Oncogenic microRNA-142-3p is associated with cellular migration, proliferation and apoptosis in renal cell carcinoma

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Abstract. MicroRNAs (miRNAs/miRs) serve an important role in the regulation of carcinogenic pathways. RCC is the most prevalent kidney cancer that occurs in adults. miRNAs have gained increasing attention due to their association with RCC tumorigenesis, serving as biomarkers for early detection and progression monitoring, and as potential targets for molecular therapy. Upregulation of miRNA-142-3p has been previously identified in RCC tissues by microarray profile, however, its expression and function in RCC have not yet been validated. In the present study, quantitative polymerase chain reaction was performed to quantify the relative expression of miR-142-3p in 53 paired RCC and adjacent normal tissues. Furthermore, wound healing, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and flow cytometry assays were performed to analyze the impacts of miR-142-3p on cellular migration, proliferation and apoptosis. The results demonstrated that miR-142-3p was significantly upregulated in RCC tissues compared with adjacent normal tissues. Downregulation of miR-142-3p, induced by chemically synthesized miR-142-3p inhibitor, significantly suppressed cell migration and proliferation, and promoted cell apoptosis in 786-O and ACHN cells, supporting the theory that miR-142-3p may function as an oncogene in RCC. The potential clinical significance of miR-142-3p, as a biomarker and therapeutic target, provides rationale for further investigation into the miR-142-3p-mediated molecular pathway and how it is associated with RCC development.

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Introduction

MicroRNAs (miRNAs/miRs) are ~22-nt, single-stranded, non-coding RNAs that serve an important role in regulating carcinogenic pathways (1,2). miRNAs may lead to messenger RNA (mRNA) degradation or translational repression by binding to the 3'-untranslated regions of target genes, and are involved in a wide range of biological and pathological processes, including cell differentiation, migration, growth, proliferation, apoptosis and metabolism (3-6). miRNA expression is often dysregulated in various types of cancer, with either an oncogenic function when overexpressed or a tumor suppressor function when downregulated (2,7,8). Whether miRNA functions as an oncogene or tumor suppressor gene is primarily dependent on the regulation of its target genes. Due to the presence of complementarity between miRNAs and target gene mRNAs, each miRNA may regulate multiple mRNAs and each mRNA may be targeted by multiple miRNAs (7). miRNAs exert their function in a temporal- and tissue-specific manner (8). In clinical practice, miRNAs may prove invaluable, being exploited as biomarkers for diagnostic, prognostic and monitoring purposes. Additionally, miRNAs may serve as possible targets for novel therapeutic approaches, particularly in patients with tumor subtypes that do not respond to therapies presently available (1).

Kidney cancer is the 14th most prevalent cancer in the world, with incidence and mortality rates that have recently plateaued in Europe and North America, but continue to increase in developing countries (9,10). Renal cell carcinoma (RCC) is the most frequent kidney cancer that develops in adults, accounting for ~90% of all renal tumors and 3.9% of all cancers (11,12). The three most prevalent histological

subtypes of RCC include clear cell RCC (ccRCC), papillary RCC and chromophobe RCC, with a prevalence of 70, 10 and 5%, respectively (13). RCCs are clinically silent at their earliest stages, therefore, at the time of the initial diagnosis, up to 30% of patients have already progressed to a locally advanced disease state or exhibit metastases (14). If detected promptly, early-stage RCC is curable in >90% of cases (15). In 20-40% of patients, a recurring disease will develop following surgical nephrectomy due to the lack of effective adjuvant therapy, including chemotherapy or radiotherapy (16).

Therefore, miRNAs have gained increasing attention as important factors associated with RCC tumorigenesis and development, serving as biomarkers for early detection and progression monitoring, and as potential targets for molecular therapy (16,17). However, to the best of our knowledge, the expression and function of miR-142-3p in RCC has not yet been fully investigated. Previous microarray chip studies have demonstrated that miR-142-3p is overexpressed in RCC tissues compared with adjacent normal or benign kidney tissues (18-21). It has also been reported that miR-142-3p is dysregulated in malignancies of the breast (22), thyroid (23), liver (24), stomach (25), lung (26), blood (27,28), colorectum (29), testes (30), esophagus (31), head and neck (32), and bone (33). The present study establishes the oncogenic role of miR-142-3p in RCC, demonstrating how it regulates cell migration, proliferation and apoptosis.

