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MiR-212-5p inhibits the malignant behavior of clear cell renal cell carcinoma cells by targeting TBX15

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Abstract. – OBJECTIVE: To investigate the role of microRNA-212-5p (miR-212-5p) in clear cell renal cell carcinoma (ccRCC) and to explore the potential underlying mechanisms.

MATERIALS AND METHODS: 32 pairs of ccRCC clinical samples were collected. Renal ccRCC cells (786-O) and embryonic kidney cells (293T) were cultured *in vitro*. The ability of cell proliferation was detected by 3-(4,5)-dimethylthiazol(-z-y1)-3,5-diphenyl tetrazolium bromide (MTT) assay. Transwell migration assay was used to detect the abilities of cell invasion and migration. The relative protein and mRNA expressions of miR-212-5p were detected by Western blot and quantitative Real-time polymerase chain reaction (qRT-PCR) analysis, respectively. Furthermore, bioinformatics online sites and luciferase reporter gene assay were performed to predict and verify the potential targets of miR-212-5p, respectively.

RESULTS: The expression level of miR-212-5p in ccRCC tissues and cell lines was significantly inhibited. Bioinformatics online sites and luciferase reporter gene assay confirmed that T-box transcription factor TBX15 (TBX15) was the potential target gene of miR-212-5p. *In vitro* experiments demonstrated that the proliferation, cell cycle, cell invasion and migration of ccRCC cells were obviously restricted after up-regulation of miR-212-5p. However, the above functional effects were significantly abolished in ccRCC cells after co-transfection with miR-212-5p mimics and LV-TBX15.

CONCLUSIONS: MiR-212-5p acted as a tumor suppressor gene in ccRCC. Through targeting TBX15, miR-212-5p significantly inhibited the malignant behavior of ccRCC cells. Our findings revealed that miR-212-5p/TBX15 axis might be a potential therapeutic target for the treatment of ccRCC.

Key Words:

Clear cell renal cell carcinoma (ccRCC), MicroRNA-212-5p (miR-212-5p), T-box transcription factor TBX15 (TBX15), Proliferation, Invasion, Migration.

Introduction

Renal cell carcinoma (RCC) is a heterogeneous malignant epithelial tumor in renal tubular epithelium. RCC accounts for over 90% of adult kidney malignancies, and about 3% of adult malignancies[1]. In the past decade, the incidence rate of RCC has increased year by year. Statistics found that RCC is the second malignancy of the urinary system, whose incidence ranks only second to bladder cancer. Nearly 200,000 people are diagnosed with RCC every year worldwide. Meanwhile, about 100,000 patients die from RCC, showing a growing trend[2,3]. Besides, RCC exerts continuously-elevated incidence rate in the world. It has also become the seventh and eighth most common cancer among men and women in the United States, respectively[4]. Clear cell RCC (ccRCC) is the most common and invasive RCC subtype, accounting for about 80% of RCC. It is characterized by highly local invasiveness, malignancy, mortality rate and resistance to chemotherapy and radiotherapy[5]. As for the treatment of ccRCC, surgery is the main treatment at present. For patients with localized ccRCC, more than 97% of them can achieve cancer-specific survival of more than 9 years through partial nephrectomy or radical nephrectomy[6]. However, the prognosis of ccRCC patients remains poor once metastasis is detected. The median survival of untreated patients with metastatic ccRCC is only 6-10 months[7,8]. Due to unsatisfactory efficacy, low response rate and evident side effects, traditional immunotherapy and chemotherapy are not recommended as first-line treatment methods[9]. Currently, with the development of molecular biological technique, breakthroughs have been made in understanding the pathogenesis of ccRCC at the molecular level. Furthermore, the treatment of ccRCC has developed into the era of targeted

