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Preliminary results indicate increased expression of miR-184 in patients with renal carcinoma

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Abstract. – OBJECTIVE: Renal carcinoma is the second most common cancer in the urinary system with an increasing trend. The major treatment for renal carcinoma is surgery, which results in unfavorable prognosis at times. As a tissue-specific marker for tumor, microRNA (miR) exerts its functions via facilitating oncogenic gene expression or suppressing tumor suppressor gene. MiR-184 is known to be abnormally expressed in various tumors. There are few studies about the lack of miR-184 expression in renal carcinoma.

PATIENTS AND METHODS: real time-Polymerase Chain Reaction (PCR) was used to measure the expression of miR-184 in 38 renal carcinoma and adjacent tissues. The *in vitro* cultured renal carcinoma cell line ACHN was transfected with miR-184 mimic or inhibitor. The expression of miR-184 was measured by real time-PCR, and the cell proliferation was measured by MTT assay. The cell colony formation was examined, and the cell invasion potency was assessed by transwell assay. The apoptotic activity was measured by flow cytometry, and the Western blot detected protein expression change of β-catenin/TCF3 pathway.

RESULTS: Compared to tumor-adjacent tissues, miR-184 and β-catenin/TCF3 showed an elevated expression in renal carcinoma tissues which were further increased with elevated RC stages ($p<0.05$). The transfection of miR-184 mimic into ACHN cells increased its expression, enhanced ACHN cell proliferation, colony formation, inhibited apoptosis, promoted tumor cell invasion, and increased the expression of β-catenin and TCF4 proteins ($p<0.05$ compared to NC control group).

CONCLUSIONS: MiR-184 is up-regulated in renal carcinoma tissues. The downregulation of miR-184 in renal carcinoma cells could facilitate cell apoptosis and inhibited tumor proliferation or invasion possibly via modulating β-catenin/TCF4 pathway.

Key Words:
Renal carcinoma, MiR-184, β-catenin/TCF4, Apoptosis, Proliferation, Cell invasion.

Introduction

Among all cancers in the urinary system, the renal carcinoma (RC) is the second most common lesion, which displays an increasing trend of incidence[1,2]. RC frequently derives from different sites of the urinary tract, mainly in the renal cortical glomerulus epithelial system. Based on pathology and typing, several subtypes of RC have been identified, such as clear cell, papillary, chromophobe, cystic-solid, collecting ducts (Bellini), medullary, as well as some other rare subtypes[3]. RC accounts for 2% of all cancers worldwide and can occur across all age groups with a high prevalence rate in the middle-aged males[4,5]. Being subjected to lifestyle and diet habit transition, the RC incidence in China is rapidly increasing, with a growing trend in the younger patient population[6]. Currently, the major treatment approaches for RC is surgery. However, lots of patients were already at terminal stage when received a primary diagnosis because of atypical early symptoms.

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Some patients also tend to developed recurrence or metastasis after surgery[7,8]. The clinical symptoms in RC patients mainly include urea blood, waist pain, and abdominal lesion, which form the triad of RC. As the common late onset of typical symptoms, the primary RC is frequently already at an advanced stage when diagnosed, leading to metastasis toward distal adrenal gland or lymph node tissues, plus distal metastasis in lung, liver or bone tissues[9]. On the other hand, RC is reported to be insensitive to the auxiliary treatments, including chemotherapy or radiotherapy, which add the chance of unfavorable prognosis in RC patients[10]. RC has complicated the pathogenesis mechanisms that have not been fully illustrated. Therefore, it is urgent to identify the effective molecular targets to provide evidence to analyze the pathogenesis mechanism and clinical treatment of RC.

MicroRNA (miR) consists of 22-23 nucleic acids and is widely distributed in eukaryotic cells in plants and animals[11]. MiR has been demonstrated to be able to bind with mRNA via targeted complementary binding, which results in the negative regulation of the gene expression at the post-transcriptional level to facilitate mRNA degradation or translational inhibition[12]. MiR participates in various biological behaviors including cell growth, proliferation, apoptosis, organogenesis, inflammation, and tumor[13,14]. As one tumor biological marker, miR has tissue sensitivity and can exert its roles via facilitating the oncogene expression or suppressing the tumor suppressor gene expression[15]. Fadejeva et al[16] found abnormal expression of miR-184 in different types of tumors. However, the mechanism by which miR-184 participates in the pathogenesis of RC remains unknown.

Patients and Methods

Patients

A total of 38 RC patients who were diagnosed by histopathology examination and were treated in Maoming People's Hospital (Maoming, Guangdong, China) for surgery between January 2017 and October 2017 were recruited in this study. Patients were aged between 31 and 69 years (average age = 47.2±9.2 years). Among these enrolled patients, there were 8 patients in T1, 14 in T2, 11 in T3 and 5 in T4. In addition, there were 19 patients in G1, 11 in G2, and 8 in G3. Based on the pathology, there were 18 patients with renal clear cell carcinoma, 14 with papillary renal cell carcinoma, 3 with medullary carcinoma and 3 with others. The tumor tissues and adjacent tissues (>3 cm from tumor edge) were collected during the surgery for pathology examination and were confirmed to be cancer-free by histological evaluation. All patients were confirmed as primary RC. The inclusive and exclusive criteria were[10]: having confirmed pathology diagnosis; primary RC that hasn't been treated with surgery, chemotherapy or radiotherapy. Those patients with recurrent or metastasis RC were excluded. Patients having received surgery, chemotherapy or radiotherapy previously, complicated with other systemic diseases such as immune disorder or malignant tumor complication, or those who had low motivation to engage with the research were also excluded from this study.

