

MiR-425 involves in the development and progression of renal cell carcinoma by inhibiting E2F6

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Abstract. – OBJECTIVE: To investigate the effect of miR-425 on the proliferation and apoptosis of clear cell renal carcinoma (ccRCA) cells, and to explore the underlying mechanism.

PATIENTS AND METHODS: A total of 80 pairs of human clear cell renal carcinoma (ccRCA) and cancer-adjacent normal tissue samples were collected in this study. Human ccRCA cell line (786-O) and normal human kidney cell line (HK-2) were used in cellular research. The expression level of miR-425 was detected in ccRCA tissues and cells, respectively. Target genes of miR-425 were predicted by bioinformatics and verified by luciferase reporter gene assay. Moreover, the role of miR-425 in regulating E2F6 as well as its effect on the proliferation and apoptosis of ccRCA cells were detected.

RESULTS: Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) results showed that the expression of miR-425 was significantly decreased in ccRCA tissues and cells. The proliferation ability and cell cycle of 786-O cells were significantly inhibited after miR-425 overexpression. The percentage of cells in G0/G1 phase was remarkably increased, while the percentage of cells in S and G2/M phases was significantly decreased. Besides, the number of apoptotic cells was significantly increased in the miR-425 intervention group. On-line target gene prediction software indicated that E2F6 was the potential downstream target gene of miR-425. RT-PCR, Western blotting and luciferase reporter gene assay demonstrated that the expression of E2F6 was negatively regulated by miR-425. In addition, subsequent experiments showed that the up-regulation of E2F6 could suppress the inhibitory effect of miR-425 on the proliferation and apoptosis of ccRCA cells.

CONCLUSIONS: Our research demonstrated the inhibitory function of miR-425 in ccRCA. Therefore, the miR-425/E2F6 axis was expected to be one of the targets of ccRCA targeted therapy.

Key Words:

MiR-425, Renal cell carcinoma (RCA), Clear cell renal carcinoma (ccRCA), E2F6.

Introduction

Renal cell carcinoma, also known as renal carcinoma (RCA), originates from renal tubular epithelial cells of the renal parenchyma histologically. The incidence of RCA is about 3% of all adult malignant tumors, ranking tenth among all adult cancers. Moreover, studies have shown that the incidence of RCA is increasing year by year¹⁻³. Among all adult urological malignant tumors, its incidence ranks second only to bladder cancer in China. However, the mortality rate of RCA is the highest, seriously threatening the survival and health of human beings^{2,4,5}. There are multiple pathological types of RCA, mainly including clear cell type, chromophobe cell type and papillary cell type. The clear cell type is the most common one, accounting for about 60-90% of all RCAs. In addition, it is also the RCA subtype with greatest invasiveness and worst prognosis⁶.

Due to the lack of specific tumor markers for early diagnosis of RCA in clinic, the occurrence of the following symptoms such as pain in waist and back, abnormal lump in abdomen and back as well as gross hematuria, is commonly known as the trilogy of RCA. Meanwhile, RCA is very likely to develop into middle or advanced stage, or even distant metastasis has already occurred. Moreover, epidemiological studies have confirmed that distant metastasis occurs in about 1/3 of patients when they are diagnosed with RCA for the first time^{7,8}. Even after surgical resection of renal tumors, about 20-40% of RCA patients may still suffer from recurrence or metastasis^{7,9}.

Therefore, it is of vital importance to study the mechanism of RCA metastasis and to find out specific molecular markers for the diagnosis, treatment and prognosis of RCA.

Micro ribonucleic acids (miRNAs), as a hot research topic in recent years, can participate in cellular biological behaviors such as tumor cell proliferation, adhesion, migration and invasion. The role of miRNAs has been confirmed in multiple biological fields, including the occurrence, progression, differentiation and drug resistance of cancer. Similar to other tumors, a large number of differentially expressed miRNAs have been found in RCA¹⁰⁻¹⁴. Previous works have explored such aspects and discovered some miRNAs with potential clinical value. As a carcinogenic miRNA, miR-425 is highly expressed in many tumor tissues, which also involves in the occurrence and development of many malignant tumors¹⁵⁻¹⁸. However, few studies have investigated the role of miR-425 in the occurrence and development of RCA.

In this study, we found that the expression level of miR-425 in clear cell renal carcinoma (ccRCA) tissues was significantly decreased. Meanwhile, the biological role of miR-425 in ccRCA was further studied.