Materials and methods

Cell culture and transfection. Human RCC cells (786-O and ACHN) and normal human embryo kidney cells (293T) from the Guangdong and Shenzhen Key Laboratory of Male Reproductive Medicine and Genetics (Shenzhen, China) were seeded and grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum, and 1% glutamine and included 100 μg/ml penicillin and 100 mg/ml streptomycin sulfates (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C, in a humidified atmosphere containing 5% CO_2. For the down-regulation of miR-142-3p, synthesized miR-142-3p inhibitor (Shanghai GenePharma, Co., Ltd., Shanghai, China) was transfected into cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), and then the cells were mixed in the Opti-MEM® I Reduced Serum Medium (Gibco; Thermo Fisher Scientific, Inc.) 24 h after plating. Quantitative polymerase chain reaction (qPCR) was used to verify the efficiency of transfection. The sequence of the miR-142-3p inhibitor was 5'-UCC AUA AAG UAG GAA ACA CUA CA-3' and the negative control was 5'-CAG UAC UUU UGU GUA GUA CAA-3'.

Human patient samples. A total of 53 paired RCC tissues and adjacent non-cancerous kidney tissues were collected from patients in the Peking University Shenzhen Hospital (Shenzhen, China), from September 2012 to November 2014. Written informed consent was provided by all patients. Histological and pathological diagnostics for patients with RCC were determined according to the 2009 American Joint Committee on Cancer staging system (34). The patients with RCC had not been treated with either chemotherapy or radiotherapy prior to tissue sampling. Tissue samples were snap-frozen and stored in a cryo freezer at -80˚C in RNAlater® RNA Stabilization Agent (Qiagen, Inc., Valencia, CA, USA) for further research. The clinicopathological information of all the patients is presented in Table I.

Table I. Clinicopathological features observed in renal cell carcinoma patients.

Characteristic	Value
Mean age (range), years	49 (27-71)
Gender, n	
Male	35
Female	18
Histological type, n	
Clear cell	42
Papillary	11
pT-stage, n	
T1	30
T2	20
T3+T4	3
Fuhrman grade, n	
I	18
II	23
III	9
IV	3
AJCC clinical stage, n	
I	31
II	19
III+IV	3

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

RNA isolation, cDNA synthesis and qPCR. Total RNA was extracted from the cell lines and tissues using TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and was purified using the RNeasy® Maxi kit (Qiagen, Inc.) according to the manufacturer's protocols. The amount of RNA was measured on a NanoDrop 2000c (Thermo Fisher Scientific, Inc.) and the RNA samples with 260/280 ratios of 1.8-2.0 were utilized for further experiments. Synthesis of cDNA with reverse transcriptase was performed with the miScript II RT kit (Qiagen, Inc.). The sequence of the forward primer for miR-142-3p was 5'-TGT AGT GTT TCC TAC TTT ATG GA-3' and the reverse primer was provided by the miScript SYBR® Green PCR kit (Qiagen, Inc.). qPCR analysis was performed using the same kit, according to the manufacturer's protocols, on the LightCycler® 480 Real-Time PCR system (Roche Diagnostics, Basel, Switzerland). The thermal cycling conditions for PCR were as follows: 95˚C for 15 min, followed by 40 cycles of 94˚C for 15 sec, 55˚C for 30 sec and 72˚C for 30 sec. Quantification cycle (Cq) values of miR-142-3p were equilibrated to U6, which was used as an internal control. The forward primer of U6 was 5'-CTC GCT TCG GCA GCA CA-3' and the reverse primer was 5'-ACG CTT CAC GAA TTT GCG T-3'. Relative expression was calculated using the $\Delta\Delta$Cq method.