This study has been approved by the Ethical Committee of Maoming People's Hospital (Maoming, Guangdong, China). All participants have acknowledged this research and have signed the informed consents.

Major Reagent and Equipment

RC cell line ACHN, 769-1, and normal renal cell line HK-2 were purchased from ATCC cell bank (Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM) medium and penicillin-streptomycin dual antibiotics were purchased from HyClone (South Logan, UT, USA). Fetal bovine serum (FBS), DMSO, and MTT powder were purchased from Gibco (Grand Island, NY, USA). The trypsin-EDTA digestion buffer was purchased from Sigma-Aldrich (St. Louis, MO, USA). The polyvinylidene difluoride (PVDF) membrane was purchased from Pall Life Sciences (Port Washington, NY, USA). The Western blot reagents were purchased from Beyotime Biotech (Nantong, China). Enhanced chemiluminescence (ECL) reagent was purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Rabbit anti-human β-catenin, TCF4 monoclonal antibody, and goat anti-rabbit horseradish peroxidase (HRP) conjugated IgG secondary antibody were purchased from Cell Signaling (Danvers, MA, USA). RNA extraction kit and reverse transcription kit were purchased from Axygen (Union City, CA, USA). The annexin V-PI apoptotic assay kit was purchased from BD (San Jose, CA, USA). MiR-184 inhibitor and miR-184 negative control (NC) sequences were synthesized by Gimma Gene (Shanghai, China). The Lipofectamine 2000 reagent was purchased from

Invitrogen (Carlsbad, CA, USA). The transwell chamber was purchased from Corning (Corning, NY, USA). The Dual-Luciferase Reporter Assay system was purchased from Promega (Madison, WI, USA). The Labsystem Version 1.3.1 microplate reader was purchased from Bio-Rad (Hercules, CA, USA). The ABI 7700 fluorescent quantitative PCR cycler was purchased from ABI (Waltham, MA, USA). The ultrapure workstation was purchased from Sutai purification Corp (Suzhou, China). The melody flow cytometry was purchased from BD Biosciences (San Jose, CA, USA). The Thermo Scientific Heraeus CO_2 incubator was purchased from Thermo-Fisher Scientific (Waltham, MA, USA). The sonics ultrasonic rupture was purchased from Sonics (Oklahoma City, OK, USA).

Grouping of RC Cell Line ACHN

The RC cell line ACHN kept in liquid nitrogen was resuscitated and was grown in Eagle's Minimum Essential Medium supplemented with 10% FBS and 1mm/L penicillin-streptomycin. Post passage, the cells were randomly assigned into 4 groups: i.e., miR-184 mimic NC group, miR-184 mimic group, miR-184 inhibitor NC group, and miR-184 inhibitor group, which were transfected with miR-184 inhibitor NC sequence or miR-184 inhibitor sequence.

Liposome Transfection of MiR-184 Inhibitor Into ACHN Cells

MiR-184 mimic (5'-CAGCUGAUATACCGT-3'), miR-184 mimic NC (5'-CGUAUGAUCCGACGAG-3'), MiR-184 inhibitor NC (5'-CGCCA GCUGA UUUAT AUAT-3') or miR-184 inhibitor sequences (5'-CCUGU ACGAU CGAGU GAGAU G-3') were transfected into ACHN cells. The cells were incubated in 6-well plate reaching 70-80% confluence. MiR-184 inhibitor NC or miR-184 inhibitor liposome was added into 200 µl serum-free DMEM medium for 15 min at room temperature incubation. The Lipofectamine 2000 was mixed for 30 min at room temperature incubation. The serum was removed from the cell culture, and the cells were gently rinsed by PBS. 1.6 ml serum-free DMEM was added into each system. The cells were kept in 5% CO_2 incubator for 6 h incubation at 37°C. The serum containing DMEM medium was switched for 48 h continuous incubation in further assays.

Real Time-PCR Measuring MiR-184 Expression in RC Tissues and ACHN Cells

TRIzol reagent was used to extract mRNA from RC tumor tissues or adjacent tissues, and from ACHN tumor cell lines in all groups. Based on the manual instruction of the test kit, the DNA reverse transcription was performed. All primers were designed by Primer Premier 6.0 based on a target gene sequence and were synthesized by Invitrogen Biotech (Table I). Real time-PCR was performed for target genes under the following conditions: 56°C for 1 min, followed by 35 cycles each consisting of 92°C for 30 s, 58°C for 40 s, and 72°C for 30 s. The data were collected for calculating CT values of all samples as per standards based on fluorescent quantification and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin housekeeping gene using built-in software. A standard curve was plotted using the CT values of standards, and semi-quantitative analysis was performed by the $2^{-\Delta\Delta Ct}$ method.

