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Original Article

microRNA-451 inhibited cell proliferation, migration and invasion through regulation of MIF in renal cell carcinoma

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Abstract: The expression and functions of microRNA-451 have been studied in many human cancers. However, up to date, there is no study of microRNA-451 in renal cell carcinoma. In the present study, we aimed to investigate the expression, biological functions and molecular mechanisms of microRNA-451 in renal cell carcinoma. microRNA-451 expression level in renal cell carcinoma tissues and cell lines was measured using quantitative Real-time PCR. By using CCK8 assay, cell migration and invasion assay, we explored the functions of microRNA-451 in renal cell carcinoma. Dual-Luciferase report assay, quantitative Real-time PCR and western blot were performed to explore the molecular mechanisms of microRNA-451 functions in renal cell carcinoma. Functional assays were also performed to explore the effects of endogenous MIF in renal cell carcinoma. In this study, we showed for the first time that microRNA-451 was significantly down-regulated in renal cell carcinomas tissues and cell lines. microRNA-451 expression level was correlated with histological grade and lymph node metastasis. In addition, microRNA-451 inhibited proliferation, migration and invasion of renal cell carcinomas cells. Moreover, MIF was identified as a target of microRNA-451, and down-regulation of MIF could mimic the suppressive functions of microRNA-451 in renal cell carcinomas, suggesting that microRNA-451 might be a novel therapeutic strategy for the treatment of renal cell carcinomas.

Keywords: Renal cell carcinoma, RCC, MIF, microRNA-451, targeted therapy

Introduction

Renal cell carcinoma (RCC), the most common subtype of kidney cancer, accounts for 2-3% of all adult malignancies [1]. In USA, it is estimated that there would be approximately 30,000 new cases and 12,000 deaths due to RCC each year [2]. Clear cell RCC (ccRCC) is the most common subtype of RCC and accounts for 80%-90% of all RCC cases [3]. Certain environmental and genetic factors have been demonstrated as risk factors for RCC. However, the molecular mechanisms contributed to RCC carcinogenesis and development are still unclear [4].

Currently, radical or partial nephrectomy remains definitive and most effective therapeutic treatment for RCC patients with noninvasive disease [5]. Even so, approximately 20%-40% RCC patients will develop recurrence or metastasis after surgical resection [6]. This mainly dues to tumor's highly resistant phenotype to chemotherapy and radiation [7]. The 5-year overall survival of RCC with non-metastatic is approximately 80%, whereas 5-year overall survival of RCC patients with metastasis is less than 10% [8]. More than 30% patients are present with local or systematic metastasis at diagnosis [9]. Therefore, a better understanding of the molecular mechanisms underlying the carcinogenesis and progression of RCC is essential for developing new targeted therapies.

Many studies have focused on expression and functions of microRNAs (miRNAs) in many kinds of human cancers, also including RCC [8, 10, 11]. miRNAs are a group of non-coding, endogenous and short single stranded ncRNA molecules that approximately 18-25 nucleotide in

length [12]. They regulate target mRNAs expression through binding to the 3'untranslated regions (3'UTRs) of mRNAs and resulting in transcript degradation, translational repression, and gene silencing [13, 14]. A growing body of evidences have demonstrated that miRNAs can regulate many normal crucial biological processes and pathological processes, such as cell proliferation, apoptosis, cell cycle, differentiation, migration, invasion and metabolism, and so on [15, 16]. miRNAs can function as tumor suppressors or oncogenes in various kinds of tumors depending on its expression and target mRNAs [17]. miRNAs, down-regulated in cancers, decrease target oncogenes expression and function as tumor suppressors. Meanwhile, up-regulated miRNAs suppress tumor suppressor genes expression and act as oncogenes [18]. Hence, further exploration of the functions and the target of miRNAs would provide insight into the mechanisms of RCC carcinogenesis and progression. It also suggested that miRNA might be a target for RCC therapy.

The purpose of this study was to investigate the expression and functions of miR-451 in RCC. In this study, we found that miR-451 was down-regulated in RCC and expression level of miR-451 was associated with histological grade and lymph node metastasis. In addition, the molecular mechanisms underlying cell proliferation, migration and invasion in RCC was also explored. Our results revealed that macrophage migration inhibitory factor (MIF) was a direct target of miR-451. These findings suggested that miR-451 could be investigated as a targeted therapy for RCC.

Material and methods

Clinical samples

This study was approved by Yidu Central Hospital of Weifang Ethics Committees. Prior written informed consent was also obtained from each RCC patients in this study. A total of 79 pairs of RCC tissues and corresponding normal adjacent tissues (NATs) were obtained from RCC patients who had undergo radical or partial nephrectomy at Yidu Central Hospital of Weifang from 2010 to 2014. All these RCC patients were not received other therapeutic treatments before surgery. All tissues were immediately snap-frozen at liquid nitrogen and transferred to -80°C refrigerator.

