

MicroRNA-451a is associated with cell proliferation, migration and apoptosis in renal cell carcinoma

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Abstract. MicroRNAs (miRNAs) are an important class of small, non-coding RNA molecules that regulate gene expression at the transcriptional or post-transcriptional level. They are involved in apoptosis, proliferation and migration and are known to have an important role in many types of cancer. Aberrant expression of miRNA-451a (miR-451a) has previously been reported in tumors, however its role in renal cell carcinoma (RCC) is currently unknown. The aim of the present study was to investigate the role of miR-451a in RCC. The expression of miR-451a was analyzed in 50 paired RCC and normal tissues by quantitative polymerase chain reaction. Furthermore, the effects of miR-451a on cell migration, proliferation and apoptosis were evaluated, using migration scratch, MTT and flow cytometric assays. The present study demonstrated that miR-451a was upregulated in RCC, as compared with paired normal tissues ($P<0.05$). Downregulation of miR-451a using a synthesized inhibitor, significantly suppressed cell migration and proliferation, and induced apoptosis of renal cancer cells *in vitro*, as compared with a negative control ($P<0.05$). In the present study, it was determined that miR-451a may have an important role as a tumor enhancer in RCC. These results imply that miR-451a may be a promising therapeutic target for the treatment of RCC.

Introduction

Renal cell carcinoma (RCC) is the second leading cause of mortality among urological tumors, accounting for ~3% of all adult malignancies (1). Clear cell RCC is the most common malignant tumor of the kidney and is associated with a poor prognosis (2). The reported incidence of RCC has increased in the United States over the past two decades (3,4). In >1/3 of patients RCC may have already metastasized prior to diagnosis, and 50% of patients may suffer from recurrence, even following nephrectomy (5). Traditional chemotherapy and radiotherapy are not effective in the treatment of advanced RCC. Furthermore, the molecular mechanisms regulating the aggressive properties of RCC remain poorly understood (6). Nevertheless, new therapeutic strategies have emerged, based on molecular and biological studies of RCC, including the potential applications of microRNAs (miRNAs) in the diagnosis, prognosis and treatment of tumors (7,8).

miRNAs are endogenous, non-coding 19-23 nucleotide RNA molecules which are involved in post-transcriptional regulation of gene expression (9). Mature miRNAs bind to the 3'-untranslated regions (3'-UTR) of target mRNAs, resulting in the degradation of mRNA or the blockade of translation. Currently ~2,000 human miRNAs have been identified, which are known to be involved in numerous biological processes, including proliferation, apoptosis, migration and differentiation (10). Numerous miRNAs have been demonstrated to function as tumor suppressors or oncogenes (11). It has been reported that miRNA-451a (miR-451a) is widely dysregulated in numerous human malignancies, including lung (12), liver (9) and breast cancer (13), and glioma (14), thus indicating that miR-451a may have an important role in oncogenesis. However, knowledge is currently limited on the mechanism of action of miR-451a in RCC. A previous miRNA microarray chip analysis showed that miR-451a was upregulated in RCC (15). The expression and function of miR-451a in RCC requires further investigation. The aim of

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the present study was to use quantitative polymerase chain reaction (qPCR) to determine the relative expression levels of miR-451a in paired RCC and normal tissues and to analyze the effects of miR-451a on cell migration, proliferation and apoptosis.

Materials and methods

Clinical sample collection and RNA extraction. The present study was approved by the Institutional Review Board and Ethical Committee of Peking University Shenzhen Hospital (Shenzhen, China). All patients provided written informed consent prior to the study. The RCC and matched normal adjacent tissues were collected from the hospitals of Guangdong and Anhui. The adjacent normal tissues were located 2.0 cm away from visible RCC lesions. The fresh tissue samples were immediately immersed in RNAlater (Qiagen, Hilden, Germany) following surgical resection, stored at 4°C overnight and then frozen in liquid nitrogen and stored at -80°C until further use. All tissue samples were reviewed and classified with hematoxylin & eosin staining and disease stages of the patients were classified according to the 2009 American Joint Committee on Cancer staging system. Total RNA was extracted from 50 paired RCC samples and normal tissue using TRIzol® (Invitrogen Life Technologies, Carlsbad, CA USA) and were purified using the RNeasy® Maxi kit (Qiagen), according to the manufacturer's instructions. The clinical and pathological characteristics of the 50 RCC patients, included in the present study, are shown in Table 1.

