

microRNA-181a-5p functions as an oncogene in renal cell carcinoma

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Received June 1, 2017; Accepted February 23, 2018

DOI: 10.3892/mmr.2018.8899

Abstract. Renal cell carcinoma (RCC) is one of the most common urinary tumors. Previous studies have demonstrated that microRNA (miR)-181a-5p has an important role in numerous types of cancer. However, the function of miR-181a-5p in RCC remains unknown. In the present study, the expression levels of miR-181a-5p in RCC tissues and cell lines were investigated using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The results of the RT-qPCR analyses suggested that the expression of miR-181a-5p was upregulated in RCC tissues and cells lines compared with adjacent normal renal tissues and normal renal cell lines. Furthermore, the effect of miR-181a-5p on cell proliferation, migration, invasion and apoptosis was investigated in the present study. Overexpression of miR-181a-5p was revealed to suppress the apoptosis of 786-O and ACHN cells, in addition to enhancing the proliferation, migration and invasion abilities of 786-O and ACHN cells *in vitro*, thus suggesting that miR-181a-5p may function as an oncogene in RCC. However, further studies are required to investigate the underlying mechanism of miR-181a-5p and its potential role as a biomarker for early detection and prognosis, in addition to as a therapeutic target in RCC.

Introduction

Renal cell carcinoma (RCC) is the 9th most common cancer diagnosis and cause of cancer-associated mortality,

and accounts for 3% of all malignancies worldwide (1,2). Considering that, at present, there are no available biomarkers for RCC screening, ~30% of patients develop metastasis at the time of diagnosis, and 20-40% patients develop recurrence or metastases following initial surgical resection (3-5). Furthermore, RCC is insensitive to conventional chemotherapy and radiotherapy (6). In total, approximately a third (~71,300) of patients diagnosed with RCC each year worldwide will ultimately succumb to metastatic disease (7). Thus, identification of novel biomarkers is important for the early diagnosis and treatment of RCC, and therapeutic strategies based on novel molecular targets are urgently required in RCC diagnosis and therapeutics.

Recently, microRNAs (miRNAs/miRs) have been revealed to have important roles in numerous carcinomas, including kidney cancer (8-12). miRNAs are short non-coding single stranded RNAs containing 20-22 nucleotides and are able to regulate gene expression at the post-transcriptional level (13). Increasing evidence has suggested that miRNAs function as oncogenes or tumor suppressors in RCC (3,14,15).

miR-181a-5p is located on chromosome 9, and its activity is dysregulated in numerous tumor types, including gastric cancer (16), pituitary adenoma (17) and hepatocellular carcinoma (18). Thus, it may be suggested that miR-181a-5p may function as either a tumor suppressor gene or an oncogene. However, the effect of miR-181a-5p on RCC remains largely undetermined. In the present study, the expression levels of miR-181a-5p in RCC tissues and cell lines were investigated, in addition to the effect of miR-181a-5p on cell function.

Materials and methods

Ethics statement. All human paired RCC samples and adjacent normal tissue samples were obtained at the Peking University Shenzhen Hospital (Shenzhen, China), between July 2015 and July 2016. All patients provided written informed consent, and the present study was approved by the Ethical Review Committee of Peking University Shenzhen Hospital (Shenzhen, China) and complied with the Declaration of Helsinki. Patient characteristics are presented in Table I.

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Key words: microRNA, microRNA-181a-5p, renal cell carcinoma, oncogene

Cell culture. 786-O, Caki-1 and ACHN are renal cell carcinoma cell lines. 293T is a normal renal cell line. Cell lines were purchased from the Guangdong and Shenzhen Key Laboratory of Male Reproductive Medicine and Genetics (Shenzhen, China). ACHN, Caki-1 and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.), 1% antibiotics (100 μ l/ml penicillin and 100 mg/ml streptomycin sulfate) and 1% glutamine, and maintained in a humidified atmosphere with 5% CO₂ at 37°C. 786-O cells were cultured in RPMI 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from excised tumor specimens using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and purified with the RNeasy Maxi kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's protocol. The concentration of RNA was determined using a NanoDrop 2000/2000c (Thermo Fisher Scientific, Inc.). Following this, RNA (1 μ g) was reverse-transcribed to cDNA using a miScript Reverse Transcription kit (Qiagen GmbH). Total RNA was converted into cDNA using the miScript II RT kit (Qiagen GmbH). The reaction was performed at 37°C for 1 h, followed by RT inactivation at 95°C. qPCR was subsequently performed to determine the expression level of miR-181a-5p using a miScript SYBR® Green PCR kit (Qiagen GmbH) on the Roche Lightcycler 480 Real Time PCR system (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's protocol. The miRNA expression was normalized to U6 expression. The thermocycling conditions of qPCR were as follows: Initial denaturation of 95°C for 2 min; followed by 40 cycles of denaturation at 95°C for 10 sec, annealing and elongation at 55°C for 30 sec, and final extension at 72°C for 30 sec. The sequences of the primers used were as follows: miR-181a-5p forward, 5'-AACAUUCAACGCUGUCGGUGAGU-3' and reverse, miScript universal primer (miScript SYBR-Green PCR kit; sequence unavailable); U6 forward, 5'-CTCGCTTCGGCAGCACCA-3' and reverse, 5'-ACGCTTCACGAATTTGCGT-3'. The expression levels of miR-181a-5p in tissues and cells were determined using the 2^{- $\Delta\Delta C_q$} method (19).

