MicroRNA-373 promotes tumorigenesis of renal cell carcinoma *in vitro* and *in vivo*

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Abstract. Renal cell carcinoma (RCC) is the most common type of malignancy in the kidney parenchyma. MicroRNAs (miRNAs) are small non-coding RNAs that serve a role in various biological processes associated with human cancer. The present study aimed to explore the potential role of miRNA (miR)-373 in the tumorigenesis of RCC. The effects of miR-373 on the proliferation and apoptosis of RCC cells were determined using MTT, colony formation and flow cytometry assays *in vitro*. The results demonstrated that miR-373 was significantly upregulated in RCC tissues and cell lines. Knockdown of miR-373 expression reduced cell proliferation and promoted cell apoptosis in 786-O and ACHN cell lines. Furthermore, an *in vivo* tumorigenicity assay revealed that knockdown of miR-373 expression reduced tumor growth in nude mice. Taken together, these data indicate that miR-373 may promote tumorigenesis in RCC, suggesting that miR-373 may act as a potential therapeutic target against RCC.

Introduction

MicroRNAs (miRNAs) are endogenous small non-coding RNA molecules, ~22 nucleotides in length, which can regulate gene expression at the transcriptional or post-transcriptional level by binding with the 3'-untranslated regions of target mRNAs (1-3). It has previously been reported that miRNAs regulate numerous cellular biological processes, including growth, migration, differentiation and apoptosis (4-6). Previous studies have indicated that miRNAs are aberrantly expressed in various types of human cancer (7-9); miRNAs may act as oncogenes or tumor suppressors in cancer depending on their tissue- and disease-specific expression patterns (10,11). In cancer, downregulated miRNAs usually serve as tumor suppressors, whereas upregulated miRNAs act as oncogenes.

Renal cell carcinoma (RCC) is the most common type of adult kidney tumor worldwide, and accounts for 2-3% of all adult malignancies (12,13). RCC originates from the renal cortex and is a highly metastatic urinary tumor (14). Despite increasingly early detection and more frequent use of surgery, the mortality rate of RCC has not significantly decreased since 1990 (15,16). Therefore, improved understanding regarding the molecular mechanisms underlying the pathogenesis and development of RCC, and developing more effective treatment options for the treatment of RCC, are required.

A previous study demonstrated that aberrant miRNA expression may serve an important role in RCC development (17). Various miRNAs have been reported to regulate cell growth, metastasis and apoptosis in RCC (18-20). The mature sequence of miRNA (miR)-373 is located at human chromosome 19q13.42. Previous studies have demonstrated an oncogenic role for miR-373 in numerous types of human cancer, including oral carcinomas, lung adenocarcinoma and breast cancer (21-23). To the best of our knowledge, the biological role of miR-373 in RCC has yet to be elucidated. The aim of the present study was to identify the potential role of miR-373 in the tumorigenesis of RCC. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to determine the upregulation of miR-373 in RCC tissues and cell lines compared with paired normal tissues and cell lines. In addition, the effects of miR-373 on cell proliferation, cell apoptosis and tumorigenicity in RCC were explored. The results revealed that miR-373 may promote the tumorigenicity of RCC.

Materials and methods

Clinical samples. A total of 52 pairs of RCC tissues and adjacent normal tissues were collected from patients with RCC who had undergo nephrectomy at The First Affiliated Hospital of Zhengzhou University (Zhengzhou, China) between June 2010 and June 2015. No treatment was administered prior to surgery. The total cohort consisted of 27 men and 25 women, with an age range of 42-76 years. The clinicopathological features of the patients are presented in Table I. The

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present study was approved by the Ethics Committees of The First Affiliated Hospital of Zhengzhou University, and written informed consent was obtained from all patients. All tissues were freshly frozen in liquid nitrogen and were subsequently stored at -80˚C for further experimentation.

Cell lines and cell culture. ACHN and 786-O human RCC cell lines and the HK-2 immortalized normal human proximal tubule epithelial cell line were obtained from the American Type Culture Collection (Manassas, VA, USA). ACHN and 786-O cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 U/ml streptomycin. HK-2 cells were maintained in keratinocyte-serum-free medium (Gibco; Thermo Fisher Scientific, Inc.). All cells were cultured in a humidified incubator containing 5% CO_2 at 37˚C.