qPCR. A previous miRNA microarray chip analysis showed that hsa-miR-451a was highly expressed in RCC tissues, as compared with the adjacent normal tissues (15). In order to validate the results of the miRNA microarray chip analysis, qPCR was performed to detect the relative expression levels of miR-451a in 50 paired RCC and adjacent normal tissues. A total of 1 µg RNA was reverse transcribed into cDNA using the miScript Reverse Transcription kit (Qiagen), according to the manufacturer's instructions. The qPCR reaction of miR-451a was performed using a LightCycler® 480 Fluorescent Quantitative PCR system (Roche Diagnostics GmbH, Mannheim, Germany) and miScript SYBR Green PCR kit (Qiagen), according to the manufacturer's instructions. Primers for the U6 non-coding small nuclear RNA were used as an internal control. The 20 µl reaction mixture contained 10 µl 2X QuantiTect SYBR Green PCR Master Mix, 2 µl 10X miScript Universal Primer, 0.4 µl specific microRNA primer, 1 µl cDNA template and RNase-free water. The following primers were used: miR-451a forward, 5'-AAACCGTTACCATTACTGAGTT-3' and the reverse miScript SYBR Green PCR kit Universal Primer; U6 forward, 5'-CTCGCTTCGGCAGCAC-3' and reverse, 5'-ACGCTTCACGAATTTGCGT-3' (Qiagen). The qPCR was performed on tumor and normal cDNA in triplicate for each set. The PCR reaction was performed as follows: 95° 15 min, followed by 40 cycles of 94°C 15 s, 55°C 30 s and 72°C 30 s.

Cell culture and transfection. 786-O and ACHN human RCC cell lines, were obtained from the Guangdong and Shenzhen Key

Table I. Clinical characteristics of 50 patients with renal cancer.

Characteristic	N (%)
Ages (years)	
≥54	29(58)
<54	21(42)
Gender	
Male	30(60)
Female	20(40)
Histological type	
Clear cell	40(80)
Papillary	10(20)
AJCC clinical stage	
I	27(54)
II	20(40)
III+IV	3(6)

N, number of patients; AJCC, American Joint Committee on Cancer.

Laboratory of Male Reproductive Medicine and Genetics. The cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies), supplemented with 10% fetal bovine serum (Shanghai ExCell Biology Inc., Shanghai, China), and maintained in a humidified incubator containing 5% CO₂, at 37°C. The miRNA oligonucleotide was chemically synthesized by GenePharma Company, Ltd. (Shanghai, China). The sequences were as follows: hsa-miR-451a inhibitor, 5'-AACUCAGUAAUGGUAACGGUUU-3'; and the hsa-miR-451a inhibitor negative control, 5'-CAGUACUUUUGUGUAGUACAA-3'. The cells were grown to 60-80% confluence, followed by a transfection with the miR-451a inhibitor or negative control using Lipofectamine® 2000 reagent (Invitrogen Life Technologies), according to the manufacturer's instructions. The transfection efficiency and miR-451a expression changes were confirmed by fluorescence microscopy (DMIRB; Leica Microsystems GmbH, Wetzlar, Germany) and qPCR. The culture medium was refreshed 6 h after transfection, and the transfection efficiency was observed in the cells that were transfected with green fluorescent protein Fam-labeled miR-451a inhibitor-negative control (GenePharma Co., Ltd.). The cells were harvested and the total RNA was extracted for qPCR analysis 24 h after transfection with a miR-451a inhibitor or the control.

