# Tumor suppressive miR-196a is associated with cellular migration, proliferation and apoptosis in renal cell carcinoma

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Abstract. Certain microRNAs (miRs) are implicated in the genesis and progression of various cancers by regulating multiple cellular processes, including apoptosis, proliferation and migration. The aim of the present study was to explore the functions of miR-196a in renal cell carcinoma (RCC). RCC and paired normal tissues we assessed for miR-196a expression by reverse-transcription quantitative PCR. Furthermore, the effects of miR-196a on renal cell proliferation, apoptosis and migration were determined using an MTT assay, flow cytometry and a scratch wound assay following restoration of miR-196a with synthetic mimics. miR-196a was found to be significantly downregulated in RCC tissues compared with that in normal tissues (P<0.05). In addition, miR-196a suppressed cell proliferation, apoptosis and migration of the 786-O and ACHN RCC cell lines. To the best of our knowledge, the present study was the first to report this tumor suppressor role of miR-196a in RCC. The results indicated that miR-196a may be a potential diagnostic biomarker for RCC and that transfection of miR-196a mimics may represent a novel treatment strategy for RCC.

## Introduction

Renal cell carcinoma (RCC) is the third most common urological cancer type after prostatic and bladder cancer, accounting for  $\sim 3\%$  of all malignant tumors in adults and almost 90% of all renal tumors (1,2). The incidence of RCC

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shows a great variation among different counties and the male-to-female ratio is >2:1 (3). Cancer statistics estimated for 2013 in the USA showed that due to its incidence of >65,150 new cases and >13,680 mortalities, RCC is among the 10 leading cancer types (4). Annual estimates of newly diagnosed cases of RCC have been gradually increasing over recent years and the most prevalent histological sub-type of RCC is clear-cell RCC with a prevalence of 85% (5). As RCC patients tend to show no symptoms at the early stage, distant metastasis is present in >30% of cases at the time of diagnosis (6,7), for which only few and ineffective treatment options are available (8). Therefore, it is urgently required to identify novel biomarkers to facilitate the diagnosis of RCC, as well as novel treatment strategies.

MicroRNAs (miRNAs/miRs) are a class of single non-coding RNAs of ~22 nucleotides in length (9). Previous studies have shown that miRNAs regulate various cellular processes, including differentiation, migration, proliferation, apoptosis and metabolism (10,11). miRNAs are epigenetic regulators which bind to the 3'-untranslated regions of their target mRNAs and degrade them or repress their translation (9,12). In various cancer types, certain miRNAs are aberrantly expressed and act as oncogenes or tumor suppressors (13-15). Due to the imperfect complementarity between miRNAs and their target mRNAs, each mRNA may be regulated by various miRNAs and each miRNA may target various mRNAs (16,17). However, the specific roles of certain miRNAs in cancer have remained elusive. miRNAs have the potential to be used as diagnostic and prognostic biomarkers, for clinical monitoring purposes and as treatment targets for cancer.

Among these miRNAs, miR-196a has been reported to be aberrantly expressed in various tumor types, including osteosarcomas (18), pancreatic cancer (19) and gastric cancer (20), while its function has remained elusive in RCC. However, certain miRNA profiling studies have indicated that miR-196a is downregulated in RCC (21,22). The purpose of the present study was therefore to assess the expression of miR-196a in RCC and normal tissues and to explore the effects of miR-196a on RCC cell proliferation, migration and apoptosis.

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### Materials and methods

Sample collection. A total of 48 paired RCC tissues and adjacent normal kidney tissues were collected from hospitals in Guangdong and Anhui province (China). Adjacent normal tissues were extracted at a 2-cm distance from visible RCC lesions. Written informed consent was obtained from all patients from Peking University Shenzhen Hospital (Shenzhen, China) between January 2012 and December 2014. Protocols for the collection and use of the samples were reviewed and approved by the ethics committee of Peking University Shenzhen Hospital (Shenzhen, China). The tissues were dissected while being immersed in RNAlater (Qiagen, Hilden, Germany) over 30 min and 1 g of tissue was stored at -80°C for further use. The tissues collected were reviewed and classified following hematoxylin and eosin staining by three independent examiners using a previously described method (23). The clinical and pathological characteristics of the tissue donors are presented in Table I.