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therapy. Micro ribonucleic acids (miRNAs), first discovered in nematodes, are a type of non-coding single-stranded RNA molecules with approximately 22-23 nucleotides in length. MiRNAs are encoded by endogenous genes, which participate in the regulation of post-transcriptional gene expression in plants and animals[10,11]. So far, tens of thousands of miRNA molecules have been found in plants and animals. MiRNAs have been shown to regulate 50% of protein-coding genes, which are also widely involved in various pathophysiological processes. In addition, previous studies have demonstrated that miRNAs participate in the development and progression of various cancers, including lung cancer[12], breast cancer[13], gastric cancer[14] and osteosarcoma[15]. Moreover, the analyses of miRNA expression and function have demonstrated that miRNAs significantly affect the development of malignancies. Meanwhile, dysregulation of miRNAs is related to malignant phenotypes of cancers. This has already made them become potential biomarkers for tumor diagnosis, prognosis, as well as potential targets for oncotherapy[16,17]. The discovery of miRNAs has provided major breakthroughs and broad prospect for researching the development and progression of RCC. As early as in 2007, Gottardo et al[18] have examined 27 RCC specimens and found that the expression levels of miR-28, 185, 27 and let-7f-2 are overtly up-regulated in tumor tissues when compared with healthy kidney tissues. Nakada et al[19] analyzed 470 patients through miRNA array as well. They have discovered that 43 miRNAs in ccRCC are differentially expressed in healthy tissues. Meanwhile, the expression levels of miR-141 and miR-200c decrease significantly in ccRCC. Petillo et al[20] have found that miR-32 is associated with poor prognosis of RCC. More and more miRNAs have been found to be dysregulated in ccRCC cells. These findings have manifested that increasing miRNAs participate in the development and progression of RCC through different targets and pathways. Some of these miRNAs serve as cancer-promoting genes, while some inhibit the development of RCC[21-24]. Hsa-miR-212-5p is located on human chromosome 17, which does not seem to be involved in the development of malignant tumors in the early stage. With the progression of researches, the role of miR-212-5p in malignant tumors has been gradually explored. In breast cancer, miR-212-5p inhibits triple-negative breast cancer (TNBC) from acquiring EMT phenotype by down-regulating Prrx2. Eventually, this inhibits cell migration and invasion during cancer progression[25]. In our study, we aimed to investigate the possible role of miR-212-5p in ccRCC and to explore the possible underlying mechanism.

Patients and Methods

Clinical Samples and Cell Lines

Clinical samples: 32 pairs of ccRCC clinical samples were provided by the Department of Urology. All collected tissue samples were stored in liquid nitrogen after surgical resection. Informed consent was obtained from all patients before the study. All operations were approved by the Ethics Committee of Peking Union Medical College.

Cell lines: renal ccRCC cell line (786-O) and embryonic kidney cell line (293T) were passaged and preserved by our laboratory. All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 15% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) (with 1% antibiotic), and maintained in an incubator with 5% CO_2 at 37°C.

Target Prediction and Luciferase Reporter Gene Assay

Target prediction: bioinformatics online sites (miRBase, TargetScan and PicTar) were used to predict the target binding sites of miR-212-5p to the regulator of T-box transcription factor TBX15 (TBX15). Luciferase reporter gene assays: 293T cells were co-transfected with luciferase reporter vector and miR-212-5p mimics or miR-NC (negative control) according to the instructions of Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA), respectively. Experimental groups included: TBX15-Wt + miR-NC group, TBX15-Wt + miR-212-5p group, TBX15-Mut + miR-NC group and TBX15-Mut + miR-212-5p group. After transfection for 4 h, the medium was replaced, followed by 48 h of culture in an incubator. Next, the cells were collected, and luciferase activity was determined by a microplate reader.

Cell Transfection

786-O cells were first inoculated into 96-well plates at a density of approximately 1×10^6/well. 0.18 mL of Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) was added to each well, followed by incubation for another 24 h. Cell transfection was performed after

the adherence of 786-O cells. MiR-212-5p mimics (40 nmol/L) or LV-TBX15 (20 nmol/L) was transfected into cells according to the instructions of Lipofectamine™ 2000. Finally relevant indicators were detected after 48 h. Three groups were established for functional assay, including: miR-NC group (negative control), miR-212-5p mimics group (786-O cells transfected with miR-212-5p mimics) and miR-212-5p + TBX15 group (786-O cell transfected with miR-212-5p mimics and LV-TBX15).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA). QRT-PCR was carried out using the Roach480 fluorescence Real-time quantitative PCR instrument (Roche, Basel, Switzerland). Specific reaction conditions were as follows: pre-denaturation at 95°C for 10 min, denaturation at 95°C for 30 s, denaturation at 55°C for 30 s, and extension at 72°C for 2 min, for a total of 35 cycles. The expression levels of miR-212-5p were quantified by the $2^{-\Delta\Delta Ct}$ method. Primer sequences used in this study were as follows: miR-212-5p, F: 5'-GGAAACATCCTCGACTG-3', R: 5'-ATTGAACGTGCCTCCGTGTTGAGG-3'; MKRN3, F: 5'-CAGCTTAGCATTTGGACAA-3', R: 5'-CGTGCGAATAGCGTTGCGTTCT-3'. U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