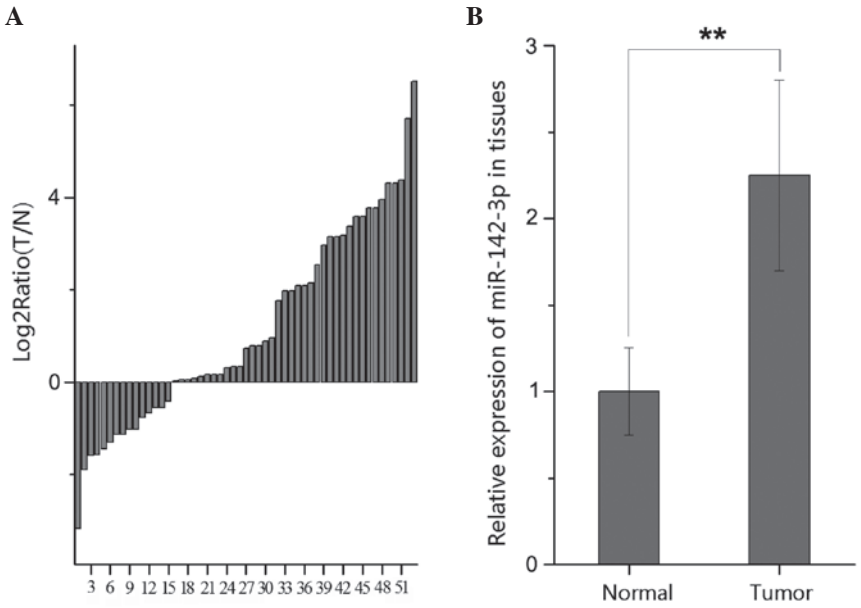

Figure 1. Expression of miR-142-3p in 53 paired RCC and adjacent normal tissues. (A) Log2 ratios of miR-142-3p expression in 53 paired RCC tumor (T) tissues to normal (N) kidney tissues. (B) Relative expression of miR-142-3p in normal and RCC tissues. miR, microRNA; RCC, renal cell carcinoma. **P<0.01.

Wound healing assay. A wound scratch assay was performed to evaluate the migratory ability of the 786-O and ACHN cells *in vitro*. A total of ~3x10^5 cells were seeded into each well within a 12-well dish, and each was transfected with either 100 pmol of chemically synthesized miR-142-3p inhibitor or a negative control, using Lipofectamine 2000. Following 6 h of transfection, a sterile 200-μl pipette tip was used to scratch a clear line through the cell layer. The cells were then rinsed with phosphate-buffered saline (PBS) and cultured in serum-free DMEM at 37˚C in a humidified chamber, containing 5% CO_2. Using a digital camera system, images were captured at 0, 24 and 48 h after the scratches were made. The experiments were performed in triplicate and repeated ≥3 times.

Cell proliferation assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT assays were conducted to analyze the proliferation ability of the 786-O or ACHN cells. A total of ~5x10^3 786-O or ACHN cells were seeded into each well within 96-well plates, with 5 replicate wells of each condition. Each well was transfected with either 5 pmol miR-142-3p inhibitor, or a negative control, and proliferation was measured at 0, 24, 48 or 72 h post-transfection. Prior to measurement, 20 μl MTT (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to each well, with the 96-well plates then incubated at 37˚C in a humidified chamber, containing 5% CO_2, for 6h. Subsequently, the MTT medium mixtures were discarded and 120 μl dimethylsulfoxide (Sigma-Aldrich, Shanghai, China) was added. Following agitation for 30 min at room temperature, the optical density values were measured by the iMark Microplate Absorbance Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a wave length of 490 nm, with 630 nm serving as the reference wave length.

Flow cytometry assay. The Coulter® Epics XL® flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) was used to quantify the early apoptosis rate of the 786-O and ACHN cells, with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining (Invitrogen; Thermo Fisher Scientific, Inc.). A total of ~3x10^5 786-O or ACHN cells were seeded into 6-well plates for the cell apoptosis assay. The cells were transfected with 200 pmol miR-142-3p inhibitor, or the negative control, for 6 h. Following a 48-h transfection, the cells, including floating cells, were harvested, washed twice with 4˚C PBS and resuspended in 100 μl 1X binding buffer at a concentration of at least 3x10^6 cells/ml. This suspension (100 μl) was stained with 5 μl Annexin V-FITC and 5 μl PI for 15 min at room temperature in the dark. Following the addition of 400 μl binding buffer to each tube, the cells were analyzed by flow cytometry. Each experiment was performed at least 3 times.