RNA extraction and reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from RCC tissues and normal adjacent tissues using TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and purified with the RNeasy Maxi kit (Qiagen) following the manufacturer's instructions. The RNA concentration was measured using a NanoDrop 2000/2000c (Thermo Fisher Scientific, Inc.) and the RNA samples with optical density (OD) ratios at 260/280 nm of 1.8-2.0 were used for further experiments. For cDNA synthesis, 1  $\mu$ g total RNA of each sample subjected to reverse transcription with the miScript Reverse Transcription kit (Qiagen) following the manufacturer's instructions. The temperature protocol for the RT reaction was 37°C for 60 min, 95°C for 5 min and storage at 4°C. Amplification of cDNA for the quantification of miR-196a was performed using the miScript SYBR®Green PCR kit (Qiagen) on a Roche Lightcycler 480 Real-Time PCR System according to the manufacturer's instructions. U6 was used as an internal control. PCR thermocycling conditions were set as follows: 95°C for 1 min, then 40 cycles of 95°C for 15 sec, 55°C for 30 sec and 72°C for 30 sec. The following primers (Invitrogen; Thermo Fisher Scientific, Inc.) were used: miR-196a forward, 5'-TAGGTAGTTTCATGTTGT TGGG-3' and reverse as provided by the miScript SYBR® Green PCR kit; U6 forward, 5'-CTCGCTTCGGCA GCACA-3' and reverse, 5'-ACGCTTCACGAATTTGCGT-3'. The expression of miR-196a was analyzed using the  $2^{-\Delta\Delta Cq}$ method (24).

Cell culture and transfection. The 786-O and ACHN human renal carcinoma cell lines (American Type Culture Collection, Manassas, VA, USA) and 293T human embryo kidney cell line (Type Culture Collection of the Chinese Academy of Medical Sciences, Beijing, China) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum, 1% antibiotics (100  $\mu$ l/ml penicillin and 100 mg/ml streptomycin sulfates) and 1% glutamine (all from Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified incubator containing 5% CO<sub>2</sub>. Cells were transfected with miR-196a

Table I. Clinicopathological features of renal cell carcinoma patients (mean age, 52 years; age range, 27-72 years).

Characteristic	Number of patients
Males	30
Females	18
Histological type	
Clear cell	39
Papillary	9
pT-stage	
T1	27
Τ2	19
T3 + T4	2
Fuhrmann grade	
Ι	15
II	22
III	8
IV	3
AJCC clinical stage	
Ι	27
II	18
III + IV	3

pT, primary tumor; AJCC, American joint committee on cancer.

mimics, synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) (5'-UAGGUAGUUUCAUGUUGUUGG GCAACAACAUGAAACUACCUAUU-3') or negative control (NC) miRNA (5'-CAGUACUUUUGUGUAGUA CAA-3') using Lipofectamine 2000 (Invitrogen) in Opti-MEM<sup>®</sup> I Reduced Serum Medium (Gibco; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. To determine the transfection efficiency and miR-196a expression, fluorescence microscopy and RT-qPCR were performed.

Wound healing assay for cell migration. The migratory capacity of 786-O and ACHN cells was assessed in vitro by performing a wound healing assay. At 24 h after seeding 3x10<sup>5</sup> cells into each well of a 12-well plate, the cells were transfected with 100 pmol of miR-196a mimics or negative control using Lipofectamine®2000. Following 6 h of transfection, the cell monolayer was scratched with a sterile 200-µl pipette tip to generate a line-shaped wound. Floating cells were removed by rinsing with phosphate-buffered saline (PBS) and the cells were further cultured. A digital camera system (Olympus Corporation, Tokyo, Japan) on a Leica DMIRB inverted fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) was used to acquire the images of the scratches at 0, 12 and 24 h. The experiments were performed in triplicate and repeated at least three times.

Cell proliferation assay. The proliferation of 786-O and ACHN cells was assessed in vitro by performing



Figure 1. Expression levels of miR-196a in 48 paired tissues. (A)  $Log_2$  ratios of miR-196a expression in 48 paired RCC tissues vs. normal kidney tissues. (B) Relative expression of miR-196a in RCC and normal tissues. Data are expressed as the mean  $\pm$  standard error. \*\*P<0.01. miR, microRNA; RCC, renal cell carcinoma; T, tumor tissues; N, normal tissues.

a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded into a 96-well plate at 5,000 cells/well and transfected with 5 pmol miR-196a mimics or negative control. At 0, 24, 48 and 72 h post-transfection, 20  $\mu$ l MTT (5 mg/ml; Sigma-Aldrich, St Louis, MO, USA) was added to the wells. followed by incubation for 4 h. The medium was replaced with 150  $\mu$ l dimethylsulfoxide (Sigma-Aldrich), followed by agitation of the plates for 30 min at room temperature. An ELISA microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was then used to measure the OD of each well at a wavelength of 490 nm.

