Tumor suppressor miR-149-5p is associated with cellular migration, proliferation and apoptosis in renal cell carcinoma

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Abstract. Several studies have recently explored the role of microRNAs (miRNAs, miRs) in the tumorigenesis of various types of cancer. miRNAs have been reported to be involved in numerous cell processes, including cell apoptosis, proliferation and migration, thus suggesting that miRNAs may have an important role in cancer progression. Downregulation of miR-149-5p has been detected in RCC tissues by microarray profiling; however, its expression and function in RCC has yet to be elucidated. In the present study, reverse transcription-quantitative polymerase chain reaction was performed to detect the expression levels of miR-149-5p in RCC tissues and paired normal tissues. In order to determine whether miR-149-5p was able to regulate cell proliferation, apoptosis or migration, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, flow cytometric and wound healing assays were conducted. The results demonstrated that miR-149-5p was significantly downregulated in RCC tissues compared with in normal tissues ($P<0.05$). The restoration of miR-149-5p expression using synthetic mimics suppressed cell proliferation and migration, and promoted cell apoptosis. These results indicated that miR-149-5p may act as a tumor suppressor in RCC. The present study is the first, to the best of our knowledge, to identify miR-149-5p as a tumor suppressor in RCC. Future studies will be focused on the potential role of miR-149-5p as a biomarker for the early detection and prognostic prediction of RCC, and as a therapeutic target in RCC. In addition, further exploration regarding the pathways underlying the effects of miR-149-5p in RCC is required.

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Introduction

MicroRNAs (miRNAs, miRs) are short non-coding, single-stranded RNAs ~22 nucleotides in length (1,2), which were initially identified as developmental mediators in *Caenorhabditis elegans* (3). Previous studies have reported that miRNAs have an important role in the regulation of gene expression (4-6), and may be dysregulated in numerous disorders, including metabolic diseases, infectious diseases and cancer (7,8). A substantial amount of evidence has suggested that miRNAs are aberrantly expressed in various types of human cancer, and previous studies have reported that miRNAs are implicated in tumor progression, including cell proliferation, migration, invasion and apoptosis (9-11). Dysregulation of miR-149-5p (also known as miR-149) has been reported to be associated with certain types of cancer, including nasopharyngeal carcinoma, colorectal cancer (CRC) and lung cancer (12-14). Furthermore, miR-149-5p has been revealed to be dysregulated in other diseases, including osteoarthritis and geriatric coronary artery disease (15,16).

Renal cell carcinoma (RCC) is the most common type of kidney cancer, which accounts for 3-5% of all malignant tumors (17,18). In addition, RCC has the highest rate of recurrence and mortality among urological malignances (7). Approximately 50% of patients with RCC suffer relapse and metastasis following curative surgical resection (17). In addition, RCC is highly resistant to chemotherapy and radiotherapy (19). The approval of several targeted therapies has led to an improvement in the survival rate of some patients; however, advanced and metastatic RCC remains difficult to treat (20). Therefore, it is important to determine the molecular mechanisms underlying RCC and to identify a novel biomarker of RCC.

miR-149-5p has been reported to be dysregulated in various types of cancer, including nasopharyngeal carcinoma, CRC and lung cancer (12-14). However, the expression and function of miR-149-5p in RCC remains to be elucidated. The present

study is the first to explore the possible role of miR-149-5p in the oncogenesis of RCC.

Materials and methods

Sample collection. A total of 32 paired RCC tissues and adjacent normal renal tissues were collected from Peking University Shenzhen Hospital (Shenzhen, China). Written informed consent was obtained from all patients. Collection and usage of the samples was reviewed and approved by the Ethics Committees of Peking University Shenzhen Hospital. Once the tissues were dissected they were immersed in RNAlater (Qiagen GmbH, Hilden, Germany) for 30 min, and were then stored at -80˚C until further use. The collected adjacent normal tissues were 2 cm away from visible RCC lesions. The collected tissues were reviewed and classified following hematoxylin and eosin staining. The clinical and pathological characteristics of the patients are presented in Table I. The ages of the patients varied between 25 and 70 years, and the mean age was 50 years old.

Table I. Clinicopathological features of patients with RCC.

Characteristic	Number of cases
Mean age, range (years)	50 (25-70)
Gender	
Male/female	20/12
Histological type	
Clear cell/papillary	28/4
pT-stage	
T1/T2/T3+T4	18/12/2
Fuhrman grade	
I/II/III/IV	9/16/5/2
AJCC clinical stages	
I/II/III+IV	18/12/2

pT, primary tumor; AJCC, American Joint Committee on Cancer.