Cell transfection. 786-O and ACHN cells were seeded into a 6-well plate (3x10⁵ cells/well). Following culture for 24 h, cells were transfected with 5 ml miR-181a-5p mimics (forward, 5'-AACAUUCAACGCUGUCGGUGAGU-3' and reverse, 5'-UCACCGACAGCGUUGAAUGUUUU-3') or negative control (NC; forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3') purchased from Guangzhou RiboBio, Co., Ltd. (Guangzhou, China) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), which was mixed with Opti-MEM® I Reduced Serum Medium (Gibco; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. In order to confirm the efficiency of transfection, RT-qPCR was performed to determine the levels of miR-181a-5p expression 24 h post-transfection.

Table I. Clinicopathological characteristics of patients with renal cell carcinoma.

Characteristic	Number
Age range (years)	27-72
Gender (male/female)	16/5
Histological type (clear cell/papillary)	18/3
Fuhrman grade (I/II/III/IV)	13/6/1/1
AJCC clinical stage (I/II/III+IV)	13/7/1
AJCC, American Joint Committee on Cancer.	

Wound healing assay. 786-O and ACHN cells were seeded into a 6-well plate (3x10⁵ cells/well) and incubated in a humidified atmosphere with 5% CO₂ at 37°C for 24 h. Following this, cells were transfected with miR-181a-5p mimics or negative control (NC) using Lipofectamine® 2000. A wound was created in a monolayer of 786-O cells or ACHN cells using a sterile 200- μ l pipette tip 6 h post-transfection. PBS was used to wash away the cell debris. Images of cells were recorded at 0 and 12 h time intervals following the initial creation of the wound using a light microscope (magnification, x100; Olympus Corporation, Tokyo, Japan).

Transwell assay. The cellular migratory and invasive ability of 786O and ACHN cells was determined using a Transwell assay. Transwell chambers (BD Biosciences, Franklin Lakes, NJ, USA) with Matrigel were used to analyze the invasive capacity of cells, whereas Transwell chambers without Matrigel were used to analyze the migratory ability of cells. At 24 h post-transfection, ~2x10⁴ cells were added to the upper chamber with serum-free DMEM, and DMEM supplemented with 10% FBS was plated in the lower chamber. Following this, the chambers were incubated for 48 h in a 5% CO₂ incubator at 37°C. Cells adhering to the upper side of the inserts were gently scraped off, and invasive cells on the lower surface were stained with 0.1% crystal violet at room temperature for 25 min and counted using a light microscope at a magnification of x100 (Olympus Corporation).

TT assay. The cytoactivity of the 786O and ACHN cells was determined using a MTT assay (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Renal cancer cells (~5,000 cells) were inoculated in a 96-well plate (5x10³ cells/well) and transfected with miR-181a-5p mimics and NC using Lipofectamine® 2000. A total of 96 h post-transfection, 20 μ l MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA) was added to each well of the 96-well plate. After 4 h, a total of 100 μ l dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added to the 96-well plate, which was shaken on a reciprocating decolorization shaking table (TSB-108; Haimen LinBair Instruments Manufacturing Co., Ltd., Haimen, China) for 10 min in the dark at room temperature. Finally, the optical density (OD) values of each well were determined using an ELISA microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a wavelength of 595 nm (620 nm as the reference wavelength).