Cell culture

The human RCC-derived cell lines (786-O, A498, Caki-1 and Caki-2) and HK-2 cell lines were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). HK-2, used as control, is originated from normal kidney cortex/proximal tubule. HEK293T cell line, used for Dual-Luciferase report assay, was purchased from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). HK-2 cells were maintained in keratinocyte-SFM (Gibco, Carlsbad, CA, USA). The other cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco Grand Island, NY) medium supplemented with 10% heat-inactivated fetal-calf-serum (FBS; Gibco, Grand Island, NY), 100 U/mL penicillin (Gibco, Grand Island, NY) and 100 mg/L streptomycin (Gibco, Grand Island, NY). All cell lines were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Transfection

miR-451 mimics, scrambled control (NC), miR-451 inhibitor, NC inhibitor and the luciferase reporter plasmid were obtained from GenePharma Co., Ltd., Shanghai, China. MIF siRNA and NC siRNA were obtained from Guangzhou RiboBio Co., Ltd (Guangzhou, China). Cell transfection and co-transfection were conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), following to the manufacturer's protocol.

RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA was isolated from tissues and cells using TRIzol (Invitrogen, Carlsbad, CA, USA), following to the manufacturer's instructions. The total RNA concentrations were measured with a biophotometer (Eppendorf). TaqMan assays (Applied Biosystems) were used to measure miR-451 expression according to the manufacturer's protocol. U6 snRNA was used as control for miR-451 expression. For MIF mRNA expression, reverse transcribed was performed using M-MLV Reverse Transcription system (Promega, USA). qRT-PCR was conducted using the SYBR Green Master Mix (Takara, China). GAPDH was used as an endogenous control for MIF mRNA expression.

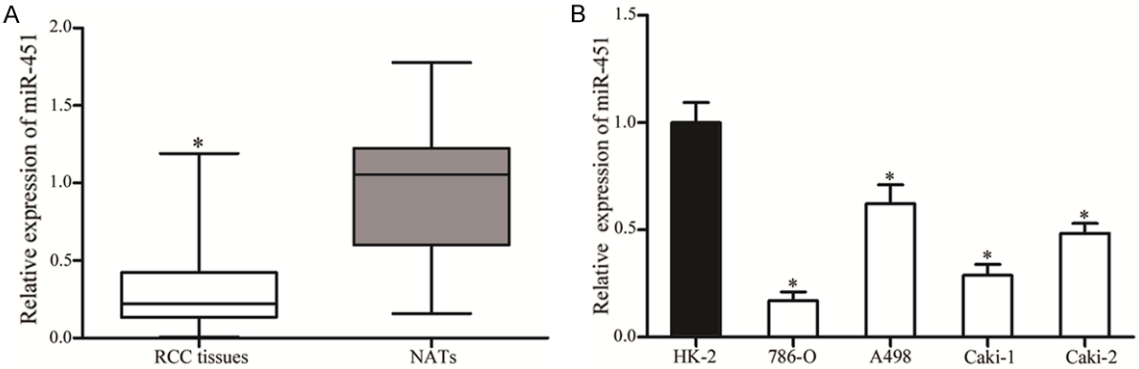

Figure 1. miR-451 expression level in RCC tissues and cell lines. A. miR-451 expression level was decreased in RCC tissues in comparison to matched NATs. B. miR-451 was down-regulated in RCC cell lines compared with HK-2. $^{*}P<0.05$ compared with their respective controls.

Table 1. A comparison of miR-451 expression in RCC and clinicopathological factors

Clinical features	Case number	miR-451 expression		*p*-value
		Low	High	
Sex				0.926
Male	45	26	19	
Female	34	20	14	
Age				0.580
<60 years	45	25	20	
≥60 years	34	21	13	
Tumor size				0.231
<4 cm	54	29	25	
≥5 cm	25	17	8	
Histological grade				0.002
I-II	49	22	27	
III-IV	30	24	6	
Tumor stage				0.986
T1-T2	36	21	15	
T3-T4	43	25	18	
Lymph node metastasis				0.013
Negative	47	22	25	
Positive	32	24	8	

CCK8 assay

Cell viability was measured using the Cell counting kit 8 (CCK8; Dojindo, Kumamoto, Japan) assay. Transfected cells were collected and seeded into 96-well plate at a cell density of 3000 cells per well. After transfection 24 h, 48 h, 72 h and 96 h, CCK8 assay was performed. In briefly, 10 μl CCK8 assay solution was added into each well and incubated for 2 h. The absorbance was measured at 450 nm for each well using a multi-well spectrophotometer.