Migration scratch assay in vitro. The migration scratch assay was used to assess the migratory ability of 786-O and ACHN cells *in vitro*. Approximately 600,000 cells were seeded per 6-well dish and transfected after 24 h with 100 pmol of either the miR-451a inhibitor or a negative control, using Lipofectamine® 2000. Following 6 h of transfection, a sterile 20 µl pipette tip and markers were used to make a scratch, at the same point in each of the samples, in the cell monolayers. The cells were then rinsed with phosphate-buffered saline (PBS) and cultured at 37°C. Images of the scratches were acquired, using a digital

camera system, 0 and 24 h after the scratches were made. The migration distances (μm) were measured using MIAS-2000 software and the experiments were performed in triplicate and repeated ≥ 3 times.

Assessment of cell proliferation by MTT assay. The cellular proliferation potential was determined by MTT assay, as previously described (16). Briefly, 786-O and ACHN cells were seeded into 96-well plates, at a cell density of 8,000 cells/well, in growth medium and transfected with 10 pmol of either the miR-451a inhibitor or a negative control. The cell growth was measured by adding 20 μl of MTT (5 mg/ml, Sigma-Aldrich, St Louis, MO, USA) to each well, followed by an incubation at 37°C for 4 h. The proliferation assay was performed for three days and the cell growth was measured at 24 h intervals. The reaction was stopped by the addition of 150 μl dimethyl sulfoxide (Sigma-Aldrich). Following agitation for 15 min at room temperature, the optical density (OD) of each sample was measured at a wavelength of 490 nm, using an Enzyme Immunoassay Instrument (Model 680; Bio-Rad, Hercules, CA, USA).

Flow cytometric analysis of apoptosis. 786-O and ACHN cells ($\sim 3,000$) were cultured at 37°C and 5% CO_2 in 6-well plates. Once the cells had reached $\sim 60\%$ confluence, they were transfected with either the miR-451a inhibitor or a negative control. For the apoptosis assays, floating and adherent cells were harvested 48 h after transfection, combined and washed twice with cold PBS, followed by resuspension in 1X binding buffer (Invitrogen Life Technologies). A total of 5 μl Alexa Fluor® 488 Annexin V (Invitrogen Life Technologies) and 3 μl propidium iodide (Invitrogen Life Technologies), was added to 500 μl of the cell suspension, and the samples were analyzed within 30 min of staining. The fluorescence was measured using a flow cytometer (Beckman Coulter, Miami, FL, USA) at an excitation of 488 nm, according to the manufacturer's instructions (8).

Statistical analysis. Statistical significance was determined using the Student's t-test. For the comparison of miR-451a expression levels between the matched RCC and normal tissue samples, a paired two-tailed t-test was used. A $P < 0.05$ was considered to indicate a statistically significant difference. Statistical analyses were carried out using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA).

Results

miR-451a is significantly upregulated in RCC tissue. Previous research determined that the expression of miR-451a was upregulated in RCC tissues (15). To confirm the results of the previous miRNA microarray chip analysis, the expression of miR-451a was determined in 50 matched RCC and adjacent normal tissues, by qPCR. The results demonstrated that miR-451a relative expression levels were significantly higher in RCC tissues, as compared with the adjacent normal tissues ($P < 0.05$; Fig. 1).

Reduction of miR-451a inhibits RCC cell migration. To explore the functions of miR-451a in renal cancer, a miR-451a inhibitor or a negative control was transfected into 786-O

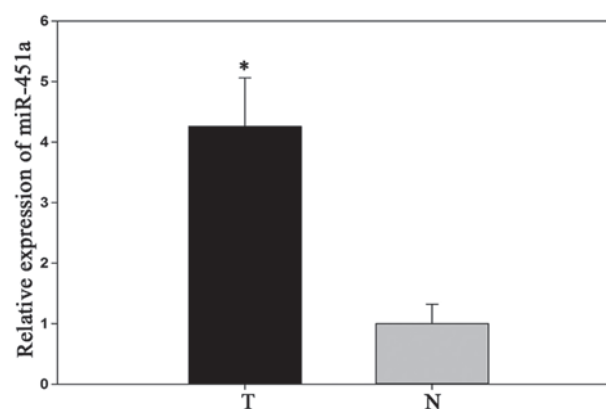


Figure 1. Analysis of relative miRNA-451a (miR-451a) expression levels, by quantitative polymerase chain reaction. The relative expression of miR-451a in RCC tumor (T) tissue samples was significantly higher, as compared with the adjacent normal tissues (N). Data are presented as the mean of three experiments and the bars present the standard deviation of the mean. $n=50$; * $P < 0.05$.

