegible]and [illegible]Luciferase® Reporter Assay System were pro[illegible]by Promega (Madison, W[illegible]A). RIPA l[illegible] was purchased from [illegible]arBio (Beijing, China). Bicinchoninic acid (BCA) protei[illegible]antification reagent was bought [illegible]Thermo[illegible]ientific Pierce (Rockford, IL, U[illegible]Ove[illegible]pression plasmid pIRES2 was deriv[illegible]BioVector (Beijing, China). FITC Annexin V/PI cell apoptosis detection kit was [illegible]d from Beyotime Biotech. (Shanghai, [illegible]). Matrigel was purchased from BD Biosciences (San Jose, CA, USA). Transwell chamber was got from Corning (Corning, NY, USA).

Cell Culture

786-O cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. CCC-HEK-1 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) medium containing 20% FBS and 1% penicillin-streptomycin. The cells were passaged at 1:4 and used for experiments during logarithmic phase.

Dual-Luciferase Reporter Gene Assay

The PCR products containing the full-length of GSK-3β gene 3'-UTR or mutant segment were cloned to pMIR. Next, it was transformed to DH5α competent cells and sequenced to select the plasmid with correct sequence. Then, pMIR-GSK-3β-wt (or pMIR-GSK-3β-mut) was co-transfected to HEK293T cells using FuGENE6 together with miR-155 mimic (or anti-miR-155, or miR-NC). After 48 h incubation, the cells were lysed by passive lysis buffer on ice for 20 min and detected using Stop&Glo solution.

GSK-3β Over-Expression Plasmid Construction

The CDS region segment of GSK-3β was amplified and recycled after gel electrophoresis. Next, it was connected to pIRES2 plasmid after double digestion and transformed to bacteria. Then, the bacterial strain was amplified and sequenced to confirm the correct GSK-3β gene segment insertion. It was named as pIRES-GSK-3β for the following experiments.

Cell Transfection and Grouping

786-O cells were divided into four groups, including miR-NC, anti-miR-155, pIRES2-blank, and pIRES2-GSK-3β groups. MiR-NC, anti-miR-155, pIRES2-blank, or pIRES2-GSK-3β at 20 nmol/l and FuGENE6 at 10 μl were diluted in serum free medium at room temperature for 20 min. Then, they were added to the cells and incubated for 72 h for the following experiments.

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using GenElute™ Total RNA Purification Kit and adopted for PCR reaction by TransScript Green On qRT-PCR SuperMix. The reaction system tained 2 μg template RNA, 0.3 μM primers, μl 2×TransStart Tip Green qPCR SuperMix, 0 μl One-Step RT Enzyme Mix, Passiv Reference Dye II, and RNase e w r. The PCR reaction was compose 45°C 5 min and 94°C for 30 s, follow 4 94°C for 5 s and 60°C f 0 s time PCR was performed on Bi ad CFX test the relative expression mer seque were listed as follows.

miR-155P$_F$: ' GCTCAG ATGCTAATC-GTGATA-3', -155P$_R$: 5′-AT ATGTTGTC-CACTGTC G-3′; U6P$_F$: 5′-ATTGGAAC-GATACA AA T-3′, U6P$_R$: 5′-GGAAC-GCTTCAC G-3′; K-3βP$_F$: 5′-GGCAG-CAT AG 5′, GSK-3βP$_R$: 5′-GG-C TC AATC-3′; β-cateninP$_F$: AAG GCTGT AGTCACTGG-3′, β-cat-P$_R$: CATTGCATACTGTCCAT-3′; β- 5′-GAACCCTAAGGCCAAC-3′, β-actin P$_R$ GTCACGCACGATTTCC-3′.

Western Blot

The total protein was extracted by RIPA from cells. A total of 50 μg protein was separated by 10% sodium lauryl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membrane. Next, the membrane was blocked by 5% skim milk at room temperature for 60 min and incubated in primary antibody at 4°C overnight (GSK-3β, β-catenin, and β-actin at 1:3000, 1:3000, and 1:10000, respectively). Then, the membrane was incubated in HR jugated secondary antibody (1:30000) after washed by PBST for three tim At last, t o-tein expression was detecte ECL (enha d chemiluminescence).