Patients and Methods

RCA Patients

A total of 80 ccRCA patients undergoing surgery in Tongde Hospital of Zhejiang Province were enrolled in this study. All these patients were confirmed as ccRCA by pathological diagnosis. Preoperative chemotherapy or radiotherapy treatment was forbidden. After resection, liquid nitrogen was used to freeze ccRCA tissues and corresponding adjacent normal tissues. All collected samples were stored in a -80°C refrigerator. Adjacent normal tissues were confirmed by biological biopsy to ensure that they did not include ccRCA cells. After all, declaration of Helsinki should be mentioned and respected. This study was approved by the Ethics Committee of Tongde Hospital of Zhejiang Province. Signed written informed consents were obtained from all participants before the study.

Cells Culture

Human ccRCA cell line (786-O) and normal human kidney cell line (HK-2) were purchased from the Chinese Academy of Sciences

(Shanghai, China). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) complemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin and 100 IU/mL penicillin (Invitrogen, Carlsbad, CA, USA), and were grown in a 37°C, 5% CO₂ incubator. MiR-425 mimics/inhibitors and negative controls (NCs) were purchased from GenePharma (Shanghai, China). Cell transfection was performed according to the instructions of Lipofectamine® 2000 (Thermo Fisher Scientific, Waltham, MA, USA). When 786-O cells were grown into the logarithmic growth phase, they were collected and inoculated into corresponding 6-well plates. After 24 h, 786-O cells were transfected with NCs or miR-425 mimics/inhibitors with the final concentration of 100 nmol/L. 6 h after transfection, cell culture medium was replaced with complete DMEM (Dulbecco's modified eagle Medium) (HyClone, South Logan, UT, USA).

Luciferase Reporter Gene Assay

In TargetScan, miRDB and microRNA websites, we found that E2F6 was a target gene of miR-425. The binding sequence of miR-425 at the 3'-end of E2F6 was mutated by using a point mutation kit (Agilent Technologies, Santa Clara, CA, USA). Subsequently, mutated E2F6 (Mut-type) and non-mutant E2F6 (WT-type) were connected with the pGL3-Basic luciferase reporter vector (Promega, Madison, WI, USA). PGL3-Basic vector with mutant E2F6 was transfected into 786-O cells after lentivirus intervention on 24-well plates. According to the procedure of the Luciferase Reporter Gene Assay Kit, the same treatment was performed for the pGL3-Basic vector connected with non-mutant E2F6. Luciferase activity was then detected by a multi-function microplate reader.

Cell Transfection

MiR-425 mimics and inhibitor were synthesized and transfected into ccRCA cells to analyze the biological function of miR-425. Next, three groups were established to elucidate the potential correlation between miR-425 and ccRCA, including: the NC group (negative control), the miR-425 mimics (786-O cells transfected with miR-425 mimics) group and the mimics + E2F6 (786-O cells transfected with miR-425 mimics and si-E2F6) group. All the stuff was purchased from RiboBio (Guangzhou, China). Cell transfection

tion was performed according to the instructions of lipofectaminRNAiMAX (Life Technologies, Gaithersburg, MD, USA).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) Analysis

The expression levels of miR-425 and E2F6 in 786-O cells were detected *via* quantitative polymerase chain reaction (qPCR), respectively. Total RNA was extracted in accordance with the manufacturer's protocol of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). SYBR green qPCR assay was used to measure the expression level of E2F6. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. TaqMan miRNA assay (Applied Biosystems, Foster City, CA, USA) was used to measure the expression level of miR-425 normalized to U6. Primers used in this study were as follows: E2F6, F: 5'-TCTGAATTCTTGTGCTGGGCCCTTGGAATC-3', R: 5'-CCCGGTACCAATGCCATCAGTTGCTTACTTCAA-3'; miR-425, F: 5'-TCGGTGTAACATCCTCGACTG-3', R: 5'-GACCGTGTCTGCTGGAGTCG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot

Radioimmunoprecipitation assay (RIPA) lysate (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was employed to extract total protein of 786-O cells. Extracted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Sigma-Aldrich, St. Louis, MO, USA) and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Subsequently, the membranes were incubated with 5% milk for non-specific binding. After that, the membranes were incubated with primary E2F6 and GAPDH antibodies [diluted at 1:1000, Cell Signaling Technology (CST) Inc. Danvers, MA, USA] at 4°C overnight. The next day, the membranes were incubated with corresponding secondary antibodies (CST, Inc. Danvers, MA, USA) at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence method (Thermo Fisher Scientific, Waltham, MA, USA). GAPDH was used as an internal reference, and relative changes of protein expression were calculated.