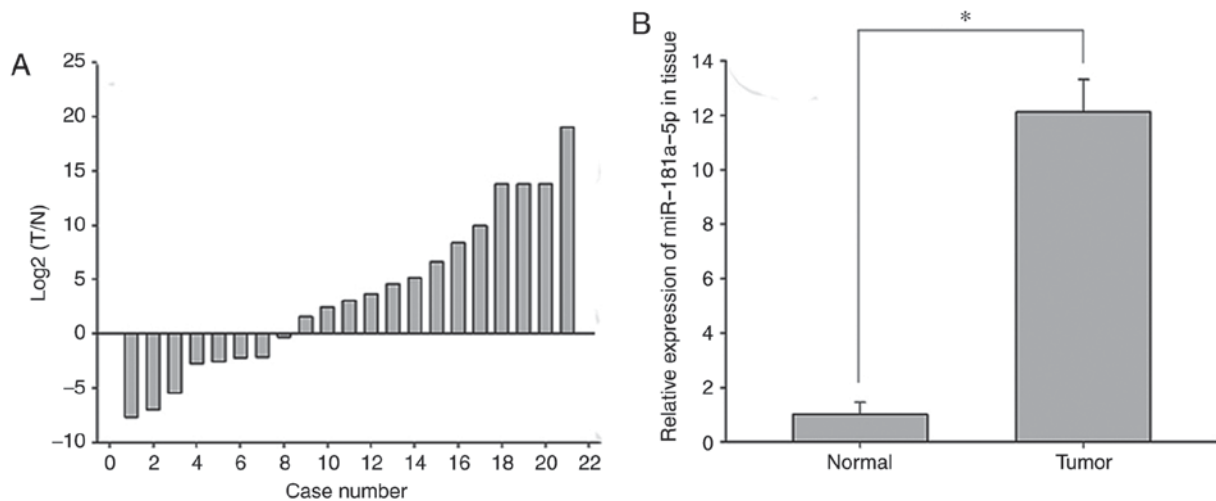


Figure 1. Expression levels of miR-181a-5p in 21 paired tissue samples. (A) Log2 ratios of miR-181a-5p expression in 21 paired tumor renal tissues and normal renal tissues. (B) The relative expression of miR-181a-5p in tumor renal tissues and normal renal tissues. * $P<0.05$. miR, microRNA; T, tumor renal tissues; N, normal renal tissues.

Cell Counting Kit-8 (CCK-8) assay. Cell proliferation was determined using a CCK-8 assay (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. A total of 5×10^3 cells/well were seeded in 96-well plates and incubated in a humidified atmosphere with 5% CO_2 at 37°C for 24 h until 30-50% confluence was reached. Cells were subsequently transfected with miR-181a-5p mimics, in addition to NC. The OD values of experimental wells were investigated at 450 nm at 0, 24, 48 and 72 h time intervals post-transfection using an ELISA microplate reader (Bio-Rad Laboratories, Inc.).

Flow cytometry assay. The apoptosis rate of 786-O and ACHN cells was determined via flow cytometry assays. Cells were added to a 6-well plate (3×10^5 cells/well) and subsequently transfected with miR-181a-5p mimics or NC. Cells were collected 48 h post-transfection and washed with cold PBS (4°C). Subsequently, the cells were resuspended in 100 μl 1X binding buffer, and 5 μl Annexin V-fluorescein isothiocyanate (Invitrogen; Thermo Fisher Scientific, Inc.) and 5 μl propidium iodide (Invitrogen; Thermo Fisher Scientific, Inc.) was added to each cell suspension. Cells were subsequently incubated at room temperature for 15 min in the dark, and 400 μl binding buffer was added to each tube. The apoptosis rate was determined using flow cytometry (EPICS, X1-4; Beckman Coulter, Inc., Brea, CA, USA) and was analyzed with FlowJo software (version 10; Flow Jo LLC, Ashland, OR, USA).

Statistical analysis. Data are presented as mean \pm standard error of the mean. All assays were repeated at least three times. Significance of differential expression was analyzed using one way analysis of variance followed by Tukey's post-hoc test. The SPSS 23.0 statistical software package (IBM Corp., Armonk, NY, USA) was used to perform statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-181a-5p is upregulated in RCC tissues and cell lines. To investigate the role of miR-181a-5p in RCC, RT-qPCR was

performed to determine the expression levels of miR-181a-5p in 21 paired RCC tissues and adjacent normal renal tissues (Fig. 1A). miR-181a-5p was revealed to be upregulated in RCC tissues compared with normal renal tissues ($P<0.05$; Fig. 1B). Furthermore, miR-181a-5p was demonstrated to be expressed in RCC cell lines and normal renal cells; however, it was revealed that the 786-O, Caki-1 and ACHN cell lines exhibited significantly increased expression levels of miR-181a-5p compared with the 293T normal kidney cell line ($P<0.05$; Fig. 2).