Western Blot (WB) Analysis

After transfection for 72-96 h, prostate carcinoma cells were collected for protein expression analysis. Collected cells were first washed with phosphate-buffered saline (PBS) solution for 3 times. After that, the cells were added with cell lysis buffer (about 100 μL/well), lysed on ice for about 30-40 min, and centrifuged at high speed for 15 min. The supernatant was then collected, and the concentration of extracted protein was determined by the bicinchoninic acid (BCA) protein assay kits. 30 μg protein sample was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) for about 120 min. After blocking with TBST solution containing 5% skim milk powder at room temperature for about 1-2 h, the membranes were incubated with primary antibodies at 4°C overnight. On the next day, the membranes were incubated with corresponding secondary antibody at room temperature for 1 h. Finally, Western blotting chromogenic reagent was added for exposure and development.

Cell Proliferation and Cell Cycle

After transfection for 24 h, the cells were digested, centrifuged and collected. Next, the cells were inoculated into 96-well plates at a density of 1×10^3 cells per well. 5 replicate wells were set in each group. PBS solution (about 200 μL/well) was added to the surrounding wells of 96-well plates to avoid evaporation. 3-(4,5)-dimethylthiahiazol (-z-yl)-3,5-di-phenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, St. Louis, MO, USA) was used to detect cell viability at 1, 2, 3, 4 and 5 d after transfection, respectively. At each time point, 96-well culture plates to be detected were taken out, and the medium was discarded. 20 μL of 5 mg/mL MTT solution was added to each well in dark, followed by incubation in a cell incubator for 4 h. Next, MTT solution was discarded, and 100 μL dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added to each well. Later, the plates were shacked on a shaker at low speed for about 10 min until crystals were completely dissolved. Thereafter, the bubbles in each well were eliminated. Optical density (OD value) at the wavelength of 490 nm was detected by a microplate reader. After detection, the 96-well plate was put back into an incubator for analysis and detection at the next time point. After transfection for about 72 h, the cells were trypsinized, centrifuged at low speed and collected. After washing with PBS solution for 3 times, the cells were incubated with 70% ethanol solution at 4°C overnight and washed twice with PBS solution again. Next, the supernatant was discarded. After that, 100 μL RNase was added to re-suspend the cells, followed by incubation in water bath at 37°C for about 30 min. 100 μL Propidium Iodide (PI) was then added, and the cells were incubated in dark at 4°C for about 30 min. Finally, red fluorescence at the excitation wavelength of 488 nm was detected by flow cytometry.

Cell Invasion and Migration Assays

Transwell migration assay: cells transfected for 72 h were digested, centrifuged and counted. The cells were diluted into cell suspension with serum-free medium at a density of 1.5×10^5

cells/mL. Subsequently, the cell suspension was added to transwell upper chambers at 200 μL/well. Meanwhile, 600 μL complete medium was added to transwell lower chambers. Then, cells were cultured in an incubator for 24 h. After fixing with formaldehyde for 15 min and staining with crystal violet for 15 min, cells on the inner membrane were gently wiped with a cotton swab. Finally, the number of cells passing through the filter membrane was counted. 4 high-power fields (×100) were randomly selected for each sample. Transwell invasion assay: 50 μL (10.2 μg/μL) matrigel was first evenly coated onto transwell chamber inner membranes, followed by incubation at 37°C for 15 min. Treatments after cell digestion, centrifugation and counting were the same as those in transwell migration assay. The assay was repeated for at least 4 times.

Statistical Analysis

Statistical analysis was performed with Student's *t*-test or *F*-test. *p*-values were two-sided, and $p<0.05$ were considered statistically significant. Prism 6.02 software (La Jolla, CA, USA) was used for all statistical analysis.

