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Original Article

MiR-216a exerts tumor-suppressing functions in renal cell carcinoma by targeting TLR4

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Abstract: MiR-216a, a tumor-related microRNA (miRNA), has been reported to be implicated in the tumorigenesis and progression of diverse types of human malignancies; however, its role in renal cell carcinoma (RCC) remains unclear. This study aimed to explore the biological role of miR-216a in RCC and clarify the potential mechanisms involved. In the present study, miR-216a was found to be significantly down-regulated in both RCC tissues and cell lines. Functional studies demonstrated that enhanced expression of miR-216a suppressed RCC cell proliferation, migration and invasion *in vitro*, inhibited tumor growth *in vivo*, and induced RCC cell cycle arrest and apoptosis. Moreover, the tumor-suppressing effects of miR-216a in RCC were abrogated by the miR-216a inhibitor treatment. Notably, toll-like receptor 4 (TLR4) was downregulated by miR-216a via direct binding to its 3' untranslated region in RCC cells. Furthermore, TLR4 expression was discovered to be markedly up-regulated and inversely correlated with miR-216a expression in RCC tissues. Mechanistic studies revealed that restoring the expression of TLR-4 alleviated miR-216a-induced inhibitory effects on proliferation, migration and invasion of RCC cells. Taken together, these findings suggest that miR-216a functions as a tumor suppressor in RCC by directly targeting TLR4 and that miR-216a might be a novel therapeutic target for RCC.

Keywords: Renal cell carcinoma, miR-216a, TLR4, tumor-suppressing

Introduction

Renal cell carcinoma (RCC), the predominant type of kidney neoplasm, has the third highest mortality among all genitourinary tumors [1]. RCC is characterized by lack of early warning signs, diverse clinical manifestations and resistance to radiation and chemotherapy [2, 3]. RCC accounts for approximately 3-4% of adult malignancies and 5% of epithelial tumors worldwide [4, 5]. Clear cell RCC (ccRCC) is the most frequent pathological subtype, representing roughly 70% of RCC cases [6, 7]. Approximately 30% of RCC patients develop metastases at the time of diagnosis; in addition, recurrence of RCC is found in roughly 40% of patients who undergo curative surgical resection [8-10]. Even though recent therapeutic developments have improved the overall survival of RCC patients, long-term prognosis for RCC remains poor. Thus, there is an urgent need to elucidate the potential mechanisms underlying RCC occurrence and progression in order to facilitate identification of novel prognostic markers and development of promising targeted therapeutic strategies.

MicroRNAs (miRNAs) are endogenous small (~22 nucleotides) single-strand and non-coding RNA molecules, exerting their functions through binding to the 3'-untranslated regions (3'UTRs) of target mRNAs in a sequence-specific manner [11, 12]. Accumulating evidence indicates that aberrant expression of miRNAs plays crucial roles in carcinogenesis and progression of multiple types of human cancers, including hepatocellular carcinoma, nasopharyngeal carcinoma, breast cancer and ovarian cancer [13-16]. Indeed, ectopic expression of miRNAs has also been found to be involved in RCC occurrence and progression [17-20].

Toll-like receptors (TLRs) are a group of pattern recognition receptors (PPRs) expressed on the cellular surface that are responsible for recognizing the conserved molecular motifs [21, 22]. TLR4 has been found to be associated with the migratory, invasive and adhesive properties of

cancer cells and involved in diverse human cancers, including lung cancer, colorectal cancer and pancreatic cancer [23-25].

miR-216a has been reported to be down-regulated and act as a tumor suppressor in multiple types of human tumors, including pancreatic cancer, colorectal cancer and oral squamous cell carcinoma [26-28]; however, its role in RCC remains unknown. The current study aimed to explore the role of miR-216a in RCC and clarify the potential mechanisms involved. In the present study, miR-216a was found to be markedly downregulated in RCC tissues and cell lines, and exert tumor-suppressing functions in RCC. Furthermore, TLR4 was identified as a direct functional target of miR-216a in RCC.

Materials and methods

Patients and tissue specimens

RCC tissues and the matched adjacent non-cancerous tissues were obtained from 27 patients who underwent nephrectomy from January 2013 to October 2015 in the Second Affiliated Hospital of Harbin Medical University (Harbin, China). Matched adjacent normal tissues were obtained at a distance of 5 cm from RCC tissues. All clinical samples were immediately frozen in liquid nitrogen and stored at -80°C for subsequent experiments. This study was approved by the Ethics and Scientific Committee of Harbin Medical University. All the patients enrolled in the present study gave written informed consent.

Cell lines and culture

Six human RCC cell lines (786-O, ACHN, Caki-1, A498, GRC-1 and OS-RC-2) and a normal human kidney cell line (HK-2) were obtained from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 medium (Gibco, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (FBS, Shanghai Sangon Biological Engineering Technology and Services, Shanghai, China). All the cells were maintained at 37°C in a humidified 5% CO_2 environment.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted from tissues and cells using TRIzol reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Following quantification with a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, USA), the extracted total RNA was reverse-transcribed using a TaqMan High-Capacity cDNA Archive kit (Applied Biosystems, Foster, USA). For mRNA analysis, reverse transcription products were mixed with TaqMan Universal PCR Master Mix (Applied Biosystems), and qRT-PCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR systems (Applied Biosystems). The specific primer sequences (Invitrogen, Shanghai, China) were as follows: TLR4, forward 5'-CTGCAATGGATCAAGGACCA-3' and reverse 5'-TCCCACTCCAGGTAAGTGTT-3'; GAPDH, forward 5'-ACAACTTTGGTATCGTGGAAGG-3', reverse 5'-GCCATCACGCCACAGTTTC-3'. GAPDH was used as an endogenous control to normalize TLR4 expression levels.