Cell transfection efficiency validation. RT-qPCR was performed to determine the transfection efficiency of miR-181a-5p mimics compared with the NC. As revealed in Fig. 3, the expression level of miR-181a-5p was significantly enhanced in cells transfected with miR-181a-5p mimics 24 h post-transfection compared with the NC (Fig. 3; $P<0.05$).

Effect of miR-181a-5p on RCC cell proliferation. The results of the MTT and CCK-8 assays revealed that the upregulation of miR-181a-5p enhanced cell proliferation. The proliferation of ACHN cells transfected with miR-181a-5p mimics was significantly enhanced at 24 (59.15%), 48 (41.73%) and 72 (50.81%) h time intervals post-transfection, respectively, compared with cells transfected with the NC (Fig. 4A; $P<0.05$, $P<0.001$). The proliferation of 786-O cells transfected with miR-181a-5p mimics significantly increased by 22.82% ($P<0.05$), 13.98% ($P<0.05$) and 43.33% ($P<0.01$) at 24, 48 and 72 h time intervals post-transfection, respectively, when compared with cells transfected with the NC (Fig. 4B). The results of the MTT assay demonstrated that the viability of ACHN and 786-O cells increased by 9.20% ($P<0.05$) and 30.3% ($P<0.05$), respectively following transfection with miR-181a-5p compared with cells transfected with the NC (Fig. 4C and D).

Effect of miR-181a-5p on RCC cell motility. A Transwell assay and wound scratch assay were performed to investigate the effect of miR-181a-5p on the motility of the 786-O and ACHN cell lines. As revealed in Fig. 5, the results of the wound

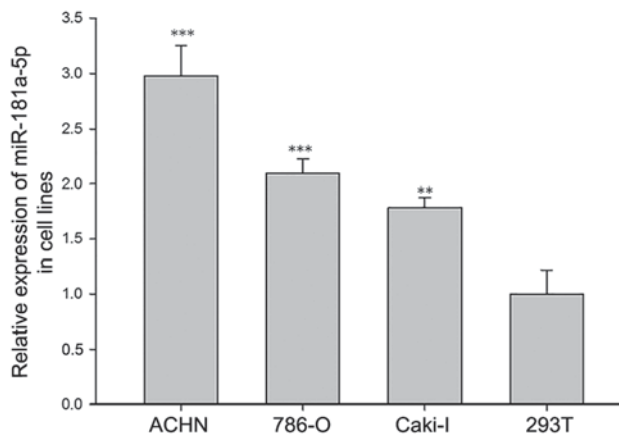


Figure 2. Relative expression levels of miR-181a-5p in renal cell carcinoma cell lines and normal renal cells. ** $P < 0.01$ and *** $P < 0.001$ vs. 293T cells. miR, microRNA.

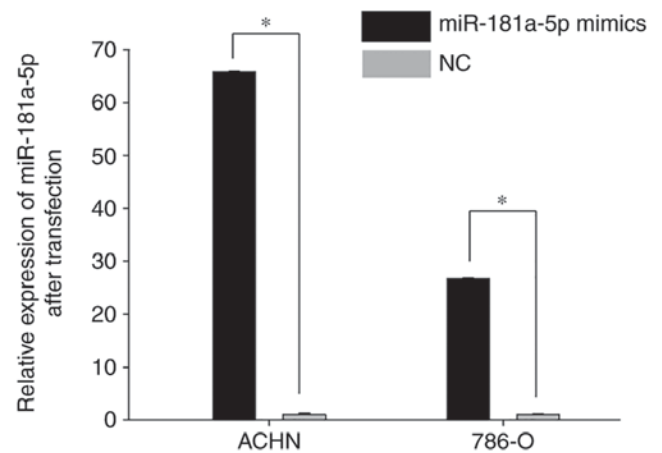


Figure 3. Relative expression levels of miR-181a-5p in ACHN and 786-O cells following transfection. * $P < 0.05$. miR, microRNA; NC, negative control.