Results

MiR-212-5p Expression in ccRCC Tissues and Cells

The expression of miR-212-5p in tissues and cells was first detected by qRT-PCR. As shown in Figure 1A, the expression of miR-212-5p was significantly reduced in ccRCC patients when compared with normal tissues. At the same time, the same results were observed at the cellular level. The expression level of miR-212-5p in ccRCC cells 786-O was reduced by nearly half when compared with 293T cells (Figure 1B). This indicated that the expression level of miR-212-5p in 789-O cells was consistent with that of clinical ccRCC samples. Therefore, 789-O cells could be used for studying the molecular mechanisms of ccRCC progression *in vitro*.

Transfection Efficiency

Transfection efficiency of miR-212-5p was detected and confirmed by qRT-PCR assay ($p<0.001$). The results of qRT-PCR were shown in Figure 2C. Compared with NC mimics group, miR-212-5p expression level increased by 2.6 times after transient transfection of miR-212-5p mimics in 786-O cells.

TBX15 was a Direct Target of miR-212-5p in ccRCC Cells

Bioinformatics online prediction database indicated that there was a miR-212-5p binding site in the 3'-UTR of TBX15 (Figure 2A). However, whether miR-212-5p exhibited significant regulatory effect on TBX15 required further research. Luciferase reporter gene assay, as a classic method of miRNA research, was the first applied. As shown in Figure 2B, the results demonstrated that in WT TBX15-3'UTR, Renilla luciferase activity of 293T cells over-expressing miR-212-5p was significantly reduced when compared

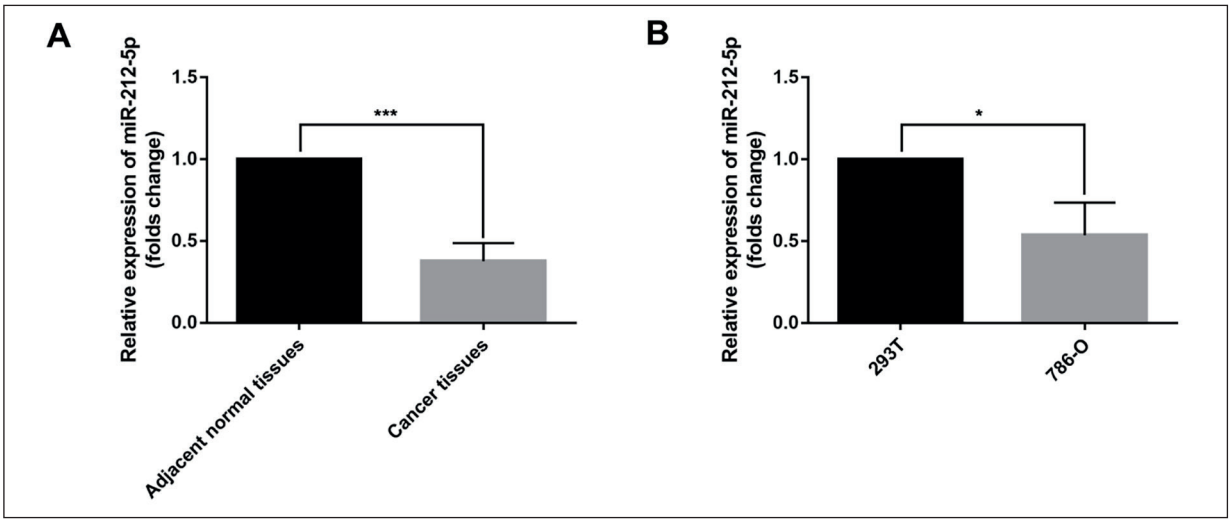

Figure 1. A, The expression of miR-212-5p in clinical samples (***$p<0.001$). **B,** The expression of miR-212-5p in cell lines (*$p<0.05$).