and ACHN cells. The Fam-labeled negative control was transfected into the cells, and the transfection efficiency was analyzed by fluorescence microscopy 6 h after transfection. As shown in Fig. 2A and B, the transfection efficiency was ~ 90 and 85% in 786-O and ACHN cells, respectively. In addition, the fold changes to the expression levels of miR-451a, as determined by qPCR, were 198.1 and 157.9 in the 786-O and ACHN cells, respectively (Fig. 2C, $P < 0.05$) (Fig. 2C, $P < 0.05$). Scratch assays were performed to observe the function of miR-451a in cell migration. Images of the scratches were captured at 0 and 24 h after transfection, using a camera coupled to a fluorescence microscope (Fig. 3). The results demonstrated that the migration distances of the cells transfected with miR-451a inhibitor were markedly shorter, as compared with the negative control group ($P < 0.05$). These results indicate that the reduction of miR-451a expression, inhibited the migration of RCC cells (Fig. 3C).

miR-451a inhibitor suppresses RCC cell proliferation. To determine the potential role of miR-451a on the proliferation of RCC cells, MTT assays were performed. The miR-451a inhibitor and negative control groups were measured at 0, 24, 78 and 72 h after transfection. The OD values demonstrated that the proliferation of 786-O cells was decreased by 4.6 ($P < 0.05$), 11.25 ($P < 0.05$) and 16.31% ($P < 0.05$), and the proliferation of ACHN cells was decreased by 5.8 ($P < 0.05$), 13.12 ($P < 0.05$) and 15.84% ($P < 0.05$) at 24, 48 and 72 h after transfection, respectively. These results suggest that the miR-451a inhibitor reduced the growth of 786-O and ACHN cells, as compared with the negative control inhibitor (Fig. 4).

Downregulation of miR-451a induces RCC cell apoptosis. The effects of miR-451a on apoptosis were determined. 786-O and ACHN cells were transfected with either the miR-451a inhibitor or a negative control for 48 h. Flow cytometric analysis demonstrated that the apoptotic rate of 786-O cells transfected with miR-451a inhibitor or a negative control was 6.6 vs. 2% ($P < 0.05$) and the apoptotic rate of ACHN cells was 7.7 vs. 4.3% ($P < 0.05$). These data demonstrate