Cell Proliferation

When cells grew to the logarithmic growth phase, they were collected and diluted into 1×10^6 cell suspension. Then the cells were seeded into 96-well plates with $5 \times 10^3/100 \mu\text{L}$ per well. The wells only added with medium were used as blank controls. Cell viability was determined *via* MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay (Beyotime, Shanghai, China). A total of $15 \mu\text{L}$ MTT reagents ($500 \mu\text{g/mL}$) were first added. Subsequently, the absorbance was measured by an enzyme-labeled spectrophotometer, followed by zero setting with blank controls.

Cell Cycle

Single cell suspension was prepared 48 h after cell transfection for each group, respectively. 3 replicates were set in each group. Prepared cell suspension was washed with pre-cooled phosphate-buffered saline (PBS) and fixed with 70% ethanol for 12 h. Subsequently, 0.5 mL 100 g/mL propidium iodide was added in the cell suspension for staining at room temperature for 10 min. The changes of DNA content in 786-O cells were detected *via* a flow cytometer and analyzed by random software Modfit LT. Finally, the changes of cell cycle were further analyzed.

Cell Apoptosis

48 h after transfection, 786-O cells were collected and washed with pre-cooled poly butylene succinate (PBS). The cells were then suspended with $300 \mu\text{L}$ binding buffer, and the concentration of 786-O cell was adjusted to 1×10^6 cells/ mL . Subsequently, $100 \mu\text{L}$ cell suspension was added in a flow tube, followed by $5 \mu\text{L}$ Annexin V-fluorescein isothiocyanate 1 (FITC) and $5 \mu\text{L}$ propidium iodide (PI) (eBioscience, San Diego, CA, USA). Next, the mixture was incubation at room temperature for 15 min in dark. $400 \mu\text{L}$ PBS was added to the reaction tube. Finally, a flow cytometer was used for cell apoptosis detection within 1 h.

Statistical Analysis

Prism 6.02 Software (La Jolla, CA, USA) was used for statistical analysis. All quantitative data were expressed as mean $\pm$ standard deviation. Student's *t*-test or *F*-test was used for comparison between two groups. $p < 0.05$ was considered statistically significant.

Results

Expression of miR-425 in ccRCA Tissues and Cells

To explore the role of miR-425 in ccRCA, we detected the expression level of miR-425 in ccRCA tissues and cells. Results indicated that the expression of miR-425 was significantly lower in ccRCA tissues than that of adjacent normal tissues (Figure 1A). Subsequently, we detected the expression level of miR-425 in 786-O cells and HK-2 cells. The same results were obtained at the cellular level (Figure 1B). Taken together, we believed that miR-425 might play a regulatory role in the progression of ccRCA.

Effect of miR-425 on the Proliferation of 786-O Cells

To investigate the effect of miR-425 on the proliferation of ccRCA, 786-O cells were transfected with miR-425 mimics, miR-425 inhibitor and miRNA NC, respectively. The changes of miR-425 expression were detected by qRT-PCR, and the efficiency of miR-425 transfection was verified (Figure 2A). Methyl thiazolyl tetrazolium (MTT) assay showed that the proliferation rate of 786-O cells was significantly decreased after miR-42 mimics transfection. However, the proliferation rate of 786-O cells was significantly increased in the miR-425 inhibitor group than that of the control group (Figure 2B), indicating that miR-425 could inhibit the proliferation of ccRCA cells.

Influence of miR-425 on the Cell Cycle of 786-O Cells

After 48 h of transfection with miR-425 mimics and inhibitor, cell cycle was detected by flow cytometry. Compared with the control group, the number of cells in G1/G2 phase was significantly increased significantly, whereas the number of cells in S and G2/M phases was significantly decreased in the miR-425 mimics group. However, the number of cells in G1/G2 phase was significantly declined and the number of cells in S and G2/M phases was remarkably increased in the miR-425 inhibitor group when compared with the control group. These results suggested that miR-425 could inhibit 786-O cells from crossing the node between G0/G1 phase and S phase, and entering S and G2/M phases for division and proliferation (Figure 2C).

Effect of miR-425 on the Apoptosis of 786-O Cells

The apoptosis rate of 786-O cells was detected by flow cytometry. As shown in Figure 2D and 2E, after transfection with miR-425 mimics, the percentage of apoptotic cells was significantly increased. However, the total apoptotic rate was significantly reduced in the inhibitor group, indicating the facilitating role of miR-425 in cell apoptosis.