scratch assay demonstrated that the distance migrated by 786-O cells transfected with miR-181a-5p mimics was significantly increased at the 12 h time interval post-transfection compared with cells transfected with the NC (69.90%; $P < 0.01$). Furthermore, the distance migrated by ACHN cells transfected with miR-181a-5p mimics was significantly increased at the 12 h time interval post-transfection compared with cells transfected with the NC (66.80%; $P < 0.01$). Furthermore, the results of the Transwell assay revealed that 786-O cells transfected with miR-181a-5p mimics exhibited a significantly increased migratory capacity compared with cells transfected with the NC (84.40%; Fig. 6; $P < 0.01$). In addition, ACHN cell migration was significantly increased in cells transfected with miR-181a-5p mimics compared with cells transfected with the NC (168.43%; Fig. 6; $P < 0.01$). Furthermore, the invasive ability of 786-O cells transfected with miR-181a-5p mimics was significantly increased compared with cells transfected with the NC (118.32%; $P < 0.01$; Fig. 6). In addition, the invasive ability of ACHN cells was significantly increased in cells transfected with miR-181a-5p mimics compared with cells transfected with the NC (127.78%; $P < 0.01$; Fig. 6). These results suggested that miR-181a-5p may enhance the motility of RCC cells.

Upregulation of miR-181a-5p reduces the apoptosis of ACHN and 786-O cells. The apoptosis rate was investigated by flow cytometry, and the results demonstrated that miR-181a-5p overexpression significantly suppressed cellular apoptosis in 786-O and ACHN cell lines (Fig. 7). The apoptosis rate of 786-O and ACHN cells transfected with miR-181a-5p significantly decreased by 49.96 and 27.82%, respectively, when compared with cells transfected with the NC (Fig. 7B; $P < 0.05$).

Discussion

miRNAs have important roles in numerous biological processes, including cell development, differentiation, metabolism, proliferation, the cell cycle and apoptosis (1). Aberrant miRNA expression has been associated with a number of chronic illnesses, including heart disease, diabetes and cancer (20). Deregulated expression of miRNAs is involved

in the initiation and progression of tumors, metastasis and therapeutic resistance (21).

Previous studies have demonstrated that miR-181a-5p serves as an oncogene or a tumor suppressor via one or more signaling pathways in certain types of tumors. In colorectal cancer, downregulation of miR-181a-5p has been demonstrated to enhance cell proliferation and chemoresistance by targeting protein Wnt/ β -catenin and transcription factor 4 (22). A previous study that investigated hepatocarcinogenesis revealed that the downregulation of miR-181a-5p activated hepatocyte growth factor receptor-mediated oncogenic signaling (18). Furthermore, miR-181a-5p has been demonstrated to be downregulated in aggressive human breast and colon cancer, which promotes cancer cell migration and angiogenesis (23). miR-181a-5p has been revealed to function as a tumor suppressor by targeting GTPase KRas in non-small cell lung cancer (24). Mi *et al* (25) demonstrated that miR-181a-5p may function as an onco-miRNA via activation of Ras association domain-containing protein 6-mediated mitogen-activated protein kinase signaling in gastric cancer. In a further study, the transcription factor SOX2/miR-181a-5p/tumor suppressor candidate 3 axis was revealed to have an important role in the proliferation, migration and invasiveness of breast cancer cells (26). Petrillo *et al* (27) demonstrated that concomitant expression of phosphorylated mothers against decapentaplegic homolog 2 and miR-181a-5p represents a biomarker for poor prognosis in patients with ovarian cancer. Furthermore, Boguslawska *et al* (28) revealed that serine/arginine-rich splicing factor 7 and miR-181a-5p form a regulatory feedback loop in renal cancer cells, which affects cell proliferation.

The results of the present study demonstrated that the expression of miR-181a-5p was upregulated in 786-O and ACHN cell lines compared with normal renal tissues. In addition, the results of the present study revealed that expression of miR-181a-5p promoted cell proliferation, invasion and migration, and suppressed cellular apoptosis.