Cell transfection. miR-373 inhibitors (5'-GGAAAGCGCCCC CAUUUUGAGU-3') and mimics (5'-GAAGUGCUUCGA UUUUGGGGUGU-3') designed to interfere and overexpress endogenous mature miR-373, as well as negative control inhibitors (inhibitors-NC, 5'-GUCAGACGGUUCAAG CGAGUAU-3') and mimics (mimics-NC, 5'-AUGGCACGA CUACUAUUTACUAU-3') were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). To perform the *in vitro* functional studies, 786-O and ACHN cells were grown to 65-75% confluence, after which they were transiently transfected with miR-373 inhibitors or inhibitors-NC, and miR-37 mimics or mimics-NC using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Cells were collected for RNA analysis 48 h post-transfection.

RT-qPCR. Total RNA was isolated from the tissues and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The miScript II RT kit (Qiagen GmbH, Hilden, Germany) was used to synthesize cDNA according to the manufacturer's protocol. PCR amplification was conducted on the Roche LightCycler 480 Real-Time PCR system (Roche Diagnostics GmbH, Mannheim, Germany) using the miScript SYBR Green PCR kit (Qiagen GmbH), according to the manufacturer's protocol. The thermocycling conditions were as follows: 95˚C for 15 min; and 40 cycles of 94˚C for 15 sec, 55˚C for 30 sec and 72˚C for 30 sec. The primers used were as follows: miR-373, sense 5'-GAAGTGCTTCGATTTTGGGGTGT-3', antisense 5'-TGCCGCCTGAACTTCACTCC-3'; and U6, sense 5'-CTC GCTTCGGCAGCACA-3' and antisense 5'-ACGCTTCAC GAATTTGCGT-3'. U6 was used as an internal control. The relative expression levels of miR-373 in tissues and cells were determined using the $2^{-\Delta\Delta Cq}$ method (24), and were normalized to the expression levels of U6.

MTT assay. Cell proliferation was evaluated using MTT (Sigma-Aldrich; Merck KgaA, Darmstadt, Germany). Briefly, ~$6x10^3$ 786-O and ACHN cells were seeded into 96-well plates and were cultured for 24 h at 37˚C in an atmosphere containing 5% CO_2. Subsequently, the cells were transfected with miR-373 inhibitors or mimics for 6 h. A total of 30 μl MTT solution (5 mg0/ml) was added to each well after 0, 24, 48 and 72 h and the plates were incubated for 4 h at 37˚C. The medium was then discarded and 150 μl dimethyl sulfoxide (Sigma-Aldrich; Merck KgaA) was added to each well to dissolve the formazan crystals. Absorbance was then measured at 480 nm using an iMark microplate reader (Model 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Colony formation assay. A total of 24 h post-transfection with the inhibitors, 786-O and ACHN cells were seeded into 6-well plates (~$2x10^3$/well) and were cultured for 10 days at 37˚C in an atmosphere containing 5% CO_2. Subsequently, the cells were fixed with methanol (Sigma-Aldrich; Merck KgaA) and stained with 0.1% crystal violet (Sigma-Aldrich; Merck KgaA). Images of the colonies were then captured and the number of colonies was counted under a light microscope (Eclipse TS100; Nikon Corporation, Tokyo, Japan).

Cell apoptosis assay. ACHN and 786-O cells were seeded in 6-well plates (~$2x10^6$/well), a total of 48 h post-transfection with miR-373 inhibitors or inhibitors-NC. Cell apoptosis was detected using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) detection kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Briefly, cells were collected and stained with 5 μl FITC-Annexin V and 3 μl PI. Subsequently, flow cytometry (Epics XL-MCL; Beckman Coulter, Inc., Brea, CA, USA) was used to evaluate the percentage of apoptotic cells following incubation for 15 min at 37˚C. The results were analyzed by Flowjo software (version 7.6.5 and version 10.4; FlowJo LLC, Ashland, OR, USA).

Tumorigenicity assay in nude mice. A total of 40 4-week-old male nude mice were obtained from the Laboratory Animals Center of South Medical University (Guangzhou, China). All mice were maintained under specific pathogen-free conditions at ~22˚C, under a 12-h light/dark cycle, with *ad libitum* access to food and water. This study was approved by the Animal Ethics Committee of The First Affiliated Hospital of Zhengzhou University.

In order to generate a mouse xenograft model, male nude mice were randomly divided into two groups (n=20/group) and were injected with 786-O or ACHN cells, which had been transfected with miR-373 inhibitors or inhibitors-NC. The nude mice were subcutaneously injected in both axillas with $3x10^6$ cells in 0.2 ml medium; the left axilla was injected with miR-373 inhibitors-transfected cells and the right axilla was injected with inhibitors-NC-transfected cells. The tumors were measured using vernier calipers every week. The tumor volume was calculated according to the following formula: Volume (mm^3)=$width^2$ x length/2. A total of 28 days post-injection, the mice were anesthetized with 10% chloral hydrate (370 mg/kg) and sacrificed by cervical dislocation. Following this step, the tumors were observed and resected.