For miRNA expression analysis, microRNAs were isolated using miRNeasy Mini kit (Qiagen, Hilden, Germany) and TaqMan MicroRNA Assay Kit was used to detect miRNA expression on an Applied Biosystems 7500 Fast Real-Time PCR systems (Applied Biosystems). The specific primer sequences (Invitrogen, Shanghai, China) were as follows: miR-216a, forward 5'-TAATCTCAGCTGGCAACTGTGA-3' and reverse 5'-TCACAGTTGCCAGCTGAGATTA-3'; U6, forward 5'-CTCGCTTCGGCAGCACA-3', reverse 5'-AACGCTTCACGAATTTGCGT-3'. The PCR conditions were as follows: denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 15 sec and 72°C for 30 sec. The universal small nuclear RNA, U6, was used as an internal control. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method. The experiments were carried out in triplicate.

Cell transfection

Cell transfection was performed using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, USA) according to manufacturer's protocols. Cells were transfected with miR-control, miR-216a mimics or miR-216a inhibitor. miR-control, miR-216a mimics and miR-216a inhibitor were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). Cells were harvested 48 h post-transfection for further experiments.

Cell viability assay

Cell proliferation was assessed using MTT Cell Proliferation and Cytotoxicity Assay Kit (Sigma-

Aldrich, St. Louis, USA) according to the manufacturer's protocols. Briefly, following transfection with miR-control, miR-216a mimics or miR-216a inhibitor, cells were seeded in each well of 96-well plates at a density of 1×10^4 cells/well. Following incubation at 37°C for different periods of time (12, 24, 48 and 72 h), the culture medium was removed and MTT (20 μL; 5 mg/mL) was added to each well. After incubation at 37°C for another 4 hours, MTT solution was removed and replaced with dimethyl sulfoxide (150 μL; 4%; Sigma-Aldrich). Absorbance was measured at 560 nm by a microplate reader (Bio-Tek Instruments, Germany).

Tumor xenograft model assay

All animal experiments were performed according to institutional and international animal regulations. The animal protocol was approved by the Institutional Animal Care and Use Committee of Harbin Medical University. Female BALB/c nude mice (6 weeks of age) were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China). 786-O and Caki-1 cells transfected with miR-control, miR-216a mimics or miR-216a inhibitor (1×10^6 cells per mouse) were subcutaneously administered into flanks of BALB/c nude mice, respectively. The length and width of tumors were measured using a caliper every 5 days. All mice were euthanized after 35 days, and their tumor nodules removed and weighed. The tumor volume was calculated according to the following equation: tumor volume (mm^3) = length (mm) × width $(mm)^2/2$.

Cell cycle and apoptosis analysis

For cell cycle analysis, the cells were harvested at 48 h post-transfection. The cells were washed with PBS and fixed in ethanol at -20°C. Then, the cells were washed with PBS, rehydrated and stained in propidium iodide (PI) (BD Biosciences, San Jose, USA). The stained cells (~1×10^5) were then examined using a flow cytometer and analyzed with ModFit software (BD Biosciences, San Jose, USA).

For apoptosis analysis, the cells were harvested, washed with ice-cold PBS and stained with Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kits (BD Biosciences). The stained cells were measured using a flow cytometer (BD Biosciences).

Wound healing assay

Cells were seeded in 6-well culture plates to grow into a monolayer (100% confluence). The cell monolayer was scraped using a pipette and washed twice with medium to form a wound. The cells were further cultured in the medium for 24 h and scratch closure was observed using an inverted microscope (Olympus, Japan). Cells were observed at 0 and 24 h after scraping under an inverted microscope and corresponding photographs were taken. The cell-free area at 24 h after wounding and original denuded area were measured using Image J software (National Institutes of Health, Bethesda, MD, USA).

Cell invasion assay

Transwell invasion assay was performed to assess cell invasion. The upper surface of the filter (pore size, 8.0 μm; Biosciences, Heidelberg, Germany) was coated with basement membrane Matrigel (BD Bioscience), at a concentration of 2 mg/ml and incubated at 4°C for 3 h. Cells (2×10^4) were seeded into the upper chamber with 200 μL serum-free medium. The lower chamber was supplemented with 750 μL medium containing 10% FBS. Following incubation for 24 h at 37°C, cells were fixed with 4% polyoxymethylene and stained with 0.5% crystal violet (Sigma-Aldrich). Then stained cells were observed and counted under a microscope. Five visual fields were selected and the average number was calculated.

Luciferase reporter assay

The 3'-UTR of TLR4 containing a binding site for miR-216a was amplified from a human cDNA library and cloned into a dual-luciferase expression vector (Promega, Madison, WI, USA). Mutant TLR4 3'-UTR was obtained by overlap-extension PCR. Cells were co-transfected with miR-216a mimics and wild-type or mutant 3' UTR of TLR4 by Lipofectamine 2000. Relative luciferase activity was measured on a dual-luciferase reporter assay system (Promega Corp., Fitchburg, USA) at 48 h post-transfection. Data were expressed as the ratio of Renilla luciferase activity to firefly luciferase activity.

Western blot analysis

Protein lysates were extracted from cells using 500 μL radioimmunoprecipitation assay (RIPA)

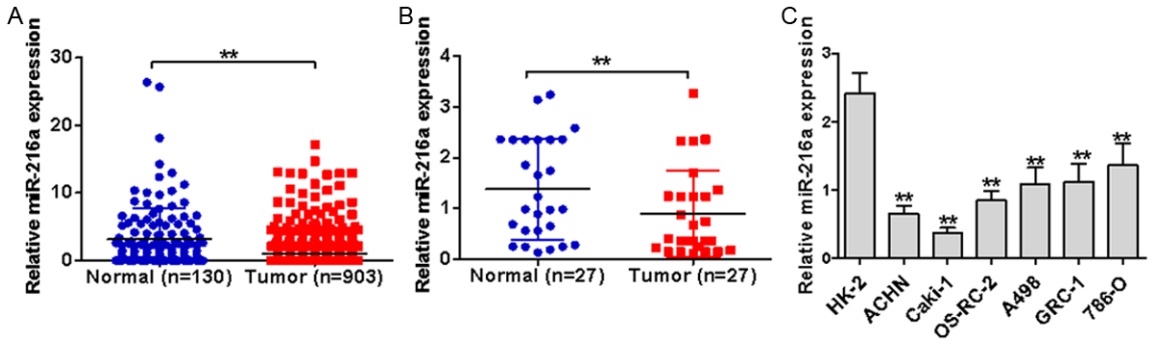


Figure 1. miR-216a is significantly downregulated in RCC tissues and cell lines. A. miR-216a expression data in RCC tissues and adjacent normal tissues were downloaded from the TCGA database. B. miR-216a expression levels in 27 pairs of RCC tissues and adjacent non-cancerous tissues were measured using qRT-PCR. C. miR-216a expression levels in six human RCC cell lines (786-O, ACHN, Caki-1, A498, GRC-1 and OS-RC-2) and normal human kidney HK-2 cells were detected via qRT-PCR. Each sample was tested three times. $^{**}P < 0.01$.

