

# MicroRNA-375 Suppresses the Tumor Aggressive Phenotypes of Clear Cell Renal Cell Carcinomas through Regulating YWHAZ

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## Abstract

**Background:** MicroRNAs (miRNAs) are key regulators during tumor initiation and progression. MicroRNA-375 (MiR-375) has been proven to play a tumor-suppressive role in various types of human malignancies; however, its biological role in clear cell renal cell carcinoma (ccRCC) remains unclear. The purpose of this study was to explore the biologic role as well as the underlying mechanism of miR-375 in ccRCC progression.

**Methods:** Quantitative polymerase chain reaction (qPCR) was applied to test the expression of miR-375 in tissues and cell lines by *t*-test. Functional experiments were used to investigate the biological role of miR-375 utilizing a gain-of-function strategy. The target of miR-375 was investigated by bioinformatic analysis and further verified by luciferase reporter assay, qPCR, Western blotting, and functional experiments *in vitro*.

**Results:** Our study demonstrated that miR-375 was significantly downregulated in ccRCC tissues (cancer vs. normal,  $0.804 \pm 0.079$  vs.  $1.784 \pm 0.200$ ,  $t = 5.531$   $P < 0.0001$ ) and cell lines, and loss of miR-375 expression significantly associated with advanced Fuhrman nuclear grades (Grade III and IV vs. Grade I and II,  $1.000 \pm 0.099$  vs.  $1.731 \pm 0.189$ ,  $t = 3.262$   $P = 0.003$ ). Functional studies demonstrated that miR-375 suppressed ccRCC cell proliferation, migration, and invasion (all  $P < 0.05$  in both 786-O and A498 cell lines). Multiple miRNA target prediction algorithms indicated the well-studied oncogene YWHAZ as a direct target of miR-375, which was further confirmed by the luciferase reporter assay, qPCR, and Western blotting. Moreover, restoration of YWHAZ could rescue the antiproliferation effect of miR-375.

**Conclusions:** The data provide the solid evidence that miR-375 plays a tumor-suppressive role in ccRCC progression, partially through regulating YWHAZ. This study expands the antitumor profile of miR-375, and supports its role as a potential therapeutic target in ccRCC treatment.

**Key words:** Clear Cell Renal Cell Carcinoma; MicroRNA-375; Proliferation; YWHAZ

## INTRODUCTION

Kidney cancer is among the ten most common cancers in both men and women worldwide. According to the latest statistics from The American Cancer Society, about 64,000 new cases will occur, and about 14,000 people will die from kidney cancer in 2017.<sup>[1]</sup> Clear cell renal cell carcinoma (ccRCC) represents the most common adult kidney cancer subtypes (7 out of 10),<sup>[2]</sup> and approximately 25% of ccRCC patients

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Website:  
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DOI:  
10.4103/0366-6999.238153

**Received:** 20-05-2018 **Edited by:** Ning-Ning Wang

**How to cite this article:** Zhang X, Xing ND, Lai CJ, Liu R, Jiao W, Wang J, Song J, Xu ZH. MicroRNA-375 Suppresses the Tumor Aggressive Phenotypes of Clear Cell Renal Cell Carcinomas through Regulating YWHAZ. Chin Med J 2018;131:1944-50.

have developed metastases at the time of diagnosis, and it is likely that 30% of the remaining patients will develop metastatic lesions even undergoing nephrectomy.<sup>[3]</sup> Despite therapeutic developments have improved the overall survival of the ccRCC patients in advanced stages, the long-term prognosis is generally poor in patients with metastatic lesions. In addition, ccRCC is generally resistant to radiotherapy and chemotherapy.<sup>[4]</sup> To develop novel therapeutic agents, a comprehensive understanding of the molecular signaling pathways of the malignant progression of ccRCC is urgently required.

MicroRNAs (miRNAs) are endogenous, conserved, small noncoding RNAs that regulate gene expression negatively by binding to the 3'-untranslated region (3'-UTR) of the target mRNAs.<sup>[5,6]</sup> It is known that miRNAs played a key role in various cellular processes including the cell cycle regulation, cell proliferation, differentiation, and carcinogenesis.<sup>[7]</sup> In addition, hundreds of miRNAs have been found in ccRCC, in which some were proven to be oncogenic, while the others function as tumor suppressors.<sup>[8-13]</sup> MicroRNA-375 (miR-375) has been identified to play a tumor-suppressive role in various types of human cancers including hepatocellular carcinoma, non-small cell lung cancer, esophageal cancer, oral squamous cell carcinoma, gastric cancer, pancreatic cancer, colorectal cancer, and prostate cancer.<sup>[14-17]</sup> However, the biological roles of miR-375 in ccRCC have by far not been clearly elucidated.

