Upregulated microRNA-16 as an oncogene in renal cell carcinoma

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Abstract. MicroRNAs (miRs) are small, endogenous noncoding RNAs that serve a significant function in various biologic processes, including those involved in cancer. The present study aimed to determine the expression and function of miR-16 in renal cell carcinoma (RCC). Quantitative polymerase chain reaction was used to quantify the expression of miR-16 in 48 paired RCC tissues and adjacent normal tissues. The impact of miR-16 on cell proliferation, migration and apoptosis was analyzed by transfecting miR-16 mature molecules into the renal cancer cell lines 786-O and ACHN. The results indicated that miR-16 was significantly upregulated in RCC tissues (P<0.05). Downregulation of miR-16 resulted in reduced cell proliferation and migration and increased levels of apoptosis, while overexpression of miR-16 resulted in accelerated cellular proliferation and migration, suggesting that miR-16 may function as an oncogene in RCC. The present study demonstrated for the first time, to the best of our knowledge, that miR-16 is upregulated in RCC and acts as an oncogene by inducing cellular proliferation, migration and reducing apoptosis. Further study of miR-16 in RCC may clarify the molecular mechanisms of RCC carcinogenesis and aid in the development of novel biomarkers and therapeutic options.

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Introduction

Renal cell carcinoma (RCC) is the most common type of malignancy in kidney parenchyma, with a high rate of recurrence and mortality (1). Over the last two decades, the incidence of RCC has progressively increased and at present, it accounts for ~3% of all cases of cancer (2). Early diagnosis of RCC is a challenge, as it presents no clinical symptoms for the majority of its course (3). Almost 30% of all patients exhibit metastatic disease at presentation, and 40% of patients with localized disease ultimately develop distant metastases following removal of the primary tumor (4). Therefore, it is critical to identify novel molecular mechanisms, including microRNAs (miRs), to elucidate RCC oncogenesis and metastasis.

miRs are non-coding RNAs of ~22 nucleotides in length, which function as regulators at the post-transcriptional level (5). Numerous studies have demonstrated the notable roles of miRs in cancer pathogenesis, including onset, progression and metastasis (6,7). Involvement of miRs in the oncogenic process has been indicated by the observation that the 13q14 deletion characterizing >50% of all cases of chronic lymphocytic leukemia results in a loss of miR-16 genes (8). This finding provided the primary evidence that miR genes may have a role in tumorigenesis. Downregulation of miR-16 has been reported in other neoplastic malignancies, including multiple myeloma (9), pituitary adenomas (10), mantle cell lymphoma (11), lung cancer (12) and prostate carcinoma (13).

miR-16 is involved in numerous types of cancer; thus, the present study investigated the potential role and function of miR-16 in RCC. Quantitative polymerase chain reaction (qPCR) was used to quantify the expression of miR-16 in RCC tissues and paired normal adjacent tissues, and the impact of miR-16 on renal cancer was assessed by MTT, wound scratch and apoptosis assays following transfection of mature miR-16 molecules. Bioinformatics analysis was also performed to predict the target genes of miR-16.

Materials and methods

Human tissue samples and cell lines. All human RCC tissues and adjacent normal tissues were collected from Anhui Medical

University (Hefei, China) and Peking University Shenzhen Hospital (Shenzhen, China), between January 2012 and August 2013. Written informed consent was obtained from each patient prior to sample collection. The collection and use of these samples were reviewed and approved by the ethics committees of the First Affiliated Hospital of Anhui Medical University (Hefei, China) and Peking University Shenzhen Hospital (Shenzhen, China). Once dissected, fresh tissues were immersed in RNAlater RNA stabilization reagent (Qiagen GmbH, Hilden, Germany) within 30 min then stored at -80˚C. The pathological and clinical characteristics of 48 RCC patients are presented in Table I. The age range of patients was 29-76 years, with a median age of 52 years.

The two human renal cancer cell lines ACHN and 786-O were used in the present study. Cells were cultured at 37˚C in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technologies, Carlsbad, CA, USA) mixed with 10% fetal bovine serum (Invitrogen Life Technologies) in an incubator with a 5% CO_2 atmosphere.