Western Blot for Measuring β-Catenin/TCF4 Protein Expression

The cellular proteins were extracted from renal carcinoma tissues, adjacent tissues, and all groups of ACHN cells. In brief, the radioimmunoprecipitation assay buffer (RIPA) lysis buffer containing proteinase inhibitor was added for 30 min cell lysis on ice. The cells were ruptured by ultrasound (5 s, 4 times), and cell lysate was centrifuged at 10000 g for 15 min under 4°C. The supernatant was saved and transferred into a new tube. The proteins were quantified by the bicinchoninic acid assay (BCA assay) method and were kept at -20°C for Western blot. The proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to PVDF membrane using semi-dry method (200 mA, 2

Table I. Primer sequence.

Gene	Forward primer 5′-3′	Reverse primer 5′-3′
GAPDH	AGTACCAGTCTGTTGCTGG	TAATAGACCCGGATGTCTGGT
β-actin	TACTTGCAGTCTGTAC	CCTACCGCATGTGGATGT
miR-184	TCGACCCCTACCTGCTTAGT	TGGGATACCAGTGTGTCATA

h). The non-specific background was removed by 5% nonfat milk powder for 2 h at room temperature incubation. The primary antibody against β-catenin (1:2000) or Transcription factor 4 (TCF-4; 1:2000) was added for 4°C overnight incubation. On the next day, the membrane was rinsed in Phosphate-Buffered Saline supplemented with Tween-20 (PBST) and was incubated with 1:2000 goat anti-rabbit secondary antibody for 30 min incubation at room temperature. After washing the membrane with PBST, the enhanced chemiluminescent (ECL) reagent was added for 1 min incubation, followed by X-ray film exposure. The protein imaging processing software and the Quantity One system were used to scan X-ray film and to measure band density. All experiments were repeated four times (n=4) for the statistical analysis.

MTT Assay for Measuring Cell Proliferation

ACHN cells at log-growth phase were inoculated into 96-well plate using DMEM medium containing 10% FBS at $5X10^3$ cells density. Post 24 h incubation, the supernatant was discarded, and the cells were randomly assigned into 2 treatment groups, as previously described. After 48 h, all test wells were added with 20 μl sterile MTT, with triplicated wells in each treatment group. After 4 h incubation, the supernatant was completely removed. 150 μl dimethyl sulfoxide (DMSO) was added into each well for 10 min vortex until the complete resolving of the violet crystal. The absorbance (A) values at 570 nm wavelength were measured under a microplate reader for calculating the proliferation rate (=experimental group/control group × 100%). All experiments were repeated for more than 3 times.

Cell Apoptosis Assay

The culture medium was discarded from all groups of cells, which were digested after rinsing in pre-cold PBS. The cells were collected and mixed for centrifugation for 5 min using 1XPBS. The cells were fixed in 75% pre-cold ethanol for 4°C overnight incubation. On the next day, 75% ethanol was discarded, and cells were rinsed in 1XPBS for 5 min under 1000 rpm. The cells were re-suspended in 800 μl 1XPBS and 1% BSA and were sequentially mixed with 100 μg/ml propidium iodide (PI) dye (3.8% sodium citrate, pH7.0) and 100 RNase A (10 mg/ml). Following 37°C dark incubation for 30 min, 300 μl 1X Binding buffer was added for assay in flow cytometry.

Transwell Chamber Assay

Following the manual instruction, the serum-free DMEM medium was switched. 24 h later, the transwell chamber was pre-coated on the bottom and upper membrane phase using 1:5 Matrigel dilution (50 mg/L), and was air-dried at 4°C. 500 μl DMEM medium containing 10% FBS and 100 μl tumor cell suspension in serum-free medium were added into the interior and exterior of the chamber. Triplicated wells were set for each group, and the chambers were placed in a 24-well plate. The control group utilized the Matrigel-free transwell chamber. After 48 h of incubation, the transwell chamber was rinsed in PBS to remove the cells on the membrane. The cells were fixed in cold ethanol and were stained in crystal violet. The cells at the lower phase of the micropore membrane were enumerated. All experiments were repeated for 3 times.

Colony Formation Assay

All cells were inoculated into 6-well plate at 1000 cell per well density and were incubated at 37°C incubator for 10 days with 5% CO_2. The cells were lastly fixed in 4% paraformaldehyde, stained in crystal violet and then, the formed colonies were counted under a microscope.

Dual-Luciferase Reporter Assay

The bioinformatics software was used to analyze the binding sequence of β-catenin and TCF4 to miR-184, which was, then, synthesized. Meanwhile, the mutant sequence of β-catenin and TCF4 was also synthesized. The PCR product of the β-catenin and TCF4 3'-UTR full-length fragment or mutant fragment was double-digested and then ligated into the GP-miRGLO vector. After sequencing, the plasmid was designated as miRGLO-Wt-β-catenin, miRGLO-Mut-β-catenin, miRGLO-Wt-TCF4 and miRGLO-Mut-TCF4 which were then transfected into HEK293T cells together with miR-184 mimic (or miR-184 inhibitor, miR-NC) by Lipofectamine 2000. Post incubation for 48 h, the relative luciferase activity was measured using the Dual-Luciferase Reporter Assay system according to the manual.