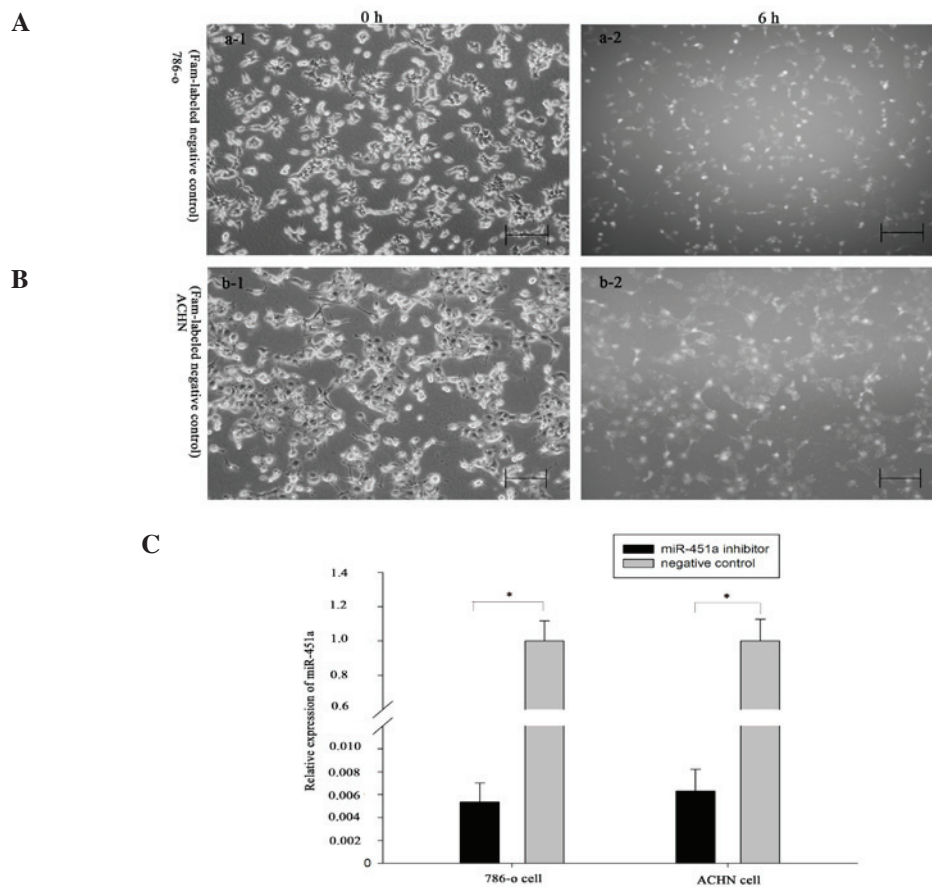


Figure 2. Analysis of transfection efficiency and miRNA-451a (miR-451a) expression levels by fluorescence microscopy and quantitative polymerase chain reaction. (A and B) The transfection efficiency was ~90 and 85% in 786-O and ACHN cells, respectively. Images of (a-1) 786-O cells transfected with the Fam-labeled negative control. (a-2) 786-O cells exhibiting green fluorescence 6 h after transfection with Fam-labeled negative control. Scale bars, 200 μm. (b-1) Images of ACHN cells transfected with the Fam-labeled negative control. (b-2) ACHN cells exhibiting green fluorescence 6 h after transfection with the Fam-labeled negative control. (C) The fold changes of miR-451a expression in 786-O and ACHN cells treated with miR-451a inhibitor or negative control were analyzed 24 h after transfection. Data are presented as the mean ± standard deviation. All experiments were performed in triplicate; *P<0.05.

that downregulation of miR-451a promoted RCC cell apoptosis (Fig. 5).

Discussion

miRNAs are a class of small, non-coding RNAs that can regulate the expression of protein-coding genes through various mechanisms, including targeted mRNA degradation and translational inhibition (7,17). Previous evidence has suggested that miRNAs have a crucial role in carcinogenesis and cancer progression. By altering cell proliferation, differentiation, invasion and apoptosis, miRNAs can function as either tumor suppressors or oncogenes (18-20). Various miRNAs have previously been shown to be significantly upregulated in RCC, including miR-210, miR-34a and miR-21, which were correlated with pathological processes, including oncogenesis (21-23). Conversely, miR-141, miR-224, miR-200c have been shown to be decreased in human RCC tissues, implying that they possess tumor suppressive activity (24,25). In the present study, the expression of miR-451a was shown to be upregulated in RCC tissues.

Previous studies have provided evidence of the effects of miR-451a, demonstrating its ability to inhibit cell

proliferation and induce apoptosis in numerous cancer cell lines. Wang *et al* (26) reported that ectopic miR-451a significantly suppressed the proliferation of non-small cell lung carcinoma cells *in vitro*; this effect was shown to be partially due to the downregulation of ras-related protein 14. Bandreset *et al* (27) reported that hsa-miR-451a was significantly underexpressed in gastric and colorectal cancer, as compared with adjacent normal tissues, and showed that the overexpression of miR-451a in gastric and colorectal cancer cells inhibited cell proliferation. The downregulation of miRNA-451a was also shown to be associated with a worse prognosis in these cancers. Previous research has also demonstrated that miR-451a may regulate LKB1/AMPK signaling and promote proliferation and migration of glioma cells (28). There is numerous evidence demonstrating that miR-451a may be up or downregulated in various cancers and function as either a tumor suppressor or oncogene. Furthermore, the present study demonstrated that miR-451a expression was upregulated in RCC tissues, as compared with adjacent nonmalignant tissues. These results suggest that miR-451a may be characterized as an oncogene in RCC.