RNA isolation and reverse transcription (RT)-qPCR. According to the manufacturer's instructions, total RNA from each sample was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and purified using an RNeasy Maxi kit (Qiagen GmbH). The RT reaction was conducted with 1 μg total RNA from each sample to obtain the cDNA templates, using a miScript RT kit (Qiagen GmbH) according to the manufacturer's instructions. The qPCR reaction was performed in a LightCycler 480 Real-Time PCR system (Roche Diagnostics, Basel, Switzerland) using miScript SYBR Green PCR kit (Qiagen GmbH) with U6 small nuclear RNA (Qiagen GmbH) as a control. The reaction mixture consisted of the following reagents: Specific microRNA primer (1 μl); cDNA template (1 μl); 2X QuantiTect SYBR Green PCR Master Mix (10 μl); 10X miScript Universal Primer (2 μl); and RNase-free water to a total volume of 20 μl. The reverse primer was provided in the miScript SYBR Green PCR kit. The other primer sequences were as follows: miR-16 forward, 5'-TAGCAG CACGTAAATATTGGCG-3'; U6 reverse, 5'-ACGCTTCAC GAATTTGCGT-3' and forward, 5'-CTCGCTTCGGCAGCA CA-3'. Amplification conditions were as follows: 95˚C for 15 min, then 40 cycles of 95˚C for 15 sec, 58˚C for 30 sec and 72˚C for 30 sec. PCR products were analyzed by agarose gel electrophoresis. The relative expression levels of miR-16 were calculated using the $2^{-\Delta\Delta Ct}$ method (14).

Cell transfection. To overexpress or downregulate miR-16, miR-16 mimics (5'-UAGCAGCACFUAAAUAUUGGCG-3' and 5'-CCAAUAUUUACGUGCUGCUAUU-3') and inhibitor (5'-CGCCAAUAUUUACGUGCUGCUA-3') were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) and transfected into the cancer cells using Lipofectamine 2000 (Invitrogen Life Technologies). Cancer cells were harvested 24 h subsequent to transfection and the relative expression of miR-16 was detected by qPCR.

Cell proliferation assay. The ability of cellular proliferation was evaluated using an MTT assay. Approximately $5x10^3$ cells were seeded into 96-well culture plates and then transfected with 5 pmol miR-16 mature molecules (mimics/inhibitor) or a negative control. At 0, 24, 48 or 72 h following transfection, the cells were incubated with 20 μl MTT solution (5 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) for 4 h at 37˚C, lysed in 150 μl dimethyl sulfoxide and agitated for 10 min at room temperature. The cell number was estimated by the measurement of optical density (OD) with a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at a dual wavelength of 490/630 nm.

Flow cytometric evaluation of apoptosis. Approximately 300,000 renal cancer cells were cultured in six-well plates. At 48 h subsequent to transfection, cells (including floating cells) were harvested, washed twice with cold phosphate-buffered saline (Invitrogen Life Technologies) and resuspended in 100 μl 1X binding buffer (Invitrogen Life Technologies), followed by the addition of 5 μl annexin V-fluorescein isothio-cyanate (Invitrogen Life Technologies) and 3 μl propidium iodide (Invitrogen Life Technologies). The fluorescence of stained cells was analyzed using flow cytometry (EPICS XL; Beckman Coulter, Brea, CA, USA) within 30 min of staining, according to the manufacturer's instructions.

Wound healing assay. A wound healing assay was used to evaluate the migration ability of renal cancer cells *in vitro*. Approximately 350,000 cells were seeded per 12-well dish, then transfected with miR-16 mature molecules (80 pmol) or negative control (80 pmol) 24 h later. Following transfection for 5 h, the cell monolayer was scraped using a P-20 micropipette

Table I. Clinical and pathological features of 48 patients.