Cell migration and invasion assay

Cell migration and invasion assays were performed using transwell chambers (Millipore, Billerica, MA, USA) with an 8-μm pore polycarbonate membrane. Transfected cells were collected and resuspended in FBS-free DMEM. 1×10^5 cells in 200 μl medium were added into the upper transwell chamber. 500 μl DMEM containing 20% FBS was added to the lower chamber. For invasion assay, transwell chambers were pre-coated with Matrigel (BD Biosciences, San Jose, CA). Except for this procedure, the migration assay and the invasion assay were performed with the same procedure. Transwell chambers were incubated in cell incubator at 37°C for 24 h. Then, chambers were fixed with 95% ethanol, stained with 0.1% crystal violet and washed with phosphate-buffered saline (PBS). Non-migrating and non-invading cells were removed carefully with a cotton swab. The migrated or invaded cells were counted with a light microscope.

Western blot

Total proteins were isolated from cells using RIPA lysis buffer. BCA assay (Thermo Fisher Scientific, Inc., Rockford, IL, USA) was adapted to measure total proteins concentration. Equal

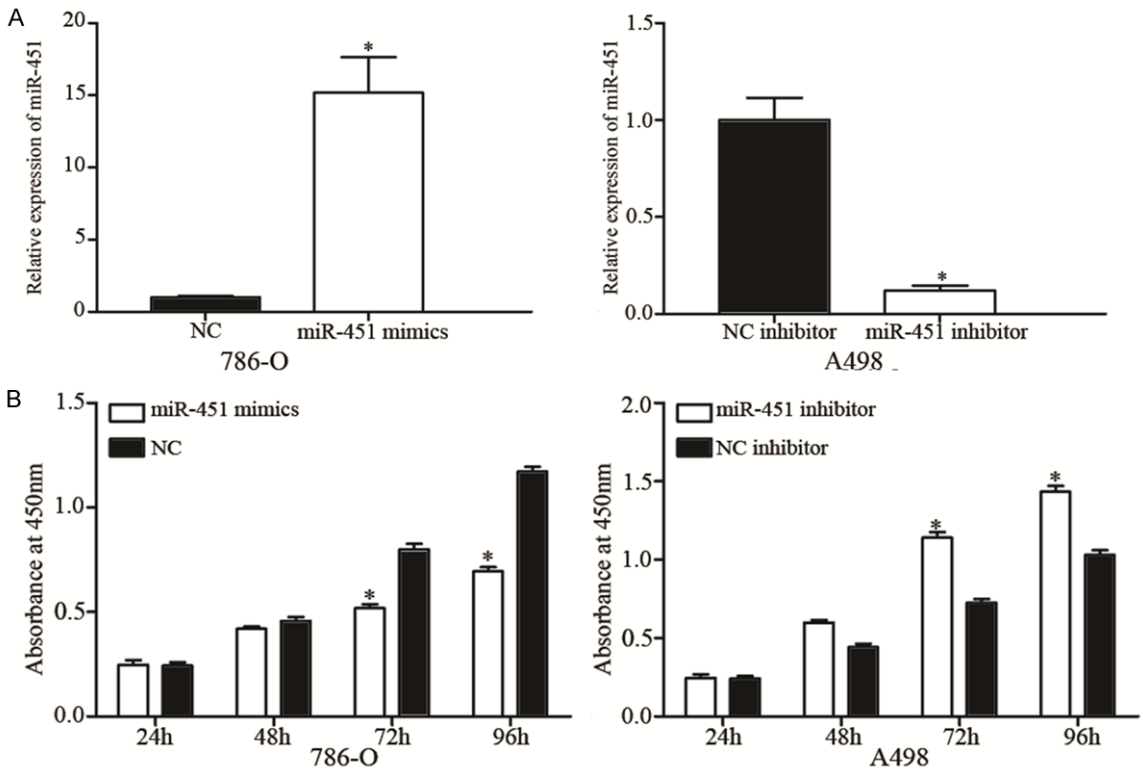

Figure 2. miR-451 inhibited proliferation of RCC cells. A. miR-451 was obviously up-regulated in 786-O cells after transfection with miR-451 mimics. miR-451 was down-regulated in A498 cells after transfection with miR-451 inhibitor. B. CCK8 assay showed that miR-451 mimics inhibited 786-O cells proliferation, whereas miR-451 inhibitor improved A498 cells growth. $^{*}P<0.05$ compared with their respective controls.

amounts of proteins were separated with 10% SDS-PAGE and then transferred onto PVDF membrane (Millipore, Billerica, MA, USA). The membranes were blocked in T-BST with 5% skim milk at room temperature for 1 hour. The membranes were incubated with primary antibodies, goat anti-human monoclonal MIF and mouse anti-human GADPH, at 4°C for overnight. Then, the membranes were incubated with appropriate peroxidase-conjugated secondary antibody. Antibody binding was visualized with ECL solution (Pierce Biotechnology, Inc., Rockford, IL, USA). GADPH was used as an endogenous control.