Statistical Analysis

All data were presented as mean±standard deviation (SD). The Student's *t*-test or Wilcoxon test was performed for comparing the means between the two groups. SPSS 11.5 software (SPSS Inc., Chicago, IL, USA) was used for the analysis. The difference among groups was

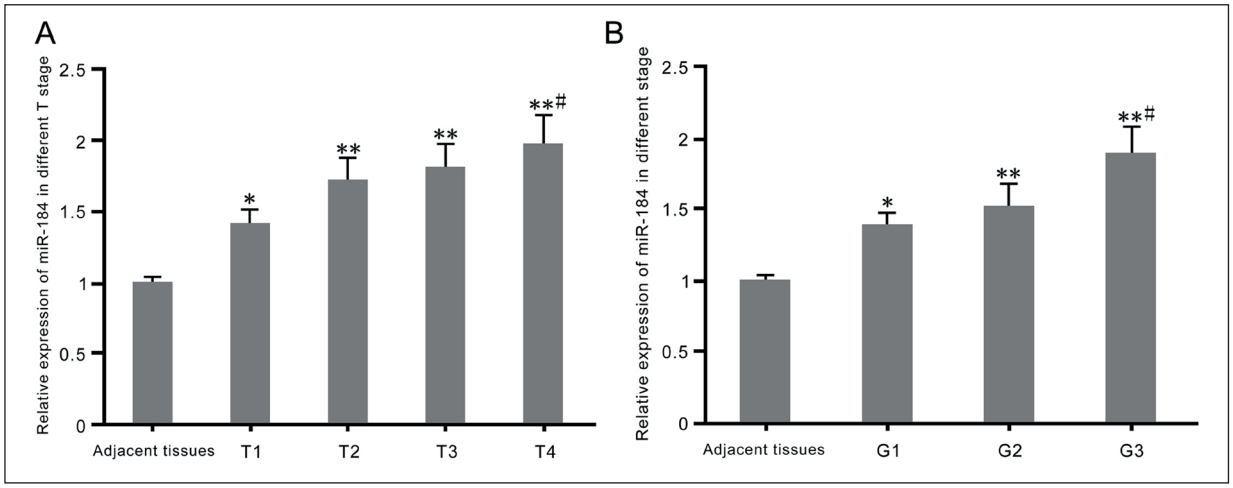

Figure 1. MiR-184 expression in RC tissues. The total RNA was isolated from renal carcinoma tissues or adjacent tissues of 38 patients with different stages ***(A)*** and ***(B)*** followed by the analysis of the miR-184 expression by quantitative RT-PCR. *$p<0.05$.

tested by the analysis of variance (ANOVA). The enumeration data were analyzed by χ^2-test. The Pearson test was employed for the correlation analysis. The statistical analysis was defined when $p<0.05$.

Results

MiR-184 Expression in RC Tissues

Real-time PCR was used to analyze the expression of miR-184 in RC tissues. Comparing to tumor-adjacent tissues, miR-184 showed a significantly elevated expression in RC tumor tissues with statistical significance ($p<0.05$, Figures 1A and 1B). We also analyzed the expression of miR-184 in patients with different stages and found with the increased stages that the miR-184 expression was further increased.

Catenin/TCF4 Expression in RC Tissues

We further showed that the expression of β-catenin/TCF4 was also significantly higher in RC tissues and further increased with the elevated stages (Figures 2A and 2B).

Considering the similar expression profile of miR-184 and β-catenin/TCF4, we performed the correlation analysis to evaluate their relationship and found a positive correlation between them (r=0.712, r=0.695, $p<0.05$).

MiR-184 Expression in RC Cells

As seen in Figure 3, a significantly higher miR-184 expression was observed in RC cell line ACHN 769-1, compared with normal renal cell line HK-2, consistent with the finding in RC tissues. As ACHN showed a considerably higher miR-184 level, we selected it for further experiments.

Regulation of MiR-184 Expression in RC Cells

RC cell line ACHN was transfected with miR-184 mimic or inhibitor, and real time-PCR results showed a significant up-regulation or down-regulation of miR-184 in RC cells respectively, compared to the control group ($p<0.05$, Figure 4).

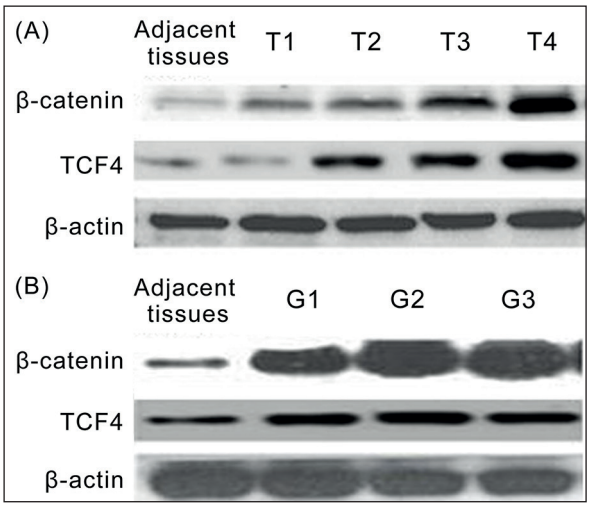

Figure 2. Expression of β-catenin and TCF4 in RC tissues. The total protein was extracted from RC patients in different T stages ***(A)*** or G stages ***(B)*** and the expression of β-catenin and TCF4 was measured by Western blot.