In the present study, qPCR was used to detect the relative miR-451a expression levels in 50 paired RCC and adjacent

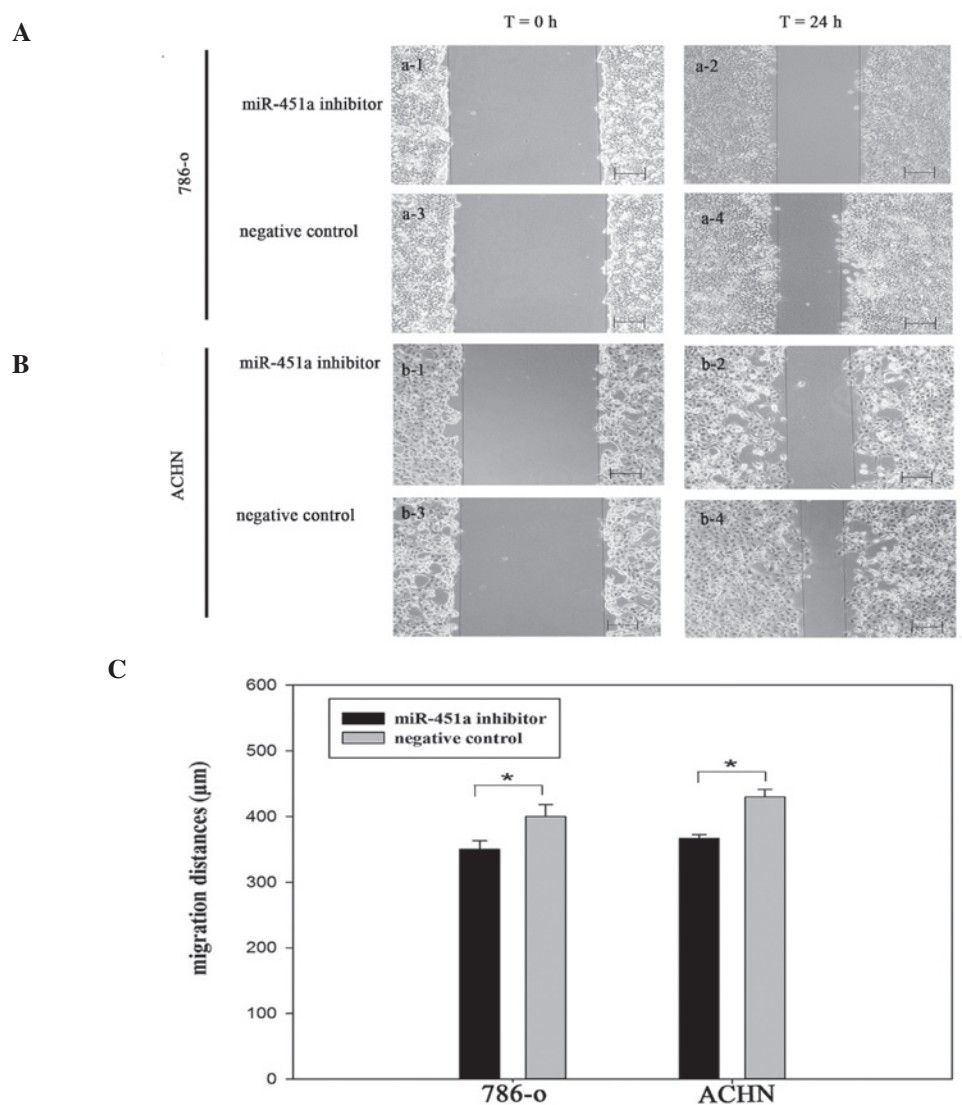


Figure 3. Migration scratch assay for 786-O and ACHN renal carcinoma cells 24 h post-transfection. (A) Images of the 786-O cells transfected with (a-1 and 2) miR-451a inhibitor or (a-3 and 4) negative control 0 h and 24 h after the scratches were made at the same point. (B) Images of the ACHN cells transfected with (b-1 and 2) miR-451a inhibitor or (b-3 and 4) negative control 0 h and 24 h after the scratches were made at the same point. The lines indicate the migration front. (C) Quantification of the migration distances (μm) using MIAS-2000 software; * $P < 0.05$. All experiments were performed in triplicate. miR, microRNA. Representative images are shown (scale bars, $200 \mu\text{m}$) and data are presented as the mean \pm standard deviation of three independent experiments.

