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# MiR-376a functions as tumor suppressor by targeting SGK3 in renal cell carcinoma

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**Abstract. – OBJECTIVE: Emerging evidence showed that microRNA-376a (miR-376a) functions as a crucial role in human cancers. However, its role in renal cell carcinoma (RCC) remains unclear.**

**MATERIALS AND METHODS: MiR-376a expression in RCC cell lines was analyzed by quantitative real time-polymerase chain reaction (qRT-PCR). The target of miR-376a was validated using the luciferase activity reporter assay, and the effects of miR-376a expression on RCC cell behaviors were investigated *in vitro*.**

**RESULTS: MiR-376a expression was downregulated in RCC cell lines in comparison with HK-2 cell line. Low miR-376a expression was correlated with poor overall survival of RCC patients. Serum/glucocorticoid regulated kinase family member 3 (SGK3) was validated as a direct target of miR-376a. The overexpression of miR-376a inhibits RCC cell proliferation, migration, and invasion through regulating SGK3.**

**CONCLUSIONS: Taken together, these results demonstrated the tumor suppressive role of miR-376a via targeting SGK3 in RCC and indicated that miR-376a might represent a novel therapeutic target for RCC treatment.**

Key Words
MiRNA, Renal cell carcinoma, Carcinogenesis, Prognosis, SGK3.

## Introduction

Renal cell carcinoma (RCC) is a common form of kidney cancer and accounts for approximately 3% of all human cancers[1]. The past two decades have witnessed the rapid increase in the morbidity of RCC due to the resistance to chemotherapy and radiotherapy of metastatic RCC[2,3]. Therefore, the advanced understanding of the molecular mechanisms regarding the initiation and progression of RCC will help us to identify novel therapeutic targets.

MicroRNAs (miRNAs) are a group of non-coding RNA molecules with a length of 20-24 nucleotides[4]. These molecules were reported to regulate the expression of approximately 30-60% of human protein-coding genes mainly through 3'-untranslated region (3'-UTR) binding[5]. Accumulating evidence has revealed that miRNAs were frequently abnormally expressed in human cancers through functioning as a tumor suppressor or promoter[6,7]. MicroRNA-376a (miR-376a), located at chromosome 14q32, was reported as a crucial participator in the initiation and progression of human cancers along with the other miR-376a family member[8,9]. For example, miR-376a was frequently found downregulated in hepatocellular carcinoma tissues and cell lines, and its overexpression was able to inhibit cell proliferation, but promoted apoptosis *in vitro*[10]. In breast cancer, miR-376a downregulation was reported to predict poor prognosis of cancer patients[11]. Moreover, the overexpression of miR-376a was able to suppress cell proliferation, migration, and invasion and promote cells apoptosis by targeting neuropilin 1[11]. The downregulation of miR-376a was also found in another solid tumor and non-small cell lung cancer[12]. In immune systems, miR-376a was reported to be downregulated in lymphoma, and the downregulation of miR-376a promotes cell proliferation but inhibits apoptosis by regulating forkhead box protein P2[13]. Besides that, miR-376a was also found to have a role in glioma, a central neutron system malignancy[14]. miR-376a expression was revealed to be markedly downregulated in glioma tissues and cell lines, and it directly targeted the expression of the specific protein 1 to result in cell proliferation and invasion inhibition. Nevertheless, the expression and function of miR-376a are still unknown in RCC.

In this study, we explored the expression and function of miR-376a in RCC. We showed that miR-376a expression was downregulated in RCC cell lines. The restoration of miR-376a expression



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inhibits RCC cell proliferation, migration, and invasion *in vitro*. Serum/glucocorticoid regulated kinase family member 3 (SGK3) was proved to be the direct target of miR-376a in RCC. The overexpression of SGK3 abolished the effects of miR-376a on cell proliferation, migration, and invasion. This study suggested a tumor suppressive role of miR-376a in RCC, and it has the potential to be used as a therapeutic target in the future.