RNA extraction, cDNA synthesis and quantitative polymerase chain reaction (qPCR). Total RNA was extracted from the samples using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and was purified with the RNeasy Maxi kit (Qiagen GmbH) according to the manufacturer's protocol. RNA concentration was measured using a NanoDrop 2000/2000c (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA), and RNA samples with 260/280 ratios of 1.8-2.0 were used for further experiments. Reverse transcription (RT)-qPCR was performed to detect the expression levels of miR-149-5p in the samples. To obtain cDNA 1 μg total RNA from each sample underwent RT using the miScript Reverse Transcription kit (Qiagen GmbH) according to the manufacturer's protocol. qPCR was performed, in order to quantify the expression levels of miR-149-5p, using 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. PCR reactions (20 μl) contained 1 μl cDNA template, 1 μl specific microRNA primer (Invitrogen; Thermo Fisher Scientific, Inc.), 2 μl 10X miScript Universal Primer, 10 μl 2X QuantiTect SYBR Green PCR Master Mix and RNase-free water. PCR was performed at 95˚C for 30 sec, 40 cycles of 95˚C for 5 sec, 60˚C for 30 sec and 72˚C for 30 sec, followed by incubation at 72˚C for 15 min. U6 was used as an internal control. The primer sequences were as follows: MiR-149-5p, forward 5'-TCTGGCTCCGTGTCT TCACTCCC-3', reverse primer was provided by the miScript SYBR® Green PCR kit; and U6, forward 5'-CTCGCTTCG GCAGCACA-3' and reverse 5'-ACGCTTCACGAATTT GCGT-3'. The expression levels of miR-149-5p were analyzed using the ΔΔCq method (21).

Cell culture and transfection. The 786-O and ACHN human RCC cell lines were used in the present study (American Type Culture Collection, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin and streptomycin, and 1% glutamine at 37˚C in a humidified incubator containing 5% CO_2. miR-149-5p expression was upregulated in these cells following transfection with synthesized miR-149-5p mimics (Shanghai GenePharma Co., Ltd., Shanghai, 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. Fluorescence microscopy (DMIRB; Leica Microsystems, Inc., Buffalo Grove, IL, USA). and RT-qPCR were performed to observe the transfection efficiency and the alterations to miR-149-5p expression. The sequence of the miR-149-5p mimics is 5'-UCUGGCUCCGUG UCUUCACUCCC-3'.

Wound healing cell migration assay. The wound healing assay was performed to assess the migratory ability of 786-O and ACHN cells *in vitro*. Cells (~$3x10^5$) were seeded into every well of a 12-well plate, and after 24 h the cells were transfected with 100 pmol miR-149-5p mimics or negative control mimics (5'-UUCUCCGAACGUGUCACGUTT-3'; Shanghai GenePharma Co., Ltd.) using Lipofectamine® 2000. A total of 6 h post-transfection, a vertical horizontal line was scratched into the cell layer using a sterile 200 μl pipette tip. To remove the floating cells, the plates were rinsed with phosphate-buffered saline and were then cultured at 37˚C in a humidified chamber containing 5% CO_2. A digital camera system (DP Controller and DP manager; Olympus Corporation, Tokyo, Japan). was used to capture images of the scratches at 0 and 24 h after generation of the scratch. The experiments were performed in triplicate and were repeated at least three times.

MTT cell proliferation assay. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to assess the proliferative ability of 786-O and ACHN cells *in vitro*. Cells (~$5x10^3$) were seeded into each well of a 96-well plate and were then transfected with 5 pmol miR-149-5p mimics or negative control mimics. A total of 20 μl MTT (5 mg/ml; Sigma-Aldrich, St Louis, MO, USA) was