In this study, we tested the expression levels of miR-375 in ccRCC tissues and the matched adjacent normal renal tissues and explored its biologic role in ccRCC carcinogenesis, as well as the underlying mechanisms.

## METHODS

### Ethical approval

The study was approved by Ethics Boards of Qilu Hospital, Shandong University. All tissue sample acquisition was carried out according to the institutional guidelines, and all subjects signed written informed consent.

### Tissue specimens and cell lines

Twenty-seven freshly frozen ccRCC tissues were acquired from patients pathologically diagnosed with ccRCC at the Qilu Hospital of Shandong University between January 2016 and January 2017. Human ccRCC cell lines A498, 786-O, Caki-1, Caki-2, and the immortalized normal renal cell line HK-2 were purchased from ATCC (Manassas, VA, USA). ccRCC cell line KRC/Y was acquired from the MTC-KI cell line collection (Stockholm, Sweden). A498 were cultured in DMEM with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA). 786-O, Caki-1, Caki-2, and KRC/Y were maintained in RPMI 1640 medium containing 10% FBS. HK-2 cells were cultured in KSFM medium (Gibco, USA). All cells were incubated in a humidified incubator with 5% CO<sub>2</sub> at 37°C.

### RNA extraction, reverse transcription-polymerase-chain reaction, and quantitative real-time polymerase chain reaction

Total RNA was extracted from cell lines transfected for 48 h and frozen surgical samples using the Trizol reagent (Invitrogen, Carlsbad, USA) and reverse transcribed into cDNA using the PrimeScript reverse transcriptase reagent kit (TaKaRa, Shiga, Japan). miRNA was reverse transcribed with the Prime-Script miRNA cDNA Synthesis Kit (TaKaRa). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in a Mastercycler RT-PCR system (Eppendorf, Germany) using the SYBR Green kit (Applied Biosystems, Foster City, USA). Hairpin-it<sup>TM</sup> miRNA qRT-PCR Primer Set and Control Primer Set (GenePharma, Shanghai, China) was used to detect the mature miR-375 expression. Other primers used in this study were as follows: YWHAZ (forward): 5'-TGTTGTAGGAGCCCGTAG-3' and YWHAZ (reverse): 5'-GACCCAGTCTGATAGGATGT-3', GADPH (forward) 5'-GAAGGTGAAGGTCGGAGT-3' and GADPH (reverse): 5'-GAAGATGGTGATGGGATTTC-3'. GADPH and U6 were used as endogenous controls. The relative expression level was computed using 2<sup>-ΔΔCt</sup> method.

### Cell transfection

miR-375 mimic and miR mimic control were purchased from GenePharma (Shanghai, China). Cells were transfected with the Lipofectamine 3000 Reagent (Invitrogen, USA) following the manufacturer's protocol.

### 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide assay

3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay was used to measure the cell proliferation capacity of cancer cells. In a 96-well plate, 1.0 × 10<sup>4</sup> cells were plated in each well the day before miR-375 mimic transfection and incubated for 72 h. After incubation for the indicated time, 20 μl of MTT (5 mg/ml in phosphate-buffered saline) was added into each well and incubated for 4 h. The medium was removed and the formazan crystals were solubilized in 150 μl of dimethyl sulfoxide. Absorbance values at 570 nm were measured on a Multiscan FC Microplate Photometer (Thermo Fisher Scientific, Rochester, NY, USA).

### Matrigel invasion assay

A Matrigel invasion chamber was used to determine cell invasive capacity. Briefly, a Transwell insert (pore size, 8 mm; Corning Inc., Corning, USA) was first coated with 1 mg/ml Matrigel (BD Biosciences, CA, USA) and cells with or without miR-375 mimic treatment were seeded onto the Matrigel with FBS-free medium. Five hundred millimeters medium containing 10% FBS were used as chemoattractants and added in the lower chamber. After incubation for 24 h, the noninvaded cells on the upper surface of the membrane were removed and the membranes were fixed and stained with 0.1% crystal violet. Stained cells were photographed using the Nikon TE2000

microscope and counted in four randomly selected areas of the membrane.

### Luciferase reporter assay

The luciferase reporter constructs with the 3'UTR of YWHAZ containing the putative or mutant miR-375 binding site were generated using the dual-luciferase miRNA Target Expression vector pmiRGlokit (Promega, WI, USA). Cells were seeded into 24-well plates and co-transfected with 500 ng of the wild-type or mutated YWHAZ 3'UTR constructs, together with 30 nmol/L of miR-375 mimics or negative controls. After 48 h, the luciferase activity was detected using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's instructions. All experiments were performed in triplicates.