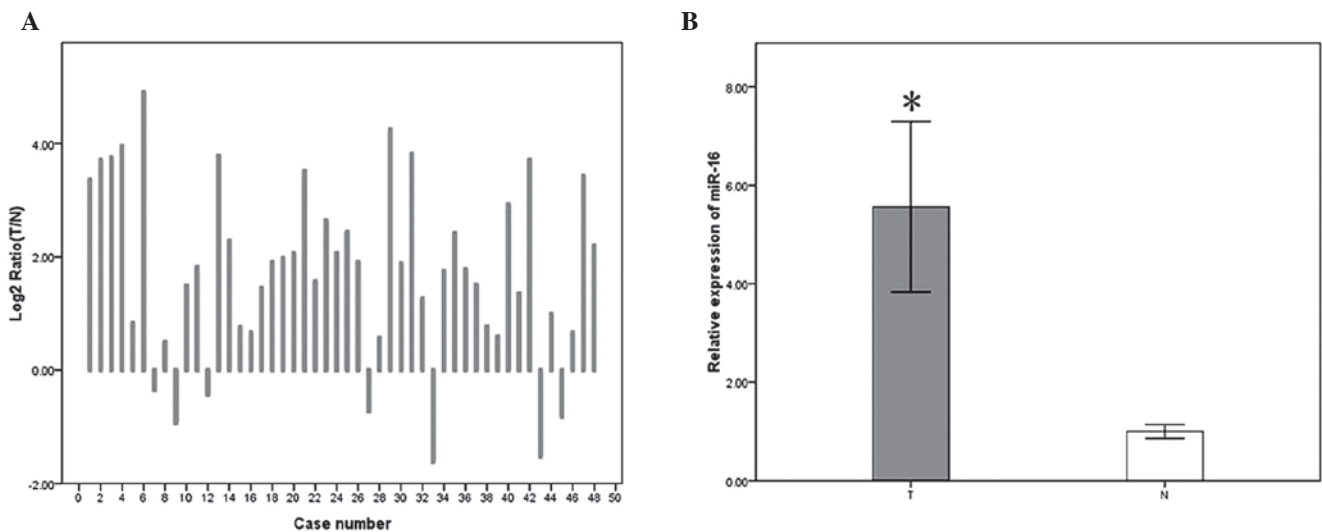| Variable	Cases (n)
Total	48
Age (years)	
≥52	29
<52	19
Gender	
Male	30
Female	18
Histological type	
Clear cell	39
Papillary	9
Primary tumor stage	
T1	27
T2	19
T3 and 4	2
AJCC clinical stages	
I	27
II	18
III+IV	3

AJCC, American Joint Committee on Cancer.

Figure 1. Expression of miR-16 in 48 paired RCC tissues and adjacent normal tissues. (A) Log2 ratios and (B) relative expression levels of miR-16 expression in 48 paired RCC and normal tissue samples. *$P \leq 0.05$ vs. N group. T, RCC tumor group; N, normal tissue group; RCC, renal cell carcinoma; miR, microRNA.
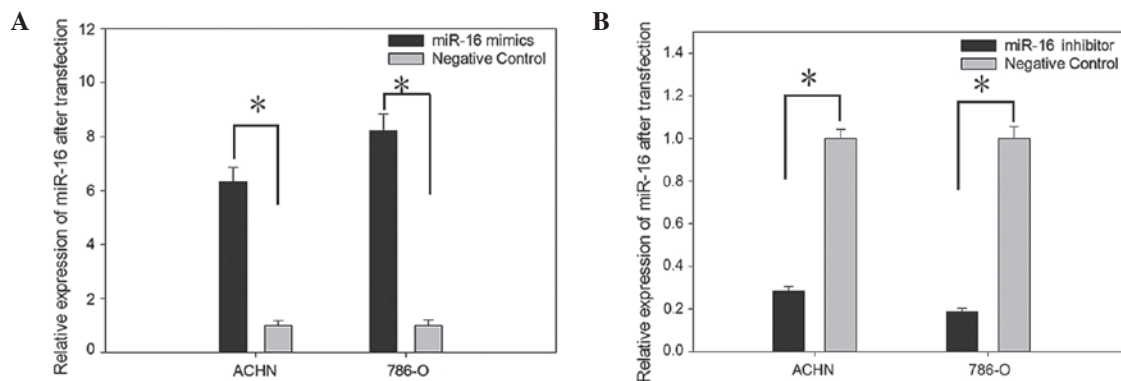