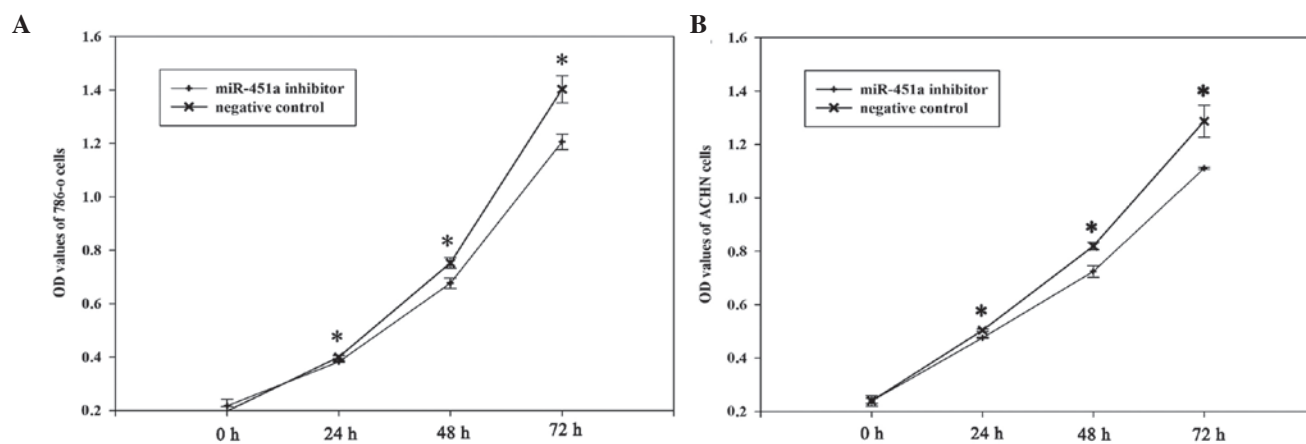


Figure 4. MTT assay for cell proliferation of 786-O and ACHN renal carcinoma cells transfected with miRNA-451a (miR-451a) inhibitor or negative control. Cell proliferation of (A) 786-o cells and (B) ACHN cells. miR, microRNA; OD, optical density. Data are presented as the mean of three measurements and the bars present the standard deviation of the mean (* $P < 0.05$).