### Western blotting analysis

Proteins extracted from the cells transfected for 72 h were immunoblotted with indicated primary antibodies using the protocol as we previously described.<sup>[18]</sup> The primary antibodies used in this study were anti-YWHAZ (1:150; Abcam, USA) and anti-β-actin (1:1500; Santa Cruz, USA).

### Statistical analysis

All cellular experiments were repeated at least three times. Experimental data were presented as the mean ± standard error (SD). Student's *t*-test or paired *t*-test was used to analyze the differences between two groups. The significance of differences among multiple groups were analyzed by one-way analysis of variance (ANOVA), followed with *post hoc* tests. All statistical analyses were performed by SPSS16.0 software (SPSS Inc., Chicago, IL, USA). A *P* < 0.05 was considered significant statistically.

## RESULTS

### MicroRNA-375 down-regulated in clear cell renal cell carcinoma tissues and cell lines

To detect miR-375 expression in ccRCCs, we applied RT-PCR to quantitatively analyze endogenous miR-375 expression in 27 ccRCC specimens and 11 adjacent normal renal tissues. It showed that miR-375 expression was significantly under-expressed in ccRCC tissues than in the normal renal tissues (cancer vs. normal,  $0.804 \pm 0.079$  vs.  $1.784 \pm 0.200$ ,  $t = 5.531$   $P < 0.001$ ; Figure 1a). In addition, miR-375 expression was downregulated in 8/11 of ccRCC tissues compared to the matched normal renal tissues from the same patients [Figure 1b]. Moreover, the expression level of miR-375 was remarkably decreased in Fuhrman nuclear Grade III and IV tumors than that in Fuhrman nuclear Grade I and II tumors (Grade III and IV vs. Grade I and II,  $1.000 \pm 0.099$  vs.  $1.731 \pm 0.189$ ,  $t = 3.262$   $P = 0.003$ ; Figure 1c). We further detected miR-375 expression levels in a series of ccRCC cell lines and got the consistent results that miR-375 is significantly under-expressed in all ccRCC cell lines (786-O, A498, KRC/Y, Caki-1, and Caki-2) than in the tumor-adjacent normal renal tissues [Figure 1d].

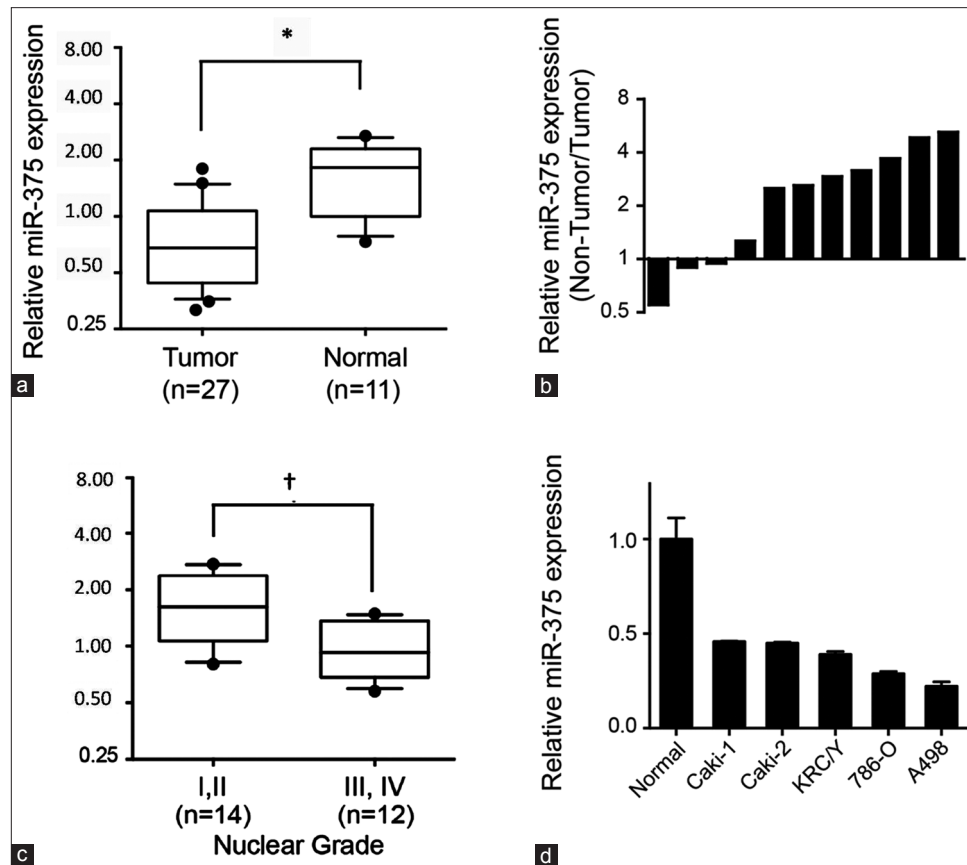
### Exogenous over-expression of microRNA-375 inhibits clear cell renal cell carcinoma cell proliferation, migration, and invasion