Statistical analysis. Data are presented as the mean ± standard deviation from at least three independent experiments. All statistical data were analyzed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). The χ^2 test was applied to determine

Table I. Association between miR-373 expression and the clinicopathological characteristics of 52 patients with renal cell carcinoma.

Characteristic	Number	miR-373 expression		P-value
		Low (%)	High (%)	
Age (years)				0.226
≤55	31	24 (46.2)	7 (13.5)	
>55	21	13 (25.0)	8 (15.4)	
Gender				0.068
Male	27	16 (30.8)	11 (21.2)	
Female	25	21 (40.4)	4 (7.7)	
Tumor size (cm)				0.160
≤4	20	12 (23.1)	8 (15.4)	
>4	32	25 (48.1)	7 (13.5)	
Histological type				0.300
Clear cell	39	26 (50.0)	13 (25.0)	
Papillary	13	11 (21.2)	2 (3.8)	
AJCC stage				0.171
I-II	35	27 (51.9)	8 (15.4)	
III-IV	17	10 (19.2)	7 (13.5)	
Lymph node metastasis				0.706
No	9	6 (11.5)	3 (5.8)	
Yes	43	31 (59.6)	12 (23.1)	

miR-373, microRNA-373; AJCC, American Joint Committee on Cancer.

the association between miR-373 expression levels and clinicopathological characteristics. Student's t-test and one-way analysis of variance followed by Dunnett's test were used to evaluate significant differences in two groups and more than two groups, respectively. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-373 expression is significantly upregulated in RCC tissues and cell lines. To determine whether miR-373 expression is associated with RCC development, the expression levels of miR-373 in 52 pairs of RCC and adjacent normal tissues were detected using RT-qPCR. Notably, there was a significant increase in the expression levels of miR-373 in RCC tissues compared with in adjacent normal tissues (Fig. 1A, $P<0.01$). In addition, the expression levels of miR-373 in the human RCC cell lines ACHN and 786-O, and in the immortalized normal human proximal tubule epithelial HK-2 cell line, were determined by RT-qPCR. The results demonstrated that the expression levels of miR-373 were markedly upregulated in ACHN and 786-O cells compared with in the HK-2 cell line (Fig. 1B, $P<0.01$).

The 52 patients with RCC were grouped into two subgroups with the average expression of miR-373 as the threshold (2.46), namely the miR-373 low expression group (n=37) and the miR-373 high expression group (n=15). The association between miR-373 expression levels and clinicopathological characteristics were evaluated using the χ^2 test. However, no significant association was identified between miR-373 expression and clinicopathological characteristics (Table I).

Knockdown of miR-373 inhibits RCC cell proliferation and colony formation. To explore the biological role of miR-373, it was suppressed or overexpressed in ACHN and 786-O cells via transfection with miR-373 inhibitors or miR-373 mimics. Transfection efficacy was detected using RT-qPCR. The results demonstrated that miR-373 expression was significantly decreased post-transfection of ACHN ($P<0.05$) and 786-O ($P<0.01$) cells with miR-373 inhibitors compared with in cells transfected with inhibitors-NC (Fig. 2A). Conversely, miR-373 expression was significantly increased in ACHN and 786-O cells post-transfection with miR-373 mimics compared with in cells transfected with mimics-NC (Fig. 2A, $P<0.01$). Subsequently, the effects of miR-373 on RCC cell proliferation were determined by MTT assay. The results revealed that knockdown of miR-373 suppressed ACHN and 786-O cell proliferation (Fig. 2B, $P<0.05$). In addition, proliferation of 786-O and ACHN cells transfected with miR-373 mimics was determined. The proliferation of RCC cells was increased in the miR-373 mimics group compared with in the mimics-NC group (Fig. 2C, $P<0.05$).

In the colony formation assay, ACHN and 786-O cells transfected with miR-373 inhibitors exhibited a significant decrease in colony formation compared with in the cells transfected with inhibitors-NC (Fig. 3, $P<0.05$).