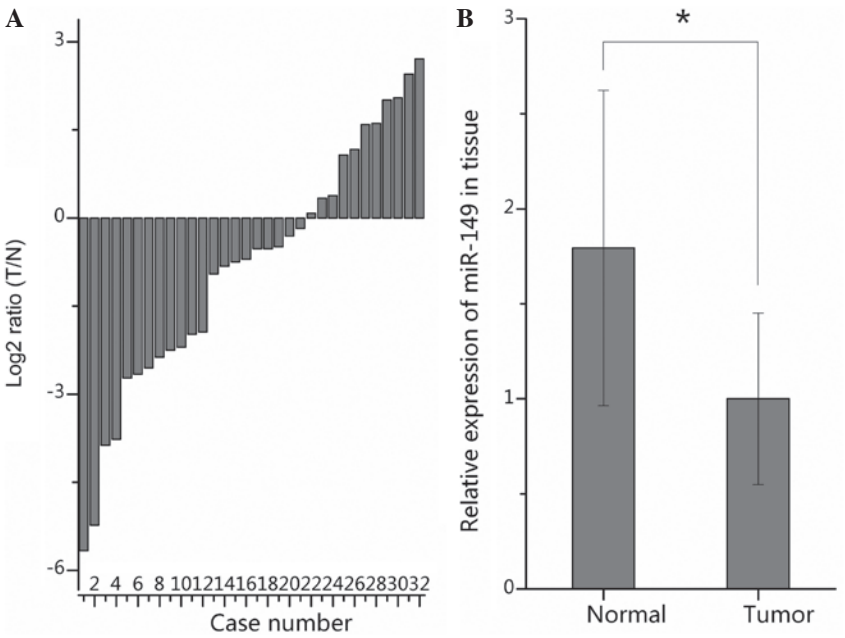

Figure 1. MicroRNA (miR)-149-5p is significantly downregulated in renal cell carcinoma (RCC) tissues compared with in normal tissues. (A) Ratios of miR-149-5p expression in 32 paired tissues [log_2Ratio (T/N)]. (B) Relative expression levels of miR-149-5p in RCC and normal tissues (*P<0.05). Data are presented as the mean ± standard error. T, tumor; N. normal.

added to each well at 0, 24, 48 and 72 h post-transfection, and the 96-well plate was incubated at 37˚C in a humidified incubator containing 5% CO_2 for 6 h. Subsequently, the medium was replaced with 150 μl dimethylsulfoxide [Sigma-Aldrich (Shanghai) Trading Co., Ltd., Shanghai, China]. The 96-well plate was then agitated for 30 min at room temperature and the optical density of each well was measured using an enzyme-linked immunosorbent assay microplate reader (model 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a wavelength of 490 nm.

Flow cytometric apoptosis assay. The apoptotic rates of 786-O and ACHN cells were measured *in vitro* by flow cytometry. Cells (~3x10^5) were seeded into each well of a 6-well plate and were then transfected with 200 pmol miR-149-5p mimics or negative control mimics. A total of 48 h post-transfection the cells were harvested and washed twice with cold PBS. Subsequently, the cells were resuspended in 100 μl 1X binding buffer, and 5 μl Annexin V-fluorescein isothiocyanate (FITC; Invitrogen; Thermo Fisher Scientific, Inc.) and 5 μl propidium iodide (PI; Invitrogen; Thermo Fisher Scientific, Inc.) were added to each cell suspension. After staining for 15 min at room temperature in the dark, 400 μl binding buffer was added to each tube. A Navios flow cytometer (Beckman, CA, USA) was used to analyze the rate of apoptosis.

Statistical analysis. Data are presented as the mean ± standard error. Paired t-test was used to compare the expression levels of miR-149-5p between the matched tumor and normal tissues. Student's t-test was used to analyze assays characterizing cell phenotypes. χ^2 test was performed to explore the correlations between pathological characteristics and the expression levels of miR-149-5p in tumor tissues. No correlations were demonstrated between pathological characteristics and the expression levels of miR-149-5p in tumor tissues (results not shown). All

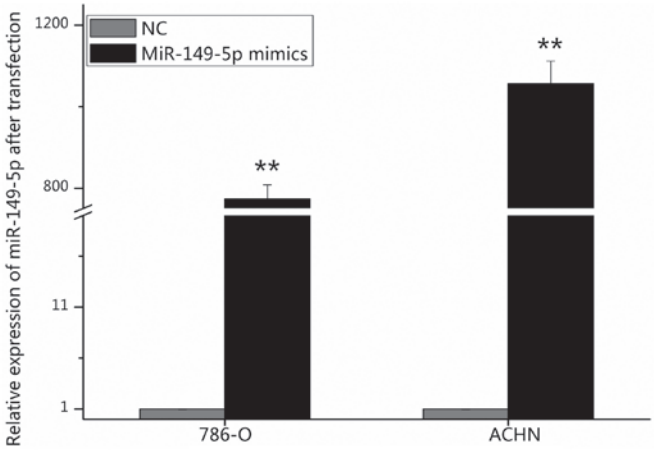

Figure 2. Relative expression levels of microRNA (miR)-149-5p post-transfection (**P<0.01). Data are presented as the mean ± standard error. NC, negative control.