Flow Cytometry

The cells were sted l rypsin and resuspended in 100 μl ffer. N t, the cells were incubat 5 nexi FITC and 5 μl PI. At la e cells d on Beckman CytoFLE ytometry valuate cell apoptosis.

E ining

he cells were added with 10 μM EdU solu- for 2 h. er incubated for 72 h, the cells digeste d collected. Next, cells were w by sphate buffered saline (PBS), fixed paraformaldehyde, and penetrated by saponin; they were incubated in 500 μl reliquid containing PBS, Catalyst solution, FAM Azide, and Buffer additive at room temperature avoid of light for 30 min. Then, the cells were washed and tested by Beckman CytoFLEX flow cytometry.

Transwell Assay

Matrigel was added to the upper chamber and incubated at 37°C for 30 min. A total of 500 μl RPMI-1640 medium contained 10% FBS was added to the 24-well plate. The

Transwell chamber paved with 100 μl Matrigel was put onto the plate and added with 786-O cells resuspended in 200 μl serum-free medium. After 48 h, the membrane was fixed in methanol and stained by 0.1% crystal violet. At last, the membrane was observed under the microscope.

Statistical Analysis

All data analyses were performed on SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were depicted as mean ± standard deviation and compared by using Student's *t*-test or ANOVA. Tukey's post hoc-test was used for comparing measurement data between groups. $p < 0.05$ was considered as statistical significance.

Results

MiR-155 Targeting Inhibited GSK-3β Expression

Bioinformatics analysis showed the targeted binding site between miR-155 and 3'-UTR of GSK-3β mRNA (Figure 1A). Dual luciferase assay revealed that miR-155 mimics or anti-miR-155 transfection significantly declined or elevated the relative luciferase activity of HEK293T cells transfected by pMIR-GSK-3β-wt (Figure 1B), while they failed to affect the relative luciferase activity of HEK293T cells transfected by pMIR-GSK-3β-mut, indicating the regulatory relationship between miR-155 and GSK-3β mRNA.

MiR-155 and GSK-3β Expression in RCC Tissue

qRT-PCR demonstrated that GSK-3β mRNA significantly declined, while miR-155 level obviously up-regulated in RCC tissue compared with control (Figure 2A). MiR-155 expression elevated following TNM staging and pathological grading (Table I). Western blot revealed that G protein level markedly decreased in RCC e compared with control and kept reducing foll ing clinical staging (Figure 2B).

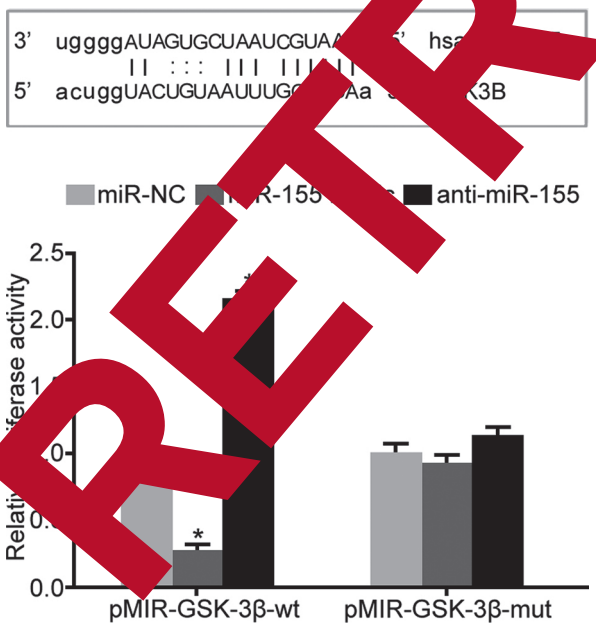

Figure 1. MiR-155 targeted inhibited GSK-3β expression. ***A,*** The binding site between miR-155 the 3'-UTR of GSK-3β mRNA. ***B,*** Dual luciferase assay. *$p < 0.05$, compared with mimic NC. miR-155: microRNA-155, NC: normal control.