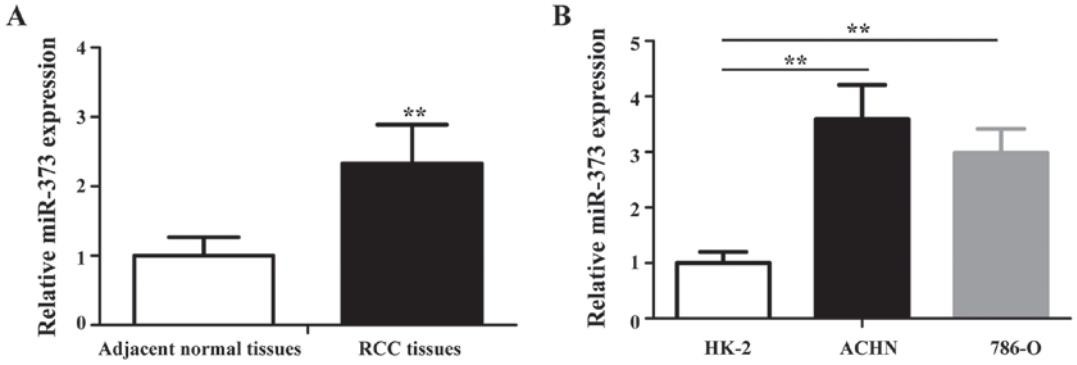

Figure 1. miR-373 expression is significantly upregulated in RCC tissues and cell lines. (A) RT-qPCR analysis of miR-373 expression levels in RCC tissues and adjacent normal tissues. (B) RT-qPCR analysis of miR-373 expression levels in ACHN, 786-O and HK-2 cell lines. U6 was used as a loading control. **P<0.01 vs. adjacent normal tissues or HK-2 cells. miR-373, microRNA-373; RCC, renal cell carcinoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