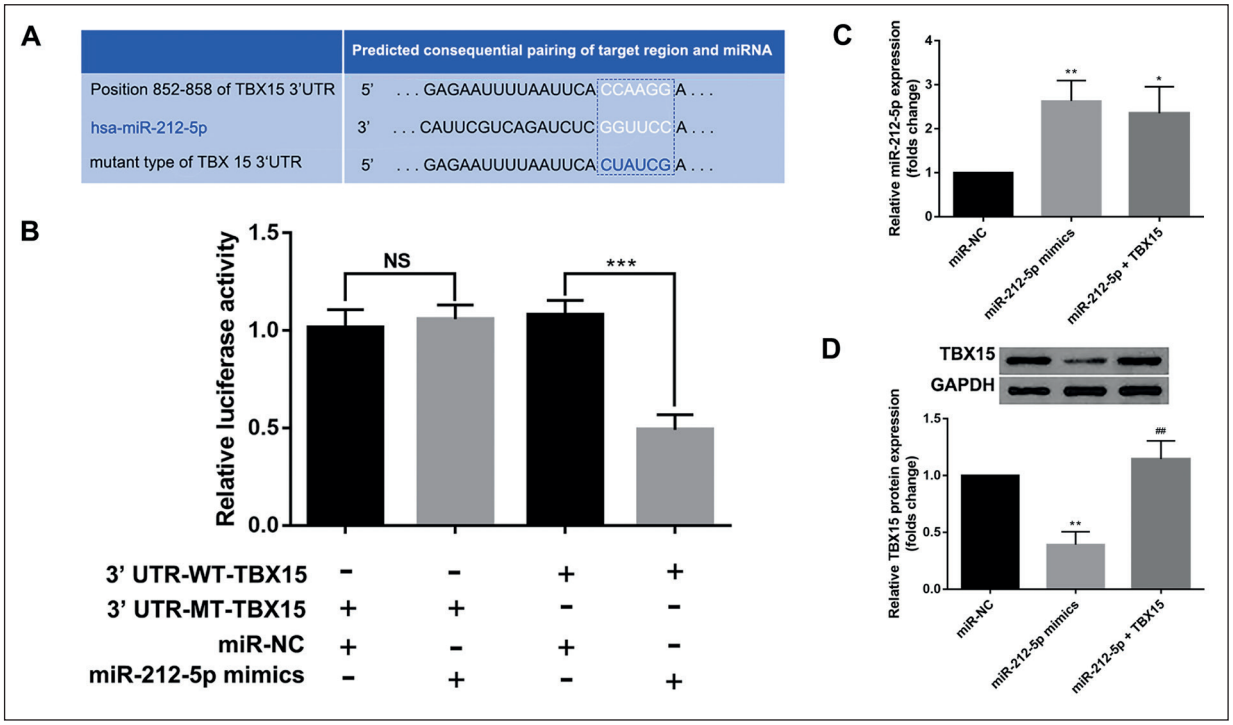

Figure 2. TBX15 was a direct and functional target of miR-212-5p. **A.** Diagram of putative miR-212-5p binding sites of TBX15. **B,** Relative activities of luciferase reporters (***p<0.01). **C,** Transfection efficiency detected by qRT-PCR (*p<0.05, **p<0.01). **D,** The protein expressions of TBX15 in ccRCC cells after different treatments. Data were presented as means ± standard deviations (**p<0.01 *vs.* NC group; ##p<0.01 *vs.* miR-212-5p mimics group).

with NC group. However, in MT TBX15-3'UTR, no statistically significant changes were found in luciferase activity after transfection of NC mimics or miR-212-5p mimics (Figure 2B). The results indicated that changes in the expression level of miR-212-5p significantly affected TBX15 expression. Furthermore, this negatively regulated TBX15 expression by targeting the 3'-UTR of TBX15 gene.

The regulation of TBX15 by miR-212-5p was also investigated by WB assay. The results indicated that the protein expression of TBX15 in 786-O cells of miR-212-5p mimics group was significantly repressed when compared with cells of control group (Figure 2D)

MiR-212-5p Suppressed the Proliferation of ccRCC Cells

The proliferation of 789-O cells was measured by MTT assay. After over-expression of miR-212-5p, the proliferation of 789-O cells was inhibited at 96 h when compared with NC group. However, the proliferation ability of cells decreased significantly at 120 h, in which the inhibition rate was 27.8% (Figure 3A). This indicated that over-expression of miR-4521 could obviously limit the proliferation ability of 786-O cells *in vitro*.