buffer with 1 mM phenylmethane sulfonyl fluoride. Samples were subsequently sonicated for 2 min and centrifuged. The supernatants were collected and used for protein analysis. Lysates were separated on 8% polyacrylamide gels and transferred onto PVDF membranes. The membranes were blocked with phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBST) and 5% nonfat milk (w/v) for 1 h at room temperature. After they were washed with PBST, the membranes were probed with antibodies overnight at 4°C. Antibodies were all obtained from Abcam (Cambridge, MA, USA) and used at the following dilutions: anti-TLR4 (1:1000) and anti-GAPDH (1:3000). The membranes were washed again with PBST, then horseradish peroxidase (HRP) labeled IgG at 1:5000 dilution was added at room temperature for 1 h, and the blots were developed using ECL western blotting reagents.

Immunohistochemistry (IHC)

Tissue specimens were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin. Then, the sections were deparaffinized and rehydrated. Hydrogen peroxide was applied to block endogenous peroxide activity for 10 min. After antigen retrieval using a microwave, the sections were treated with 1% bovine serum albumin to block nonspecific binding. The sections were then incubated with rabbit anti-TLR4 (Abcam, Cambridge, MA, USA) in a humidified chamber overnight at 4°C. After washing three times with phosphatebuffered saline (PBS) for 5 min each time, tissue sections were treated with a biotinylated anti-rabbit secondary antibody (Abcam, Cambridge, MA, USA), followed by further incubation with streptavidin-horseradish peroxidase complex. After rinsing, diaminobenzidine (DAB; Abcam, Cambridge, MA, USA) was used as a chromogen, and the sections were counterstained with hematoxylin. Samples incubated with PBS instead of the primary antibody served as negative controls.

Statistical analysis

Data were presented as mean ± standard deviation (SD). Statistical analysis was performed using SPSS 16.0 software (SPSS, Chicago, IL, USA). A two-tailed student's *t*-test was applied to compare the differences between two groups and one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison was employed to compare the differences among three independent groups. Correlation between miR-216a expression and TLR4 expression in RCC tissues was determined using Pearson's correlation analysis. $P < 0.05$ was considered statistically significant.

Results

miR-216a is significantly downregulated in RCC tissues and cell lines

Although miR-216a has been reported to be involved in diverse types of human cancers, its role in RCC remains unknown. Data from the TCGA database demonstrated that the majority of RCC tissues displayed lower expression levels of miR-216a than corresponding non-can-