statistical analyses were carried out using SPSS 19.0 statistical software package (IBM SPSS, Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-149-5p is downregulated in RCC tissues compared with in adjacent normal tissues. A total of 32 paired RCC and adjacent normal tissues were used to detect the expression levels of miR-149-5p by qPCR. The ratios of miR-149-5p expression in the 32 paired tissues [log_2Ratio (T/N)] are presented in Fig. 1A, which indicated that miR-149-5p was downregulated in 21 RCC tissues. The expression levels of miR-149-5p were significantly lower in RCC tissues compared with in paired normal tissues (P=0.034; Fig. 1B). These results suggest that

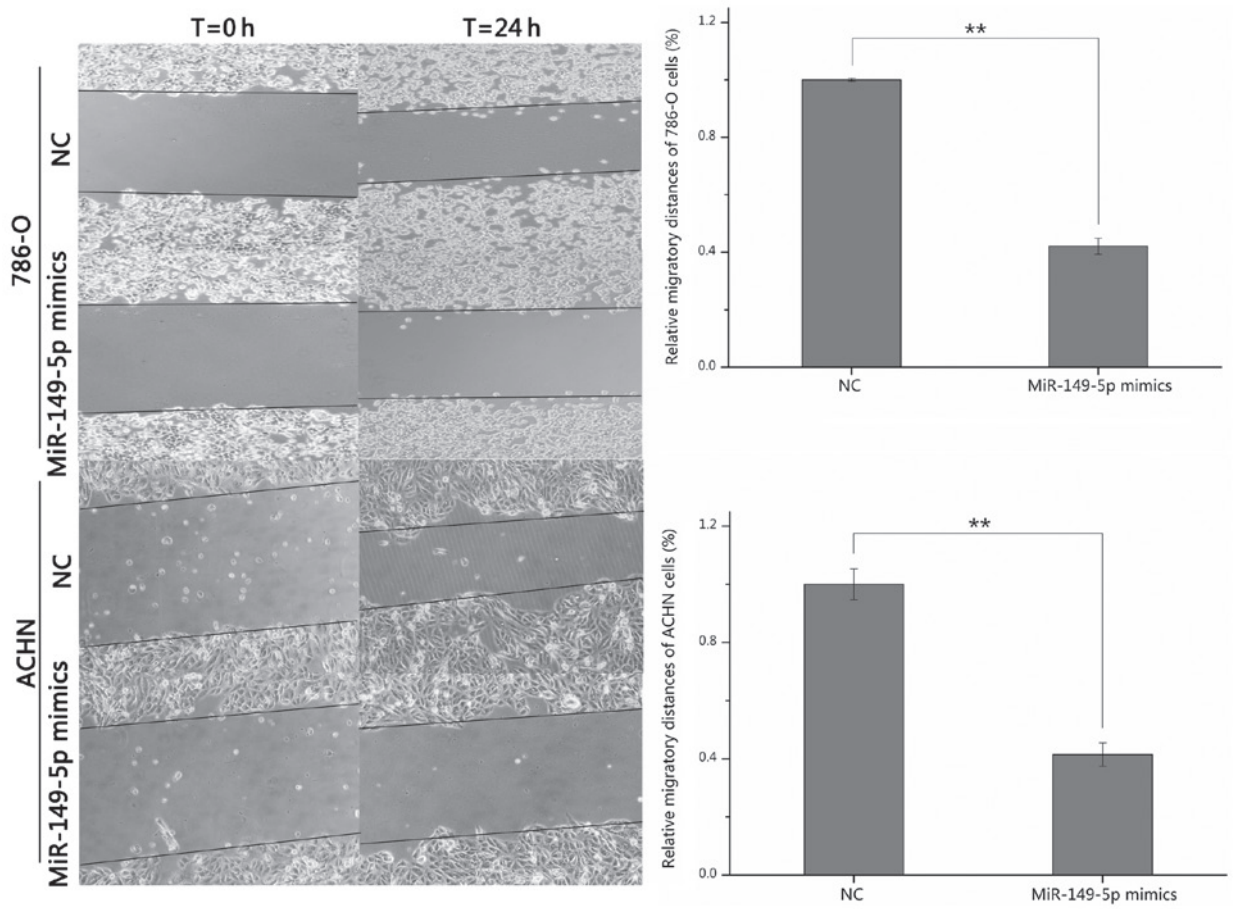

Figure 3. Images of the wounds at 0 and 24 h post-transfection (**P<0.01). Magnification, x100. Data are presented as the mean ± standard error. miR, microRNA; NC, negative control; T, time.

miR-149-5p may function as a tumor suppressor in RCC; however, the mechanism underlying the effects of miR-149-5p in RCC remains unclear.

