

Downregulated microRNA-510-5p acts as a tumor suppressor in renal cell carcinoma

DUQUN CHEN^{1,3}, YUCHI LI^{1,2,4}, ZUHU YU¹⁻³, YIFAN LI¹⁻³, ZHENGMING SU^{1,2,4},
LIANGCHAO NI^{1,2}, SHANGQI YANG^{1,2}, YAOTING GUI² and YONGQING LAI^{1,2}

¹Department of Urology, Peking University Shenzhen Hospital, Shenzhen, Guangdong 518036;

²The Guangdong and Shenzhen Key Laboratory of Male Reproductive Medicine and Genetics, Institute of Urology of Shenzhen PKU-HKUST Medical Center, Shenzhen, Guangdong 518036; ³Department of Urology, Anhui Medical University First Affiliated Hospital, Hefei, Anhui 230032; ⁴Department of Urology, Shantou University Medical College, Shantou, Guangdong 515041, P.R. China

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Abstract. MicroRNA (miR)-510-5p has been demonstrated to be involved in a number of types of malignancy; however, the function of miR-510-5p in renal cancer remains unclear. The present study aimed to determine the expression of miR-510-5p in renal cell carcinoma (RCC) specimens and analyzed the impact of miR-510-5p on renal cancer by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, wound scratch and apoptosis assays. The results showed that miR-510-5p was significantly downregulated in RCC specimens compared with normal renal specimens. Overexpression of miR-510-5p by synthetic mature mimics reduced cell proliferation and migration and induced an increase in cell apoptosis, indicating that miR-510-5p may act as a tumor suppressor in RCC. The present study firstly revealed that downregulated miR-510-5p functioned as a tumor suppressor by reducing cellular proliferation and migration, and inducing apoptosis in RCC. Further research is required to define target genes of miR-510-5p to determine the cellular mechanism of miR-510-5p in the carcinogenesis of RCC.

Introduction

Renal cell carcinoma (RCC) is the most common neoplasm of the adult kidney with the highest rate of recurrence and mortality

among malignancies in urologic systems (1). Nearly 30% of RCC patients present at advanced stages, and ~40% of patients that undergo surgical resection experience recurrence during subsequent follow-up (2,3). Metastatic RCC has an extremely poor prognosis and remains an incurable disease despite the great improvement in surgery and personalized treatments (4,5). The high rates of recurrence and mortality of RCC create an urgent requirement for personalized care and reliable biomarkers for early detection and prognosis prediction (6). Therefore, exploring the molecular mechanisms underlying the disease and identifying novel molecular biomarkers is important (7).

In recent years, miRNAs have emerged as important molecules in the complex networks of gene regulation (8). These small, endogenous non-coding RNA molecules that regulate the expression of protein coding genes at a post-transcriptional level have been implicated in a variety of human disorders, such as infectious diseases, metabolic diseases and cancer (9,10). Aberrantly expressed miRNAs are prevalent in a number of types of human cancer, and are important in cancer initiation, development and metastasis (11,12). Certain highly expressed miRNAs may function as oncogenes by repressing tumor suppressors, whereas downregulated miRNAs may function as tumor suppressors by negatively regulating oncogenes (13). Their stable expression in the blood render them reliable biomarkers for early detection, diagnosis and prognosis prediction in various diseases, including cancer (14,15).

miR-510 has been demonstrated to be involved in lung cancer, breast cancer, gastric cancer and ovarian serous carcinoma (16-19); however, the expression and function of miR-510-5p in renal cancer remains unclear. The present study aimed to determine the expression of miR-510-5p in RCC tissues and paired normal adjacent tissues, and analyzed the impact of miR-510-5p on renal cancer by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), wound scratch and apoptosis assays.

Materials and methods

Clinical specimens. The present study was approved by the Ethics Committee of Peking University Shenzhen Hospital

Correspondence to: Professor Yongqing Lai, Department of Urology, Peking University Shenzhen Hospital, 1120 Lianhua Road, Shenzhen, Guangdong 518036, P.R. China
E-mail: yqlord@163.com

Professor Yaoting Gui, The Guangdong and Shenzhen Key Laboratory of Male Reproductive Medicine and Genetics, Institute of Urology of Shenzhen PKU-HKUST Medical Center, 1120 Lianhua Road, Shenzhen, Guangdong 518036, P.R. China
E-mail: gyt@pkusz.com

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(Shenzhen, China) and Anhui Medical University First Affiliated Hospital (Hefei, China). Written informed consent was obtained from every patient prior to sample collection. A total of 48 paired renal cell carcinoma (RCC) and adjacent normal specimens were collected from patients receiving radical nephrectomies at Peking University Shenzhen Hospital or Anhui Medical University First Affiliated Hospital. All samples were processed and stored at -80°C in RNAlater (Qiagen, Valencia, CA, USA) until RNA isolation. The clinical and pathological information of all the patients is summarized in Table I. These samples were staged according to the American Joint Committee on Cancer (AJCC) staging system (20).