To further study the proliferative ability of 789-O cells, we analyzed cell growth cycle of each group. We found that the reason why the proliferation curve of miR-212-5p mimics group was different from other groups was that miR-212-5p led to G0/ G1 phase cells increased significantly due to G0/G1 phase arrest (Figure 3B).

In summary, we believed that miR-212-5p prevented the progression of cell cycle in G0/ G1 phase, which in turn affected cell proliferation.

MiR-212-5p Inhibited the Invasion and Migration of ccRCC Cells

Transwell assay results were shown in Figure 4. The number of trans-membrane cells in miR-212-5p mimics group was reduced by 39.2% (invasive assay) and 44.9% (migration assay) compared with NC group, respectively. These results indicated that miR-212-5p over-expression attenuated the invasion and migration capacities of 786-O cells *in vitro*.

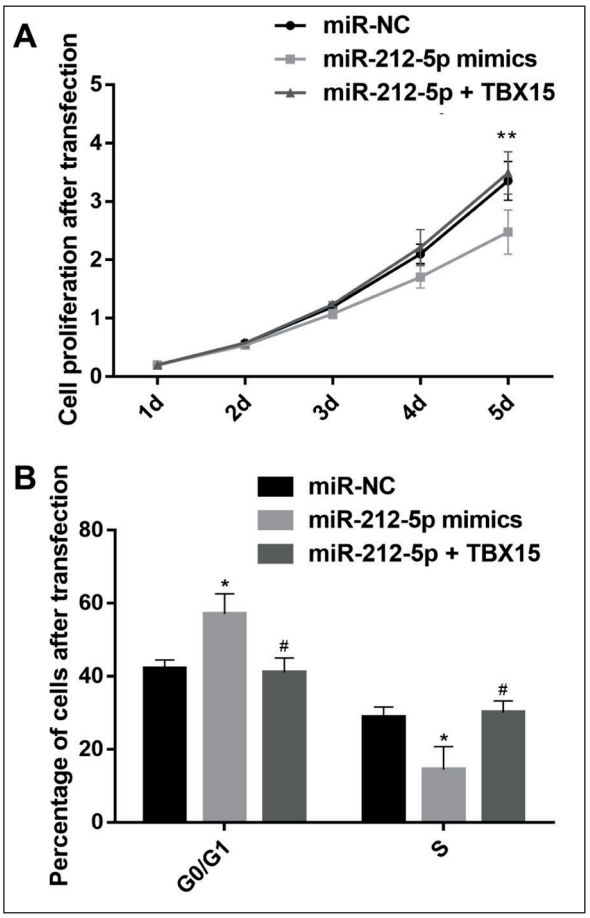

Figure 3. A, MiR-212-5p inhibited the proliferation of ccRCC cells (**p<0.01 *vs.* NC group). **B,** Cell cycle phase of ccRCC cells was analyzed using flow cytometry (*p<0.05 *vs.* NC group; $^{\#}p$<0.05 *vs.* miR-212-5p mimics group). All data were presented as means ± standard deviations.

The Role of TBX15 after Reset

To rigorously verify the role of TBX15 in 789-O cells, we established a TBX15 exogenous intervention group in miR-212-5p over-expressing cells in each experimental session.

Co-transfection of miR-212-5p and TBX15 significantly counteracted the effect of miR-212-5p (Figure 3 and Figure 4). In addition, our findings confirmed the role of miR-212-5p/TBX15 in ccRCC cells.

Discussion

RCC, the most common renal solid tumor, is second only to urinary epithelial cancer in terms of incidence rate among urinary malignancies. Meanwhile, it accounts for about 3% of all cancers. The incidence rate of RCC is increasing year by year, and the annual incidence rate has elevated by 2% all over the world[9]. In 2012, there were 84,400 new cases of RCC in Europe, with 34,700 deaths[9, 26]. In 2015, 66,800 patients were newly diagnosed with kidney cancer in China, with 23,400 deaths[27]. RCC is considered as an indolent tumor; however, it is difficult to predict its natural course[28,29]. Many patients with RCC have already been in advanced stage when first diagnosed. In some cases, primary lesions are small, but metastatic lesions can also be detected. Moreover, the therapeutic effect is very poor, thus seriously threatening the health of patients[30].