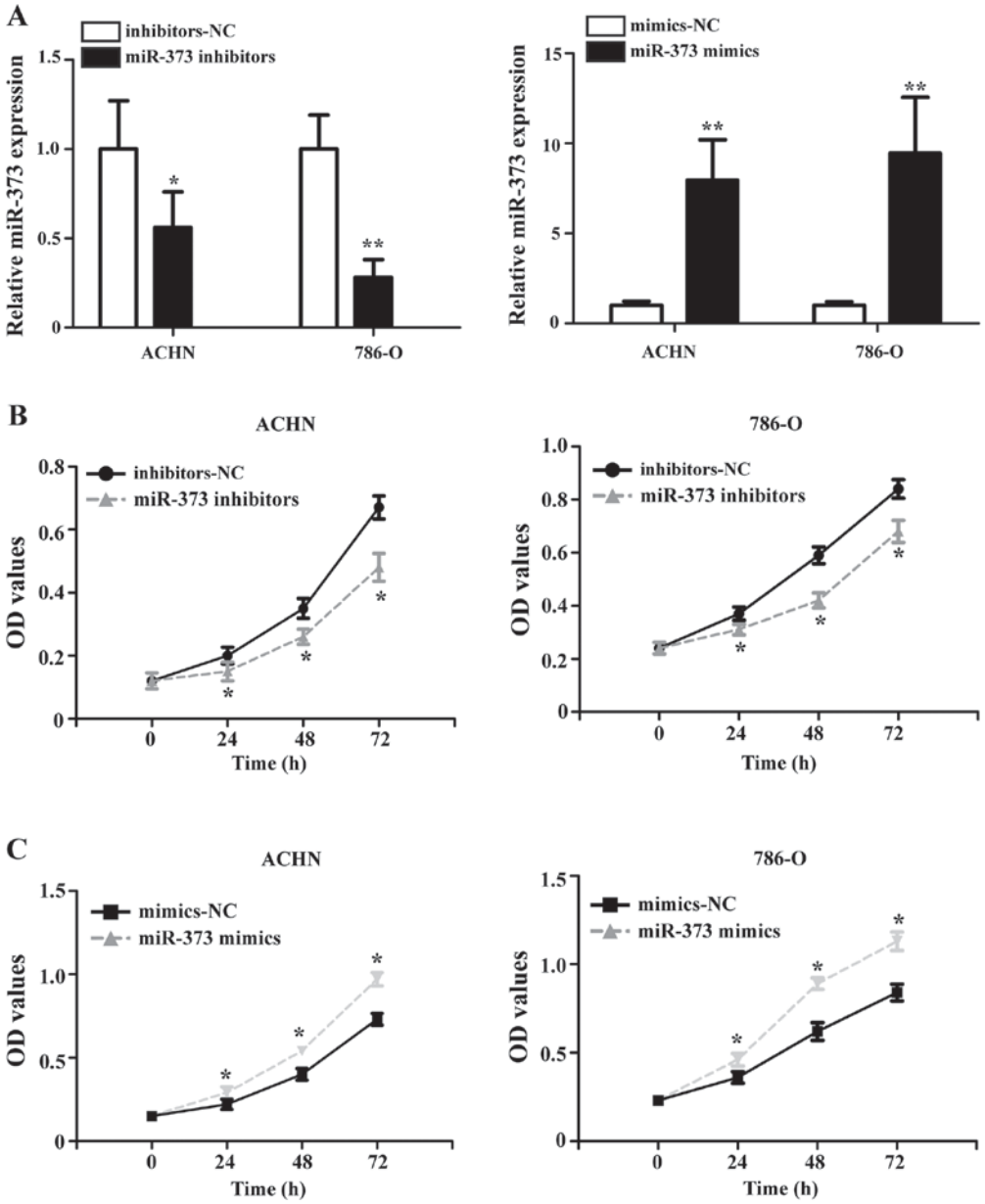

Figure 2. Knockdown of miR-373 inhibits RCC cell proliferation. (A) Expression levels of miR-373 were detected in ACHN and 786-O cells by reverse transcription-quantitative polymerase chain reaction post-transfection with miR-373 inhibitors or mimics. Cell proliferation was determined at 0, 24, 48 and 72 h in ACHN and 786-O cells by MTT assay, post-transfection with (B) miR-373 inhibitors or inhibitors-NC and (C) miR-373 mimics or mimics-NC. *P<0.05, **P<0.01 vs. inhibitors-NC group or mimics-NC group. RCC, renal cell carcinoma; miR-373, microRNA-373; NC, negative control; OD, optical density.

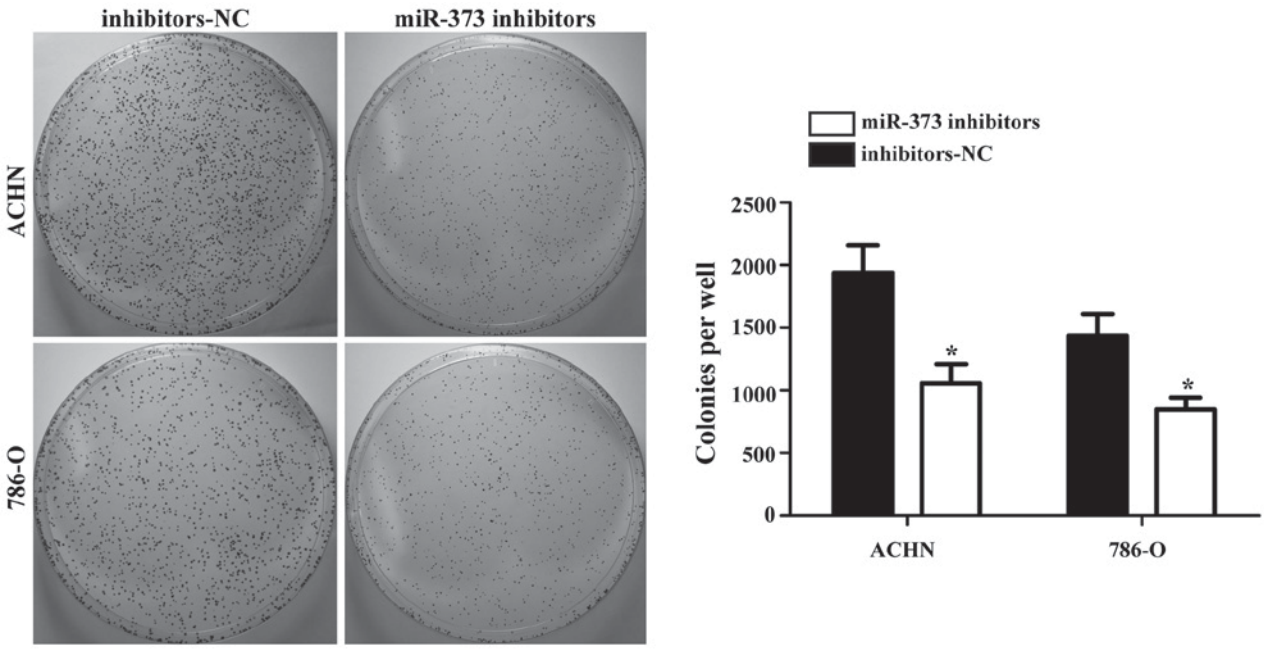

Figure 3. Knockdown of miR-373 inhibits RCC cell colony formation. The effects of miR-373 on the colony forming-abilities of ACHN and 786-O cells were determined using a colony formation assay. *P<0.05 vs. inhibitors-NC group. RCC, renal cell carcinoma; miR-373, microRNA-373; NC, negative control.