Statistical analysis. For the comparison of miR-142-3p expression levels in matched tumor and normal samples, a paired t-test was performed. The relative expression of miR-142-3p in tissues was presented as the mean ± standard error. All other data were presented as the mean ± standard deviation from the three independent experiments. Statistical analysis was performed using SPSS software, version 19.0 (IBM SPSS, Armonk, NY, USA). Statistical significance was determined with Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-142-3p is upregulated in RCC tissues compared with adjacent normal tissues. A total of 53 paired RCC and adjacent normal tissues were analyzed using qPCR in order to quantify the expression pattern of miR-142-3p in each tissue type. The results demonstrated that the expression of miR-142-3p [Log2 (T/N)] was generally increased (38/53) in the tumors in comparison with the paired normal samples (Fig. 1A). The

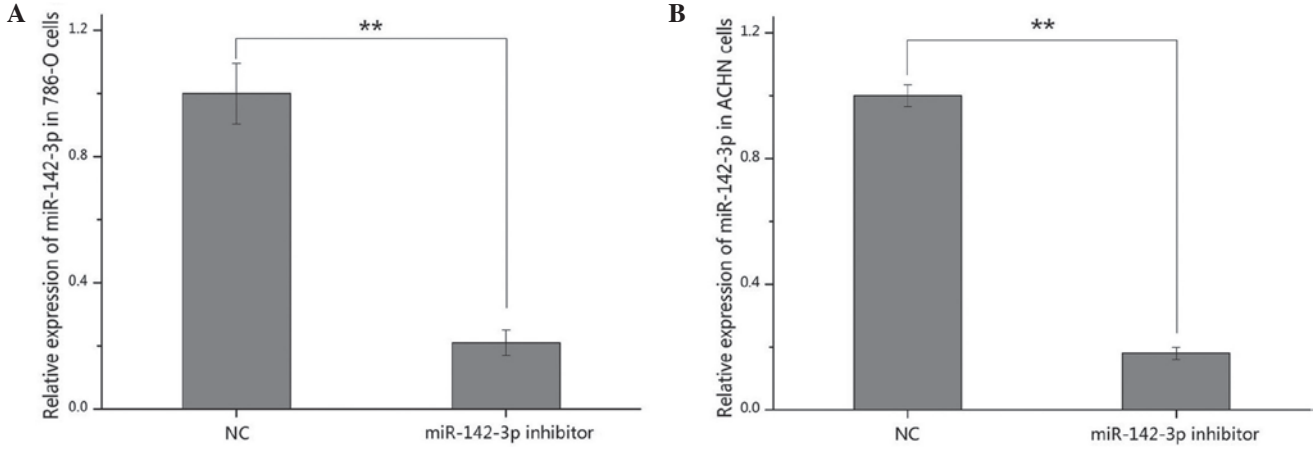

Figure 2. Validation of cell transfection efficiency. Relative expression of miR-142-3p in (A) 786-O and (B) ACHN cells transfected with either miR-142-3p inhibitor or a negative control. miR, microRNA; NC, negative control. **P<0.01.