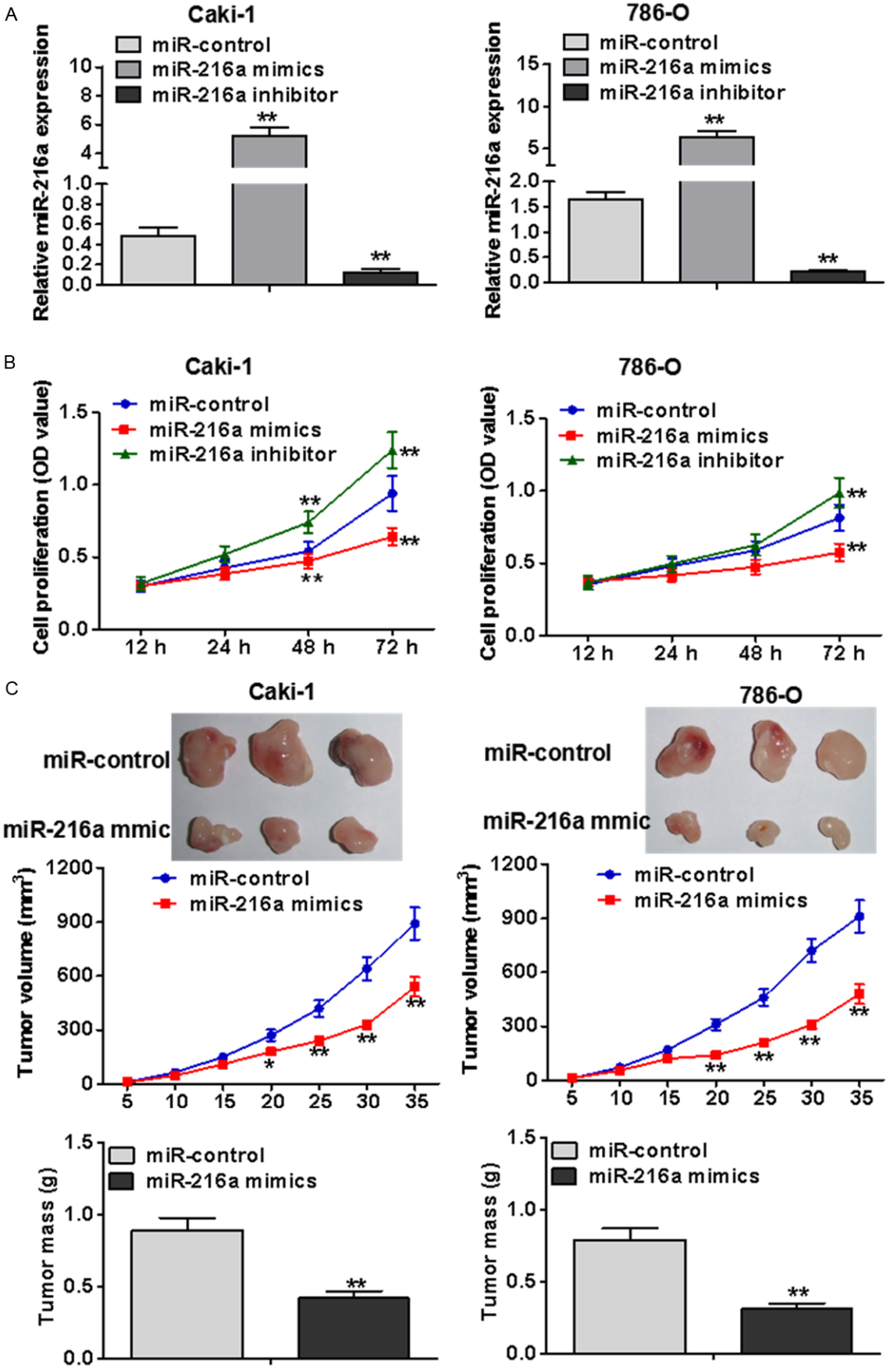

A
Caki-1
786-O
miR-control
miR-216a mimics
miR-216a inhibitor
Relative miR-216a expression
B
Caki-1
786-O
Cell proliferation (OD value)
12 h
24 h
48 h
72 h
C
Caki-1
786-O
miR-control
miR-216a mmic
Tumor volume (mm3)
Tumor mass (g)

Figure 2. miR-216a suppresses RCC cell proliferation *in vitro* and tumorigenesis *in vivo*. A. miR-216a expression levels in 786-O and Caki-1 cells were assessed by qRT-PCR after transfection with miR-control (negative control), miR-216a mimics or miR-216a inhibitor. B. Cell viability was evaluated using MTT assay after transfection with miR-control, miR-216a mimics, or miR-216a inhibitor. C. Tumor volumes were measured every 5 days using a slide caliper; at day 35 post-implantation, mice were sacrificed under anesthesia and tumors were weighed (n=3). $*P < 0.05$, $**P < 0.01$.

cerous tissues (**Figure 1A**). Subsequently, we examined the expression of miR-216a in 27 pairs of RCC tissues and adjacent normal tissues using qRT-PCR. qRT-PCR. qRT-PCR analysis showed that the expression of miR-216a in RCC tissues was markedly lower than that in adjacent normal tissues (**Figure 1B**). Consistently, RCC cell lines exhibited lower expression levels of miR-216a than normal human kidney HK-2 cells (**Figure 1C**). 786-O (highest endogenous miR-216a expression) and Caki-1 (lowest endogenous miR-216a expression) cells were selected for subsequent study.

miR-216a suppresses RCC cell proliferation in vitro and tumorigenesis in vivo

To explore the potential role of miR-216a in RCC, 986-O and Caki-1 cells were transfected with miR-control, miR-216a mimics or miR-216a inhibitor. The transfection efficiency was assessed by qRT-PCR (**Figure 2A**). As evident from the MTT assays, miR-216a overexpression dramatically repressed cell proliferation compared with the miR-control group, whereas the miR-216a inhibitor markedly promoted cell proliferation (**Figure 2B**). Tumor xenograft model assay was performed to investigate the effect of miR-216a on tumorigenesis *in vivo*. The results demonstrated that enhanced expression of miR-216a notably inhibited tumor growth *in vivo* compared with the miR-control treatment, whereas the miR-216a inhibitor dramatically promoted tumor growth *in vivo* (**Figure 2C**). Moreover, a significant reduction in tumor weight was observed in the miR-216a mimics group compared with the miR-control group, whereas a marked increase in tumor weight was seen in miR-216a inhibitor group (**Figure 2C**). Our data indicates that miR-216a inhibits RCC cell proliferation *in vitro* and tumorigenesis *in vivo*.

miR-216a induces RCC cell cycle arrest and facilitates cell apoptosis

Since a marked decrease in cell viability was caused by miR-216a mimics, we aimed to explore whether this decrease was associated with cell cycle progression and apoptosis. Flow cytometry was employed to detect cell cycle and apoptosis. A dramatic increase in the proportion of G1-phase cells and a notable decrease in the proportion of S-phase cells were observed in the miR-216a mimics group compared with the miR-control treatment (**Figure 3A**), which indicated that miR-216a induced G1 phase arrest. Cells transfected with miR-216a mimics exhibited a dramatic increase in the apoptotic rate compared with miR-control treatment, whereas a marked decrease in the apoptotic rate was observed in the miR-216a inhibitor treatment (**Figure 3B**). These results suggest that miR-216a induces cell cycle arrest and apoptosis.

miR-216a represses RCC cell migration and invasion

To determine whether miR-216a influences the mobility of RCC cells, we detected migration and invasion capabilities of 786-O and Caki-1 cells after transfection with miR-control, miR-216a mimics or miR-216a inhibitor. The results demonstrated that migration and invasion capabilities of 786-O and Caki-1 cells were dramatically weakened by the miR-216 mimics treatment compared with miR-control treatment, whereas miR-216 inhibitor notably enhanced the migration and invasion abilities of 786-O and Caki-1 cells (**Figure 4A** and **4B**). These findings indicate that miR-216a exerts inhibitory effects on migration and invasion of RCC cells.

TLR4 is a direct target of miR-216a

To elucidate the potential mechanism by which miR-216a suppresses RCC cell proliferation, migration and invasion, TargetScan and miRanda algorithms were applied to predict the potentital targets of miR-216a. TLR4, frequently reported to be involved in the occurrence, metastasis and progression of multiple types of human cancers, was selected as a candidate target of miR-216a (**Figure 5A**). To validate whether TLR4 is regulated via direct binding of