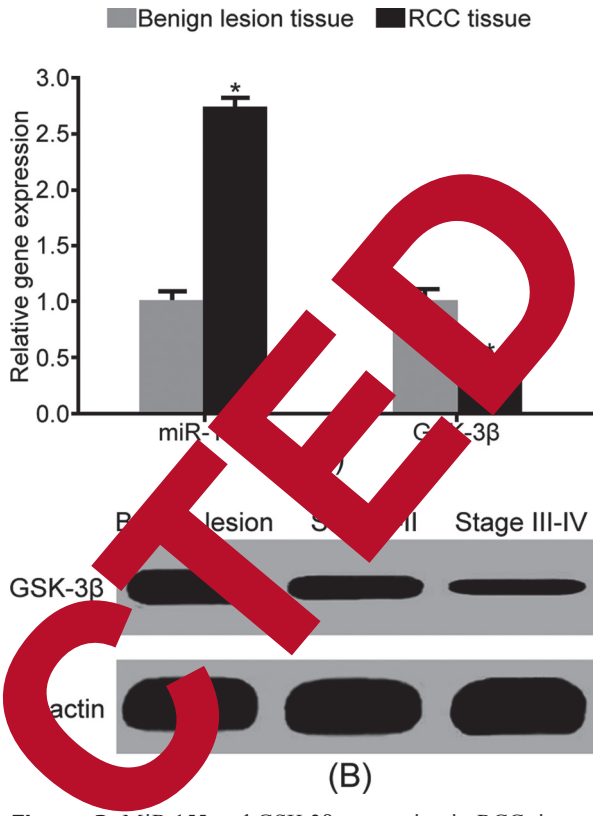

Figure 2. MiR-155 and GSK-3β expression in RCC tissue. RT-PCR detection of miR-155 and GSK-3β mRNA ns in renal tissue. ***B,*** Western blot detection of GSK-3β protein expression in renal tissue. miR-155: microRNA-155, GSK-3β: glycogen synthase kinase-3β, RCC: renal cell carcinoma.

MiR-155 Levels Were Increased and GSK-3β Levels Were Reduced in Renal Carcinoma Cells

qRT-PCR results demonstrated that GSK-3β mRNA was apparently declined, whereas miR-155 and β-catenin mRNA were significantly enhanced in renal carcinoma 786-O cells compared with HK-2 cells (Figure 3A). Western blot data exhibited that GSK-3β protein was attenuated, while β-catenin protein was significantly upregulated in 786-O cells compared with HK-2 cells (Figure 3B).

Inhibition of miR-155 Expression Attenuated Rcc Cell Proliferation and I nvasion, and Promoted Cell Apoptosis

Anti-miR-155 or pIRES2-GSK-3β transfection was significantly up-regulated GSK-3β expression, attenuated β-catenin level (Figure 4A), restrained cell invasion (Figure 4B) and proliferation (Figure 4C), and enhanced cell apoptosis (Figure 4D).

Table I. MiR-155 expression in RCC tissue with different clinical characteristics.

Group	Cases	miR-155 expression	*p*-value
Age			0.215
≤ 50 years old	15	1.58 ± 0.13	
> 50 years old	21	1.62 ± 0.16	
Gender			
Male	20	1.63 ± 0.15	
Female	16	1.61 ± 0.18	
Pathological grading			0.00
G1	10	1.43 ± 0.18	
G2	15	1.57 ± 0.18	
G3	11	1.73 ± 0.21	
Clinical stage			01
I-II	22	1.36 ± 0.15	
III-IV	14	1.81 ± 0.1	