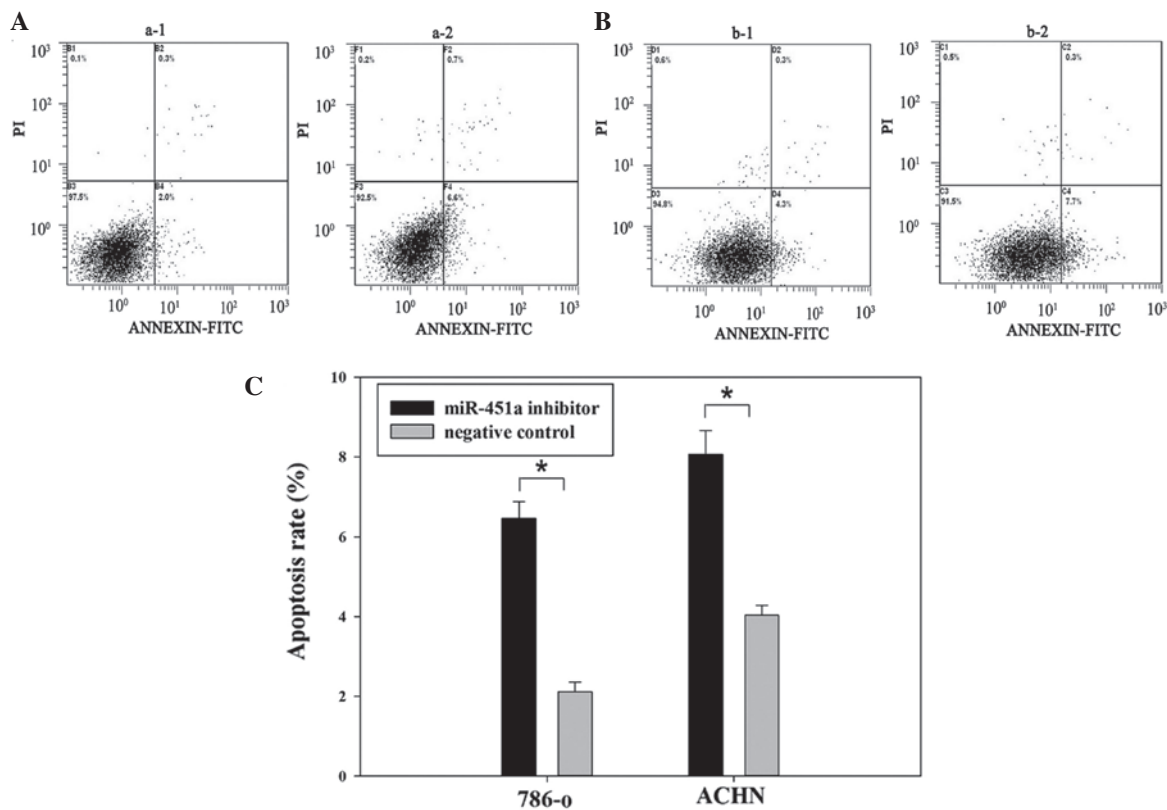


Figure 5. Flow cytometric analysis of apoptosis of 786-O and ACHN renal carcinoma cells transfected with miRNA-451a (miR-451a) inhibitor or negative control. (A) 786-O cells transfected with (a-1) miR-451a inhibitor and (a-2) negative control. (B) ACHN cells transfected with (b-1) miR-451a inhibitor and (b-2) negative control. (C) Comparison of the apoptosis rates of cells transfected with miR-451a inhibitor or negative control; $P < 0.05$. FITC, fluorescein isothiocyanate; PI, propidium iodide; miR, microRNA. Data are presented as the mean of three measurements and the bars present the standard deviation of the mean.

normal tissues. The results were consistent with the previous miRNA microarray chip analysis, which demonstrated that miR-451a expression was significantly upregulated in RCC (15). Furthermore, the functions of miR-451a on cell migration, proliferation and apoptosis were analyzed by transfecting a miR-451a inhibitor into 786-O and ACHN RCC cell lines. The results demonstrated that cells transfected with a miR-451a inhibitor had reduced cell migration and proliferation, and increased apoptosis, as compared with the cells transfected with a negative control. To the best of our knowledge, the results of the present study provide a novel insight into the roles and possible mechanisms of miR-451a in the occurrence and development of RCC.

Observed phenotypical changes may be mediated by miRNA-regulated genes. Previous research has determined that miRNAs post-transcriptionally regulate the expression of >30% of protein coding genes by translational repression. miRNAs have also been shown to regulate the expression of numerous putative target genes, by binding to a complementary sequence found predominantly in the UTR, however the binding sequences are not always completely complementary, especially in mammals (6,29). miRNA-associated post-transcriptional regulation, within the context of tumor development, has been reported for the regulation of genes that have an impact on cell differentiation, apoptosis and neoplastic transformation (30,31). The results of the present study may seem contradictory to other research, as miR-451a has previously been characterized as a tumor suppressor in

some cancers and an oncogene in others. This contradiction may be explained by the 'imperfect complementarity' of the interactions between miRNAs and target genes (8). Further research should be conducted to determine the roles and target genes of miR-451a in RCC.

In conclusion, the results of the present study demonstrate that miR-451a may be significantly upregulated in human RCC tissues and be involved in cell proliferation, migration and apoptosis in RCC cell lines. In addition, the data suggests that miR-451a may be a promising therapeutic target for the treatment of RCC. Further research is required to explore the roles and target genes of miR-451a.

Acknowledgements

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