Dual-luciferase reporter assay

The HEK293T cells were transfected with miR-451 mimics or NC, and PGL3-MIF-3'UTR Wt or PGL3-MIF-3'UTR Mut using Lipofectamine 2000, according to manufacturer's protocol. After transfection 48 h, firefly and renilla luciferases activities were detected with Dual-Luciferase Reporter Assay System (Promega, Manheim, Germany). The renilla luciferase activities were used as an endogenous control. Each sample was assayed in triplicate.

Statistical analysis

Data were presented as mean ± S.D., and compared using student t test in SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA). Double-tailed *P* value less than 0.05 was considered to be statistically significant.

Results

miR-451 expression level in RCC and its association with clinicopathological factors

miR-451 has been found down-regulated in multiple human cancers. In this study, we measured miR-451 expression level in RCC using qRT-PCR. As shown in **Figure 1A**, miR-451 expression level was significantly decreased in RCC tissues than NATs (P<0.05). Furthermore, we analyzed miR-451 expression in RCC cell

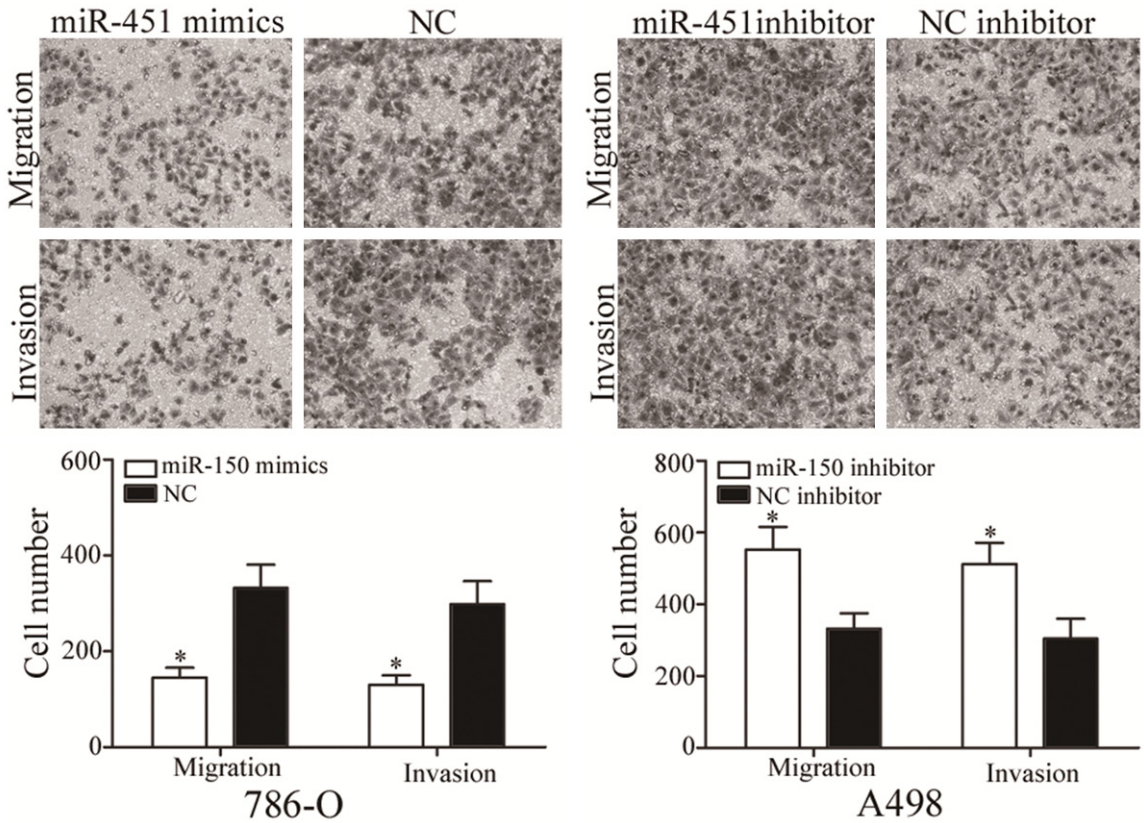

Figure 3. miR-451 inhibited migration and invasion of RCC cells. miR-451 mimics results in a reduced migration and invasion rate in 786-O cells compared with NC. Migration and invasion of A498 cells were improved by miR-451 inhibitor. $^{*}P<0.05$ compared with their respective controls.

lines. We found that miR-451 was down-regulated in RCC cell lines compared with HK-2 (shown in **Figure 1B**, P<0.05).