## Materials and Methods

### Cell Culture

Human RCC cell lines (769-P, ACHN, and Caki-1) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Human kidney cells (HK-2) were purchased from the American Type Cell Culture Collection (ATCC; Rockville, MD, USA). Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) were used to culture these cell lines at a 37°C in a humified incubator containing 5% $CO_2$.

### Cell Transfection

miR-376a mimic or negative control (NC-mimic) were purchased from GenePharm (Shanghai, China). The pcDNA3.1-SGK3 constructs and the empty vectors (NC-vector) were purchased from GenScript (Nanjing, China). Lipofectamine 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to transfect the synthetic miRNAs or the expression constructs into RCC cell lines according to the manufacturer's instructions.

### RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from cultured cells using TRIzol reagent (Beyotime, Haimen, Jiangsu, China) in line with the supplied protocols. Complementary DNA (cDNA) was synthesized from the isolated RNAs using the PrimerScript 1st Strand cDNA Synthesis kit (TaKaRa, Dalian, Liaoning, China). qRT-PCR was performed at ABI 7300 (Applied Biosystems, Thermo Fisher Scientific, Inc.) using SYBR Green PCR Master Mix kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). Relative miR-376a levels were calculated using the $2^{-\Delta\Delta Cq}$ method with U6 small nuclear RNA (U6 snRNA) as internal control. The primers used in this work were as follows: miR-376a, forward 5'-GTGCAGGGTCCGAGGT-3', reverse 5'-ATCATAGAGGAAAATCCACG-3'; U6 snRNA, forward 5'-ATTGGAACGATACAGAGAAGATT-3', reverse 5'-GGAACGCTTCACGAATTTG-3'.

### Protein Extraction and Western Blot

The total protein was extracted from cultured cells using RIPA lysis buffer supplemented with protease inhibitor (Beyotime, Haimen, Jiangsu, China). The protein concentration was determined using the bicinchoninic acid assay (Beyotime, Haimen, Jiangsu, China). Then, an equal amount of protein samples was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membranes (Beyotime, Haimen, Jiangsu, China) and probed with primary antibodies (anti-SGK3: ab153981; anti-GAPDH: ab181602; both from Abcam, Cambridge, MA, USA) at 4°C for overnight. After being washed three times with TBST, the membranes were incubated horseradish peroxidase-conjugated secondary antibody (ab6721, Abcam) for 1 h at room temperature. The protein bands were visualized with BeyoECL Plus kit (Beyotime).

### Cell Proliferation Assay

The Cell proliferation was measured using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. The cells ($5 \times 10^3$ cells/well) were seeded into 96-well plates. At various time points (0, 24, 48, and 72 h), 20 µl, 5 mg/ml MTT solution (Beyotime Haimen, Jiangsu, China) was added to the medium and cultured for other 4 h at 37°C. Subsequently, 150 µl dimethyl sulfoxide was added to each well to dissolve the crystals and then to measure the absorbance at 570 nm using absorption spectrophotometer (Olympus Corporation, Tokyo, Japan).

### Wound-Healing Assay

The wound healing assay was conducted to measure cell migration. Cells were seeded into 6-well plates and incubated until 80% confluency, and then a scratch was created by a 200 µl pipette tip. The wound images were captured at 0 h and 48 h after wounding.

### Cell Invasion Assay

The cell invasion was analyzed using transwell invasion assay with the transwell chamber (Corning, Tewksbury, MA, USA) coated with Matrigel (BD Biosciences, San Jose, CA, USA) on the upper surface. $1 \times 10^4$ cells were seeded in the upper

chamber in FBS-free medium. The lower chamber was filled with medium containing 10% FBS. After incubation for 24 h, the invasive cells on the lower membranes were fixed with 100% methanol at room temperature for 15 min, stained with 0.5 % crystal violet solution at room temperature for 15 min. Invasive cell numbers were counted under an Olympus IX53 inverted fluorescent microscope (Olympus Corporation, Tokyo, Japan) from five independent fields.