Cell culture and RNA extraction. Two human RCC cell lines, ACHN and 786-O (Guangdong and Shenzhen Key Laboratory of Male Reproductive Medicine and Genetics, Shenzhen, China) were used in this study. They were incubated in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies) and maintained in a humidified incubator containing 5% CO_2 at 37°C . Total-RNA of each sample was isolated with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and purified with an RNeasy Maxi kit (Qiagen) according to the manufacturer's instructions.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). To obtain the cDNA templates, 1 μg total RNA of each sample was isolated for reverse transcription with the miScript Reverse Transcription reagent (Qiagen).

PCR amplification was performed using 1 μl cDNA in a 20 μl reaction system, containing 10 μl QuantiTect SYBR Green PCR Master mix, 2 μl miScript Universal Primer, 0.5 μl specific microRNA primer and 6.5 μl RNase-free water. The sequence of the miR-510-5p forward primer was 5'-TAGCAGCACGTAAATATTGGCG-3' and the reverse primer was provided by the miScript SYBR Green PCR kit. The sequence of the U6 forward primer was 5'-CTCGCTTCGGCAGCACACA-3' and reverse primer was 5'-ACGCTTCACGAATTTGCGT-3'. PCR amplification conditions were set as: 95°C for 2 min, then 40 cycles of 95°C for 15 sec, 58°C for 30 sec and 72°C for 30 sec. The relative expression levels of miR-510-5p were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method (21).

Mature miRNA and negative control transfection. For the restoration of miR-510-5p, ACHN and 786-O cells were transfected with synthetic mature molecules (miR-510-5p mimics; Shanghai GenePharma Co., Ltd., Shanghai, China) with Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. Mature miR-510-5p mimics and negative control were used in the gain-of-function experiments. The cancer cells were harvested and RNA was isolated for RT-qPCR to analyze the fold changes of miR-510-5p 24 h after transfection.

MTT assay. The capacity for cellular proliferation was determined using an MTT assay, according to the manufacturer's instructions. Approximately 5×10^3 cells were

seeded into 96-well culture plates and transfected with 5 pmol miR-510-5p mimics or negative control. At 0, 24, 48 or 72 h after transfection, the cells were incubated with 20 μl MTT (5 $\mu\text{g}/\text{ml}$) for 4 h, followed by the addition of 150 μl dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) and shaking for 15 min at room temperature to solubilize the crystals. The optical density (OD) was determined using a microplate reader (Model 680; Bio-Rad) at dual wavelength of 490/630 nm.

Flow cytometric analysis of apoptosis. Approximately 300,000 renal cancer cells were cultured in 6-well plates at 37°C and transfected with miR-510-5p mimics or negative control within 24 h. Cancer cells, including floating cells, were harvested 48 h after transfection, washed twice with cold phosphate-buffered saline and resuspended in 100 μl of 1X binding buffer (Invitrogen Life Technologies), followed by the addition of 5 μl Annexin V-fluorescein isothiocyanate (Invitrogen Life Technologies) and 3 μl propidium iodide (PI). The fluorescence of stained cells was then analyzed by flow cytometry (Beckman Coulter, Miami, FL, USA) using 488 nm excitation within 30 min of staining, according to the manufacturer's instructions.

Migration scratch assay. Wound scratch assay was used to assess the migration ability of 786-O and ACHN renal cancer cells *in vitro*. Approximately 350,000 cells were seeded per 12-well dish and transfected with 80 pmol miR-510-5p mimics or 80 pmol negative control 24 h later using Lipofectamine 2000.

After 5 h of transfection, the cell monolayer was scraped using a P-20 micropipette tip. The initial gap length (0 h) and the residual gap length 24 h after wound-healing were calculated using the software program MIAS-2000 (Leica Microsystems GmbH, Wetzlar, Germany). The experiments were performed in triplicate, repeated at least three times, and analyzed in a double-blind manner by at least two observers.