Validation of cell transfection efficiency. qPCR was performed to determine the transfection efficiency of miR-149-5p mimics compared with negative control mimics. The expression levels of miR-149-5p were 773.74 times higher in 786-O cells (P=0.000324) and 1,056.45 times higher in ACHN cells (P=0.000605) transfected with miR-149-5p mimics, compared with in the negative control group (Fig. 2).

miR-149-5p mimics inhibit 786-O and ACHN cell migration. To explore the function of miR-149-5p in cell migration, a wound healing assay was conducted. Images of the wounds were captured at 0 and 24 h post-transfection using a digital camera system (Fig. 3). The wound widths of the cells transfected with miR-149-5p mimics were wider compared with the cells transfected with the negative control mimics. A total of 24 h post-transfection the distance of migration in the miR-149-5p mimics group was decreased by 57.88% in 786-O cells and 58.54% in ACHN cells compared with the negative control group (P<0.01). These results indicate that the upregulation of miR-149-5p may suppress RCC cell migration.

miR-149-5p mimics inhibit 786-O and ACHN cell proliferation. To determine whether miR-149-5p was able to affect RCC cell proliferation, an MTT assay was conducted. Post-transfection with miR-149-5p mimics the proliferation of 786-O cells was decreased by 7.43% (24 h; P=0.0258), 11.90% (48 h; P=0.00801) and 19.46% (72 h; P=0.00288) compared with the negative control group (Fig. 4A). In the ACHN cells, proliferation was decreased by 6.93% (24 h; P=0.0347), 17.29% (48 h; P=0.00178) and 21.20% (72 h; P=0.000278) (Fig. 4B). These results suggest that upregulation of miR-149-5p may inhibit RCC cell proliferation.

miR-149-5p mimics promote 786-O and ACHN cell apoptosis. To explore the role of miR-149-5p in RCC cell apoptosis, a flow cytometric analysis was conducted. The average early apoptotic rate of negative control-transfected 786-O cells was 1.89%, which increased to 17.15% in cells transfected with miR-149-5p mimics (P=0.00112; Fig. 5A). Furthermore, the average apoptotic rate of ACHN cells transfected with miR-149-5p mimics or negative control mimics was 21.30 and 3.21%, respectively (P=0.00106; Fig. 5B). These results indicate that miR-149-5p may promote the apoptosis of RCC cells.

Discussion

The mechanism underlying oncogenesis and cancer development remains unclear, and various oncogenes and tumor suppressor genes have a role in the process of oncogenesis. The most familiar genes associated with RCC tumorigenesis