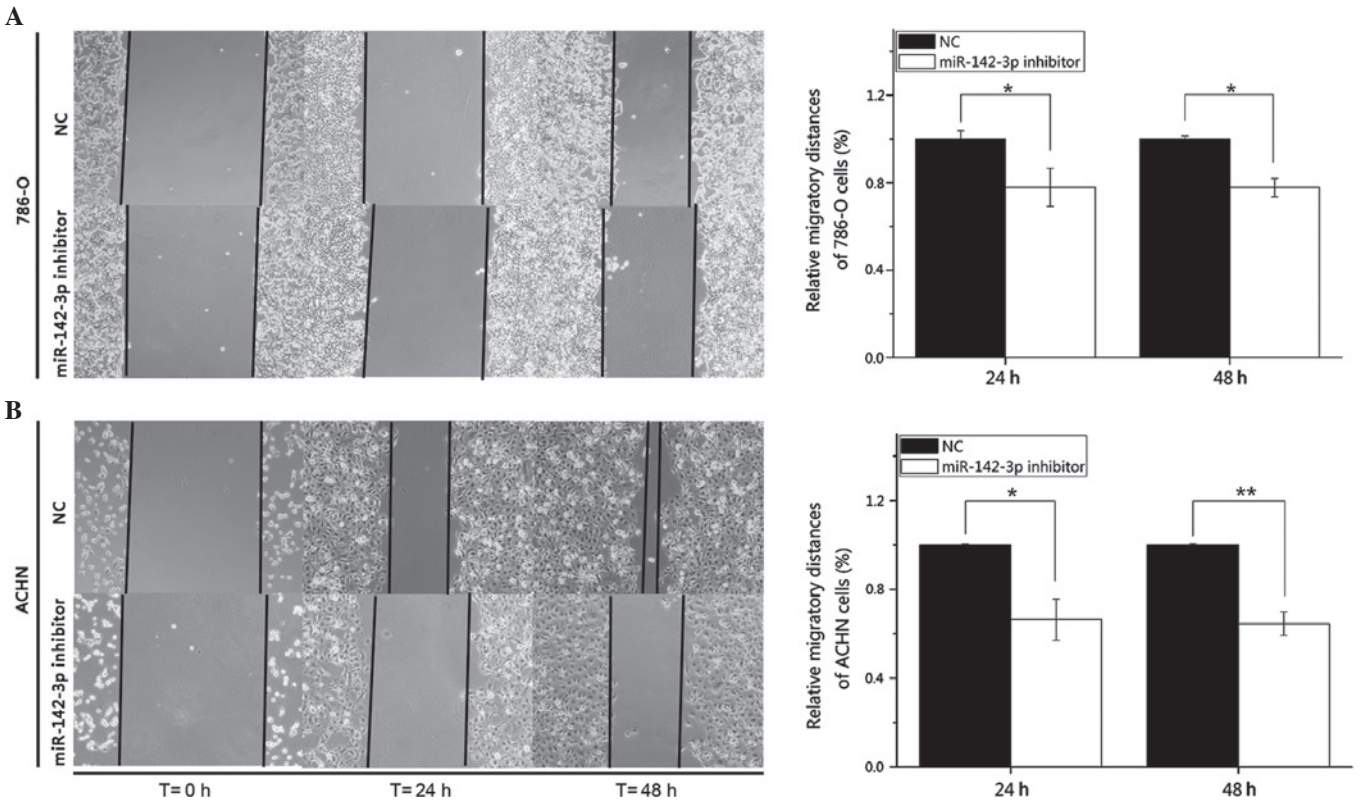

Figure 3. Wound healing assay. (A) Images of 786-O cells transfected with miR-142-3p inhibitor or negative control at 0 h, 24 h and 48 h after the scratches were made at the same point and statistical analysis of relative migratory distance at 24 h (P<0.05) and 48 h (P<0.05). (B) Images of the ACHN cells transfected with miR-142-3p inhibitor or negative control at 0 h, 24 h and 48 h after the scratches were made at the same point and statistical analysis of relative migratory distance at 24 h (P<0.05) and 48 h (P<0.01). miR, microRNA; NC, negative control. *P<0.05 and **P<0.01.

present study demonstrated that the relative expression of miR-142-3p in the RCC tissues was significantly overexpressed when compared with the adjacent normal tissues (P<0.01), as presented in Fig. 1B. Such results indicated that miR-142-3p may act as an oncogene during RCC development. However, the function of miR-142-3p required further investigation.

Validation of cell transfection efficiency. The transfection efficiency of miR-142-3p inhibitor was quantified by qPCR, whilst comparisons were made with a negative control. The results indicated that miR-142-3p was downregulated by 79.04 and 82.02% compared with the negative control, following transfection in the 786-O (P<0.01; Fig. 2A) and ACHN cells (P<0.01; Fig. 2B), respectively.

miR-142-3p inhibitor suppresses 786-O and ACHN cell migration. Wound healing assays were performed to observe the function of miR-142-3p in cell migration. Images of each wound were captured at 0, 24, and 48 h post-transfection using a digital camera system (Fig. 3). The wounds of cells transfected with miR-142-3p inhibitor were wider than those of cells transfected with the negative control. Statistical