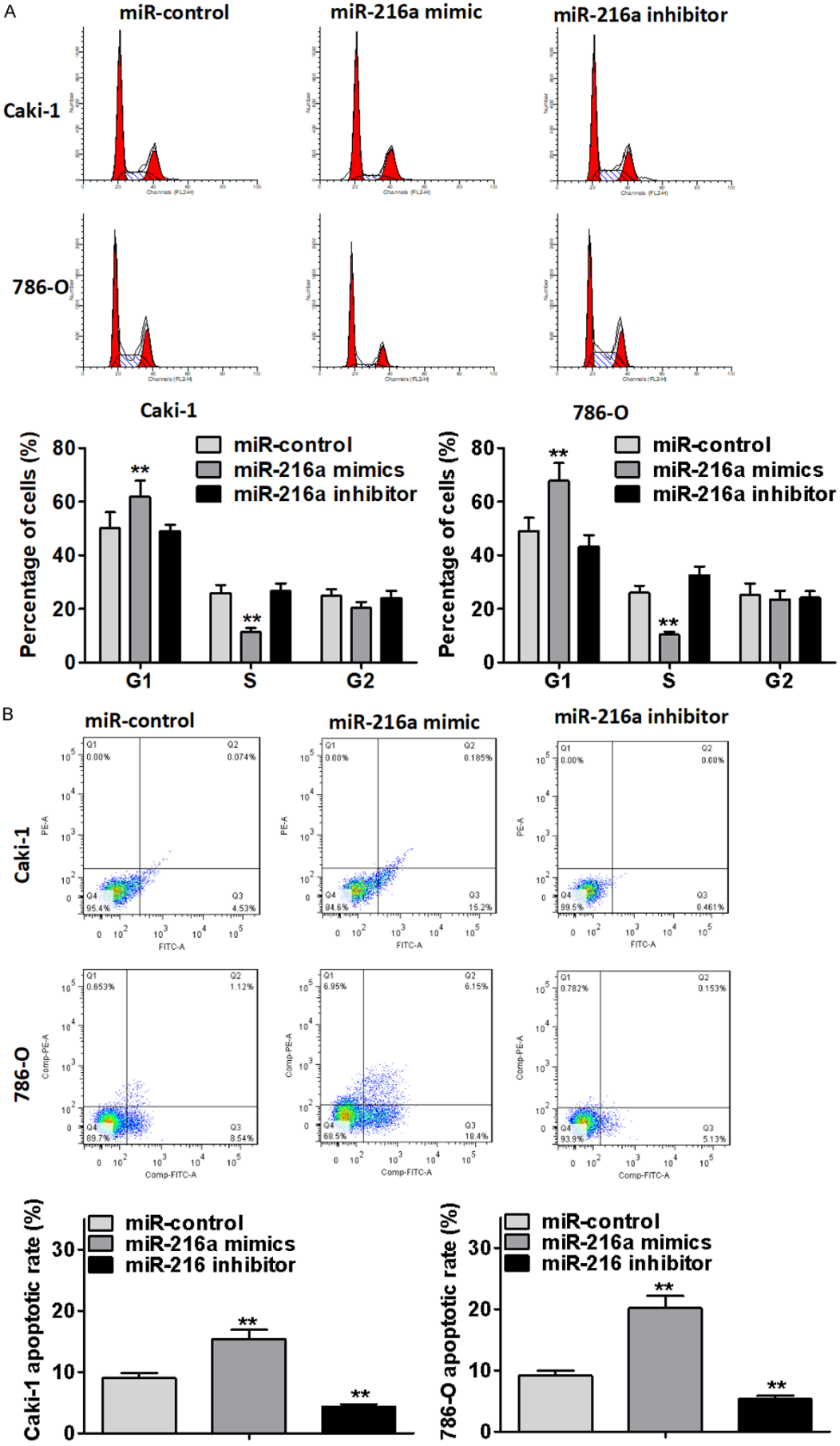

A
miR-control
miR-216a mimic
miR-216a inhibitor
Caki-1
786-O
Caki-1
786-O
Percentage of cells (%)
miR-control
miR-216a mimics
miR-216a inhibitor
G1
S
G2
B
miR-control
miR-216a mimic
miR-216a inhibitor
Caki-1
786-O
PE-A
FITC-A
Comp-PE-A
Comp-FITC-A
Caki-1 apoptotic rate (%)
786-O apoptotic rate (%)
miR-control
miR-216a mimics
miR-216 inhibitor

Figure 3. miR-216a induces RCC cell cycle arrest and promotes cell apoptosis. A. Cell cycle was analyzed using flow cytometry after transfection with miR-control, miR-216a mimics or miR-216a inhibitor. B. Cell apoptosis was detected via flow cytometry after transfection with miR-control, miR-216a mimics or miR-216a inhibitor. $^{**}P < 0.01$.