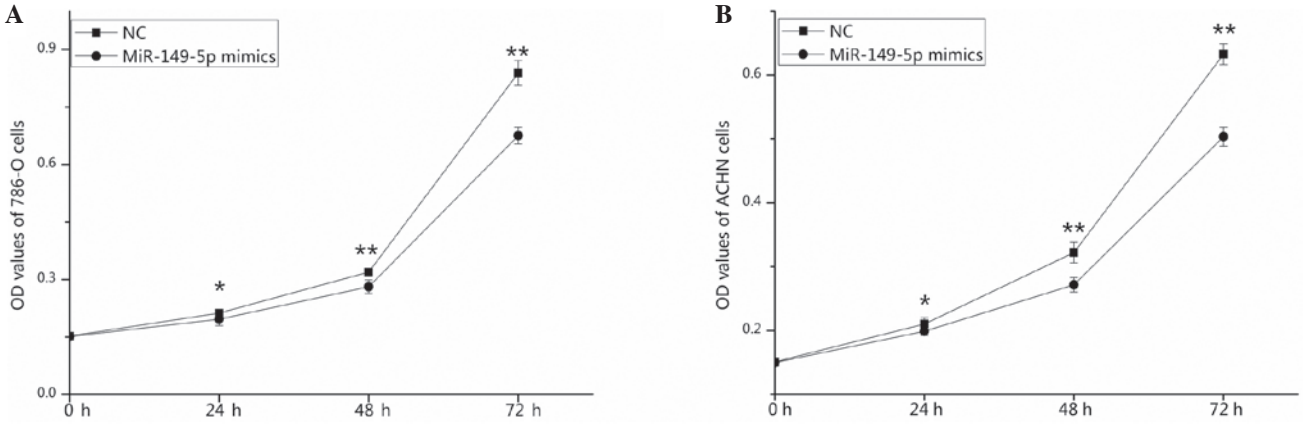

Figure 4. Results of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. (A) Optical density (OD) values of 786-O cells at 0, 24, 48 and 72 h post-transfection. (B) OD values of ACHN cells at 0, 24, 48 and 72 h post-transfection *P<0.05, **P<0.01 vs. NC. Data are presented as the mean ± standard error. miR, microRNA; NC, negative control.

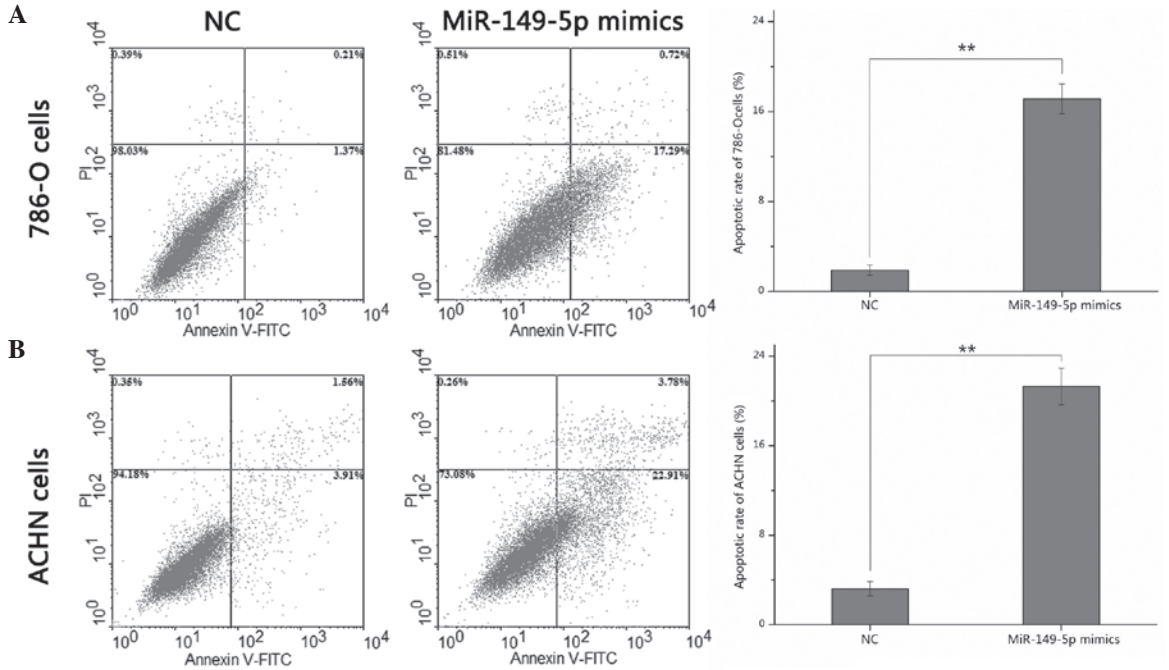

Figure 5. Early apoptotic rate of cells post-transfection. Apoptotic rate of (A) 786-O and (B) ACHN cells transfected with negative control (NC) or microRNA (miR)-149-5p mimics. (**P<0.01). Data are presented as the mean ± standard error. FITC, fluorescein isothiocyanate; PI, propidium iodide.

are von Hippel-Lindau (VHL), hypoxia-inducible factor (HIF), vascular endothelial growth factor (VEGF) and the mammalian target of rapamycin (mTOR) pathway. At present, various miRNAs have been revealed to be associated with the regulation of gene expression, and it is estimated that ~50% of human genes are the target of miRNAs (7,22). Notably, VHL, HIF, VEGF and mTOR are regulated by miRNAs, thus suggesting that miRNAs may have a crucial role in RCC tumorigenesis and development (23,24).