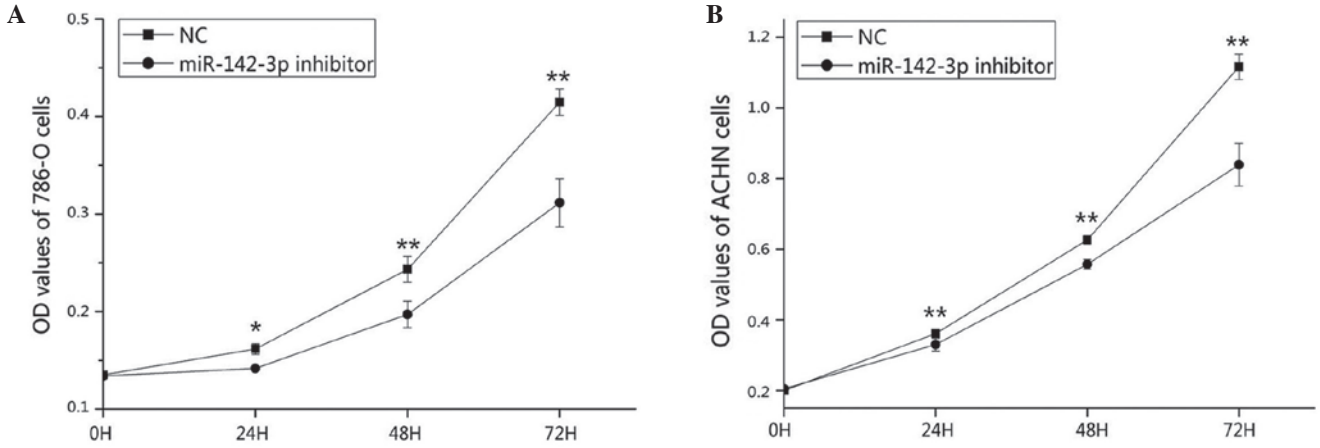

Figure 4. Cell proliferation of (A) 786-O and (B) ACHN measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay at different time intervals. miR, microRNA; NC, negative control; OD, optical density. *P<0.05 and **P<0.01.