To determine whether miR-451 expression level was associated with clinicopathological factors, statistical analysis was performed. Significantly, miR-451 expression level was correlated with histological grade and lymph node metastasis (shown in **Table 1**, P<0.05). However, no correlation was found between miR-451 expression level and age, sex, tumor stage and differentiated (P>0.05).

miR-451 inhibited proliferation of RCC cells

To explore the biological functions of miR-451, we transfected RCC cells with miR-451 mimics or miR-451 inhibitor. In RCC cell lines, miR-451 expression level in 786-O was the lowest, while its expression in A498 was the highest. Hence, 786-O was chosen to be trasnfceted with miR-451 mimics and A498 was transfected with miR-451 inhibitor. After transfection 48 h, qRT-PCR was adopted to analyze miR-451 expression. As shown in **Figure 2A**, miR-451 was obviously up-regulated in 786-O cells, whereas miR-451 was down-regulated in A498 cells after transfection (P<0.05).

CCK8 assay was performed to explore the influence of miR-451 on RCC cell growth. As shown in **Figure 2B**, miR-451 mimics significantly inhibited RCC cell growth in 786-O cells, while miR-451 inhibitor enhanced A498 cells growth (P<0.05). These results indicated that miR-451 acted as a tumor growth suppressor in RCC.

miR-451 inhibited migration and invasion of RCC cells

We further investigated the effect of miR-451 on cell migration and invasion. As shown in **Figure 3**, cell migration and invasion assays showed that miR-451 mimics results in a reduced migration and invasion rate in 786-O cells compared with NC.

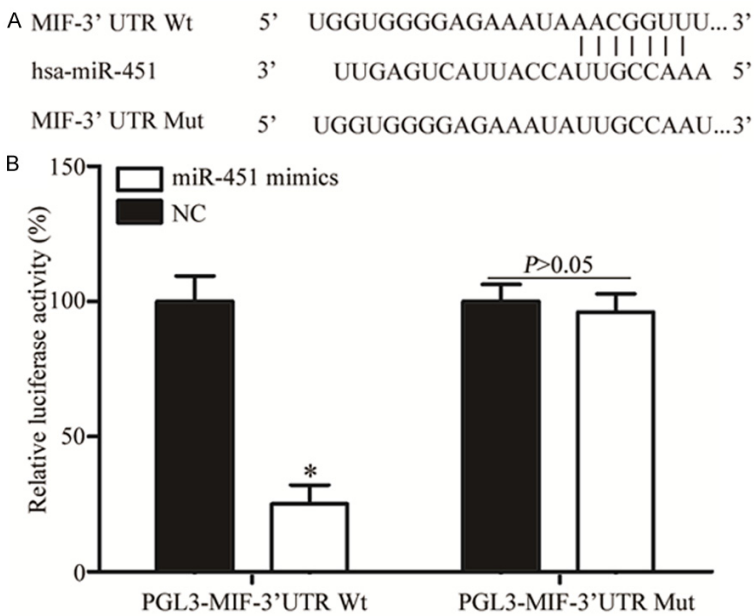

Figure 4. MIF was a direct target gene of miR-451. A. The miR-451 binding site in the 3'UTR of MIF and the MIF 3'UTR mutant sequence. B. Dual-Luciferase report assays revealed that miR-451 significantly suppressed the PGL3-MIF-3'UTR Wt but not the PGL3-MIF-3'UTR Mut luciferase activity in HEK293T cells. *P<0.05 compared with their respective controls.

Furthermore, migration and invasion of A498 cells were improved by miR-451 inhibitor (P<0.05). These results also suggested that miR451 contributed to the migration and invasion inhibition of RCC cells.

MIF was a direct target gene of miR-451.

miRNAs are known to target hundreds of mRNAs and result in expression changes of mRNAs. Initially, TargetScan (http://www.targetscan. org) was used to identify potential target of miR-451. As shown in **Figure 4A**, MIF was predicated as a potential target of miR-451. MIF mRNA contained a miR-451 seed match at position 102-108 of the MIF 3'UTR.

Furthermore, Dual-luciferase reporter assays were conducted to explore whether MIF was a direct target of miR-451 in vitro. As shown in **Figure 4B**, co-transfection of HEK293T cells with miR-451 mimics and PGL3-MIF-3'UTR resulted in obviously decrease in luciferase activity compared with NC (P<0.05). However, luciferase activity was no difference when miR-451 mimics was co-transfection with PGL3-MIF-3'UTR Mut (P>0.05). This result indicated that MIF was a direct target of miR-451 in vitro.

miR-451 negatively regulated MIF expression in RCC cells

To further confirm that MIF was a direct target of miR-451, qRT-PCR and western blot were performed to analyze MIF expression at mRNA and protein level regulated by miR-451 in RCC cells. As presentation in **Figure 5A**, MIF mRNA expression was decreased in 786-O cells after transfection with miR-451 mimics (P<0.05). Meanwhile, miR-451 inhibitor improved MIF mRNA expression in A498 cells (P<0.05). In addition, similar effect was observed in MIF protein expression (shown in **Figure 5B**; P<0.05). Overall, miR-451 regulated MIF expression at mRNA and protein level, and suggesting that MIF was a target gene of miR451.