In conclusion, the results of the present study demonstrated that miR-181a-5p was upregulated in RCC tissues and cell lines, and that miR-181a-5p was associated with cell migration, proliferation and apoptosis in RCC. The results of the present study additionally suggested that miR-181a-5p may function as

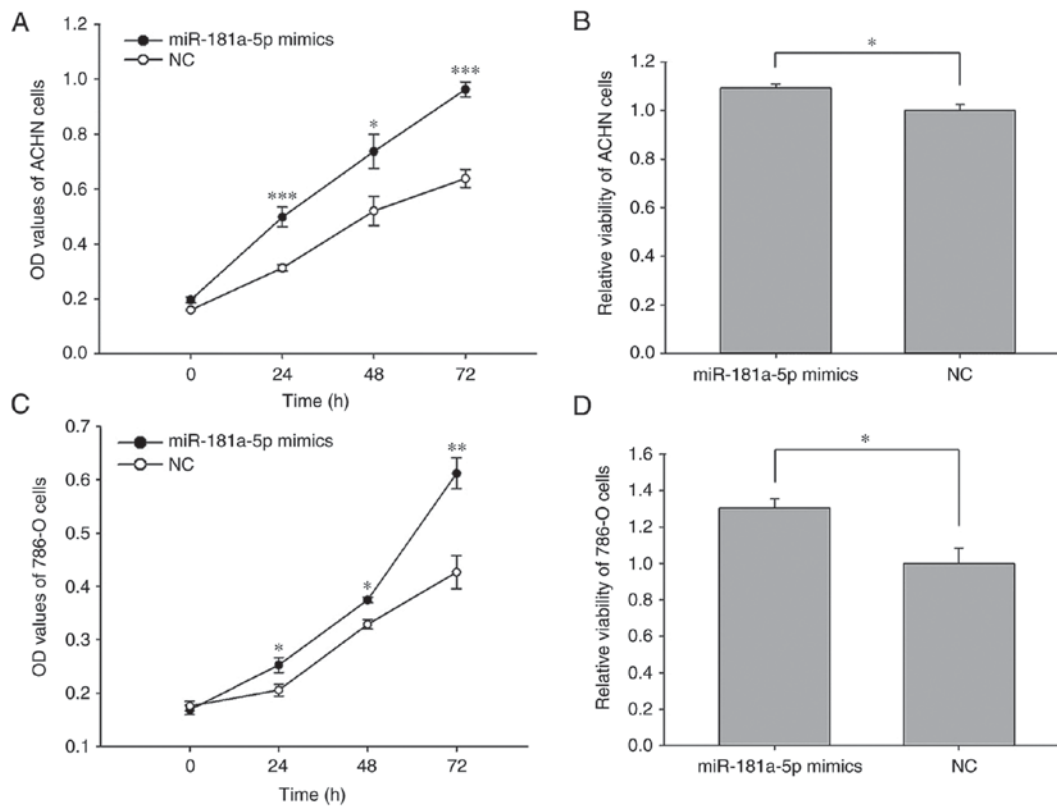


Figure 4. Effect of miR-181a-5p mimic transfection on cell proliferation and viability. (A) ACHN cell proliferation and (B) viability, as well as (C) 786-O cell proliferation and (D) viability was determined by MTT and cell counting kit-8 assays, respectively. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. NC. OD, optical density; miR, microRNA; NC, negative control.