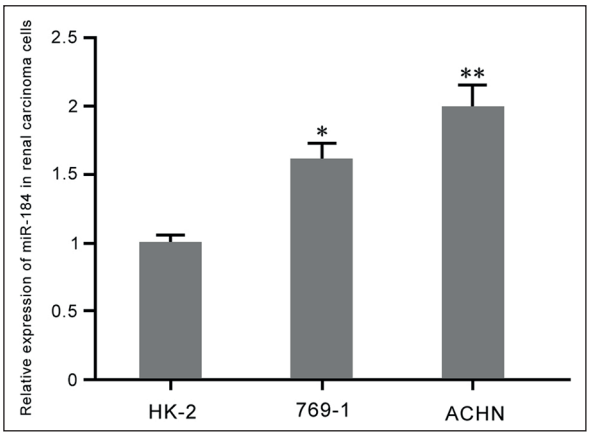

Figure 3. Expression of miR-184 in RC cell lines. The total RNA was extracted from normal renal cell HK-2, renal carcinoma cell lines 769-1 or ACHN, and the expression of miR-184 was measured by quantitative RT-PCR. *p<0.05, **p<0.01 compared to HK-2 group.

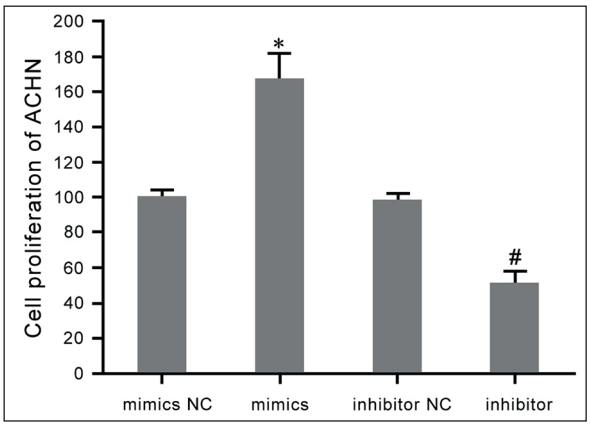

Figure 5. The effect of miR-184 modulation on RC cell proliferation. After the transfection with miR-184 mimic or inhibitor, the RC cell proliferation was measured by MTT assay. *p<0.05 compared to mimic NC group; #p<0.05 compared to inhibitor NC group.

Effects of MiR-184 Regulation on RC Cell Proliferation

MTT assay was used to measure the effect of miR-184 on the proliferation of RC cell line ACHN. The results showed that the transfection of miR-184 mimic or inhibitor into ACHN cells significantly enhanced or suppressed miR-184 expression, as well as significantly increased or inhibited ACHN cell proliferation, respectively (p<0.05 comparing to control group, Figure 5).

Effects of MiR-184 Modulation on RC Cell Colony Formation

The transfection of miR-184 inhibitor in RC cell line ACHN remarkably inhibited the cell colony formation with statistical significance, compared to the control group (p<0.05, Figure 6).

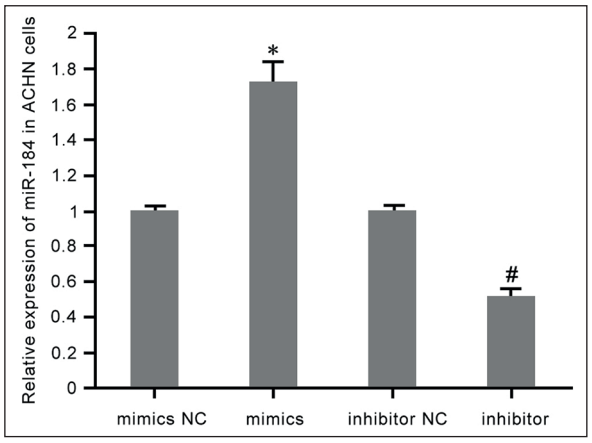

Figure 4. Expression of miR-184 after transfection of miR-184 mimic or inhibitor. *p<0.05 comparing to mimic NC group; #p<0.05 compared to inhibitor NC group.

Effects of MiR-184 Modulation on RC Cell Invasion

The transwell chamber was used to measure the effect of miR-184 modulation on RC cell invasion. The transfection of miR-184 mimic or inhibitor into RC cell line ACHN significantly enhanced or inhibited the invasion potency of cells, respectively (p<0.05 comparing to control group, Figure 7).

Effects of MiR-184 Modulation on RC Cell Apoptosis

The flow cytometry was used to examine the effect of miR-184 regulation on RC cell apoptosis. The transfection of miR-184 mimic or inhibitor into RC cell line ACHN significantly inhibited or facilitated cell apoptosis, respectively (p<0.05 compared to the control group, Figure 8).