Flow cytometric assay for apoptosis. The apoptotic rates of 786-O and ACHN cells were measured *in vitro* by flow cytometry. In each well of a six-well plate,  $3x10^5$  786-O or ACHN cells were seeded and subsequently transfected with 200 pmol miR-196a mimics or negative control. At 48 h post-transfection, all cells were harvested, washed with cold PBS twice and re-suspended in 100  $\mu$ l 1X binding buffer. To each cell suspension, 5  $\mu$ l Annexin V-fluorescein isothiocyanate (Invitrogen) and 5  $\mu$ l propidium iodide (Invitrogen) were added, followed by incubation at room temperature in the dark for 15 min. Following addition of 400  $\mu$ l binding buffer to each tube, flow cytometry (EPICS XI-4, Beckman Coulter, Brea, CA, USA) was used to assess the apoptotic rate.

Statistical analysis. Paired Student's t-test was used to compare the expression levels of miR-196a in matched tumor/normal tissues. Student's t-test was used to analyze assays for characterizing phenotypes of cells. The  $\chi^2$  test was used to explore the correlations between the pathological characteristics and the expression levels of miR-196a in tumor tissues. All data are expressed as the mean ± standard error. All statistical analyses were preformed using the SPSS 19.0 statistical software package



Figure 2. Relative expression of miR-196a after transfection. Data are expressed as the mean  $\pm$  standard error. \*\*P<0.01. miR, microRNA; NC, negative control.

(IBM SPSS, Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

#### Results

miR-196a is downregulated in RCC tissues compared with normal adjacent tissues. To explore the expression of miR-196a in 48 paired RCC and normal adjacent tissues, RT-qPCR was performed. As shown in Fig. 1A, miR-196a was downregulated in the RCC tissues of 35 out of 48 patients. Furthermore, the mean expression levels of miR-196a in RCC tissues were significantly lower than those in the paired normal tissues (P<0.01) (Fig. 1B). However,  $\chi^2$  analysis revealed that no correlation was present between the pathological characteristics of the patients and the expression levels of miR-196a in tumor tissues (results not shown).



Figure 3. A scratch wound assay was performed to examine the migratory capacity of 786-O and ACHN cells after transfection with miR-196a mimics or NC. Data are expressed as the mean  $\pm$  standard error. \*P<0.05, \*\*P<0.01. NC, negative control; miR, microRNA.



Figure 4. Proliferation of (A) 786-O and (B) ACHN cells after transfection with miR-196a mimics or NC as determined by an MTT assay. Data are expressed as the mean ± standard error. \*P<0.05, \*\*P<0.01 for NC vs. miR-196a mimics. NC, negative control; miR, microRNA; OD, optical density.

*Validation of cell transfection efficiency*. The transfection efficiency of miR-196a mimics was determined by RT-qPCR, revealing that following transfection with miR-196a mimics, miR-196a levels were increased by 100.08% in 786-O and 287.02% in ACHN cells compared with those in NC-transfected cells (Fig. 2).

miR-196a inhibits RCC cell migration. The effects of miR-196a on RCC cell migration were explored using a wound healing assay. The results showed that the migration of cells into the wounded area was reduced in the group transfected with miR-196a mimics compared to that in the NC group (Fig. 3). Compared with the NC group, the width of the wound in the miR-196a mimics group was decreased by 35.70% (P=0.01) and 47.97% (P<0.01) for 786-O cells and by 57.42% (P<0.01) and 44.27% (P<0.01) for ACHN cells at 12 and 24 h of incubation, respectively. These results indicated that miR-196a mimics had an inhibitory effect on RCC cell migration.

*miR-196a mimics suppress RCC cell proliferation*. The effects of miR-196a on RCC cell proliferation were assessed using an MTT assay. Compared with the NC control group, the cell proliferation of 786-O cells was decreased by 9.91% (P<0.05), 12.08% (P<0.05) and 18.08% (P<0.01) and that of ACHN cells was decreased by 16.41, 28.93 and 38.37% (P<0.01 for all) at 24, 48 and 72 h after transfection with miR-196a mimics, respectively (Fig. 4). These results revealed that miR-196a mimics inhibited RCC cell proliferation.