Knockdown of MIF inhibited proliferation, migration and invasion of RCC cells

To explore the functions of MIF on proliferation, migration and invasion of RCC cells, MIF was knocked down by MIF siRNA. After transfection 72 h, western blot showed that MIF was significantly downregulated in 786-O and A498 cells (shown in **Figure 6A**; P<0.05).

Consistently, MIF siRNA inhibited proliferation, migration and invasion of RCC cells (shown in **Figure 6B** and **6C**; P<0.05). These results suggested that functions of MIF siRNA were similar to those induced by miR-451 in RCC, rendering MIF as a functional target of miR-451 in RCC.

Discussion

It is well known that RCC remains to be one of the leading cause of death [4]. Although great advances have been developed in RCC therapeutic treatments, major limitations in managing RCC still existed [19]. Hence, new targets for diagnosis, prognosis and therapy are still the top priority. Recently, miRNAs have been identified to involve in cancer initiation and progression [20]. Abnormal expression of miRNAs

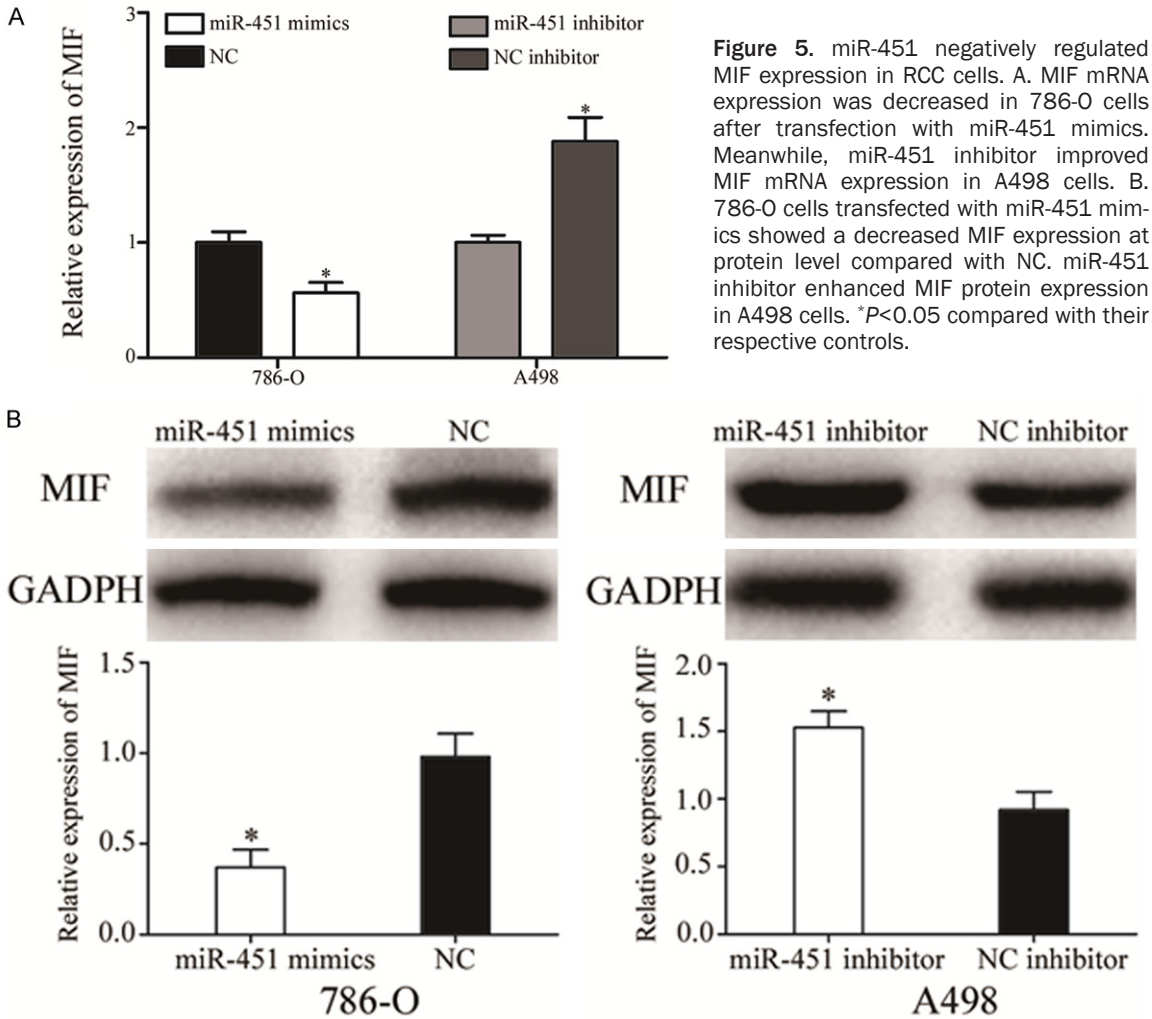

Figure 5. miR-451 negatively regulated MIF expression in RCC cells. A. MIF mRNA expression was decreased in 786-O cells after transfection with miR-451 mimics. Meanwhile, miR-451 inhibitor improved MIF mRNA expression in A498 cells. B. 786-O cells transfected with miR-451 mimics showed a decreased MIF expression at protein level compared with NC. miR-451 inhibitor enhanced MIF protein expression in A498 cells. $^{*}P<0.05$ compared with their respective controls.

has been found between normal and cancer tissues, suggesting a feasible association between miRNAs and carcinogenesis [21]. Increasing evidences have demonstrated that global downregulation of miRNAs expression was a rising feature of RCC [8, 10, 22]. In this study, we demonstrated for the first time that miR-451 was significantly down-regulated in RCC tissues as well as RCC cell lines. The low level of miR-451 was associated with histological grade and lymph node metastasis. Furthermore, miR-451 robustly decreased RCC cells growth, migration and invasion. More importantly, an important molecular link between miR-451 and MIF was observed in RCC. Therefore, miR-451 could be investigated as a targeted therapy for RCC.

miR-451 has been found down-regulated in many human cancers, including hepatocellular carcinoma [23], osteosarcoma [24], bladder cancer [25], lung cancer [26], esophageal carcinoma [27], nasopharyngeal carcinoma [28], glioma [29] and colorectal carcinoma [30]. In functional studies, miR-451 was identified as a tumor suppressor. For example, in non-small cell lung caner, miR-451 expression level was associated with tumor differentiation, pathological stage and lymph-node metastasis. In addition, low miR-451 expression level was correlated with poor prognosis in non-small cell lung caner patients. Enforced miR-451 expression inhibited non-small cell lung caner cell growth, colony formation, invasion and metastasis by targeting PSMB8, NOS2 and RAB14 [26, 31]. In esophageal carcinoma, ectopic miR-451 expression significantly suppressed cell proliferation, invasion, metastasis and enhanced cell apoptosis via blockade of Bcl-2, AKT, CDKN2D and MAP3K1 [27, 32]. In glioma,