Discussion

Wnt/β-catenin is a highly conserved signaling pathway in evolution that plays an important role in regulating cell proliferation, cycle, differentiation, apoptosis, and invasion. Wnt/β-catenin excessive activation plays a stimulate role in the occurrence and development of colorectal cancer[16] and prostate cancer[17]. β-catenin is a key p in Wnt/β-catenin signaling that mediates s al nuclear transfer and activates the pathway Abnormal up-regulation of β-catenin may e hance Wnt/β-catenin signaling p which is closely associated with var s tu s' occurrence, progression, and p stasis[2]. During Wnt/β-catenin activation, Wn bi zled (F rece and low-density lipoprotein receptor related p n 5 and 6 (LRP5/6) on th membrane, i ng to disheveled (Dvl) sphorylation. It further transduces signal to K-3β to i it the phosphorylation-degrad- role of G 3β on β-catenin and deactivate A xin-G -3β complex, resulting in the enhanc β-catenin stability and increase of β-catenin level in nucleus. It binds with TCF/LEF ote target gene transcription and expres- ion . In the negative regulatory mechanism of Wnt/β-catenin signaling pathway, GSK-3β can form complex with axin and adenomatous polyposis coli (APC) to phosphorylate β-catenin. Phosphorylated β-catenin is further degraded by β-transducin repeat-containing protein (β-TrCP)

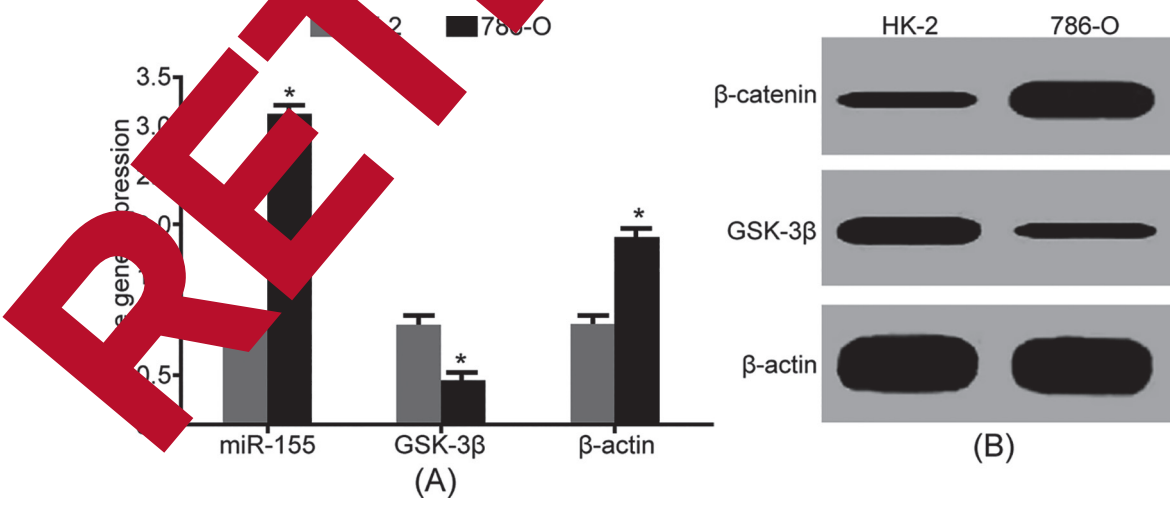

Figure 3. MiR-155 increased, while GSK-3β reduced in renal carcinoma cells. ***A,*** qRT-PCR detection of miR-155, GSK-3β, and β-catenin mRNA expressions. ***B,*** Western blot detection of GSK-3β and β-catenin protein expressions. *$p < 0.05$, compared with HK-2 cells. miR-155: microRNA-155, GSK-3β: glycogen synthase kinase-3β, qRT-PCR: quantitative RT-PCR.