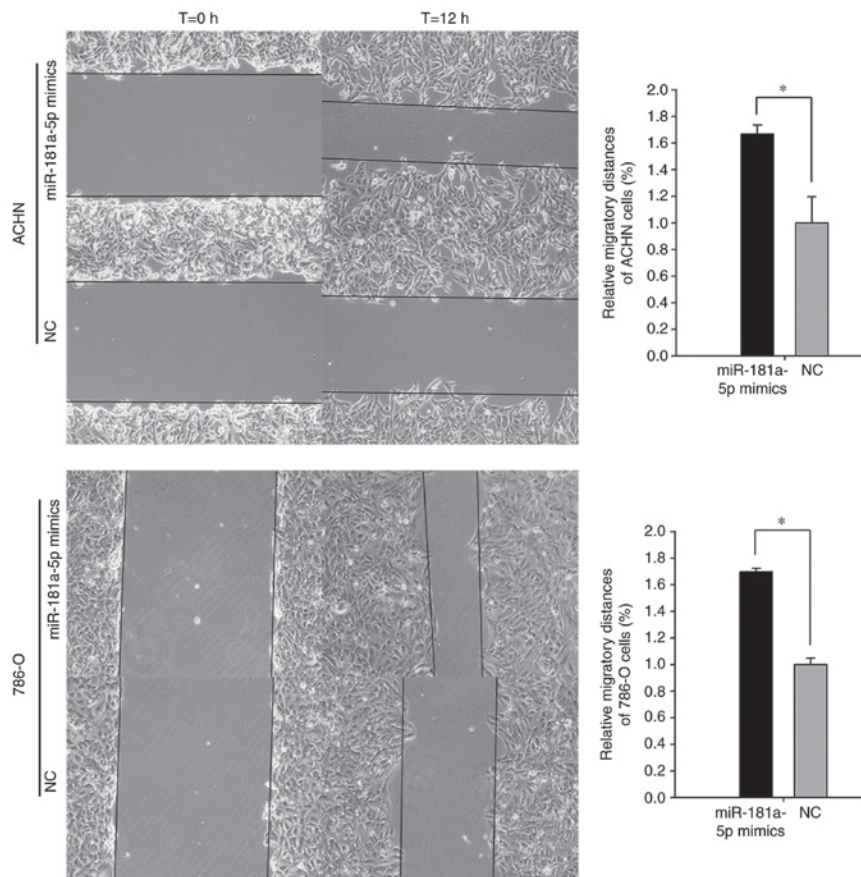


Figure 5. Wound scratch assay reveals the migratory ability of ACHN and 786-O cells following transfection with either miR-181a-5p mimics or NCs. Magnification, $\times 100$. * $P<0.05$. miR, microRNA; NC, negative control; T, time.

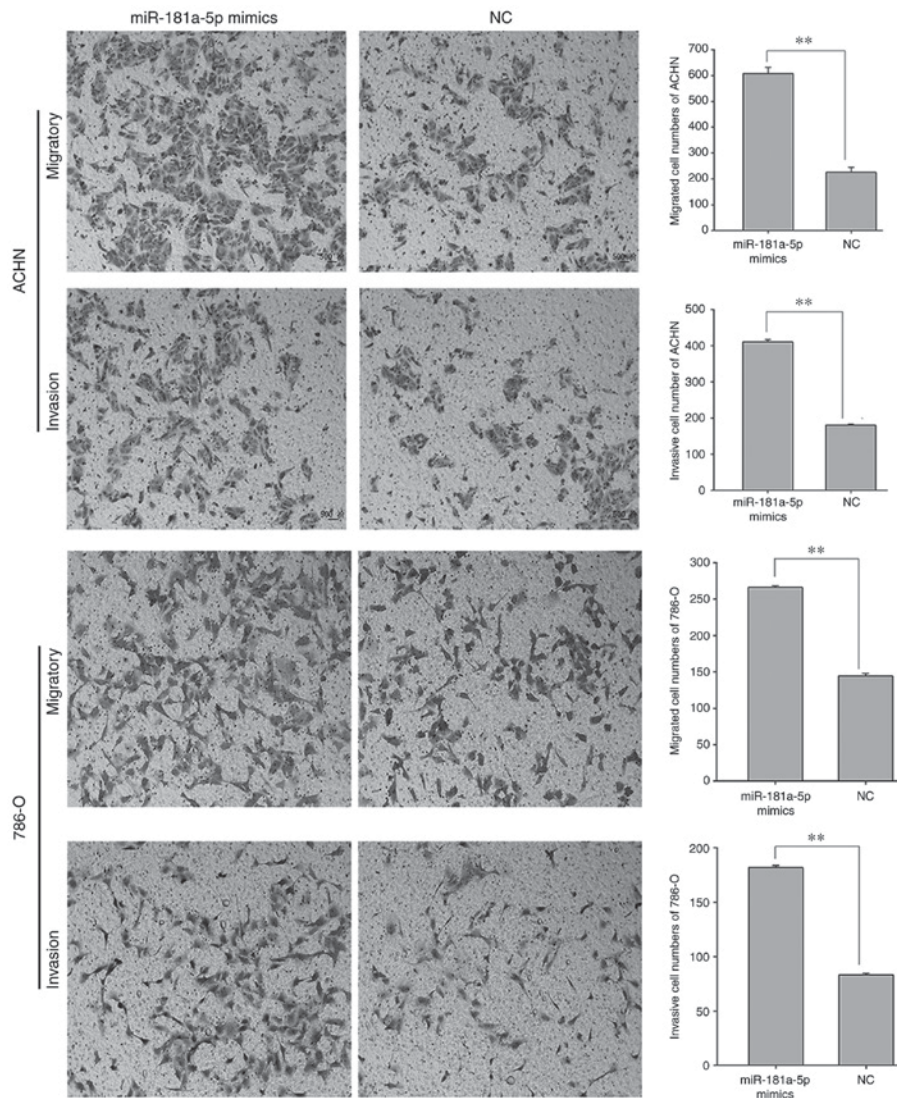


Figure 6. miR-181a-5p promotes the cell migration and invasion of ACHN and 786-O cells. Magnification, x100. ** $P < 0.01$. miR, microRNA; NC, negative control.