### Bioinformation Analysis and Dual-Luciferase Activity Reporter Assay

TargetScan (http://www.targetscan.org/vert_72/) was used to predict the targets of miR-376a. Among all these predicted targets, SGK3 contains a binding site in its 3'-UTR for miR-376a and was selected for further investigations. The wild-type (wt) or mutant (mut) 3'-UTR of SGK3 was cloned into pGL3 vector (Promega, Madison, WI, USA). Cells ($1.5 \times 10^5$ cells/well), were seeded into 24-well plate, and cultured overnight at 37°C before the co-transfection with pGL3-SGK3-wt or pGL3-SGK3-mut and miR-376a mimic or NC-mimic, using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The luciferase activities were measured with the Dual-Luciferase Reporter Assay system (Promega) after 48 h of transfection with Renilla luciferase activity as internal control.

### Kaplan-Meier (KM) Plotter Analysis

KM plotter analysis (http://kmplot.com/analysis/index.php?p=background) was done to evaluate the correlation between miR-376a expression and the overall survival of RCC patients. A cut-off value was auto-selected in the algorithm.

### Statistical Analysis

Data were presented as means ± SD and the differences between two groups or among three or above groups were evaluated using the Student's *t*-test or ANOVA and Tukey post-hoc test, respectively. GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) was used for all data analyses. $p < 0.05$ was regarded as statistical significance.

## Results

### MiR-376a Expression Was Downregulated in RCC Cell Lines

To verify the expression pattern of miR-376a in RCC, we analyzed its expression in three RCC cell lines using qRT-PCR. The results indicated that the expression level of miR-376a was significantly downregulated in RCC cell lines (769-P, ACHN, and Caki-1) compared to HK-2 cell line (Figure 1A). The 769-P and ACHN cell lines were used for the following studies as they have the first and second lowest miR-376a expression in the RCC cell lines investigated (Figure 1A).

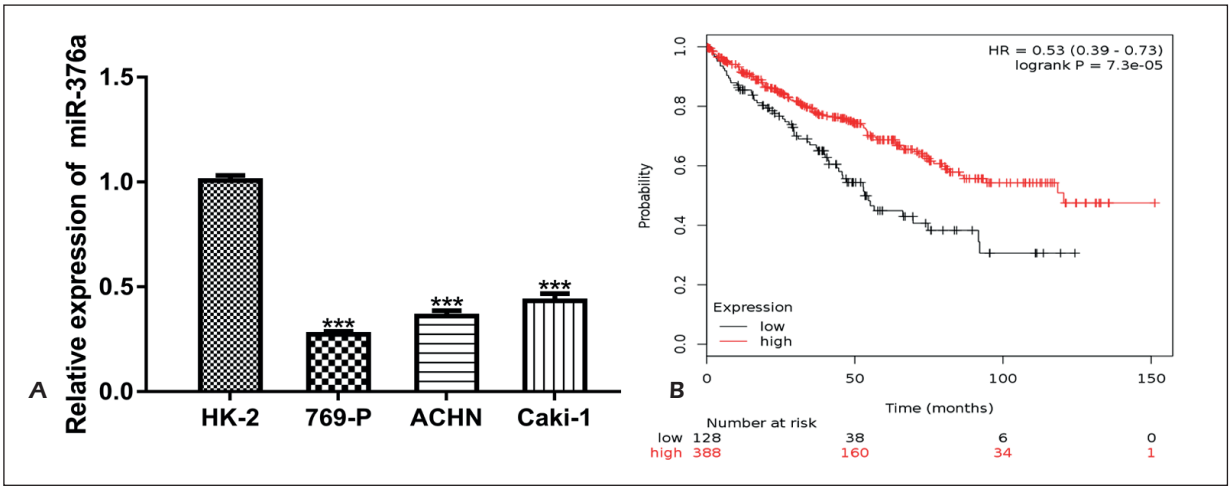


**Figure 1.** Downregulation of miR-376a in RCC. **A**, Expression of miR-376a in human RCC cell lines (769-P, ACHN, and Caki-1) and human kidney cells (HK-2). **B**, Low miR-376a expression predicts worse overall survival of RCC patients. (***$p$<0.001); miR-376a: microRNA-376a; RCC: renal cell carcinoma.