The development of ccRCC is a complex process involving changes of various genes. MiRNAs can modulate the expression of multiple functional genes at post-transcriptional level. Meanwhile, they play important roles in biological processes, such as the differentiation, proliferation and apoptosis of cells. Evidence has proved that abnormal expression of miRNAs contributes to the development of renal tumors. Previous

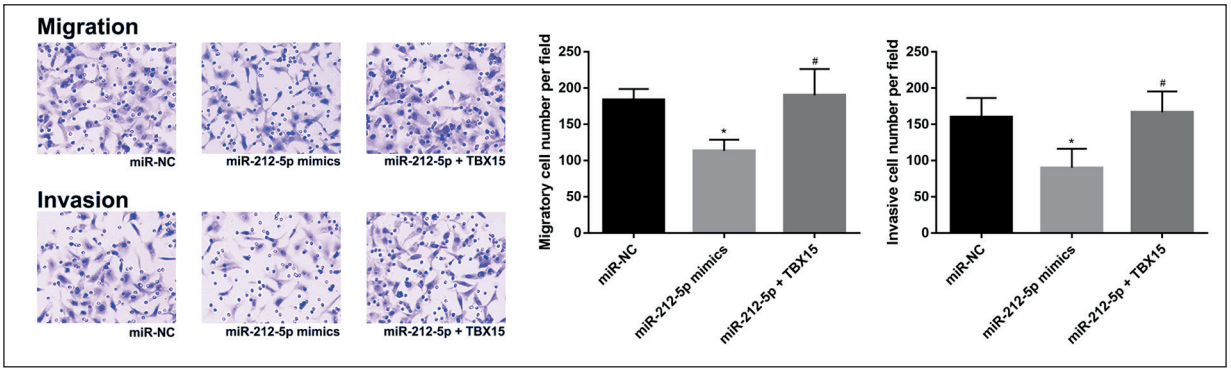

Figure 4. MiR-212-5p decreased the expression level of TBX15 and inhibited the invasion and migration of ccRCC cells. The invasion and migration were analyzed using transwell assay and detected by microscope (× 200). Data were presented as means ± standard deviations (*p<0.05 *vs.* NC group; $^{\#}p$<0.05 *vs.* miR-212-5p mimics group).

studies[31-33]have manifested that some miRNAs repress RCC by suppressing certain cytokines. One miRNA may have hundreds of target genes, and a single gene can be regulated by multiple different miRNAs. Therefore, the prediction and verification of miRNA target genes have generally been considered as key points to study relevant molecular mechanisms and biological functions. Furthermore, the rapid development of bioinformatics has facilitated the research of miRNAs by humans[34,35]. In this study, miRNA target gene prediction software (such as Targetscan, miRanda, PicTar and DIANA-microT), luciferase reporter gene assay and WB were simultaneously applied to verify that TBX15 was a downstream functional target of miR-212-5p.

TBX15, as a member of the conserved T-box gene family, is essential for many developmental processes in the human body[36]. T-box gene is involved in various physiological processes, including the growth and development of humans. Loss or mutation of T-box is one of the leading causes of congenital disease development and progression[37-39]. Overexpression of T-box enables tumor cells to exhibit unlimited proliferative capacity and induces apoptosis avoidance of tumor cells, thereby accelerating the malignant transformation of malignancies[40-42]. Increasing evidence has shown that T-box gene participates in cell differentiation, proliferation and apoptosis associated with carcinogenesis. In addition, changes of T-box gene expression have been detected in various tumors. Recently, it has been reported that TBX15 is involved in adipocyte differentiation, mitochondrial respiration and gene development of mesodermal derivatives. In ovarian cancer and other female reproductive system tumors, lowly-expressed TBX15 is closely correlated with the development and progression of malignant tumors[43].

Conclusions

We demonstrated that miR-212-5p, as a tumor suppressor, affected the malignant behavior of 786-O cells by targeting and regulating the expression of TBX15 *in vitro*. Our results provided new evidence for the role of miR-212-5p in tumor research, which also provided some basic research for subsequent *in vivo* studies. Furthermore, a new therapeutic target or direction for clinical treatment of ccRCC could be referenced.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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