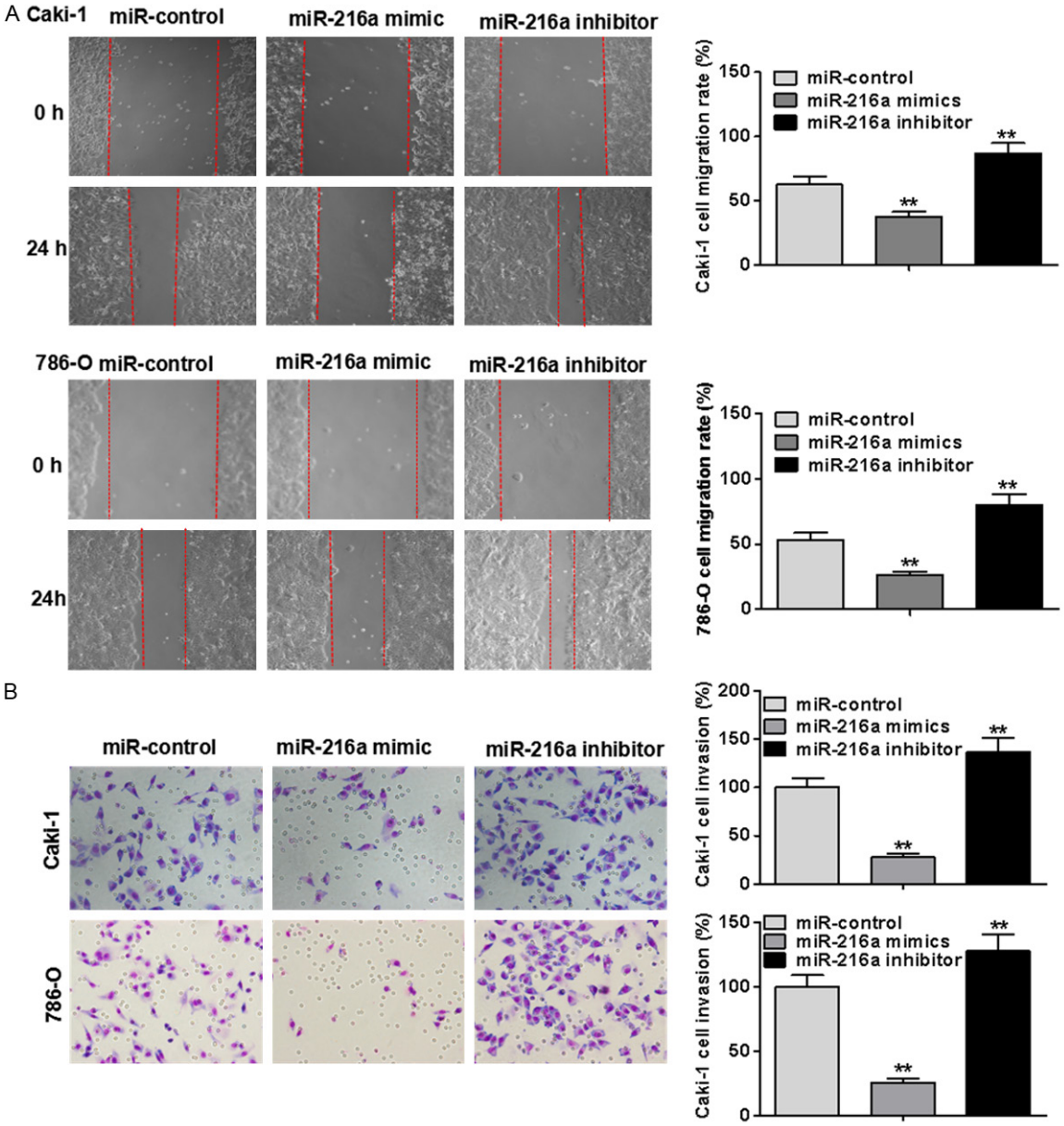


Figure 4. miR-216a represses RCC cell migration and invasion. A. Cell migration was measured using wound healing assays after transfection with miR-control, miR-216a mimics or miR-216a inhibitor. B. Cell invasion was identified via transwell invasion assays after transfection with miR-control, miR-216a mimics or miR-216a inhibitor. $^{**}P < 0.01$.

miR-216a to its 3'UTR, luciferase reporter assays were performed. TLR4 3'UTR fragments containing miR-216a targeting sequences and its corresponding mutant fragments were subcloned into firefly luciferase vectors, respectively. The results demonstrated that luciferase activity was dramatically decreased in the cells cotransfected with miR-216a mimics and wild-type TLR4 3'UTR in comparison with miR-control treatment, whereas co transfection of miR-216a mimics and mutant TLR4 3'UTR failed to repress luciferase activity (**Figure 5B**).