Knockdown of miR-373 induces RCC cell apoptosis. To explore the effects of miR-373 on the apoptosis of RCC cells, flow cytometry was used to determine the apoptotic rate (Fig. 4). Compared with the cells transfected with inhibitors-NC (apoptotic rate, 6.32%), the ACHN cell apoptotic rate was significantly increased post-transfection with miR-373 inhibitors (10.77%; Fig. 4A and C; P<0.05). In addition, the apoptosis assay revealed that 786-O cells transfected with miR-373 inhibitors exhibited increased apoptosis, which was increased from 4.65% (inhibitors-NC group) to 21.46% (miR-373 inhibitors group) (Fig. 4B and C; P<0.05).

Knockdown of miR-373 suppresses tumor growth in vivo. The present study demonstrated that miR-373 may function as an oncogene in RCC by promoting cell proliferation and inhibiting apoptosis *in vitro*; therefore, whether miR-373 exerts a similar tumor-promoting effect was investigated *in vivo*. ACHN and 786-O cells transfected with miR-373 inhibitors or inhibitors-NC were subcutaneously injected into nude mice (n=20). Total RNA was then isolated from tissues as aforementioned and the expression levels of miR-373 were detected in xenograft tumor tissue by RT-qPCR. The results indicated that the expression levels of miR-373 were downregulated in tumors from the miR-373 inhibitors group compared with in the inhibitors-NC group (Fig. 5A, P<0.01). Tumor volume in the injected mice was measured weekly with calipers. The growth curves generated for tumor volume in the nude mice demonstrated that knockdown of miR-373 significantly inhibited tumor volume compared with in the inhibitors-NC group (Fig. 5B and C, P<0.05). The tumors were extracted 28 days post-injection, and tumor volume was measured. Representative images of the extracted tumors on day 28 are presented in Fig. 5D. Knockdown of miR-373 significantly reduced tumor volume compared with in the inhibitors-NC group (Fig. 5E, P<0.05). In addition, tumor tissue weight in the miR-373 inhibitors group was markedly lower than in the inhibitors-NC group (Fig. 5F, P<0.05). These results strongly indicated that knockdown of miR-373 may suppress RCC growth *in vivo*.

Discussion

RCC remains the third leading cause of urological tumor-associated mortality worldwide (12,13). Although advances have been made regarding the therapeutic strategies used to treat RCC, the treatment of metastatic RCC remains a challenge (15,16). Previous studies have demonstrated that miRNAs are associated with cancer development and progression (7-9). Abnormal expression of miRNAs has been detected in cancer tissues and cell lines, thus indicating a feasible association between miRNAs and tumorigenesis. For example, miR-92a is upregulated in cervical cancer, and promotes tumor cell proliferation and invasion by targeting F-box and WD repeat domain-containing 7 (25). Downregulation of miR-182 is associated with the proliferation and invasion of osteosarcoma cells via regulating T-cell lymphoma invasion and metastasis 1 expression (26). In addition, increased expression of miR-222 is associated with unfavorable prognosis in bladder cancer (27).

Previous studies have demonstrated that miR-373 is involved in the tumorigenesis of numerous types of cancer, including oral carcinomas, lung adenocarcinoma and breast cancer (21-23). However, the present study is the first, to the best of our knowledge, to demonstrate that miR-373 may participate in the carcinogenesis of RCC. Compared with the HK-2 immortalized normal human proximal tubule epithelial cell line, the expression levels of miR-373 were significantly upregulated in RCC cell lines. In addition, concordant with the results in RCC cells, miR-373 expression was also markedly increased in RCC tissues compared with in adjacent normal tissues.