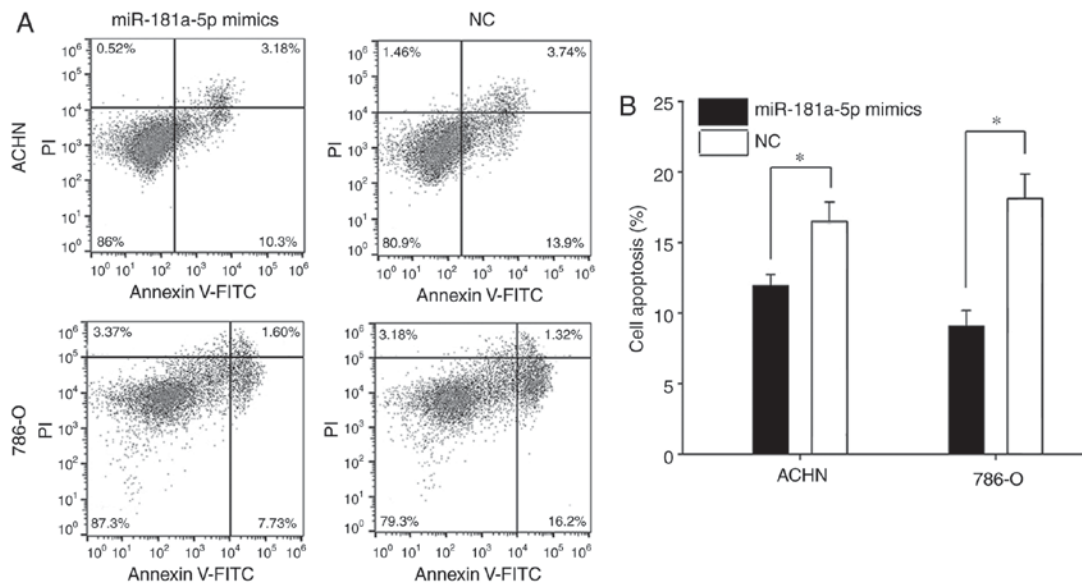


Figure 7. The apoptotic rate of ACHN and 786O cells transfected with miR-181a-5p mimics or NCs. (A) Flow cytometry plots of ACHN and 786-O cells transfected with either miR-181a-5p or NCs. (B) Quantitative analysis of the apoptosis rate of ACHN and 786-O cells transfected with miR-181a-5p mimics compared with cells transfected with NCs. * $P < 0.05$. NC, negative control; miR, microRNA; FITC, fluorescein isothiocyanate; PI, propidium iodide.

an onco-miRNA in RCC. However, Brodaczevska *et al* (29) revealed that RCC cell lines used in *in vitro* experiments may be unable represent the full pathological features of RCC, which remains a limitation that merits further study. In addition, 293T cells have been demonstrated to exhibit many features of neuronal cells (29); therefore, caution is required when interpreting the results (30,31). Furthermore, considering that renal cancer cells are adherent cells, a small number of surviving cells may have undergone mechanical death rather than apoptosis in the apoptosis assays. Therefore, these limitations require consideration in future studies aiming to investigate the effect of miR-181a-5p on RCC. Future studies investigating the effect of miR-181a-5p on RCC may focus on determining the underlying role of miR-181a-5p, including its potential application and use in early diagnosis and prognostic prediction in RCC. Further studies are required to determine the cellular mechanism of miR-181a-5p in RCC tumorigenesis, and its potential use in targeted therapy for RCC.

Acknowledgements

Not applicable.

Funding

This work was supported by the National Natural Science Foundation of China (grant no. 81101922), Science and Technology Development Fund Project of Shenzhen (grant nos. JCYJ20150403091443329 and JCYJ20170307111334308), the fund of 'San-ming' Project of Medicine in Shenzhen and the fund of Guangdong Key Medical Subject.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LN and YoL conceptualized and refined the study design. YuL, LZ and JH collected the literature data. JX, XG, JQ and PC performed the experiments. YuL evaluated and selected the data. YuL and LZ drafted the manuscript. YuL edited the manuscript. JX and XG designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients provided written informed consent, and the present study was approved by the Ethical Review Committee of Peking University Shenzhen Hospital (Shenzhen, China) and complied with the Declaration of Helsinki.

Consent for publication

All patients provided written informed consent.

Competing interests

The authors declare that they have no competing interests.

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