Bioinformatics. The potential targets of miR-510-5p were predicted by combining four public algorithms, miRanda (<http://www.targetscan.org/>), TargetScan (<http://www.targetscan.org/>), PicTar (<http://pictar.mdc-berlin.de/>) and miRWalk (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/>). Putative genes predicted by all four algorithms were accepted and candidates were selected based on the gene function.

Statistical analysis. All statistical analysis was conducted with SPSS 17.0 statistical software package (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference. The different expression of miR-510-5p in RCC and paired normal samples was analyzed by a paired t-test.

Results

miR-510-5p is downregulated in RCC tissues quantified by RT-qPCR. Previous miRNA expression profiles of RCC indicated that miR-510-5p was downregulated (22,23). In order

Table I. Clinical and pathological features of 48 patients.

Variable	Number of cases
Total	48
Age (years)	
≥52	29
<52	19
Gender	
Male	30
Female	18
Histological type	
Clear cell	39
Papillary	9
Primary tumor stage	
T1	27
T2	19
T3 and T4	2
AJCC clinical stage	
I	27
II	18
III+IV	3

AJCC, American Joint Committee on Cancer.

to confirm the results of former studies, RT-qPCR was used to quantify the expression of miR-510-5p in RCC and paired adjacent normal tissues from 48 patients. The results showed the expression of miR-510-5p decreased in 81.25% (39/48) of RCC tissues, compared with paired normal tissues, with an average reduction in expression of 0.4283-fold (Fig. 1A). To investigate the effects of miR-510-5p on renal cancer cells, synthetic miR-510-5p mature mimics and negative controls were transfected into ACHN and 786-O cell lines. As shown in Fig.1B, RT-qPCR analysis indicated the fold changes of miR-510-5p in ACHN and 786-O cells after transfection were 9.58 and 11.32, respectively.

Overexpression of miR-510-5p inhibits RCC cell proliferation. The impact on cell proliferation was analyzed by an MTT assay, OD values of two groups (miR-510-5p mimics and negative control) were measured at 0, 24, 48 and 72 h after transfection. The present results showed the proliferation of ACHN cells decreased by 5.16% ($P>0.05$), 15.26% ($P<0.05$) and 29.06% ($P<0.05$) while the proliferation of 786-O cells decreased by 5.06 ($P>0.05$), 12.42 ($P<0.05$) and 21.78% ($P<0.05$) (Fig. 2), suggesting that miR-510-5p can reduce the proliferation of cancer cells.

Restoration of miR-510-5p induces RCC cell apoptosis. To demonstrate the effect of miR-510-5p on cell apoptosis, a flow cytometry assay was performed to detect the apoptosis rates of ACHN and 786-O cells after transfection. The results revealed that apoptosis rates of ACHN transfected with miR-510-5p mimics and negative control were 13.7 versus 5.0 while the apoptosis rates of 786-O cells were 10.8 versus 6.1 ($P<0.05$),

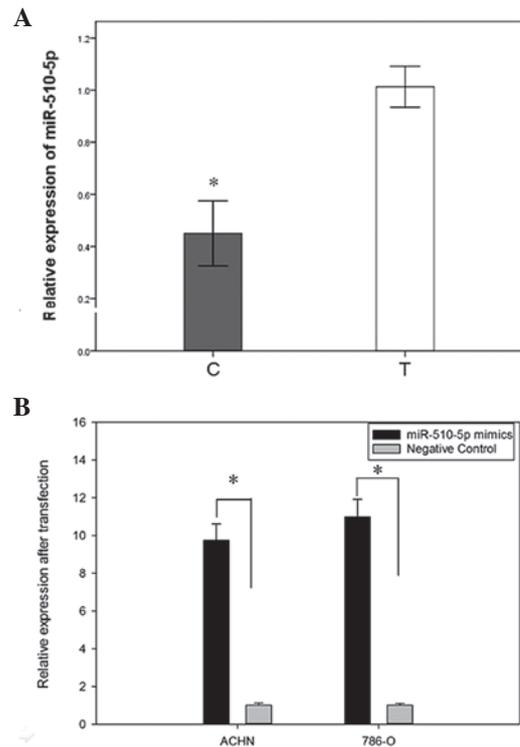


Figure 1. (A) Relative expression of miR-510-5p in renal cell carcinoma (C) and normal tissues (N) by reverse transcription-quantitative polymerase chain reaction. (B) Relative expression of miR-510-5p in ACHN and 786-O cells after transfection with miR-510-5p mimics. * $P<0.05$ vs. negative control.