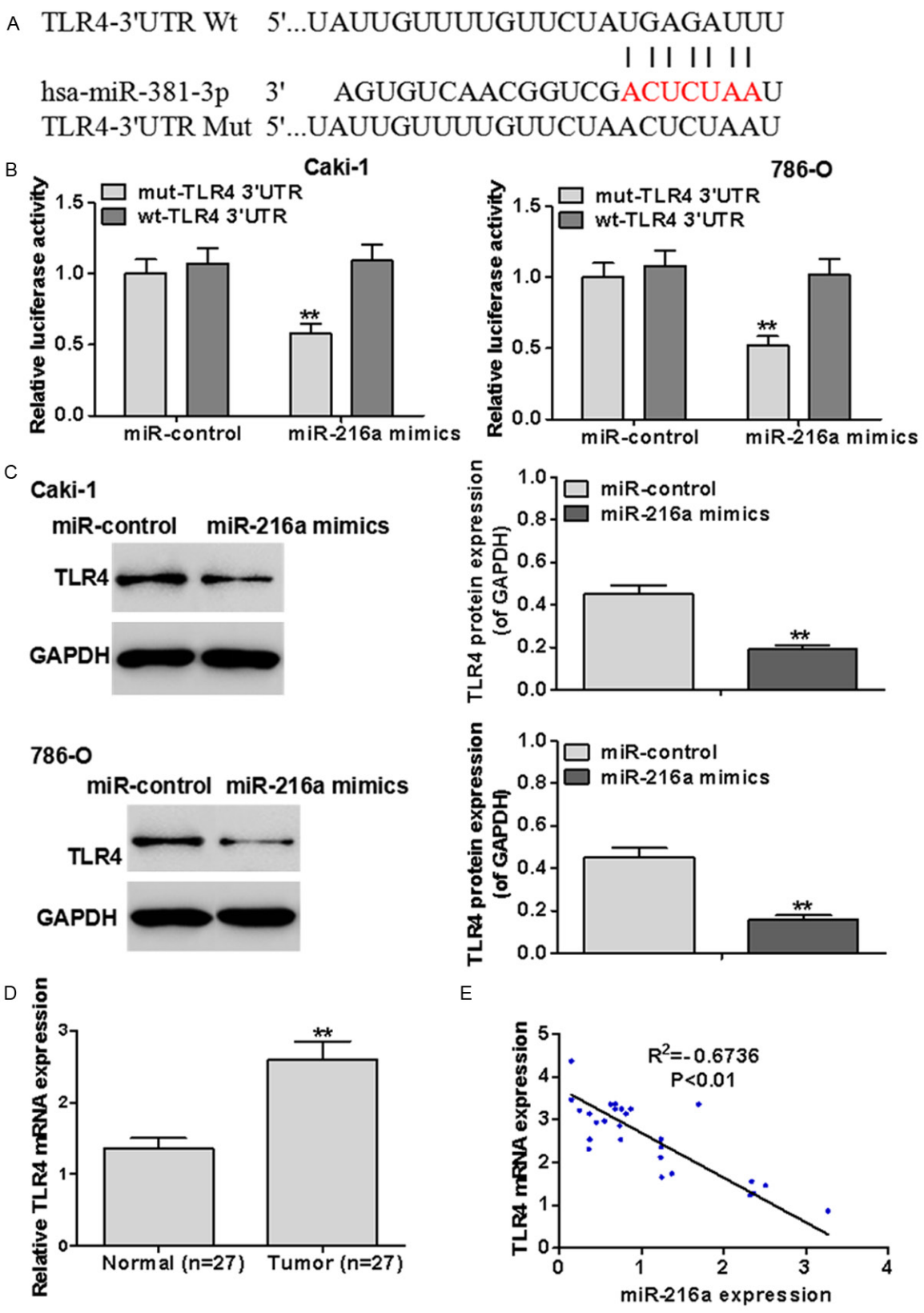


Figure 5. TLR4 is a direct target of miR-216a. A. A putative binding site of miR-216a in the 3'UTR of TLR4 was predicted by TargetScan and miRanda algorithms. B. Luciferase activity of reporter vectors carrying wild-type or mutant TLR4 3'UTR was detected in the presence of miR-216a. C. Expression of TLR4 in 786-O and Caki-1 cells transfected

with miR-control or miR-216a mimics was assessed by western blot. D. TLR4 mRNA expression in 29 pairs of RCC tissues and adjacent normal tissues was measured using qRT-PCR. E. Correlation between miR-216a expression and TLR4 expression in RCC tissues was determined using Pearson's correlation analysis.

Western blot analysis showed that TLR4 was downregulated in cells transfected with miR-216a mimics compared with miR-control treatment (**Figure 5C**). Moreover, TLR4 mRNA levels in RCC tissues and adjacent normal tissues were examined using qRT-PCR. RCC tissues displayed higher TLR4 mRNA levels than adjacent normal tissues (**Figure 5D**). In addition, Pearson's correlation analysis revealed that miR-216a expression was inversely correlated with TLR4 expression (**Figure 5E**). These results indicate that miR-216a directly binds to the 3'UTR of TLR4 to suppress its expression.

Restoring TLR4 expression alleviates the inhibitory effects of miR-216a on proliferation, migration and invasion of RCC cells

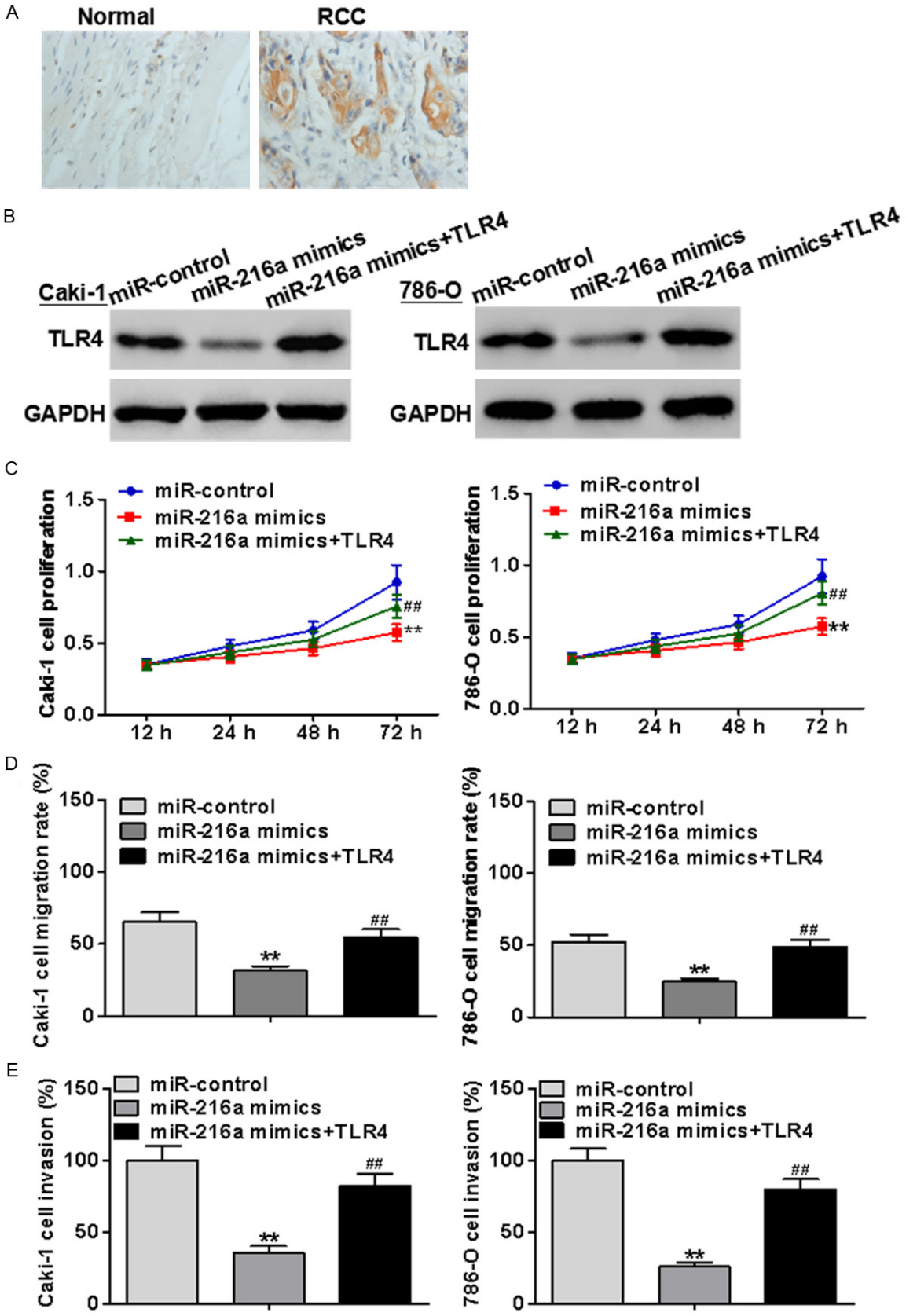
Given that TLR4 is closely associated with carcinogenesis and metastasis, TLR4 expression in RCC tissues and adjacent non-cancerous tissues was evaluated by immunohistochemistry (IHC). IHC analysis revealed that RCC tissues exhibited higher expression levels of TLR4 than adjacent normal tissues (**Figure 6A**). To identify the functional connection between miR-216a and TLR4, we rescued the expression of TLR4 in 786-O and Caki-1 cells transfected with miR-216a mimics (**Figure 6B**). Restoring the expression of TLR4 was found to accelerate the proliferation of RCC cells transfected with miR-216a mimics, and enhance their migration and invasion capabilities (**Figure 6C-E**), which suggests that restoring TLR4 expression alleviates miR-216a-induced inhibitory effects on proliferation, migration and invasion of RCC cells. These results indicate that miR-216a exerts its inhibitory effects on proliferation, migration, and invasion of RCC cells via targeting TLR4.