Prediction and Verification of miR-425 Targets

The miRNA target gene prediction software indicated that miR-425 could act on the 3'-UTR

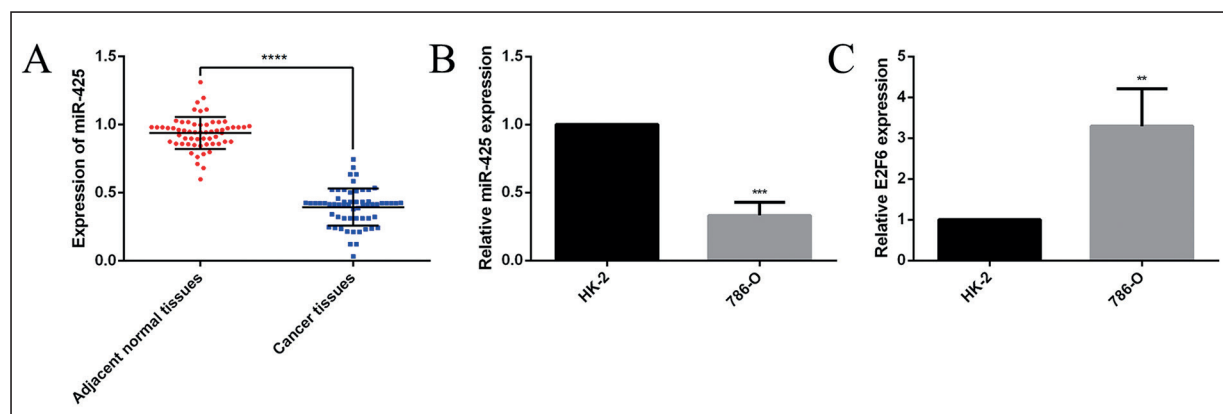


Figure 1. The expression levels of miR-425 and E2F6 in ccRCA tissues and cells. **A**, The expression of miR-425 in ccRCA tissues and corresponding adjacent normal tissues. (**** $p < 0.0001$ compared with adjacent normal tissues). **B**, The expression of miR-425 and E2F6 in ccRCA cells (786-O) and normal human kidney cells (HK-2). (** $p < 0.01$, **** $p < 0.001$ compared with HK-2s).