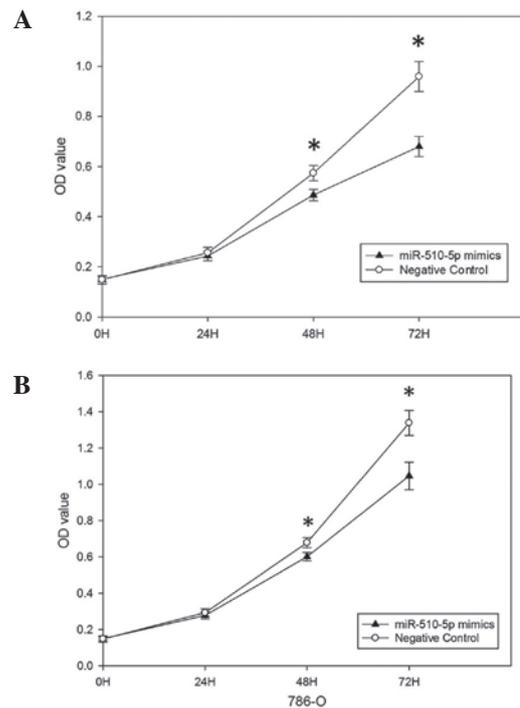


Figure 2. MTT assay for cell proliferation of ACHN and 786-O cells transfected with miR-510-5p mimics vs. negative control. (A) Cell proliferation of ACHN cells. (B) Cell proliferation of 786-O cells. * $P<0.05$, vs. negative control.

suggesting that restoration of miR-510-5p induces apoptosis of renal cancer cells (Fig. 3).

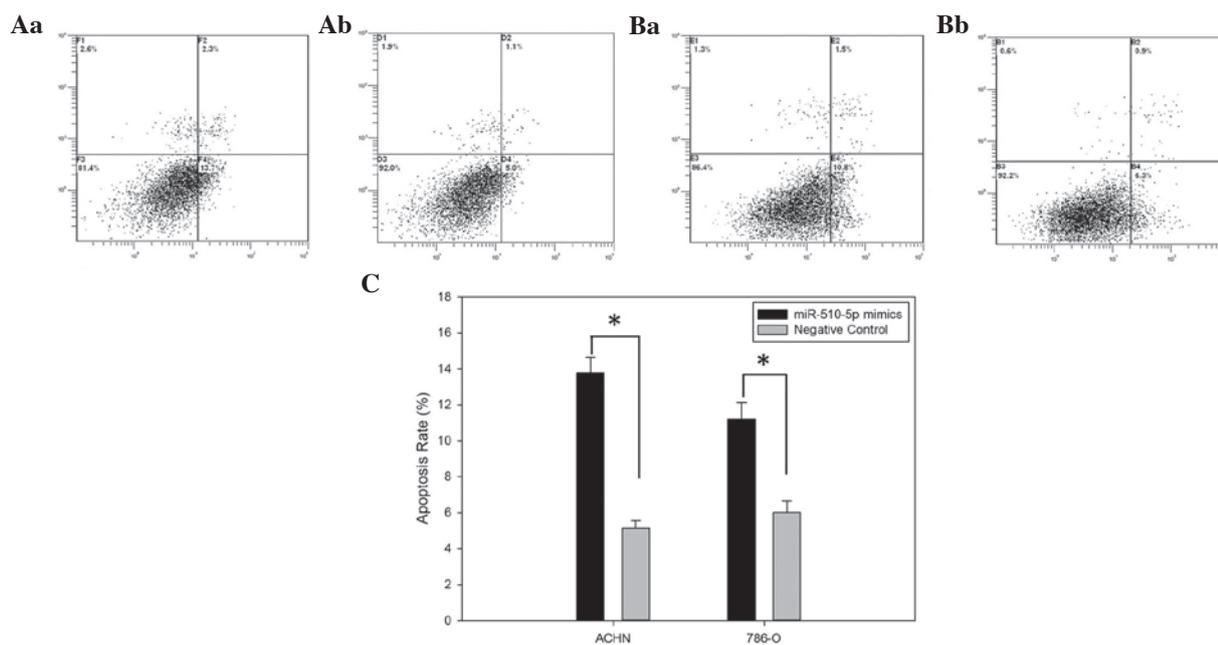


Figure 3. Flow cytometry to observe cell apoptosis of ACHN and 786-O cells after transfection with miR-510-5p mimics or negative control. (A) ACHN cells transfected with (Aa) miR-510-5p mimics and (Ab) negative control. (B) 786-O cells transfected with miR-510-5p mimics (Ba) and negative control (Bb). (C) Comparison of apoptosis rates of ACHN and 786-O cancer cells transfected with miR-510-5p mimics and negative controls. ** $P < 0.01$ vs. negative control.