Dual-Luciferase Assay Analysis of the Relationship Between MiR-184 and β-Catenin/TCF4

The bioinformatics analysis showed a complementary binding between miR-184 and the 3'-UTR of either β-catenin or TCF4 (Figure 9A). Through the dual-luciferase assay analysis, we further discovered that the transfection of miR-184 inhibitor significantly inhibited the relative

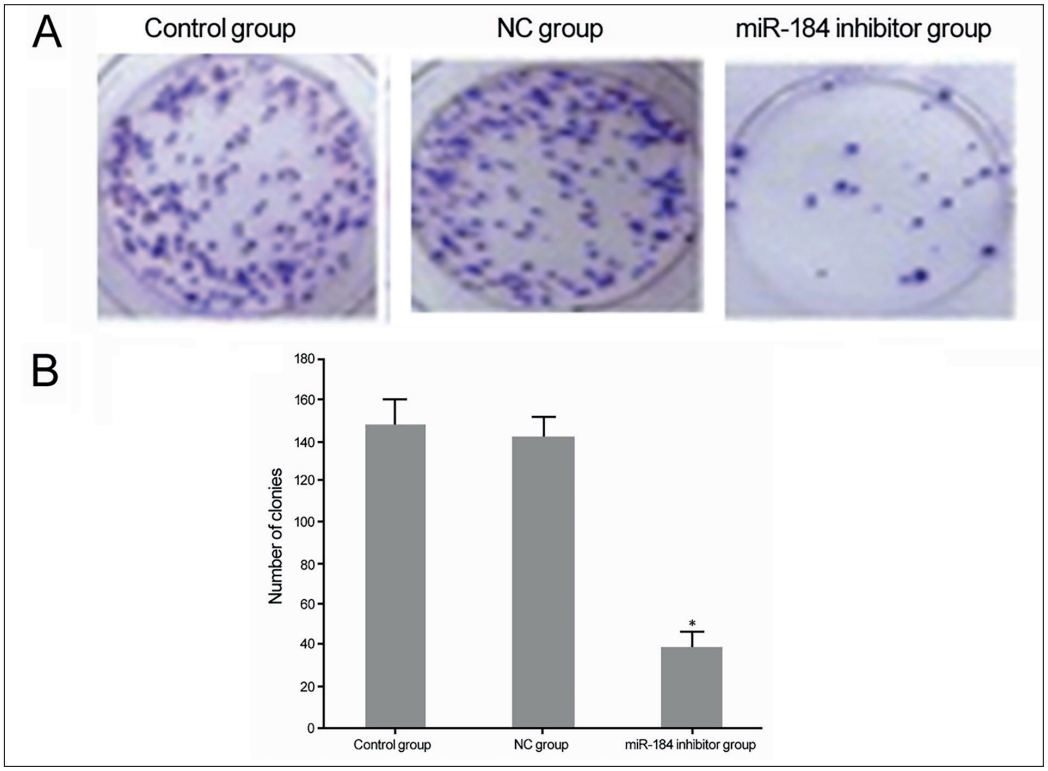

Figure 6. The effects of miR-184 modulation on RC cell colony formation. ***A,*** Effects of miR-184 modulation on colony formation of RC cells. ***B,*** Analysis for the effect of miR-184 modulation on RC cell colony formation. **p*<0.05 comparing to control group.

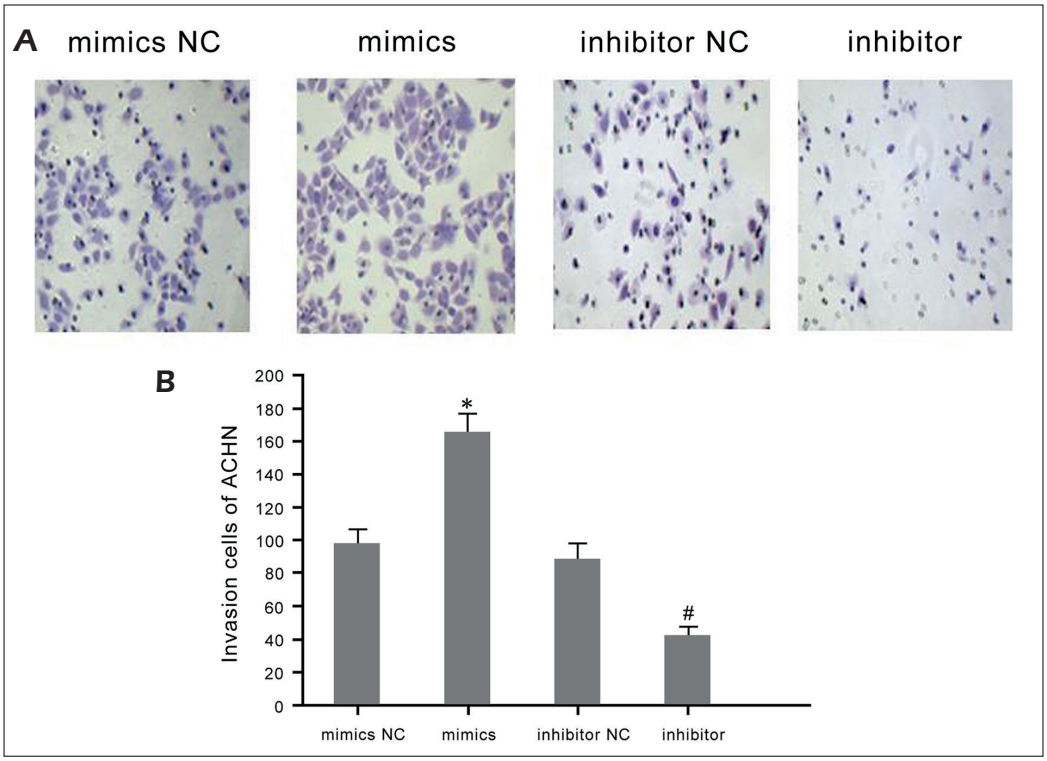

Figure 7. The effects of miR-184 on RC cell invasion. ***A,*** Transwell chamber for measuring the effect of miR-184 on RC cell invasion. ***B,*** Analysis for the effect of miR-184 on RC cell invasion. **p*<0.05 comparing to mimic NC group; #*p*<0.05 comparing to the inhibitor NC group.