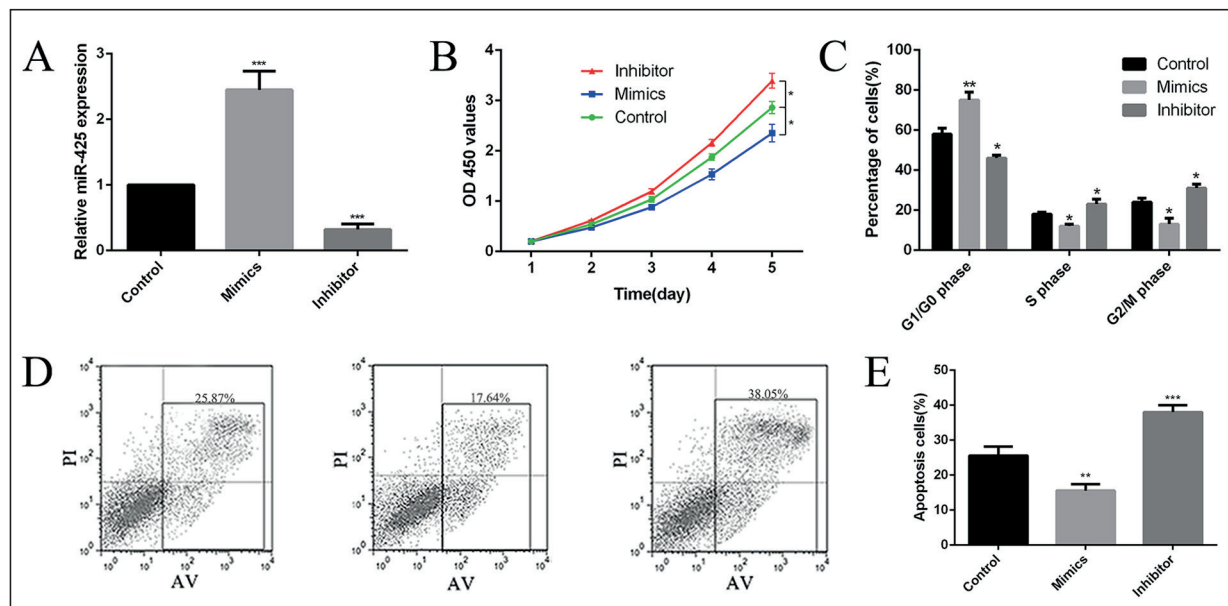


Figure 2. MiR-425 inhibited the biological function of ccRCA cells. 786-O cells were transfected with miR-425 mimics and inhibitor. **A**, The changes of miR-425 expression after cell transfection were analyzed by qRT-PCR. **B**, Effect of miR-425 on cell proliferation. MTT assay was performed to detect the proliferation of ccRCA cells transfected with mimics or inhibitor. **C**, Effect of miR-425 on cell cycle. Flow cytometry was applied to detect the cell cycle phases of ccRCA cells. **D-E**, Effect of miR-425 on cell apoptosis. Annexin V/PI staining was used to detect the apoptosis of ccRCA cells. Data were presented as means $\pm$ standard deviations. (* p < 0.05, ** p < 0.01, *** p < 0.001 vs. the control group; # p < 0.01, ### p < 0.001 vs. the mimics group).

of E2F6 (Figure 3A). After co-transfection of constructed fluorescent report vector (pmirGLO/E2F6-3'UTR and pmirGLO/E2F6-3'UTR mut) with miR-425 into 786-O cells, the luciferase activity was detected. Results revealed that over-expression of miR-425 could down-regulate the luciferase activity of the report vector of wild-type E2F6 3'UTR. However, the luciferase activity of the report vector of wild-type E2F6 3'UTR was up-regulated after inhibiting the expression of miR-425, suggesting that miR-425 had a targeting effect on E2F6. However, the luciferase activity in the report vector group transfected with mutant E2F6 3'UTR was not significantly decreased. These experimental results indicated that miR-425 could complement and bind to the 3'UTR of E2F6 (Figure 3B).

RT-PCR and Western blot showed that the expression of E2F6 was significantly reduced after miR-425 overexpression (Figure 4A-4C), further confirming the regulatory effect of miR-425 on E2F6 expression. Subsequently, we explored the correlation between of E2F6 and miR-425 in ccRCA cells. 3 groups were constructed (the miR-NC group, the miR-425 mimics group and the mimics + E2F6 group) and similar experiments were performed in 786-O cells. As expected, the

restoration of E2F6 had the reverse force on the inhibitory effect of miR-425 on the proliferation (Figure 4D), cell cycle (Figure 4E), cell apoptosis (Figure 4F-4G) of ccRCA cells. These results indicated that miR-425 could inhibit ccRCA by targeting E2F6 expression.

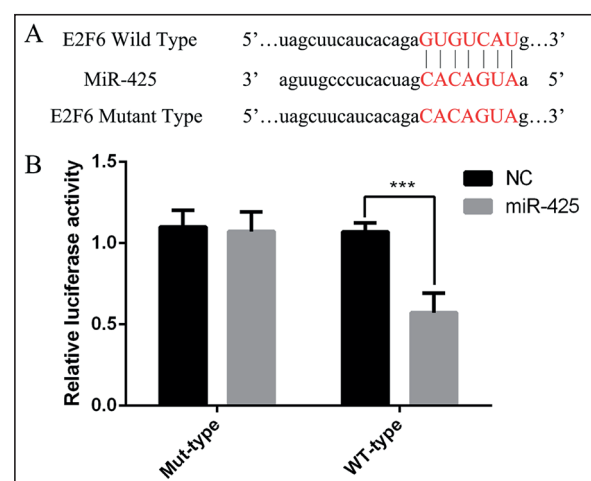


Figure 3. E2F6 was a direct and functional target of miR-425. 786-O cells were transfected with miR-425 mimics and inhibitor. **A**, Diagram of putative miR-425 binding sites of E2F6. **B**, Relative activities of luciferase reporters (***) p < 0.001).