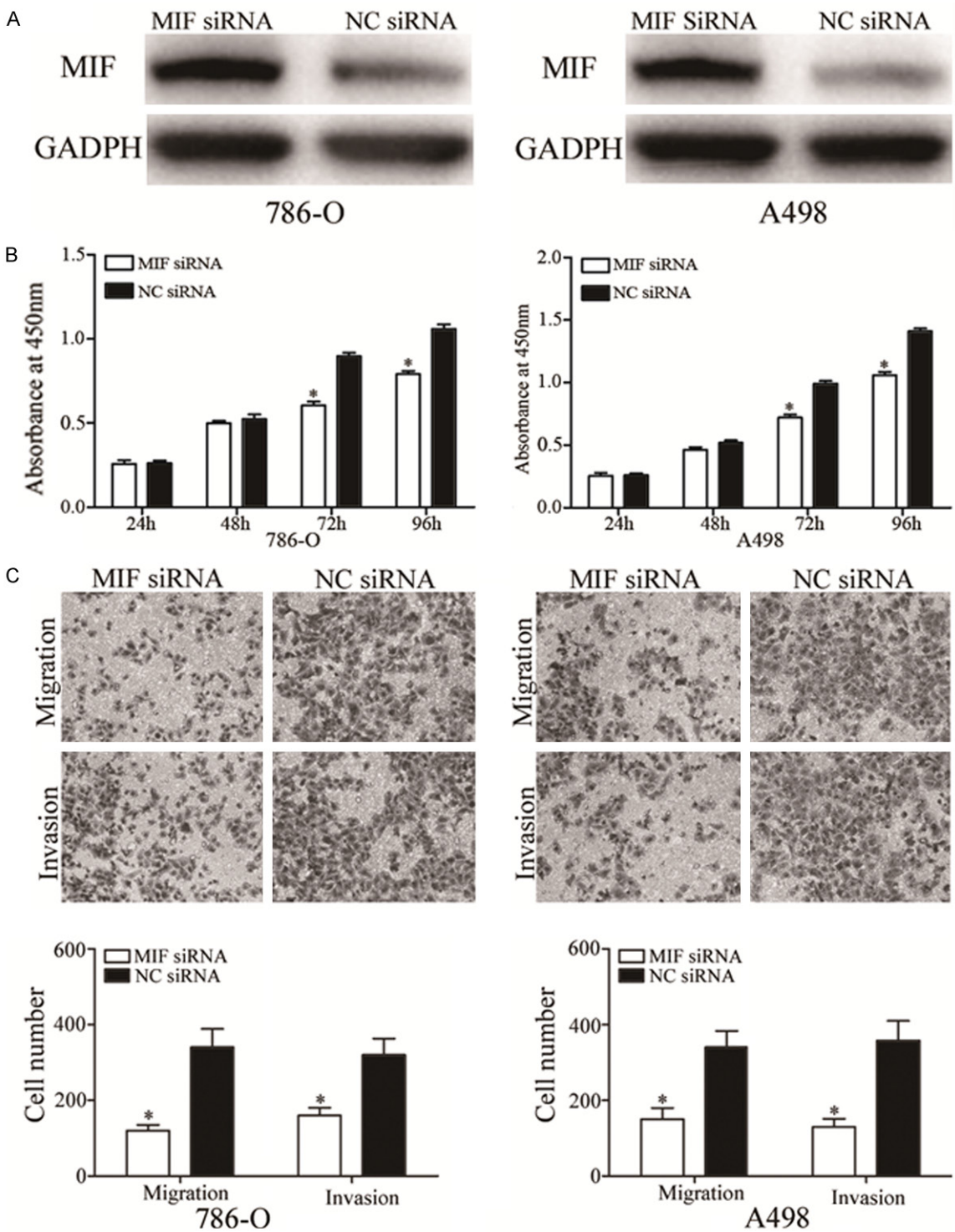

Figure 6. Knockdown of MIF inhibited proliferation, migration and invasion of RCC cells. A. Western blot showed that MIF was down-regulated in 786-O and A498 cells after transfection with MIF siRNA. B. RCC cells transfected with MIF siRNA showed significantly decreased cell proliferation compared with cells transfected with NC siRNA. C. Cell migration and invasion assays showed that MIF siRNA group markedly inhibited cell migration and invasion abilities in 786-O and A498 cells.

miR-451 inhibited glioma cell growth, invasion and induced apoptosis, probably through regulation of PI3K/Akt signaling pathway [29]. Liu and his colleague reported that nasopharyn-

geal carcinoma patients with low miR-451 expression level had poorer overall survival and disease-free survival in comparison to patients with high miR-451 expression. Up-regulation of miR-451 decreased cell proliferation, colony formation, cell migration and invasion in vitro, and suppressed xenograft tumor growth in vivo by directly targeting MIF. These findings indicated that miR-451 played important roles, and could be investigated as a potential targeted therapies for these cancers [28].

Identification of miR-451 target genes is essential for exploring its roles in tumorigenesis and tumor progression. It is also important for developing new targeted therapies. In the present study, an important molecular link between miR-451 and MIF was observed. MIF, an evolutionarily highly conserved pleiotropic chemokine, was first identified as a protein secreted by T cells and inhibiting the migration of macrophages in 1966 by Bloom and Bennett [33]. MIF gene is located at chromosome 22 and MIF protein is relatively small (12.5 kDa) with 115 amino acids [34]. In the following studies, MIF was found that MIF also could be extracted by other type of