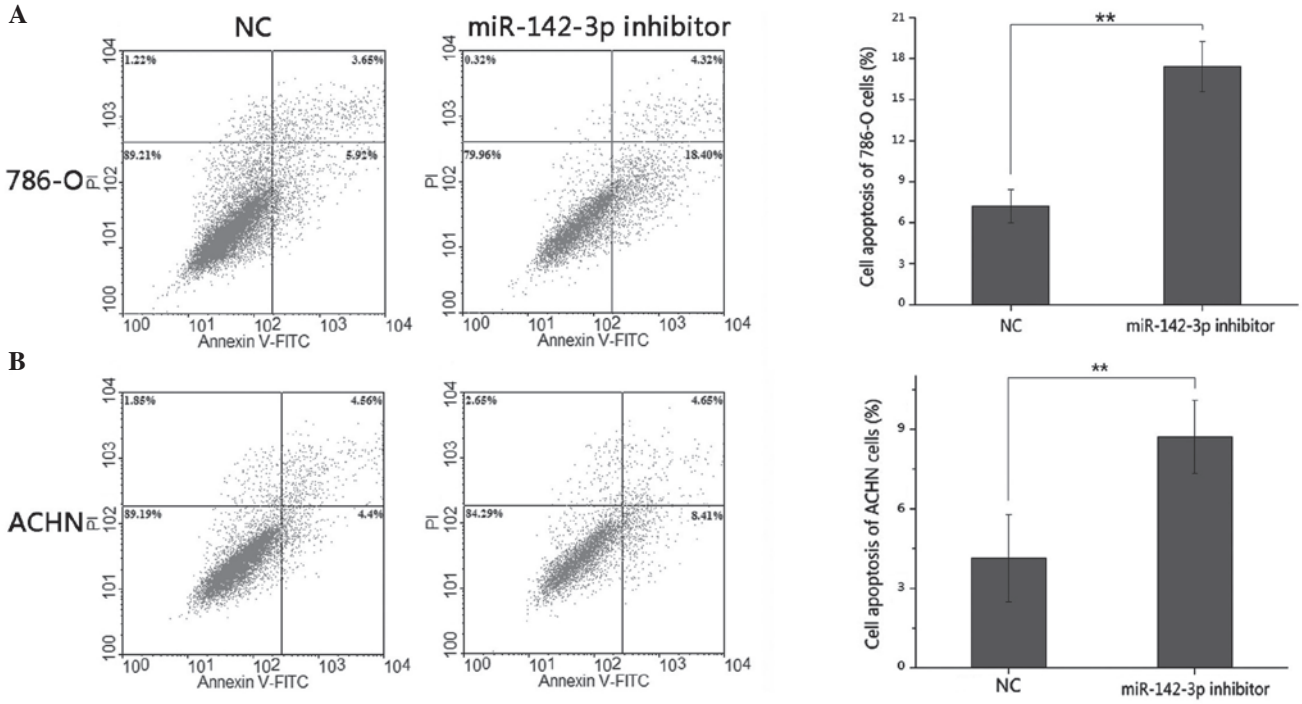

Figure 5. Cell apoptosis (%) of (A) 786-O and (B) ACHN was measured by flow cytometry. miR, microRNA; NC, negative control; FITC, fluorescein isothiocyanate; PI, propidium iodide. **P<0.01.

analysis demonstrated that the migratory distances of the miR-142-3p inhibitor group were significantly decreased by 22.11% (P<0.05) and 22.26% (P<0.05) for the 786-O cells, and by 33.66% (P<0.05) and 35.47% (P<0.01) for the ACHN cells at 24 and 48 h post-transfection, in comparison to the negative control group. Such results suggested that the downregulation of miR-142-3p inhibited the migratory ability of the RCC cells.

miR-142-3p inhibitor inhibits 786-O and ACHN cell proliferation. MTT assays were performed to determine if the downregulation of miR-142-3p had an impact on the proliferation of the RCC cells. The results demonstrated that the proliferation of the 786-O cells decreased by 10.15% (P<0.05), 19.04% (P<0.01) and 24.84% (P<0.01), and that the proliferation of the ACHN cells decreased by 8.59% (P<0.01), 11.02% (P<0.01) and 24.82% (P<0.01), at 24, 48 and 72 h post-transfection of the miR-142-3p inhibitor, as compared with the negative control. The results indicated that the inhibition of miR-142-3p expression significantly reduced the proliferation of the RCC cells (Fig. 4).

miR-142-3p inhibitor promotes 786-O and ACHN cell apoptosis. The effects of the miR-142-3p inhibitor on apoptosis were determined by flow cytometric analysis. The results demonstrated that the average early apoptosis rate of the 786-O cells, transfected with miR-142-3p inhibitor or negative control, was 17.40 vs. 7.20% (P<0.01), whilst the average apoptotic rate of the ACHN cells was 8.71 vs. 4.14% (P<0.01).

This data indicated that the downregulation of miR-142-3p promoted RCC cell apoptosis (Fig. 5).

Discussion

The initiation and development of cancer involves the activation of a number of oncogenes and the dysfunction of numerous tumor suppressor genes. miRNAs have been identified to serve important roles in various types of cancer, regulating ~50% of human genes (5). Recent genomic studies of ccRCC have advanced our understanding of the molecular background and pathways, and the possible genetic alterations, implicated in ccRCC tumorigenesis (35). The most notable gene pathways involved in RCC tumorigenesis include the Von Hippel-Lindau (VHL)/hypoxia inducible factor (HIF), vascular endothelial growth factor (VEGF) and mechanistic target of rapamycin (mTOR) pathways. Therapies targeting these genes have been introduced into clinical practice. miRNAs target numerous genes, including HIF, mTOR, VEGF and VHL, through translational inhibition or the induction of mRNA degradation, which suggests that miRNAs function as important factors in RCC initiation and development (36,37).