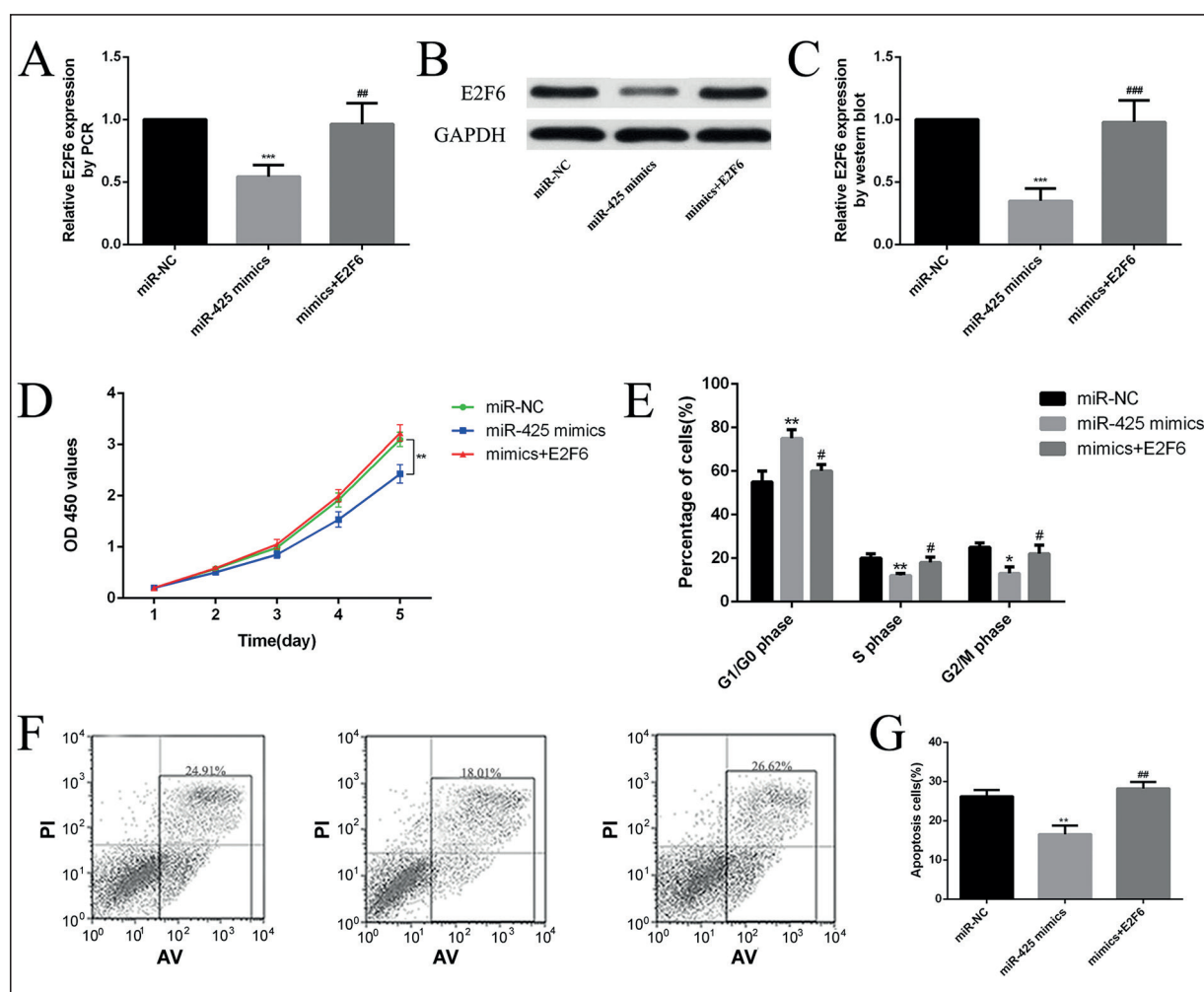


Figure 4. E2F6 was a direct and functional target of miR-425. E2F6 overexpression attenuated the inhibitory effect of miR-425 on 786-O cells. **A**, The mRNA expression level of E2F6 was detected by qRT-PCR. **B**, Protein expression of E2F6. **C**, The protein expression level of E2F6 was detected by Western blot. **D**, Cell proliferation was measured by MTT assay. **E**, Cell cycle was detected by flow cytometry. **F-G**, Cell apoptosis of ccRCA cells was analyzed by Annexin V/PI staining. Data were presented as means $\pm$ standard deviations. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the NC group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. the mimics group).

Discussion

Differential expression (up-regulated or down-regulated expression) of miRNAs can often be found in cancer tissues. Previous studies have proved that these differentially expressed miRNAs can participate in the regulation of tumor biological function, which may further affect the progression of malignant tumors. Considering the obvious down-regulation of miR-425 expression in ccRCA tissues and cells, we explored the biological role of miR-425 in ccRCA by cell transfection in this study. Results indicated that knocking down the expression of miR-425 could significantly enhance the malignant phenotype of

ccRCA cells and accelerate the growth of 786-O cells. However, over-expression of miR-425 could significantly inhibit the proliferation and promote the apoptosis of ccRCA cells. These experimental results confirmed that miR-425 exhibited an obvious inhibitory effect on the metastasis of ccRCA. Researches have also found that miR-425 also acts as an anti-oncogene in malignant tumors of most other organs. Based on the complete or incomplete principle of base complementary pairing, miRNAs usually work through binding to the 3'UTR of target genes. This may directly degrade target gene mRNAs or inhibit the protein translation process, eventually preventing the corresponding protein expression from oc-

currence¹⁹. Therefore, finding out the functional targets of miRNAs is of great significance in identifying the corresponding biological function mechanisms of miRNAs in tumors²⁰. In this study, E2F6, a potential target gene of miR-425, was selected through three websites (TargetScan, miRDB and miRanda). It was confirmed that E2F6 could be directly targeted by binding to the corresponding 3'UTR. Meanwhile, results of reporter gene assay, Western blot and qRT-PCR indicated that the expression of E2F6 was negatively regulated by miR-425.