Figure 2. miR-16 expression changes after transfection were confirmed by quantitative polymerase chain reaction. Relative expression of miR-16 in ACHN and 786-O cells 24 h subsequent to transfection with (A) miR-16 mimics or (B) miR-16 inhibitor. *$P<0.05$. miR, microRNA.

tip (Owens Corning, Toledo, OH, USA). The initial gap length (0 h) and the residual gap length after 24 h of wound-healing were calculated using the software program MIAS-2000 P3 (Leica Microsystems GmbH, Wetzlar, Germany). The experiments were performed in triplicate, repeated at least three times, and analyzed in a double-blind fashion by a minimum of two observers. The images were visualized and captured using an XSP-63XDV (Shanghai Optical Instrument Factory, Shanghai, China).

Bioinformatics analysis. The potential targets of miR-16 were predicted by combining four public algorithms, miRanda (www.microrna.org), TargetScan (www. targetscan.org), PicTar (pictar.mdc-berlin.de) and miRWalk (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. Statistical analysis was performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). A paired *t*-test was used for the comparison of miR-16 expression in matched cancer and normal samples. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Upregulation of miR-16 in RCC tissues. Previous miR expression profiles of renal cancer have indicated that miR-16 was upregulated in these tissues (15,16). In order to assess this hypothesis in the present study, qPCR was used to quantify the expression of miR-16 in 48 paired RCC tissues and adjacent normal tissues. The relative expression of miR-16 [$\log_2$ (T/N)] is exhibited in Fig. 1A. The results demonstrated that miR-16 expression levels in the RCC tissues were significantly higher than those in the paired normal tissues (P<0.05), as presented in Fig. 1B.

Transfection efficiency. To analyze the function of miR-16 in renal cancer, miR-16 mature molecules (mimics/inhibitor) or negative control were transfected into the ACHN and 768-O renal cancer cell lines. The relative expression of miR-16 following transfection of miR-16 mimics and inhibitor
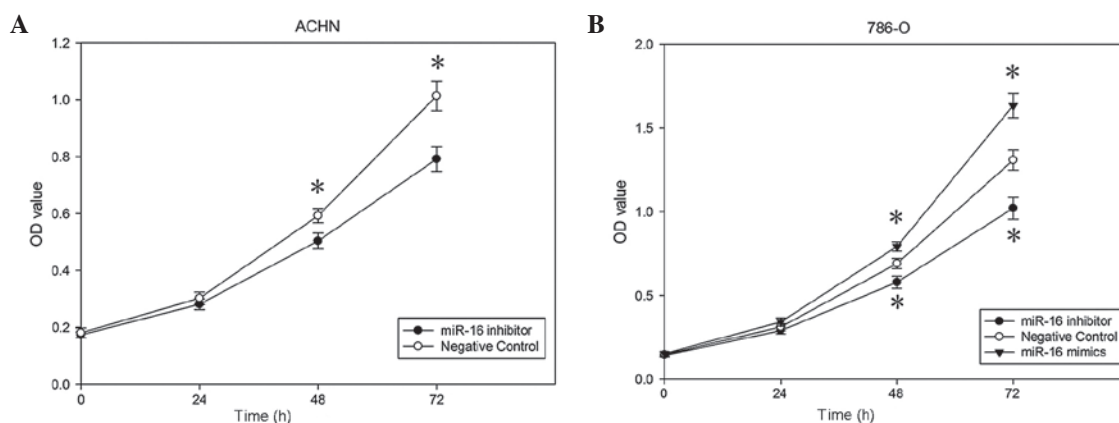
Figure 3. MTT assay indicating cell proliferation of ACHN and 786-O cells subsequent to transfection. (A) ACHN cells transfected with miR-16 inhibitor or negative control. (B) 786-O cells transfected with miR-16 mature molecules (mimics/inhibitor) or negative control. *P<0.05 vs. negative control. miR, microRNA; OD, optical density.
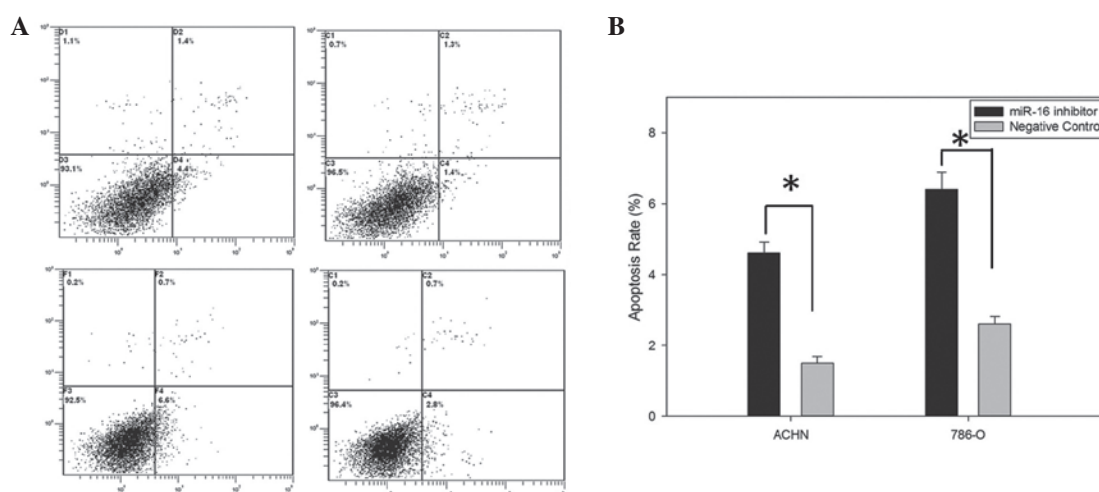