Previous studies have reported that miR-142-3p is dysregulated in various types of cancer (22-33), and may therefore function as a biomarker. The expression of miR-142-3p in RCC has not yet been validated by qPCR. However, recent microarray analyses comparing RCC and adjacent normal or benign kidney tissues demonstrated that miR-142-3p was significantly overexpressed in tumors when compared with paired control groups (18-21), suggesting that miR-142-3p may function as an oncogene in RCC development. Therefore, in the present study, qPCR was performed to quantify the relative expression of miR-142-3p in 53 paired RCC and adjacent normal tissues. Furthermore, the impacts of miR-142-3p on cellular migration, proliferation and apoptosis were analyzed through the use of wound healing, MTT and flow cytometry assays. The results demonstrated that miR-142-3p was significantly upregulated in RCC tissues in comparison to adjacent normal tissues. Downregulation of miR-142-3p, induced by the chemically synthesized miR-142-3p inhibitor, significantly suppressed cell migration and proliferation, and induced cell apoptosis in the 786-O and ACHN cells, suggesting that miR-142-3p may function as an oncogene during RCC tumorigenesis. However, the miR-142-3p-mediated molecular pathways that affect cell migration, proliferation and apoptosis remain unclear.

The various molecular mechanisms of miR-142-3p have been reported to affect tumor initiation, development, metastasis and invasion differentially, depending on the type of cancer that is present. This is believed to be due to the target genes involved being dependent on the type of cancer. In addition, different stages of cancer development exhibit the tissue- and temporal-specific manner of the function of miRNAs (8). For example, in breast cancer, overexpressed miR-142-3p regulates the properties of breast cancer stem cells (BCSCs), at least in part by activating the WNT signaling pathway and miR-150 expression. Previous *in vivo* experiments demonstrated that the enforced expression of miR-142 in normal mouse mammary stem cells resulted in the regeneration of hyperproliferative mammary glands, whilst the knockdown of endogenous miR-142 effectively suppressed organoid formation by BCSCs, and also slowed tumor growth initiated by human BCSCs (22). Downregulation of miR-142-3p in thyroid neoplastic tissues contributes to thyroid follicular tumorigenesis through the targeting of ASH1L and MLL1, and subsequently promotes its tumor suppressive function (23). In non-small cell lung cancer, overexpression of miR-142-3p represses transforming growth factor (TGF)-induced growth inhibition through the repression of TGFβ receptor 1 (26). However, a previous study investigating miR-142-3p in human acute lymphoblastic leukemia demonstrated that miR-142-3p inhibits cell proliferation by targeting the MLL-AF4 oncogene (27). miR-142-3p-mediated loss of protein tyrosine phosphatase non-receptor type 23 (PTPN23) expression may also be a key event in the pathogenesis of testicular germ cell tumors (30). By targeting the oncogenes cluster of differentiation 133, ATP-binding cassette, subfamily G, member 3 and leucine-rich repeat-containing G protein-coupled receptor 5, miR-142-3p functions as a tumor suppressor in colon cancer cells (38). Other than its roles in tumorigenesis, miR-142 is also important for megakaryopoiesis, with the genetic ablation of miR-142 responsible for impaired megakaryocyte maturation, the inhibition of polyploidization, abnormal proplatelet formation and thrombocytopenia, through the orchestration of an actin cytoskeleton network (39).

In clinical application, the reduced expression of miR-142-3p in hepatocellular carcinoma and its overexpression in esophageal squamous cell carcinoma was significantly associated with reduced survival, suggesting that miR-142-3p may function as a prognostic predictor (24,31). Serum miR-142-3p was identified to be associated with a high recurrence risk in patients with early-stage lung adenocarcinoma, and was a putative serum marker for risk assessment (40). The expression levels of miR-142-3p and miR-29a in peripheral blood mononuclear cells may be used as novel diagnostic markers with ~90% sensitivity and ~100% specificity for the diagnosis of acute myeloid leukemia (41). During human bronchial squamous carcinogenesis, miR-142-3p, which is typically upregulated during lung development, was first downregulated at the earliest stages of carcinogenesis, and was also subsequently upregulated during later stages, suggesting that the expression of miR-142-3p may be monitored to assess cancer development (42).

In conclusion, the present study demonstrated the expression and function of miR-142-3p in RCC tumorigenesis. The oncogenic function of miR-142-3p was indicated by its upregulated expression in RCC tissues, its inhibition of cellular migration and proliferation, and the reduction in cell apoptosis induced by an miR-142-3p inhibitor. Further investigation is required to analyze the miR-142-3p-mediated molecular pathway and its role in RCC development, with its potential to aid early disease detection and prognosis prediction, whilst serving as a therapeutic target, thus proving its clinical significance.

Acknowledgements

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