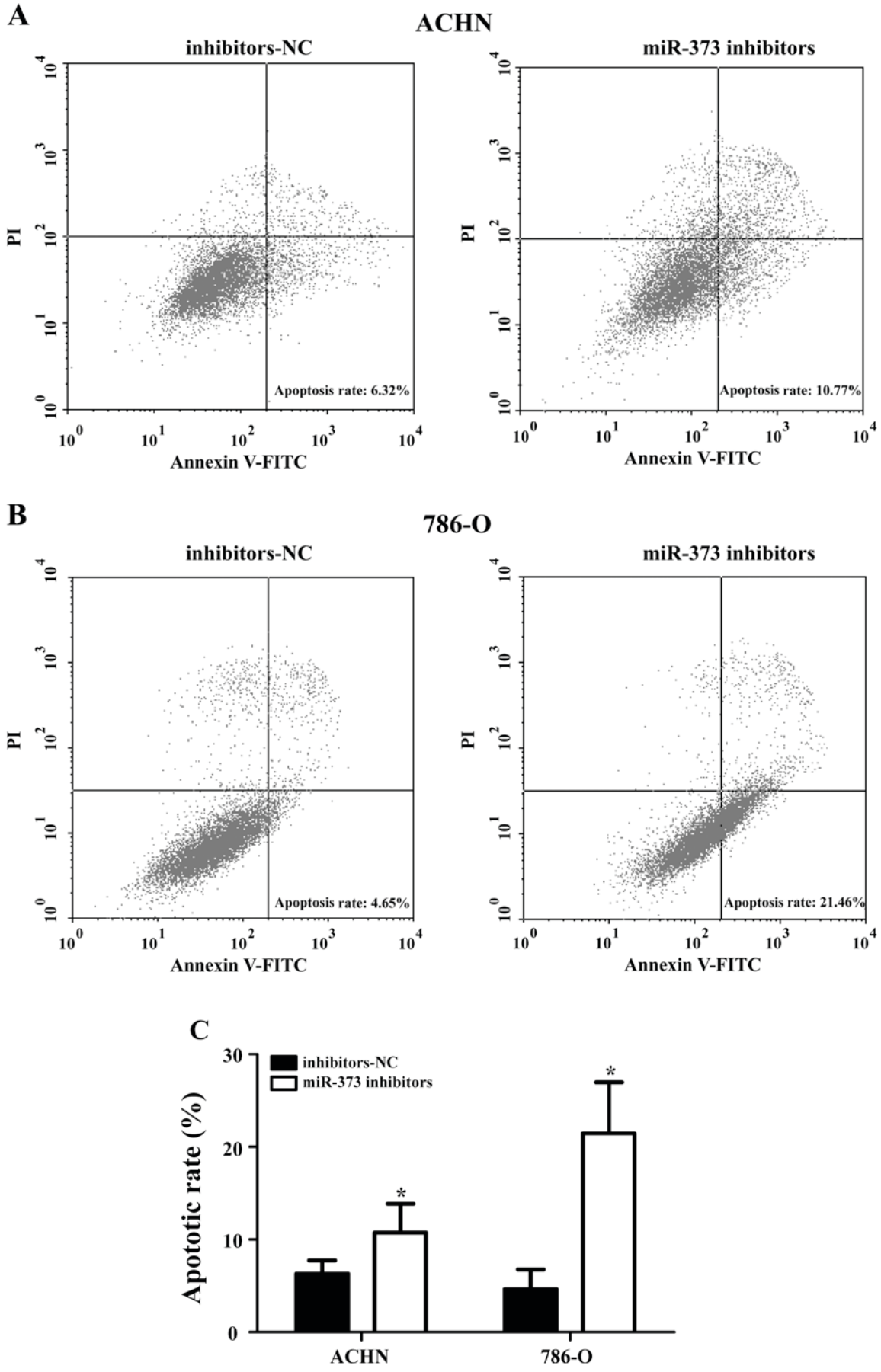

Figure 4. Knockdown of miR-373 induces RCC cell apoptosis. Effects of miR-373 on the apoptosis of (A) ACHN and (B) 786-O cells. (C) Flow cytometry demonstrated that 786-O and ACHN cells transfected with miR-373 inhibitors exhibited a significantly increased apoptotic rate compared with the inhibitors-NC group. *P<0.05 vs. inhibitors-NC group. RCC, renal cell carcinoma; FITC, fluorescein isothiocyanate; miR-373, microRNA-373; NC, negative control; PI, propidium iodide.