### Low MiR-376a Expression Predicts Poor Prognosis of RCC Patients

After having established that miR-376a expression was downregulated in RCC we, therefore, investigated the effects of miR-376a on the overall survival of RCC patients using Kaplan-Meier plotter. We found that the low expression of miR-376a was a predictor for the poor prognosis of RCC patients ($p$ = 7.3e-05, Figure 1B).

### MiR-376a Overexpression Inhibits Cell Proliferation, Migration, and Invasion

To explore the role of miR-376a in RCC, we transfected the miR-376a mimic and NC-mimic into 769-P and ACHN cell lines. The transfection of miR-376a mimic significantly enhanced the expression of miR-376a in RCC cells compared with NC-mimic transfection (Figure 2A). As shown in Figure 2B, the upregulation of miR-376a significantly suppressed 769-P and ACHN cell proliferation. The wound-healing assay showed that cell migration was significantly inhibited by miR-376 mimic (Figure 2C). Moreover, the transwell invasion assay revealed that cell invasion was remarkedly inhibited by miR-376a mimic compared with NC-mimic (Figure 2D).

### SGK3 Was a Direct Target of MiR-376a

The bioinformatic analysis showed that the 3'-UTR of SGK3 contains a binding site for miR-376a (Figure 3A). To confirm this prediction, a luciferase activity reporter assay was conducted. We showed that the luciferase activity of cells transfected with pGL3-SGK3-wt but not pGL3-SGK3-mut was suppressed by miR-376a mimic (Figure 3B). Western blot assay showed that the SGK3 protein expression could be suppressed by miR-376a mimic transfection (Figure 3C). These results demonstrated that SGK3 was a direct target of miR-376a.

### MiR-376a Inhibits RCC Cell Behaviors Through Targeting SGK3

To investigate whether miR-376a elicits the inhibitory effects on RCC cell behaviors through SGK3, we transfected the pcDNA3.1-SGK3 con-