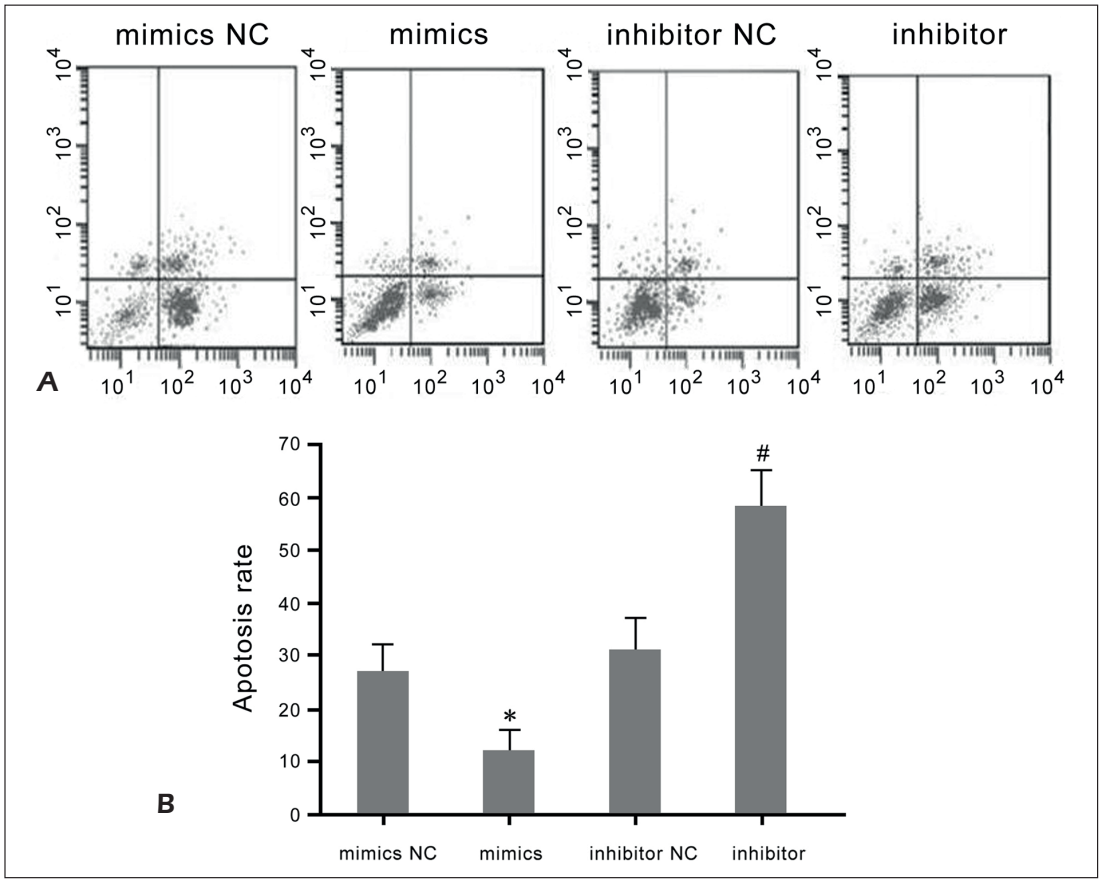

Figure 8. The effects of miR-184 modulation on RC cell apoptosis. ***A,*** Transwell chamber measuring the effect of miR-184 on RC cell apoptosis. ***B,*** Analysis for the effect of miR-184 modulation on RC cell apoptosis. *p<0.05 comparing to mimic NC group; #p<0.05 comparing to the inhibitor NC group.