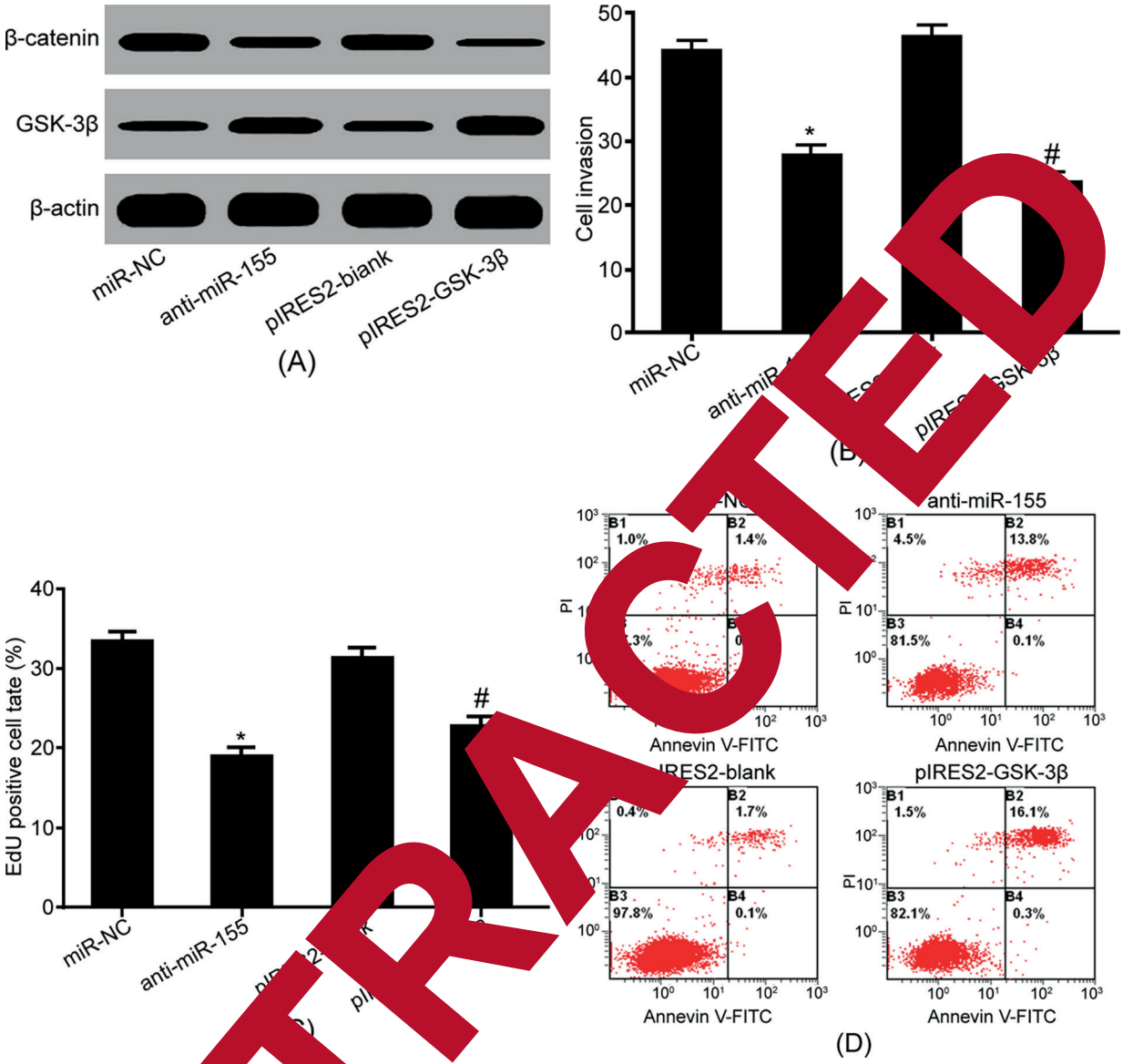

Figure 4. Inhibiti[on] of miR-155 [expr]ession attenuated RCC cell proliferation and invasion, and promoted cell apoptosis. ***A,*** qRT-PCR det[ec]tion of gene expres[sion]. ***B,*** Transwell assay detection of cell invasion. ***C,*** EdU staining detection of cell proliferation. ***D,*** [Fl]ow cytometry detection of cell apoptosis. *$p < 0.05$, compared with miR-NC; #$p < 0.05$, compared with pIRES2-blan[k]. miR-155: [m]iRNA-155, circRNA-155, RCC: renal cell carcinoma, NC: normal control.