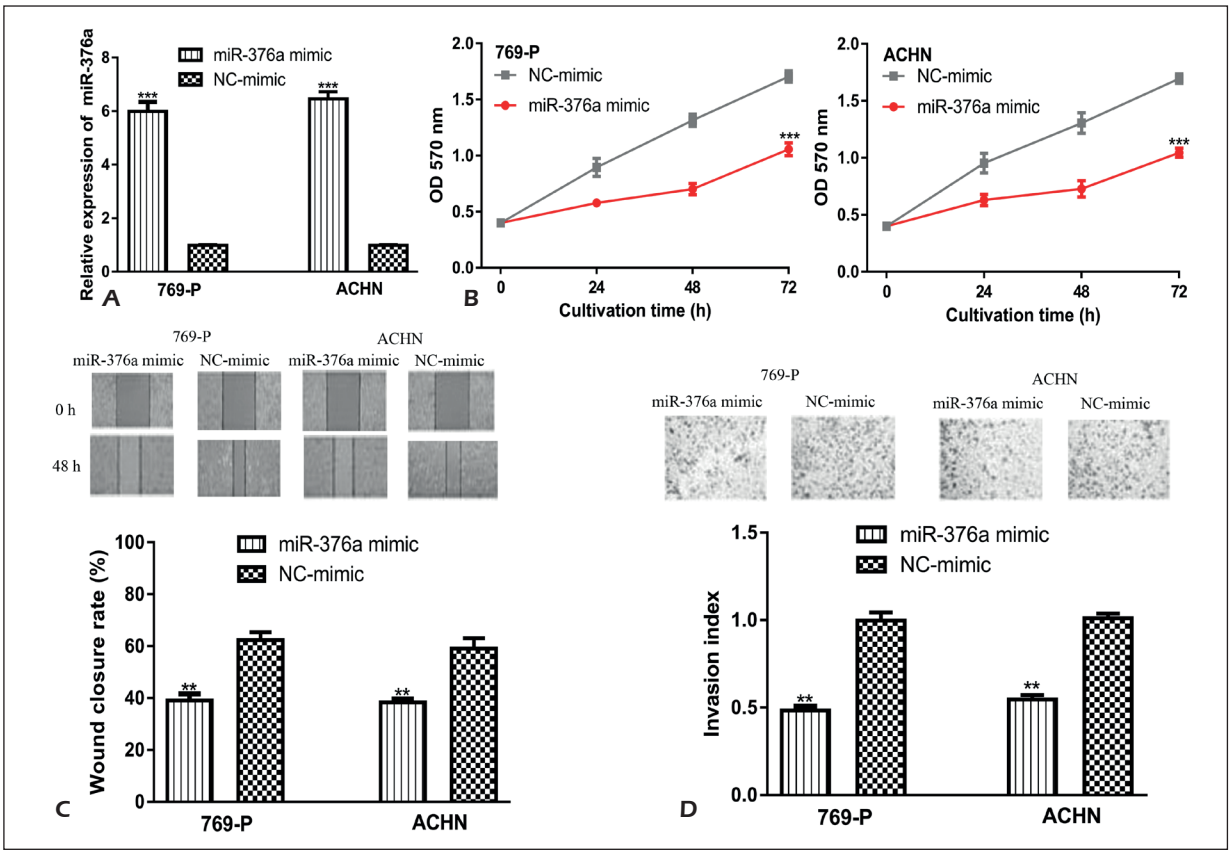


**Figure 2.** miR-376a inhibits RCC cell proliferation, migration, and invasion *in vitro*. **A**, miR-376a expression; **B**, Cell proliferation; **C**, Cell migration, and (**D**) Cell invasion in cells transfected with miR-376a mimic and NC-mimic. (**$p$<0.01; ***$p$<0.001); miR-376a: microRNA-376a; RCC: renal cell carcinoma; NC-mimic: negative control.

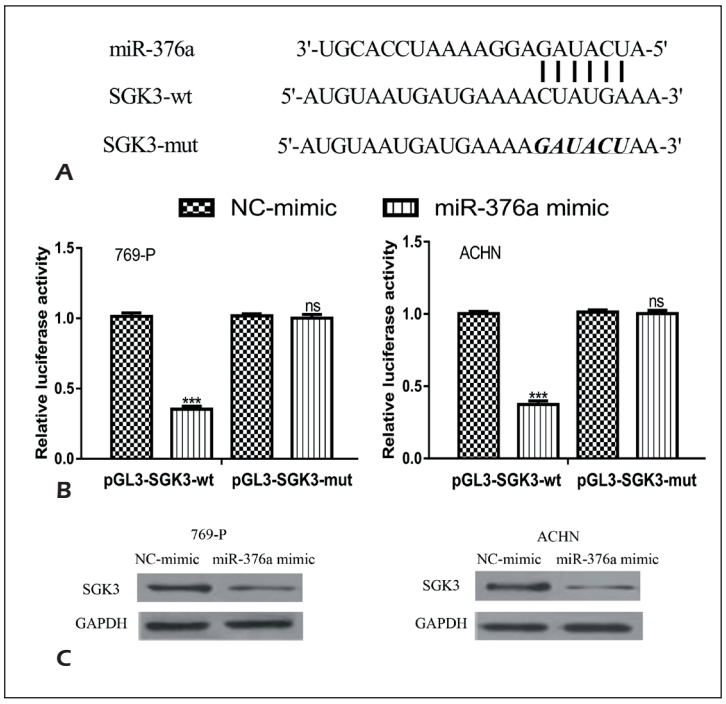


**Figure 3.** SGK3 was a direct target of miR-376a. **A**, Bioinformatic result of TargetScan in prediction the interaction between SGK3 and miR-376a. **B**, Luciferase activity in cells transfected with miR-376a mimic or NC-mimic and pGL3-SGK3-wt or pGL3-SGK3-mut. C, SGK3 expression in cells transfected with miR-376a mimic or NC-mimic. (ns not significant; ***$p$<0.001); miR-376a: microRNA-376a; NC-mimic: negative control; SGK3: Serum/glucocorticoid regulated kinase family member 3; UTR: untranslated region; wt: wild-type; mut: mutant.

struct and miR-376a mimic into the cells. Not surprisingly, the transfection of pcDNA3.1-SGK3 construct enhanced the levels of SGK3 (Figure 4A). MTT assay showed that pcDNA3.1-SGK3 construct abolished the inhibitory effect of miR-376a mimic on cell proliferation (Figure 4B). The wound-healing assay and transwell invasion assay demonstrated that SGK3 overexpression reversed the suppression effects of miR-376a mimic on cell migration and invasion (Figure 4C and 4D). These results indicated that SGK3 overexpression restored the suppressive function of miR-376a on RCC behaviors.