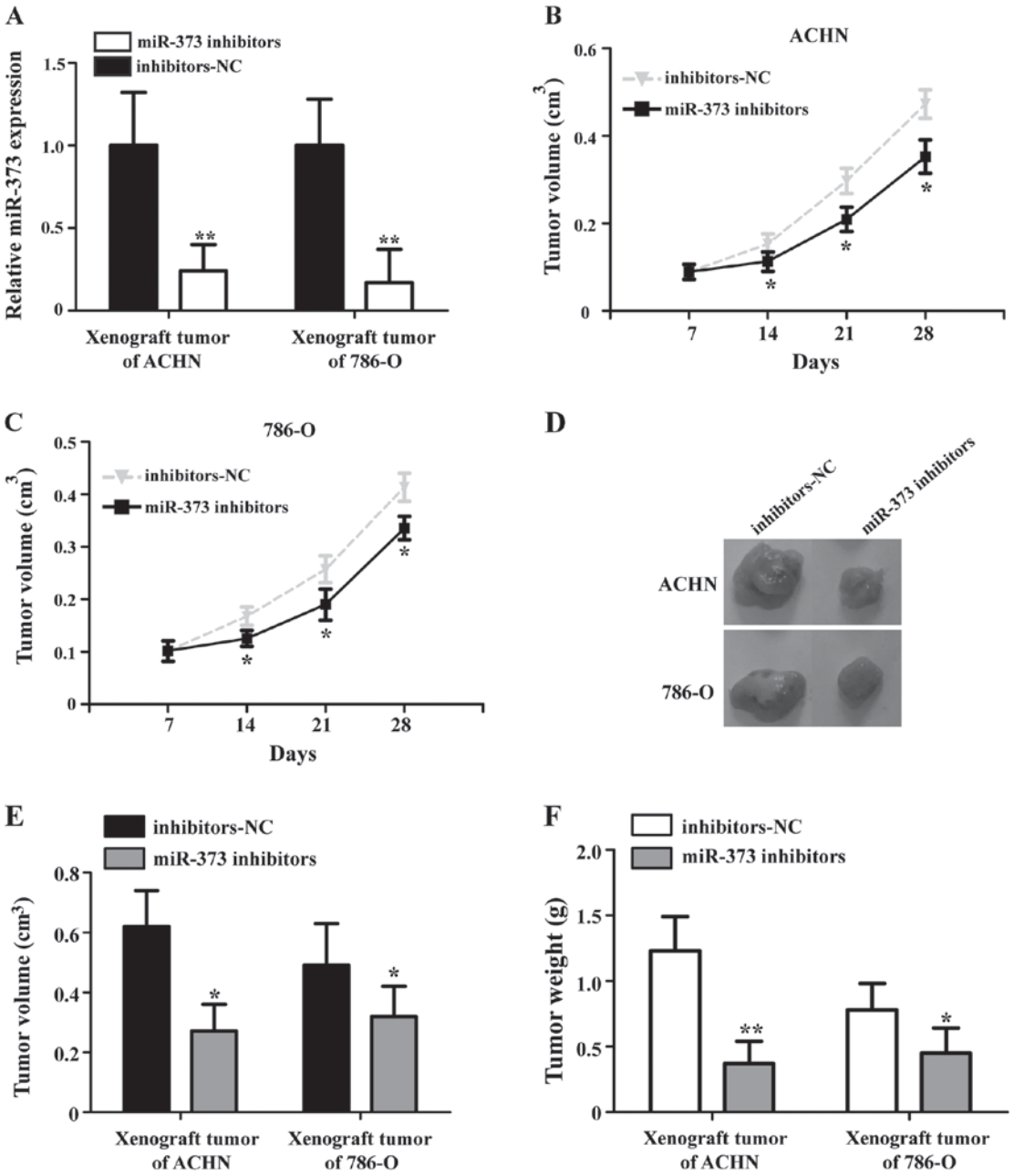

Figure 5. Knockdown of miR-373 suppresses tumor growth *in vivo*. (A) Reverse transcription-quantitative polymerase chain reaction was used to analyze miR-373 expression in tumor tissues extracted from a mouse xenograft model. Nude mice were subcutaneously injected with $3x10^6$ transfected cells. (B and C) Growth curves for tumor volumes in the nude mice. (D) Representative images of extracted tumors. Measurements of the final (E) volume and (F) weight of the extracted tumors on day 28. $^*P<0.05$, $^{**}P<0.01$ vs. inhibitors-NC group. miR-373, microRNA-373; NC, negative control.

The increased expression of miR-373 in RCC prompted the performance of experiments to determine whether miR-373 may function as an oncogene. Further experiments demonstrated that knockdown of miR-373 may suppress cell growth and colony formation, and induce cell apoptosis. In addition, an *in vivo* tumorigenicity assay revealed that suppression of miR-373 expression reduced tumor growth in nude mice. These findings suggested that miR-373 may serve an oncogenic role in RCC, and may therefore be considered a novel therapeutic target for the treatment of RCC. Recently, Liu *et al* reported that miR-373 may promote migration and invasion in human esophageal squamous cell carcinoma by regulating TIMP metallopeptidase inhibitor 3 expression (28). Wang *et al* indicated that miR-373 targeted the YOD1 deubiquitinase gene and functioned as an oncogene in cervical cancer (29). Furthermore, Zhang *et al* demonstrated that miR-373 was upregulated and contributes to tumorigenesis in human gastric cancer by targeting tumor necrosis factor-α-induced protein 1 (30). These previous findings supported the results of the present study regarding RCC.

In conclusion, the results of the present study demonstrated that miR-373 is upregulated in RCC tissues and cell lines. In addition, knockdown of miR-373 exerted a significant suppressive effect on RCC proliferation and colony formation *in vitro*, and tumor growth in a xenograft nude mouse model, strongly suggesting that miR-373 acts as an oncogene in RCC. These

findings indicated that miR-373 may serve as a promising diagnostic biomarker and therapeutic target for patients with RCC.

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