E2F6 is a member of the nuclear transcription factor E2Fs family. It is also a key molecule that can regulate important cellular biological activities, such as the proliferation, apoptosis and differentiation of cells. Scholars have proved that E2F6 can inhibit the apoptosis induced by deoxyribonucleic acid (DNA) damage^{21,22} and participate in the occurrence and development of cancers, including breast cancer²³ and lung cancer²⁴.

In our study, we found that E2F6 overexpression could significantly affect the inhibitory function of miR-425 on ccRCA cells in cell proliferation, cell cycle and cell apoptosis. This indicated that miR-425 could target the expression of E2F6. Therefore, the miR-425/E2F6 axis might be an important mechanism for the development of ccRCA.

Conclusions

For RCA, there is a lack of effective molecular markers for the early diagnosis, prognosis and treatment of sensitivity prediction of RCA. Meanwhile, we also lack RCA specific target molecules that can be used as a target for drug therapy, which are great difficulties in the clinical diagnosis and treatment of RCA. Through this study, we found that miR-425 was lowly expressed in ccRCA tumor tissues and could inhibit the growth and promote the apoptosis of ccRCA cells by targeting E2F6. This study provided a basic research for the application of miR-425 in RCA in the future.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

References

- 1) SIEGEL RL, MILLER KD, FEDEWA SA, AHNEN DJ, MEESTER R, BARZI A, JEMAL A. Colorectal cancer statistics, 2017. *CA Cancer J Clin* 2017; 67: 177-193.
- 2) CHEN W, ZHENG R, BAADE PD, ZHANG S, ZENG H, BRAY F, JEMAL A, YU XQ, HE J. Cancer statistics in China, 2015. *CA Cancer J Clin* 2016; 66: 115-132.
- 3) MCLAUGHLIN JK, LIPWORTH L. Epidemiologic aspects of renal cell cancer. *Semin Oncol* 2000; 27: 115-123.
- 4) GORE ME, SZCZYLIK C, PORTA C, BRACARDA S, BJARNASON GA, OUDARD S, HARIHARAN S, LEE SH, HAAANEN J, CASTELLANO D, VRDOLIAK E, SCHOFFSKI P, MAINWARING P, NIETO A, YUAN J, BUKOWSKI R. Safety and efficacy of sunitinib for metastatic renal-cell carcinoma: an expanded-access trial. *Lancet Oncol* 2009; 10: 757-763.
- 5) RINI BI, CAMPBELL SC, ESCUDIER B. Renal cell carcinoma. *Lancet* 2009; 373: 1119-1132.
- 6) KROEGER N, ZIMMERMANN U, BURCHARDT M, PANTUCK AJ. Prognostication in localised renal cell carcinoma. *Lancet Oncol* 2015; 16: 603-604.
- 7) NOVARA G, FICARRA V, ANTONELLI A, ARTIBANI W, BERTINI R, CARINI M, COSCIANI CS, IMBIMBO C, LONGO N, MARTIGNONI G, MARTORANA G, MINERVINI A, MIRONI V, MONTORSI F, SCHIAVINA R, SIMEONE C, SERNI S, SIMONATO A, SIRACUSANO S, VOLPE A, CARMIGNANI G. Validation of the 2009 TNM version in a large multi-institutional cohort of patients treated for renal cell carcinoma: are further improvements needed? *Eur Urol* 2010; 58: 588-595.
- 8) PICHLER M, HUTTERER GC, CHROMECKI TF, JESCHE J, KAMPF-KETTNER K, REHAK P, PUMMER K, ZIGEUNER R. External validation of the Leibovich prognosis score for nonmetastatic clear cell renal cell carcinoma at a single European center applying routine pathology. *J Urol* 2011; 186: 1773-1777.
- 9) HENG DY. Combination therapy in metastatic renal cell carcinoma. *Lancet Oncol* 2011; 12: 613-614.
- 10) SHU X, HILDEBRANDT MA, GU J, TANNIR NM, MATIN SF, KARAM JA, WOOD CG, WU X. MicroRNA profiling in clear cell renal cell carcinoma tissues potentially links tumorigenesis and recurrence with obesity. *Br J Cancer* 2017; 116: 77-84.
- 11) HE H, WANG L, ZHOU W, ZHANG Z, WANG L, XU S, WANG D, DONG J, TANG C, TANG H, YI X, GE J. MicroRNA expression profiling in clear cell renal cell carcinoma: Identification and functional validation of key miRNAs. *PLoS One* 2015; 10: e125672.
- 12) WENG L, WU X, GAO H, MU B, LI X, WANG JH, GUO C, JIN JM, CHEN Z, COVARRUBIAS M, YUAN YC, WEISS LM, WU H. MicroRNA profiling of clear cell renal cell carcinoma by whole-genome small RNA deep sequencing of paired frozen and formalin-fixed, paraffin-embedded tissue specimens. *J Pathol* 2010; 222: 41-51.