## Discussion

Emerging evidence suggested that the abnormal expression of miRNAs may contribute to the carcinogenesis[6,7,15-17]. With all these advancements, new diagnostic, prognostic, and therapeutic strategies have been developed, but few of them came into clinical stage[6,7]. In RCC, miR-543 was found to be overexpressed in tumor tissues and cell lines and therefore promoted cell proliferation and metastasis by regulating the expression of Dickkopf 1 through the Wnt/β-catenin signaling pathway, suggesting an oncogenic role of miR-543[18]. There is also a study which showed the tumor suppressive role of miRNA in RCC. miR-200-3p was demonstrated to be downregulated in RCC tissues, and cell lines and the ectopic expression of miR-200a-3p suppressed cell proliferation and migration but enforced cell apoptosis by directly inhibiting the expression of casitas-b-lymphoma[19].

In this study, miR-376a expression was found to be downregulated in RCC cell lines, suggesting that miR-376a might play a tumor suppressive role in the progression of RCC. More importantly, we found that the low miR-376a expression was a predictor for poor prognosis of RCC patients by analyzing the overall survival of RCC patients. Functional analysis showed that the overexpression of miR-376a inhibits RCC cell proliferation, migration, and invasion *in vitro*. Collectively, these results validated the tumor suppressive role of miR-376a in RCC.

Previous studies have demonstrated that miRNAs modulate the expression of specific genes to function as either tumor suppressor or promoter in different tumors[10-19]. Given the finding of miR-376a functions as a tumor suppressor in RCC, we are therefore interested to investigate the downstream target of miR-376a. The prediction algorithm showed that SGK3 might be a potential target of miR-376a and this link was not demonstrated in previous studies. SGK3 was previously reported to be overexpressed and reg-