Figure 4. Flow cytometric analysis indicating levels of cell apoptosis in ACHN and 786-O cells subsequent to transfection. (A) ACHN cells transfected with miR-16 inhibitor (top left) or negative control (top right) and 786-O cells transfected with miR-16 inhibitor (bottom left) or negative control (bottom right). (B) Comparison of cell apoptosis rates in cells transfected with miR-16 inhibitor or negative control. *P<0.05 vs. negative control. miR, microRNA.

were 6.35 and 0.2638 in ACHN cells, and 8.26 and 0.1876 in 786-O cells, respectively (Fig. 2).

Effect of miR-16 on cell proliferation. Using an MTT assay, it was observed that the proliferation of 786-O cells transfected with miR-16 inhibitor was reduced by 6.06, 11.84 and 17.24% (all P<0.05 vs. negative control), while in cells transfected with miR-16 mimics, proliferation was increased by 5.82, 15.57 and 22.42% (all P<0.05 vs. negative control) at 24, 48 and 72 h after transfection, respectively. In ACHN cells, proliferation was reduced by 5.2 (P>0.05 vs. negative control), 10.61 and 18.76% (P<0.05 vs. negative control) following transfection with miR-16 inhibitor for 24, 48 and 72 h, respectively. This indicated that miR-16 promotes cell proliferation in renal cancer cells (Fig. 3).

Impact of miR-16 on apoptosis. To analyze the influence of miR-16 on renal cancer cell apoptosis, a flow cytometry assay was performed to detect the rates of apoptosis of ACHN and 786-O cells following the transfection. The results indicated that apoptotic rates of ACHN cells transfected with miR-16 inhibitor were 4.4 vs. 1.4% in the negative control group, while

the rates in 786-O cells were 6.6 vs. 2.8% (P<0.05 vs. negative control), indicating that downregulation of miR-16 induced apoptosis in renal cancer cells (Fig. 5). Therefore, miR-16 may reduce apoptosis in renal cancer.

Influence of miR-16 on cell migration. Wound healing assays were used to assess the migration ability of renal cancer cells following transfection with miR-16 mature molecules (mimics/inhibitor) or negative control. As presented in Fig. 5, wound widths of cells transfected with miR-16 inhibitor were greater than those in the negative control group (P<0.05), while cells transfected with miR-16 mimics displayed increased migration, suggesting miR-16 may accelerate the migration of renal cancer cells *in vitro* (Fig. 5C).