miR-149-5p has been reported to be dysregulated in various types of cancer, including non-small-cell lung cancer (NSCLC) (25), CRC (26) and astrocytoma (27). However, the expression levels of miR-149-5p in RCC have not yet been validated by qPCR. A recent microarray study between RCC and normal tissues indicated that miR-149 was downregulated, and demonstrated that the possible mRNA targets of miR-149 in clear cell RCC were lysyl oxidase (LOX), voltage-gated potassium channel subunit beta-1 and calcium-activated potassium channel subunit alpha-1 (28). Therefore, in the present study, qPCR was performed to quantify the expression levels of miR-149-5p in RCC tissues and paired adjacent normal tissues. In addition, the present study aimed to determine whether miR-149-5p could affect RCC cell proliferation, migration or apoptosis, as determined by MTT, wound healing and flow cytometric assays, respectively. The results revealed that miR-149-5p was downregulated in RCC tissues compared with in adjacent normal tissues. Furthermore, overexpression of miR-149-5p with miR-149-5p mimics significantly inhibited RCC cell (786-O and ACHN cells) proliferation and migration, and promoted RCC cell apoptosis. These results suggested that miR-149-5p may have a role as a tumor suppressor in RCC.

The mechanisms underlying how miR-149-5p affects cell progression remain unclear, and have been described differently in various types of cancer. For example, miR-149 was revealed to inhibit NSCLC cells epithelial-mesenchymal transition by targeting forkhead box M1 (25). In glioblastoma cells, miR-149 was able to increase chemosensitivity to temozolomide (27). Furthermore, the expression levels of miR-149 were gradually decreased in astrocytomas of different World Health Organization grades (27), thus indicating that miR-149 may be a biomarker of astrocytoma. In CRC, overexpression of miR-149 inhibited CRC cell growth and invasion by targeting specificity protein 1 (26). In addition, lower miR-149 expression was associated with a greater depth of invasion and reduced 5-year survival rate in CRC (26). These data revealed that miR-149 may have an important role in the tumorigenesis of CRC and may be used as a biomarker to predict prognosis. He *et al* (29) reported that miR-149 was downregulated in adriamycin-resistant human breast cancer cells, and was involved in chemoresistance by targeting GlcNAc N-deacetylase/N-sulfotransferase-1. In gastric cancer, miR-149 was reported to inhibit proliferation and induce cell cycle arrest by targeting zinc finger and BTB domain containing 2 (30). These studies suggested that miR-149 is associated with the tumorigenesis or chemoresistance of cancer; however, the role of miR-149 in RCC remains unclear. Therefore, the present study performed MTT, wound healing and apoptosis assays, in order to explore the function of miR-149-5p in RCC cells. The results suggested that miR-149-5p functions as a tumor suppressor in RCC.

The association between miR-149 rs2292832 polymorphism and cancer risk has been explored in previous studies. Genetic polymorphisms within a miRNA sequence are thought to have a functional impact on how miRNAs function in tumors (31). Huang *et al* (32) reported that the CC genotype of miR-149 contributes to the progression and development, rather than the initiation, of nasopharyngeal carcinoma. Furthermore, the miR-149 rs2292832 polymorphism may be involved in the susceptibility and local progression of papillary thyroid cancer in Chinese patients (33). However, studies failed to describe the predictive ability of the polymorphism when lung cancers or breast cancers were examined (34,35). As well as in cancer, miR-149 rs71428439 polymorphism may affect miR-149 maturation, and this polymorphism may contribute to the risk of myocardial infarction via the miR-149-Puma axis (36). Nevertheless, the associations between miR-149 polymorphism and RCC risk have yet to be elucidated, and the mechanisms by which miR-149 regulates RCC biological processes remain ill-defined. Therefore, knowledge regarding the mechanisms underlying RCC tumorigenesis and development may improve by researching the pathways or target genes of miR-149 in RCC.

In conclusion, the present study detected the expression levels of miR-149-5p in RCC and paired adjacent normal tissues. miR-149-5p was downregulated in RCC, and cellular processes were altered following the upregulation of miR-149-5p, thus suggesting that miR-149-5p acts as a tumor suppressor in RCC. Further studies will be focused on the potential role of miR-149-5p as a biomarker for the early detection and prognostic prediction of RCC, and as a therapeutic target in RCC. In addition, further exploration regarding the pathways underlying the effects of miR-149-5p in RCC is required.

Acknowledgements

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