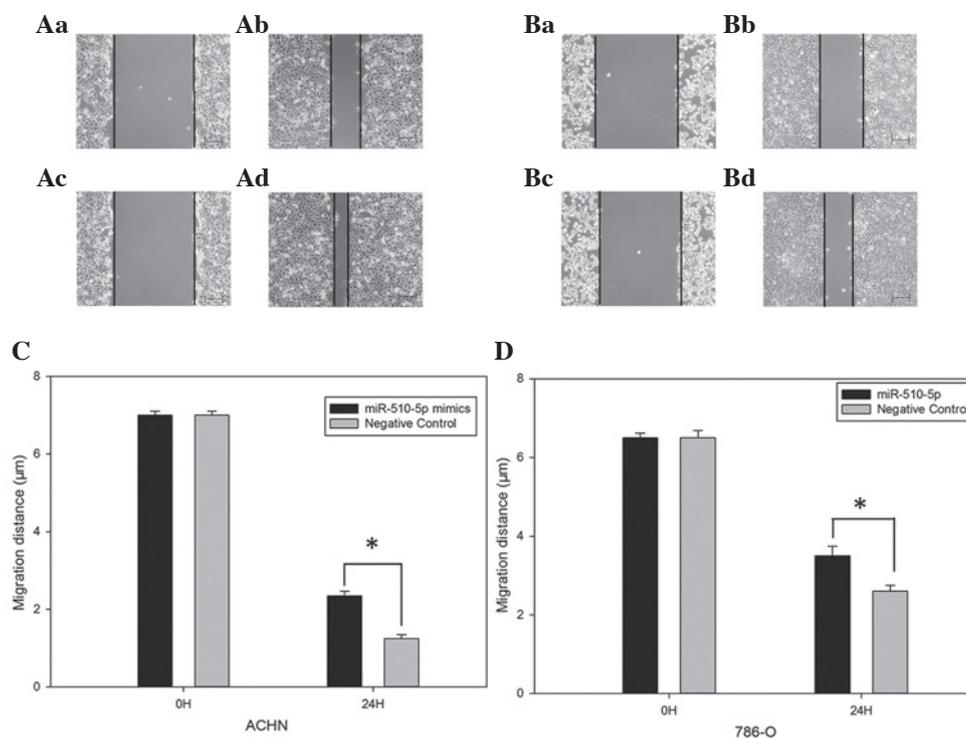


Figure 4. Wound scratch assay for ACHN and 786-O cells 24 h after transfection. (A) Images of the ACHN cells. Cells were transfected with negative control (Aa) 0 h and (Ab) 24 h after the wounds were made or with miR-510-5p mimics (Ac), 0 h and (Ad) 24 h. (B) Images of the 786-O cells. Cells were transfected with negative control (Ba) 0 h and (Bb) 24 h after the wounds were made, or with miR-510-5p mimics at (Bc) 0 h and (Bd) 24 h. Comparison of wound widths (μm) in (C) ACHN cells and (D) 786-O cells using a standard caliper.

miR-510-5p mimics inhibited cell migration. The influence of miR-510-5p on cell migration was observed by a wound scratch assay. As presented in Fig. 4, wound width of the group transfected with miR-510-5p mimics was greater than that of the negative control group ($P < 0.05$), suggesting that overex-

pressed miR-510-5p inhibited the ability of migration of renal cancer cells (Fig. 4).

Target gene prediction. To investigate the downstream target genes of miR-510-5p, miRanda, TargetScan, PicTar and

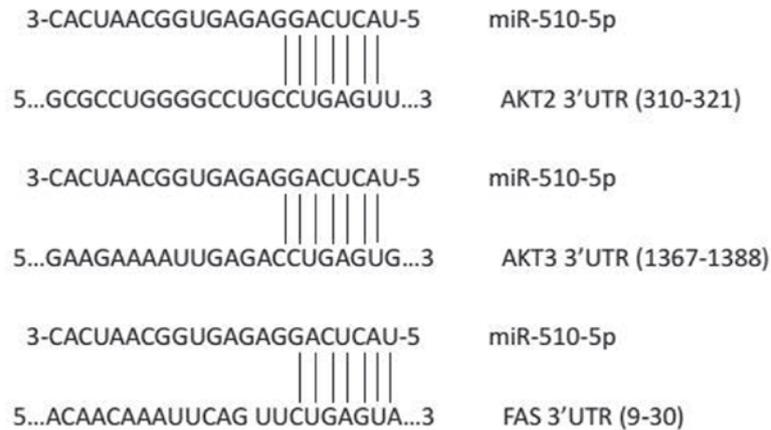


Figure 5. Bioinformatics prediction of the target genes of miR-510-5p with complementary sites.