Target gene prediction. To explore the downstream target genes of miR-16, four algorithms were combined to predict the putative targets. BAP1 was the potential target predicted by all four algorithms simultaneously. The BAP1 3'-untranslated region (3'UTR) of the mRNA contained a complementary site for the seed sequences of miR-16 (Fig. 5D).
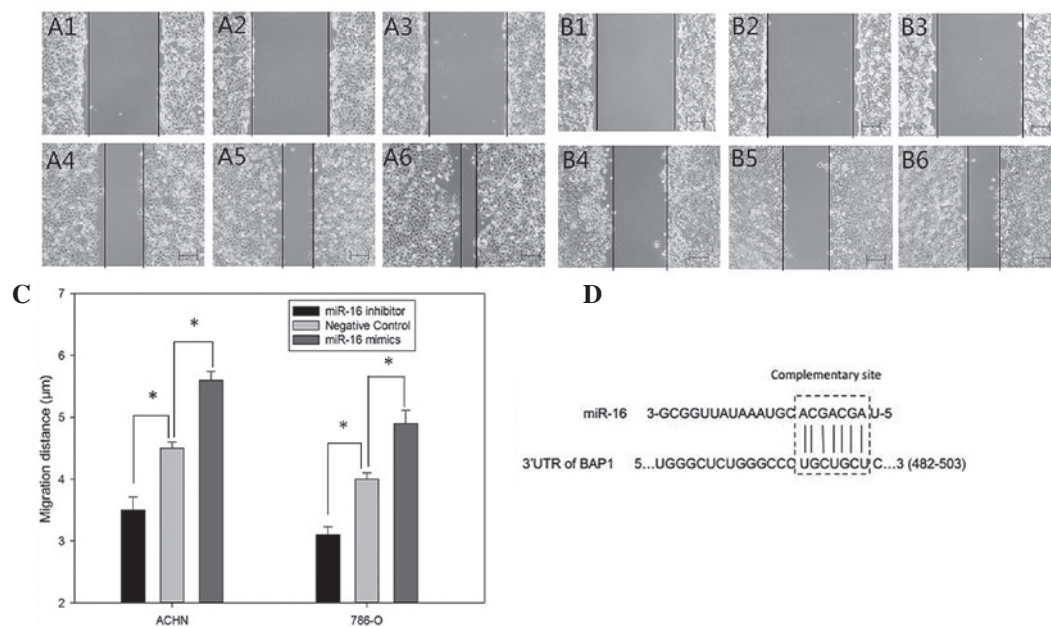
Figure 5. Wound healing assay of ACHN and 786-O cells 24 h subsequent to transfection. (A1) ACHN cells transfected with miR-16 inhibitor at 0 h; (A2) ACHN cells transfected with negative control at 0 h; (A3) ACHN cells transfected with miR-16 mimics at 0 h; (A4) ACHN cells transfected with miR-16 inhibitor 24 h after the wounds were generated; (A5) ACHN cells transfected with negative control 24 h after the wounds were generated; (A6) ACHN cells transfected with miR-16 mimics 24 h after the wounds were generated. (B1) 786-O cells transfected with miR-16 inhibitor at 0h; (B2) 786-O cells transfected with negative control at 0 h; (B3) 786-O cells transfected with miR-16 mimics at 0 h; (B4) 786-O cells transfected with miR-16 inhibitor 24 h after the wounds were generated; (B5) 786-O cells transfected with negative control 24 h after the wounds were generated; (B6) 786-O cells transfected with miR-16 mimics 24 h after the wounds were generated. (C) Comparison of migration distance in ACHN and 786-O cells. Data are represented as the means ± standard deviation; *P<0.05. (D) Bioinformatics prediction of the target gene of miR-16 with complementary sites. miR, microRNA; UTR, untranslated region.

Discussion

Cancer is characterized by multiple genetic changes affecting different oncogenes or tumor suppressor genes involved in cell cycle control, proliferation and apoptosis (17). The discovery of miRs and their ability to regulate multiple downstream genes indicates that they may be associated with cancer formation (18). It was estimated that >50% of miR genes are located in cancer-associated genomic regions or in fragile sites, suggesting that miRs may serve an important function in the pathogenesis of human cancer (19).