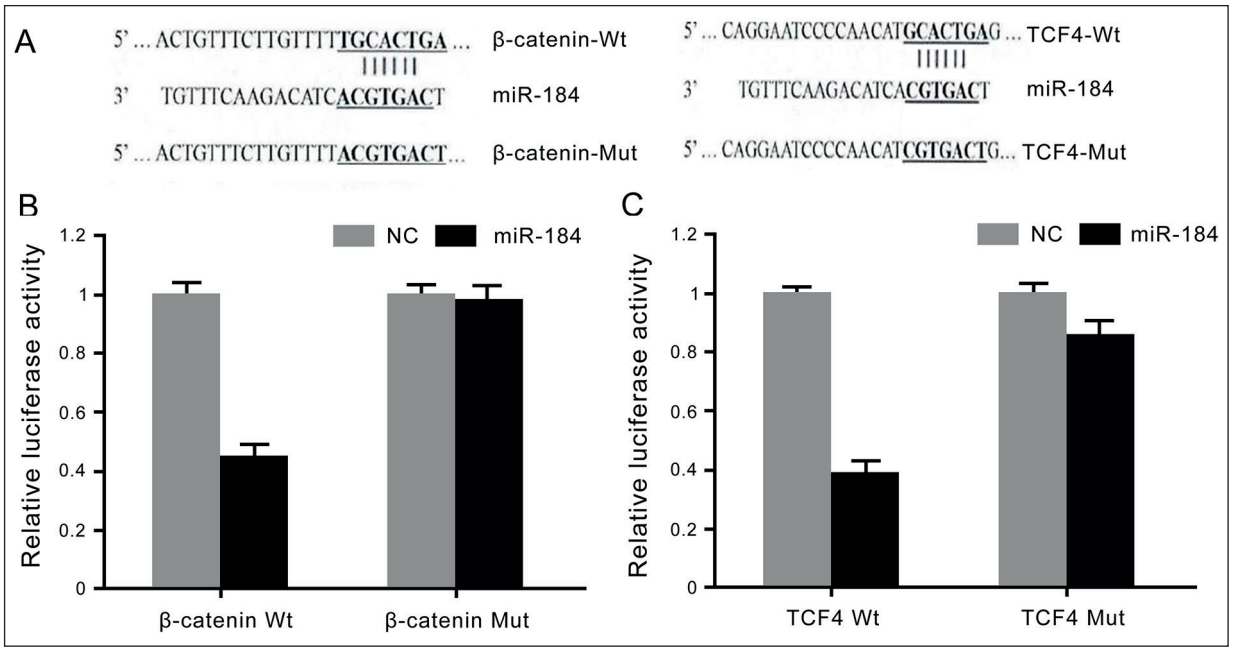

Figure 9. The Dual-Luciferase assay analysis of the relationship between miR-184 and β-catenin/TCF4. ***A,*** Bioinformatic analysis of the complementary binding site between β-catenin/TCF4. The effect of miR-184 on the relative Luciferase activity of the cells transfected with β-catenin ***(B)*** or TCF4 ***(C)***. *p<0.05 compared to NC group.

luciferase activity of the cells transfected with β-catenin or TCF4 without affecting the luciferase activity of cells transfected with mutant β-catenin or TCF4 (Figure 9B).

Effects of MiR-184 Regulation on β-Catenin/TCF4 Pathway of RC Cells

The Western blot was used to analyze the effect of miR-184 regulation on β-catenin/TCF4 expression in RC cells. The transfection of miR-184 mimic or inhibitor into RC cell line ACHN significantly increased or suppressed β-catenin and the TCF4 protein expression respectively ($p<0.05$ compared to the control group, Figure 10).

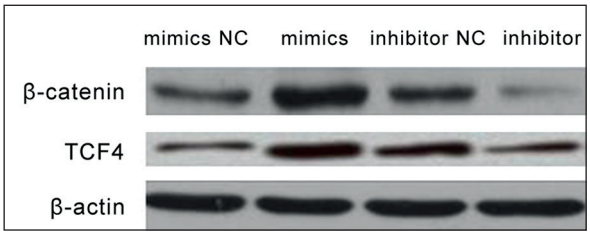

Figure 10. The effect of miR-184 regulation on β-catenin/TCF4 pathway of RC cells. The total protein was isolated after the transfection of miR-184 mimic or inhibitor followed by the Western blot analysis of the effect of miR-184 on β-catenin/TCF4 expression.

Discussion

The abnormal proliferation of tumor cells is one important factor leading to tumorigenesis and progression. The over-proliferation and suppressed death of tumor cells make it difficult to manage RC progression. Apoptosis plays an indispensable role in RC occurrence and can inhibit tumor overgrowth and retard tumor progression[17,18]. RC has a complicated pathogenesis mechanism and molecular biological progression, which have not been fully illustrated. The occurrence and progression of RC are rapid with a range of histology and morphology diversity[19]. RC has high post-op recurrent incidence with more metastatic cases that lead to higher treatment difficulty[20]. As one of the newly identified miRNA, miR-184 has been shown to have pluripotent pathology and physiology activities and can participate in metabolic disorders on top of tumorigenesis[16]. This study also showed the elevated expression of miR-184 in RC tumor tissues, which was further increased along with elevated RC stages, suggesting the involvement of the abnormal miR-184 expression in RC regulation. This study also demonstrated that the overexpression of miR-184 could inhibit RC cell apoptosis, promote RC cell proliferation, colony formation or invasion potency via *in vitro* cell culture assay.

β-catenin/TCF4 pathway participates in the occurrence and progression of various tumors[21]. As the pivotal protein in the Wnt signaling pathway, β-catenin activation facilitates T cell factor 4 (TCF4) activation, modulates target gene expression, and enhances the expression of anti-apoptotic protein Survivin. As the most potent anti-apoptotic protein, Survivin can modulate the upstream signal mechanism of tumors and exerts dual roles for apoptotic inhibition and cell division facilitation, which lead to accelerating tumor pathogenesis and progression[22,23]. Further studies on the functional mechanism of miR-184 in RC confirmed that the downregulation of miR-184 in RC cells can inhibit β-catenin/TCF pathway, thus participating in RC progression. The current study, however, only measured miR-184 expression in clinical samples and investigated the related functional mechanism in a preliminary stage. Further studies can be performed to amplify the sample size for confirming miR-184 expression and to analyze the related functional target, in order to provide novel molecular targets for clinical diagnosis and treatment of RC.

Conclusions

We found that miR-184 and β-catenin/TCF4 is overexpressed in RC tissues and further is increased with elevated RC stages. The downregulation of miR-184 in RC cells can facilitate tumor cell apoptosis, possibly via modulating β-catenin/TCF pathway, thus suppressing tumor proliferation and invasion.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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