human cells, such as macrophages, endothelial and epithelial cells [35]. A growing evidence demonstrated that MIF was involved in cancer initiation and development. It was found up-regulated in cancer tissues and cell lines, such as head and neck cancer [36], hepatocellular carcinoma [37], ovarian cancer [38], breast cancer [39], pancreatic cancer [40], lung cancer [41] and gastric cancer [42]. Moreover, MIF was also found to be highly expressed in RCC [43].

Currently, there is a general studies revealed that MIF was associated with prognosis in cancers. For example, in ovarian cancer, MIF expression level was not only correlated with tumor stage and platinum sensitivity, but also with CD8 T- and NK-cell infiltration in tumor tissue [38]. Liao et al. reported that high expression level of MIF in nasopharyngeal carcinoma was significantly associated with increased microvessels, lymph node metastasis, overall survival, disease-specific survival and locoregional failure [44]. Furthermore, experimental studies indicated that MIF could have a multifunctional roles in cancer, such as proliferation, migration, microenvironment modulation, neoangiogenesis, autophagy and drug resistance [45]. Therefore, it is worthwhile to investigate novel targeted therapy to against MIF.

Collectively, our study showed that miR-451 was down-regulated in RCC tissues and cell lines. miR-451 expression level was correlated with histological grade and lymph node metastasis. In addition, miR-451 obviously suppressed RCC cell proliferation, migration and invasion. MIF was identified as a target of miR-451, and down-regulation of MIF could mimic the suppressive functions of miR-451 in RCC, suggesting that miR-451 might be a novel therapeutic strategy for the treatment of RCC.

Disclosure of conflict of interest

None.

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