Located at 13q14.3, miR-16 has been confirmed to be downregulated in chronic lymphocytic lymphoma (8). Further study revealed miR-16 expression is inversely correlated to B-cell lymphoma 2 (Bcl2) expression in this disease and that miR-16 negatively regulates Bcl2 at the post-transcriptional level (5). A similar mechanism has also been suggested in gastric (20) and hepatic (21) cancer. miR-16 also targets the CCND1 gene (encoding cyclin D1) and WNT3A in prostate cancer (22). These findings highlight the importance of miR-16 in carcinogenesis.

As miR-16 is involved in multiple types of neoplastic malignancy, the present study aimed to explore the potential role and effect of miR-16 in RCC. qPCR was used to quantify the expression of miR-16 in 48 samples of RCC and adjacent normal tissues. The impact of miR-16 on cell proliferation, migration and apoptosis was analyzed by transfecting miR-16 mature molecules (mimics/inhibitor) into the renal cancer cell lines 786-O and ACHN. In addition, bioinformatics analysis was performed in order to predict the target genes of miR-16.

The results of the present study demonstrated that miR-16 was upregulated in renal cancer tissue samples (P<0.05), which is in accordance with previous studies of miR expression profiles in RCC (15,16). Downregulation of miR-16 resulted in reduced cellular proliferation, migration and increased apoptosis, while overexpression resulted in accelerated cellular proliferation and migration, suggesting that miR-16 may be characterized as an oncogene in RCC. Furthermore, bioinformatics prediction of the target gene of miR-16 indicated that BAP1 was one potential target.

In a previous study, BAP1 was observed to be mutated in ~15% of clear cell RCC (ccRCC) cases and located within a 50-Mb region on the short arm of chromosome 3p that encompasses VHL and is deleted in ~90% of ccRCC (23). In a study by Gossage et al (24) BAP1-mutated tumors were associated with metastatic disease at presentation, advanced clinical stage and a trend towards shorter recurrence-free survival (24). Another study confirmed that the expression of BAP1 may serve as a powerful marker to predict poor outcomes in patients with cancer (25). These results indicated the critical role of BAP1 in the carcinogenesis of RCC. Upregulated miR-16 may induce cellular proliferation and migration, and reduce apoptosis by suppressing the function of BAP1.

miR-16 acts as a tumor suppressor in certain types of cancer, whereas it is an oncogene in RCC. A similar situation is observed with regards to miR-7, which has been described as a tumor suppressor in several types of human cancer, including glioblastoma, breast and lung cancer (26); however, it acts as an oncogene in RCC as previously demonstrated (27). This inconsistency may be explained by the 'imperfect complementarity' of the interactions between miRs and their target genes.

It has been suggested that one miR can target ~200 mRNAs, which may be responsible for multiple different proteins, and one mRNA can be regulated by several miRs (28).

In addition to gene regulatory functions, miRs have also been demonstrated to present significant diagnostic, prognostic and therapeutic potential. It was previously confirmed that high expression of miR-16 was associated with a significantly improved survival in advanced non-small cell lung cancer (29), and associated with ovarian cancer survival and recurrence (30). Reid *et al* (31) used synthetic mimics to restore miR-16 expression, which led to growth inhibition in malignant pleural mesothelioma (MPM) cell lines. Intravenous administration of miR-16 mimics in xenograft-bearing nude mice led to consistent and dose-dependent inhibition of MPM tumor growth, suggesting that restoration of expression of miR-16 may be a novel therapeutic approach for MPM.

In conclusion, the present study demonstrated for the first time, to the best of our knowledge, that miR-16 is upregulated in RCC and acts as an oncogene by accelerating cellular proliferation and migration, and by reducing levels of apoptosis. Future research of miR-16 in RCC should aim to clarify the molecular mechanism underlying RCC carcinogenesis and aid in the development of novel biomarkers and therapeutic options.

Acknowledgements

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