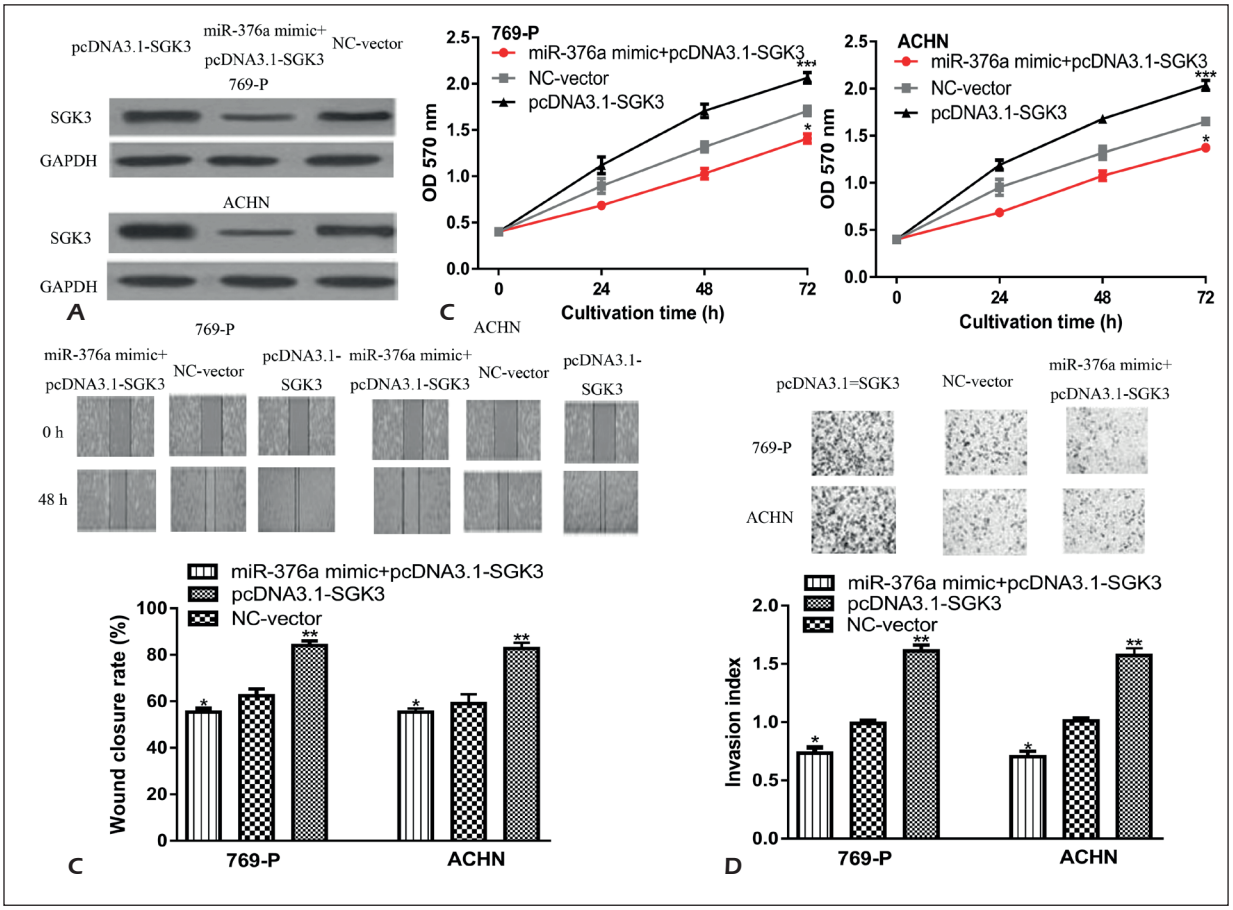


**Figure 4.** Overexpression of SGK3 reversed the inhibitory effects of miR-376a on RCC cell behaviors. **A**, SGK3 expression; **B**, Cell proliferation; **C**, Cell migration, and (**D**) Cell invasion in cells transfected with pcDNA3.1-SGK3, NC-vector, or pcDNA3.1-SGK3 and miR-376a mimic. (*$p$<0.05; ***$p$<0.001); miR-376a: microRNA-376a; NC: negative control; SGK3: Serum/glucocorticoid regulated kinase family member 3; RCC: renal cell carcinoma.

ulated by miRNAs in human cancers including hepatocellular carcinoma and glioblastoma[20,21]. The luciferase activity reporter assay and Western blot confirmed that SGK3 was also a target of miR-376a. Besides that, functional assays showed that the overexpression of SGK3 could abolish the inhibitory effects of miR-376a on RCC cell proliferation, migration, and invasion.

## Conclusions

We detected the expression of miR-376a in RCC cell lines and investigated the effects of miR-376a on overall survival of RCC patients. We also found that SGK3 was a direct target of miR-376a in RCC. The miR-376a/SGK3 axis plays a crucial role in regulating RCC cell behaviors *in vitro*. These results indicated that the miR-376a/SGK3 axis may be used as therapeutic targets for RCC in the future.

## Conflict of Interest

The Authors declare that they have no conflict of interests.

## References

1) Jonasch E, Gao J, Rathmell WK. Renal cell carcinoma. BMJ 2014; 349: g4797.
2) Ljungberg B, Campbell SC, Choi HY, Jacqmin D, Lee JE, Weikert S, Kiemeney LA. The epidemiology of renal cell carcinoma. Eur Urol 2011; 60: 615-621.
3) Escudier B, Albiges L, Sonpavde G. Optimal management of metastatic renal cell carcinoma: current status. Drugs 2013; 73: 427-438.
4) Bushati N, Cohen SM. MicroRNA functions. Annu Rev Cell Dev Biol 2007; 23: 175-205.
5) Hammond SM. An overview of microRNAs. Adv Drug Deliv Rev 2015; 87: 3-14.
6) Kala R, Peek GW, Hardy TM, Tollefsbol TO. MicroRNAs: an emerging science in cancer epigenetics. J Clin Bioinforma 2013; 3: 6.