- 13) JUNG M, MOLLENKOPF HJ, GRIMM C, WAGNER I, ALBRECHT M, WALLER T, PILARSKY C, JOHANNSEN M, STEPHAN C, LEHRACH H, NIETTFELD W, RUDEL T, JUNG K, KRISTIANSEN G. MicroRNA profiling of clear cell renal cell cancer identifies a robust signature to define renal malignancy. *J Cell Mol Med* 2009; 13: 3918-3928.
- 14) LAWRIE CH, LARREA E, LARRINAGA G, GOICOECHEA I, ARESTIN M, FERNANDEZ-MERCADO M, HES O, CACERES F, MANTEROLA L, LOPEZ JI. Targeted next-generation sequencing and non-coding RNA expression analysis of clear cell papillary renal cell carcinoma suggests distinct pathological mechanisms from other renal tumour subtypes. *J Pathol* 2014; 232: 32-42.
- 15) HE B, LI T, GUAN L, LIU FE, CHEN XM, ZHAO J, LIN S, LIU ZZ, ZHANG HQ. CTNNA3 is a tumor suppressor in hepatocellular carcinomas and is inhibited by miR-425. *Oncotarget* 2016; 7: 8078-8089.
- 16) LIU L, ZHAO Z, ZHOU W, FAN X, ZHAN Q, SONG Y. Enhanced expression of miR-425 promotes esophageal squamous cell carcinoma tumorigenesis by targeting SMAD2. *J Genet Genomics* 2015; 42: 601-611.
- 17) YAN YF, GONG FM, WANG BS, ZHENG W. MiR-425-5p promotes tumor progression via modulation of CYLD in gastric cancer. *Eur Rev Med Pharmacol Sci* 2017; 21: 2130-2136.
- 18) ZHU W, MA Y, ZHUANG X, JIN X. MicroRNA-425 is downregulated in nasopharyngeal carcinoma and regulates tumor cell viability and invasion by targeting hepatoma-derived growth factor. *Oncol Lett* 2018; 15: 6345-6351.
- 19) BARTEL DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004; 116: 281-297.
- 20) POLISENO L, SALMENA L, ZHANG J, CARVER B, HAVEMAN WJ, PANDOLFI PP. A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. *Nature* 2010; 465: 1033-1038.
- 21) YANG W/W, WANG ZH, ZHU Y, YANG HT. E2F6 negatively regulates ultraviolet-induced apoptosis via modulation of BRCA1. *Cell Death Differ* 2007; 14: 807-817.
- 22) KIKUCHI J, SHIMIZU R, WADA T, ANDO H, NAKAMURA M, OZAWA K, FURUKAWA Y. E2F-6 suppresses growth-associated apoptosis of human hematopoietic progenitor cells by counteracting proapoptotic activity of E2F-1. *Stem Cells* 2007; 25: 2439-2447.
- 23) TANG H, LIU P, YANG L, XIE X, YE F, WU M, LIU X, CHEN B, ZHANG L, XIE X. MiR-185 suppresses tumor proliferation by directly targeting E2F6 and DNMT1 and indirectly upregulating BRCA1 in triple-negative breast cancer. *Mol Cancer Ther* 2014; 13: 3185-3197.
- 24) TANG H, LIU P, YANG L, XIE X, YE F, WU M, LIU X, CHEN B, ZHANG L, XIE X. MiR-185 suppresses tumor proliferation by directly targeting E2F6 and DNMT1 and indirectly upregulating BRCA1 in triple-negative breast cancer. *Mol Cancer Ther* 2014; 13: 3185-3197.