miRWalk were used in combination to predict the putative targets. AKT2, AKT3 and FAS were three of the potential targets predicted by all four algorithms simultaneously, of which the 3' untranslated region (UTR) of the mRNA contained a complementary site for the seed sequences of miR-510-5p (Fig. 5).

Discussion

Carcinogenesis is a complicated process that involves numerous genetic aberrations and signaling pathways. The recent identification of miRNAs and their capability of simultaneously regulating multiple downstream genes may be important in explaining the complex mechanisms underlying cancer formation (24). These short RNAs of 19-25 nucleotides are key in a wide variety of biological processes, including cell fate specification, proliferation, migration, apoptosis and tumorigenesis (25,26). A number of studies have validated that miRNAs contribute to the development of various types of malignancies, as well as to their invasive and metastatic capacities, including RCC (27). For example, miR-204 was confirmed to be a Von hippel-Lindau-regulated tumor suppressor acting by inhibiting macroautophagy, with MAP1LC3B (LC3B) as a direct and functional target (28). In addition, miR-21 regulates PTEN to force the canonical oncogenic Akt/TORC1 signaling conduit to drive renal cancer cell proliferation and invasion (29). Further research into the function and interaction with the target genes of deregulated miRNAs may reveal the molecular mechanisms underlying the tumorigenesis of RCC.

Aberrant expression of miR-510 has been observed in several types of cancer, as described above. In breast cancer, miR-510 directly binds to the 3'UTR of peroxiredoxin1 and prevents its protein expression, thereby suppressing the migration of cancer cells (17). While in ovarian serous carcinoma (OSC), low miR-510 expression was significantly associated with poorer overall survival, indicating that miR-510 may be considered a novel-candidate clinical biomarker for predicting OSC outcome (18). However, the expression and function of miR-510 in RCC remains unclear.

In the present study, the expression of miR-510-5p in 48 paired RCC and normal renal specimens was quantified by

RT-qPCR and found that miR-510-5p was downregulated in RCC. The present results of decreased expression of miR-510-5p was in accordance with the results of recent miRNA expression profiles of RCC (30,31). To investigate whether miR-510-5p was important for the tumorigenesis of RCC, MTT and wound scratch assays, as well as flow cytometry were used to analyze the impact of miR-510-5p on renal cancer by transfecting synthetic miR-510-5p mimics. The results show that cancer cells transfected with miR-510-5p mimics displayed less cellular proliferation and migration and more cellular apoptosis compared with the negative control groups, indicating that miR-510-5p may act as a tumor suppressor by reducing cell proliferation and migration, and inducing cell apoptosis in RCC.

It is generally acknowledged that miRNAs are important in various biological processes by 'imperfect' complementary binding to the 3'UTR of the downstream genes. To determine the target genes of miR-510-5p, several algorithms were combined to predict putative target genes and AKT2, AKT3 and FAS were identified as potential targets of miR-510-5p. AKT is a major transducer of the phosphoinositide 3-kinase (PI3K) pathway and is crucial in the regulation of cellular processes, such as growth, metabolism and survival. Mammalian cells are characterized by the expression of three different AKT isoforms (AKT1, AKT2 and AKT3), encoded by distinct genes (32). Emerging evidence has shown that AKT2 and AKT3 serve as significant contributors to malignancy (33). While FAS is a member of the TNF-receptor superfamily, which has been shown to be central in the physiological regulation of programmed cell death, and has been implicated in the pathogenesis of various malignancies (34). It has been reported that FAS expression is a surrogate biomarker of active cancer cell proliferation and accurately predicts RCC patient survival (35). Decreased expression of miR-510-5p may regulate cellular proliferation, migration and apoptosis by targeting oncogenes AKT2, AKT3 and FAS; however, this requires further research.

In conclusion, the present study revealed that downregulated miR-510-5p functioned as a tumor suppressor by reducing cellular proliferation and migration and inducing apoptosis in RCC. Further research is required to define target genes of miR-510-5p to elucidate the cellular mechanism underlying the effect of miR-510-5p in the carcinogenesis of RCC.

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