7) Drusco A, Croce CM. MicroRNAs and cancer: a long story for short RNAs. Adv Cancer Res 2017; 135: 1-24.
8) Nadal E, Zhong J, Lin J, Reddy RM, Ramnath N, Orringer MB, Chang AC, Beer DG, Chen G. A MicroRNA cluster at 14q32 drives aggressive lung adenocarcinoma. Clin Cancer Res 2014; 20: 3107-3117.
9) Tekirdag KA, Akkoc Y, Kosar A, Gozuacik D. MIR376 family and cancer. Histol Histopathol 2016; 31: 841-855.
10) Zheng Y, Yin L, Chen H, Yang S, Pan C, Lu S, Miao M, Jiao B. MiR-376a suppresses proliferation and induces apoptosis in hepatocellular carcinoma. FEBS Lett 2012; 586: 2396-2403.
11) Zhang L, Chen Y, Wang H, Zheng X, Li C, Han Z. MiR-376a inhibits breast cancer cell progression by targeting neuropilin-1 NR. Onco Targets Ther 2018; 11: 5293-5302.
12) Wang Y, Cong W, Wu G, Ju X, Li Z, Duan X, Wang X, Gao H. MiR-376a suppresses the proliferation and invasion of non-small-cell lung cancer by targeting c-Myc. Cell Biol Int 2018; 42: 25-33.
13) Yao S, Liu Y, Yao Z, Zhao Y, Wang H, Xu Y, Zhang J, Li J, Yang S. MicroRNA-376a regulates cell proliferation and apoptosis by targeting forkhead box protein P2 in lymphoma. Oncol Lett 2018; 16: 3169-3176.
14) Li Y, Wu Y, Sun Z, Wang R, Ma D. MicroRNA-376a inhibits cell proliferation and invasion in glioblastoma multiforme by directly targeting specificity protein 1. Mol Med Rep 2018; 17: 1583-1590.
15) Qi R, Wang DT, Xing LF, Wu ZJ. MiRNA-21 promotes gastric cancer growth by adjusting prostaglandin E2. Eur Rev Med Pharmacol Sci 2018; 22: 1929-1936.
16) Kuang WB, -C Deng Q, Deng CT, Li WS, Zhang YG, Shu SW, Zhou MR. MiRNA regulates OCT4 expression in breast cancer cells. Eur Rev Med Pharmacol Sci 2018; 22: 1351-1357.
17) Liu N, Jiang F, Han XY, Li M, Chen WJ, Liu QC, Liao CX, Lv YF. MiRNA-155 promotes the invasion of colorectal cancer SW-480 cells through regulating the Wnt/β-catenin. Eur Rev Med Pharmacol Sci 2018; 22: 101-109.
18) Chen ZY, Du Y, Wang L, Liu XH, Guo J, Weng XD. MiR-543 promotes cell proliferation and metastasis of renal cell carcinoma by targeting Dickkopf 1 through the Wnt/β-catenin signaling pathway. J Cancer 2018; 9: 3660-3668.
19) Ding M, Sun X, Zhong J, Zhang C, Tian Y, Ge J, Zhang CY, Zen K, Wang JJ, Zhang C, Wang C. Decreased miR-200a-3p is a key regulator of renal carcinoma growth and migration by directly targeting CBL. J Cell Biochem 2018; 119: 9974-9985.
20) Wu M, Huang C, Huang X, Liang R, Feng Y, Luo X. MicroRNA-144-3p suppresses tumor growth and angiogenesis by targeting SGK3 in hepatocellular carcinoma. Oncol Rep 2017; 38: 2173-2181.
21) Liu H, Li C, Shen C, Yin F, Wang K, Liu Y, Zheng B, Zhang W, Hou X, Chen X, Wu J, Wang X, Zhong C, Zhang J, Shi H, Ai J, Zhao S. MiR-212-3p inhibits glioblastoma cell proliferation by targeting SGK3. J Neurooncol 2015; 122: 431-439.