Discussion

Mounting evidence reveals that abnormal expression of miRNAs exerts crucial roles in RCC occurrence and progression [17-20]. A better understanding of the biological functions of miRNAs may be useful for developing effective therapeutic strategies and identifying novel diagnosis markers for RCC patients. Previous studies have shown that abnormal expression of miR-216a is involved in tumorigenesis and tumor progression of various human cancers. Hou *et al* found that miR-216a was downregulated in pancreatic cancer tissues and functioned as a tumor suppressor in pancreatic cancer via targeting JAK2 [26]. Wang *et al* demonstrated that miR-216a inhibited colorectal cancer cell proliferation through the downregulation of COX-2 and ALOX5 [27]. Additionally, miR-216a was reported to be downregulated in oral squamous cell carcinoma (OSCC) tissues and to inhibit the growth and metastasis of OSCC by targeting EIF4B [28]. However, the role of miR-216a in RCC remains unclear.

In the present study, we initially examined the expression of miR-216a and found that miR-216a was markedly downregulated in RCC tissues and cell lines. To better understand the role of miR-216a in RCC, functional studies were conducted. The results demonstrated that enhanced expression of miR-216a inhibited RCC cell proliferation, migration and invasion *in vitro*, suppressed tumor growth *in vivo*, and induced RCC cell cycle arrest and apoptosis. Moreover, the tumor-suppressing effects of miR-216a in RCC were abolished by miR-216a inhibitor treatment. These findings indicate that miR-216a functions as a tumor suppressor in RCC.

To explore the potential molecular mechanisms by which miR-216a functions as a tumor suppressor in RCC, we performed bioinformatics analysis using TargetScan and miRanda algorithms and predicted that TLR4 was a candidate target of miR-216a. Subsequently, dual luciferase reporter assays identified TLR4 as a direct target of miR-216a in RCC. TLR4, expressed on the cellular surface, includes a domain of leucine-rich repeats (LRR) [29]. TLR4, associated with the migratory, invasive and adhesive properties of cancer cells, has been reported to be involved in the development and progression of multiple types of human cancers. Ikebe *et al* found that TLR4 enhanced the invasion ability of pancreatic cancer (PC) cells and promoted PC progression [25]. Xu *et al* reported that TLR4 promoted tumor cell proliferation and migration in human


A
Normal
RCC
B
Caki-1
miR-control
miR-216a mimics
miR-216a mimics+TLR4
786-O
miR-control
miR-216a mimics
miR-216a mimics+TLR4
TLR4
GAPDH
TLR4
GAPDH
C
Caki-1 cell proliferation
miR-control
miR-216a mimics
miR-216a mimics+TLR4
1.5
1.0
0.5
0.0
12 h
24 h
48 h
72 h
##
**
786-O cell proliferation
miR-control
miR-216a mimics
miR-216a mimics+TLR4
1.5
1.0
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0.0
12 h
24 h
48 h
72 h
##
**
D
Caki-1 cell migration rate (%)
150
100
50
0
miR-control
miR-216a mimics
miR-216a mimics+TLR4
**
##
786-O cell migration rate (%)
150
100
50
0
miR-control
miR-216a mimics
miR-216a mimics+TLR4
**
##
E
Caki-1 cell invasion (%)
150
100
50
0
miR-control
miR-216a mimics
miR-216a mimics+TLR4
**
##
786-O cell invasion (%)
150
100
50
0
miR-control
miR-216a mimics
miR-216a mimics+TLR4
**
##

Figure 6. Restoring the expression of TLR4 alleviates miR-216a-induced inhibitory effects on migration and invasion of RCC cells. A. Expression of TLR4 in RCC tissues and adjacent normal tissues was identified by immunohistochemistry. B. Expression of TLR4 in 786-0 and Caki-1 cells was identified using western blot analysis after rescuing the expression of TLR4. C-E. Proliferation, migration, and invasion of 786-0 and Caki-1 cells were examined after rescuing the expression of TLR4. $^{**}P < 0.01$ vs the miR-control group; $^{\#\#}P < 0.01$ vs the miR-216a mimics group.

colorectal carcinoma [30]. TLR4 was demonstrated to promote tumor growth in ovarian carcinoma [31].

In the current study, western blot analysis demonstrated that TLR4 expression level was notably decreased in 986-0 and Caki-1 cells transfected with miR-216a mimics compared with miR-control treatment. To further determine the link between miR-216 expression and TLR4 expression, we measured the expression of miR-216a and TLR4 in RCC tissues using qRT-PCR. TLR4 was found to be dramatically upregulated in RCC tissues and inversely correlated with miR-216a expression.

To further determine the functional correlation between miR-216a and TLR4, we rescued the expression of TLR4 in 986-0 and Caki-1 cells overexpressing miR-216a. Restoring TLR4 expression was observed to alleviate the inhibitory effects of miR-216 on proliferation, migration and invasion of RCC cells. These results indicate that miR-216a functions as a tumor suppressor partially via targeting directly TLR4.

In conclusion, miR-216a is downregulated in RCC tissues and cell lines and exerts tumor-suppressing functions via directly targeting TLR4. The present study may provide new insights to the molecular mechanism underlying RCC development and progression. Thus, miR-216a may be used as a promising therapeutic target for RCC.

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Disclosure of conflict of interest

None.

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