*miR-196a mimics induce apoptosis in RCC cells.* Flow cytometric analysis was used to determine the effects of miR-196a on the apoptotic rate of RCC cells at 48 h post-transfection. The results indicated that the average early apoptotic rate of 786-O cells was 2.95% in the NC group, which was increased to 10.19% in the miR-196a mimics group (P<0.01). Furthermore, the average early apoptotic rate of ACHN cells was 1.74% in the NC group, while it was increased to 13.69%



Figure 5. Apoptosis of 786-O and ACHN cells after transfection with miR-196a mimics or NC was assessed by Annexin V/PI double staining and flow-cytometric analysis. Data are expressed as the mean ± standard error. \*\*P<0.01. NC, negative control; miR, microRNA; PI, propidium iodide; FITC, fluorescein isothiocyanate.

in the miR-196a mimics group (P<0.01) (Fig. 5). These results indicated that miR-196a mimics induced apoptosis in RCC cells. The late apoptotic rate of 786-O and ACHN cells transfected with miR-196a mimics was significantly higher than cells transfected with NC (P<0.01), while the late apoptotic rate of 786-O and ACHN was low in comparison; therefore, mechanical error should not be excluded. Nevertheless, the main effect of miR-196a on RCC cells was manifested in early apoptosis.

# Discussion

Tumorigenesis is associated with the activation of cancer-promoting genes and the inactivation of a number of tumor suppressor genes. >50% of genes encoding for miRNAs are located at fragile genomic sites and genomic regions associated with multiple cancer types, which indicates the relevance as well as complex roles of miRNAs in cancer. An increasing number of studies have shown that miRNAs have dual roles as oncogenes or tumor suppressor genes in different types and stages of tumor (10,25,26). For instance, miR-31 acts as an oncogene, as it has been implicated in the development and drug resistance of tumors, and is a biomarker associated with poor prognosis (27).

miR-196a was recently reported as an oncogene in various tumor types (28-30). However, previous miRNA profiling studies have indicated that miR-196a is downregulated in RCC (21,22), therefore indicating its tumor suppressor role in this type of cancer. The present study therefore aimed to clarify the roles of miR-196a in RCC. RT-qPCR was performed to quantify the relative expression of miR-196a in 48 paired RCC and adjacent normal tissues. Furthermore, the effects of miR-196a mimics on RCC cell lines were assessed *in vitro*. Wound healing, MTT and flow cytometric assays were performed to assess the effects of miR-196a on cellular migration, proliferation and apoptosis. The results confirmed that miR-196a was downregulated in RCC tissues compared with that in paired normal tissues. Furthermore, transfection with miR-196a mimics suppressed cellular migration and proliferation and increased apoptosis in 786-O and ACHN cells, further confirming the tumor suppressor role of miR-196a in RCC. Possibly due to the limited number of RCC samples used in the present study, no correlation was found between miR-196a expression and clinicopathological variables. Therefore, miR-196a expression should be detected in a larger cohort of RCC patients. Further studies will also be performed to elucidate the underlying molecular mechanisms and target genes of miR-196a in RCC.

Previous studies have reported on the roles of miR-196a in cancer types other than RCC. In head and neck squamous cell carcinoma, overexpression of miR-196a was found to produce an oncogenic effect, while its knockdown resulted in decreased cell proliferation, migration and invasion via suppression of annexin A1 (28). Another study demonstrated that miR-196a was highly expressed in non-small cell lung cancer and in which it regulates cell proliferation, migration and invasion, partially via down-regulation of homeobox (HOX)A5 (31). Furthermore, Sun *et al* (32) reported that miR-196a was upregulated in gastric cancer tissues and promoted cell proliferation by downregulating p27 (kip1).

Previous studies have also described miRNAs as novel biomarkers. In pancreatic ductal adenocarcinoma, overexpression of miR-196a was observed to be associated with disease progression and patient prognosis (33), and another study reported its potential use as a biomarker for the early detection of familial pancreatic cancer (19). Aso *et al* (34) reported that miR-196a detected in the pancreatic juice is a diagnostic biomarker for intestinal-type intraductal papillary mucinous neoplasm. Furthermore, miR-196a and miR-196b have been indicated to be correlated with aggressive progression and unfavorable clinical outcome in colorectal cancer patients (35).

miR-196a was also found to have a role in certain other diseases or cellular processes. Zhang *et al* (36) revealed that the urine levels of miR-196a are associated with focal segmental glomerulosclerosis. In addition, miR-196a was found to regulate the differentiation and proliferation of human adipose tissue-derived mesenchymal stem cells by modulating the levels of the transcription factor HOXC8 (37).

In conclusion, the present study revealed that miR-196a was downregulated in RCC tissues compared with that in normal adjacent tissues. Transfection with miR-196a mimics inhibited the proliferation and migration of the 786-O and ACHN RCC cell lines, while inducing apoptosis. These results suggested that miR-196a may function as a tumor suppressor in RCC. The results in our study supported that miR-196a may not only be a promising diagnostic biomarker but also a potential therapeutic target in RCC. Further studies will identify target genes of miR-196a in RCC.

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