m[od]ulated [u]biquiti[n]-proteasome pathway, thus [re]pressing the activity of Wnt/β-catenin signaling pathway. GSK-3β plays a tumor suppressor role in multiple cancers because of its attenuation of proliferation acceleration and anti-apoptosis mediated by Wnt/β-catenin signaling pathway activation. It was showed that GSK-3β down-regulation mediated Wnt/β-catenin enhancement is closely related to a variety of cancers' occurrence, progression, metastasis, and poor prognosis, such as thyroid cancer[7], intestinal cancer[8], prostate cancer[9], and breast cancer[10]. However, its role in RCC has not been clarified. It was reported that miR-155 markedly increased in RCC tumor tissue, indicating that RCC may be treated as an oncogene of RCC[14,15]. Bioinformatics analysis revealed the complementary binding site between miR-155 and 3'-UTR of GSK-3β. This study investigated the influence of miR-155 in regulating GSK-3β expression, Wnt/β-catenin signaling pathway activity, and renal carcinoma cell proliferation, invasion, and apoptosis.

Dual luciferase assay revealed that miR-155 mimics or anti-miR-155 transfection significantly declined or elevated the relative luciferase activity of HEK293T cells indicating the regulatory relationship between miR-155 and GSK-3β mRNA. GSK-3β was significantly declined, while miR-155 level was obviously upregulated in RCC tissue compared with control. MiR-155 expression elevated following TNM staging and pathological grading. GSK-3β apparently declined, whereas miR-155, β-catenin, and survivin significantly enhanced in renal carcinoma 786-O cells compared with HK-2 cells, suggesting that miR-155 up-regulation may play a role in reducing GSK-3β expression, enhancing Wnt/β-catenin signaling pathway activity, and promoting RCC tumorigenesis. Gao et al[14] reported that miR-155 abnormally elevated in RCC tissue compared with normal renal tissue and increased following TNM staging, which was similar with our results. Jung et al[21] showed that miR-155 increased for 3.2 times in RCC tissue compared with adjacent normal control. Li et al[15] demonstrated that miR-155 in RCC tissue was obviously higher than that in para-carcinoma tissue. In addition, it was higher in RCC tissue with higher TNM stage than wi TNM stage. MiR-155 expression was ma higher in RCC cell lines ACHN and KC c pared with normal renal cell HK-2, which w in accordance with our study[15]. S ly, Mer hautova et al[22] found that miR up lation was related to the decrease emo itivity to sunitinib, presenting as 155 higher in patients with lo DF et al[23] observed that miR-155 e ssion incr in RCC tissue. Silva-Santos evealed tha R-155 level was higher i CC than that in normal renal tissue d was hig patients with worse surviv White et al[25] d nstrated that miR-155 si icant evated in RCC tissue and was posit co ted w tumor size. It was showed that 5 pla an oncogene role in RCC h wa ance with our results. Y et obse that GSK-3β reduction relate epithelial mesenchymal transition T) and invasion enhancement o In this paper, GSK-3β expression reduced CC tissue and cell line, indicating that GSK-3β elated to the pathogenesis of RCC, which was similar with Yuan et al findings[26]. Further investigation revealed that anti-miR-155 or pIRES2-GSK-3β transfection significantly attenuated β-catenin level, restrained cell proliferation and invasion, and enhanced cell apoptosis.

Gao et al[14] presented that miR-155 promoted RCC cell lines ACHN and 786-O cell migration and invasion through targeting E2F2 expression. Li et al[15] observed that downregulation of miR-155 suppressed ACHN cell proliferation, induced cell apoptosis, and restrained cell ion by increasing BACH1. Yuan et a that GSK-3β enhancement obviou inhibited T, migration, and invasion i C. It exh d that both miR-155 and GSK- ay a ro in the pathogenesis of R . This r led that inhibition of m 155 upregula SK-3β expression, atten d W -catenin activity, restrained cell p at and asion, and enhanced cell opte Howe the specific target gene miR-155 Wnt/β-catenin signaling y in regula RCC cell proliferation, vasio d apoptosis is still unclear.

Conclusions

We showe hat miR-155 promoted renal ca ma ogenesis. Inhibition of miR-155 d GSK-3β expression, attenuated Wnt/β-catenin signaling pathway, weakened pro- on and invasion, and facilitated apoptosis renal carcinoma cells.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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