As miR-375 is frequently downregulated in ccRCC tissues and cell lines, to explore its biological functions in ccRCC cells, the gain-of-function experiments were adopted. First, the highly upregulated expression of miR-375 followed mimics transfection, compared to the control group, was confirmed by qRT-PCR in both 786-O and A498 cells [Figure 2a]. MTT experiments revealed that the cell proliferation was significantly inhibited in the miR-375 overexpression group in both cell lines, as compared with the control group ( $P = 0.029$ , Figure 2b).

Given that the expression of miR-375 was significantly associated with the Fuhrman nuclear grade, we wondered whether miR-375 might play a vital role in the aggressive behavior of ccRCC cells. To test this idea, Transwell assay with or without Matrigel was adopted to examine the effect of miR-375 on cell migration and invasion. Moreover, results showed that the miR-375 overexpression group exhibited significantly inhibited migratory (miR-375 mimics vs. control,  $P = 0.008$  in 786-O;  $P = 0.003$  in A498) and invasive capacity (miR-375 mimics vs. control,  $P = 0.004$  in 786-O;  $P = 0.008$  in A498; Figure 2c and 2d). Above findings suggest a potential tumor suppressor role of miR-375 in ccRCC cells.

### YWHAZ as a direct target of microRNA-375

To explore the underlying molecular mechanism of miR-375-mediated suppression of cell proliferation and invasion, we performed bioinformatic analysis to search for potential targets of miR-375 using TargetScan, miRanda, and miRWalk 2.0. All three prediction algorithms revealed that the 3'UTR of YWHAZ harbored a putative miR-375 binding site [Figure 3a]. More importantly, the sequence of the binding site was consistent among different prediction algorithms, which was highly evolutionarily conserved. To verify the binding of miR-375 on YWHAZ, luciferase reporter assay was adopted. We synthesized the 3'UTR of YWHAZ containing the putative miR-375 binding sites without (wild-type [WT]) or with (mutant [MUT]) mutations, then inserted it into a pmiRGlo vector. Next, luciferase reporter assays were performed following co-transfecting with pmiRGlo-YWHAZ-WT or pmiRGlo-YWHAZ-MUT together with miR-375 mimic or NC [Figure 3b]. As shown in Figure 3c, the relative luciferase activity was remarkably reduced in the miR-375 mimic group when pmiRGlo-YWHAZ-WT was present (control vs. mimic,  $1.020 \pm 0.042$  vs.  $0.533 \pm 0.027$ ,  $P = 0.001$ ). However, no remarkable changes in luciferase activity were observed in the pmiRGlo-YWHAZ-MUT and miR-375 mimic group. In parallel, we performed qRT-PCR and Western blotting to investigate the perturbation of miR-375 on the YWHAZ expression. Compared with the control group, a significant downregulation of YWHAZ mRNA ( $P = 0.025$  in 786-O cells;  $P = 0.011$  in A498 cells) and protein levels were observed in the miR-375 mimic-treated groups of both cell lines [Figure 3d and 3e].



**Figure 1:** miR-375 was downregulated in ccRCC tissues and cell lines. (a) The expression of miR-375 in ccRCC tissues ( $n = 27$ ) and normal renal tissues ( $n = 11$ ) tested by qRT-PCR (cancer vs. normal,  $0.804 \pm 0.079$  vs.  $1.784 \pm 0.200$ ,  $*P < 0.001$ ). (b) Comparison of miR-375 expression level between ccRCC and their matched normal renal tissues. (c) The expression of miR-375 in Fuhrman nuclear Grade III and IV tumors ( $n = 12$ ) versus I and II tumors ( $n = 14$ ) tested by qRT-PCR (Grade III and IV vs. Grade I and II,  $1.000 \pm 0.099$  vs.  $1.731 \pm 0.189$ ,  $†P = 0.003$ ). (d) Relative miR-375 expression levels in ccRCC cell lines and the normal renal tissues ( $n = 11$ ) by qRT-PCR. ccRCC: Clear cell renal cell carcinoma; qRT-PCR: Quantitative real-time polymerase chain reaction; miR-375: MicroRNA-375.

To explore whether miR-375-mediated tumor-suppressor effect of ccRCC cells could be reversed by restoration of YWHAZ expression, we transfected a vector-expressing YWHAZ without its 3'UTR, which could constitutively express YWHAZ. MTT assay showed that overexpression of YWHAZ could abrogate miR-375-mediated suppression of cell proliferation ( $P = 0.029$  in both cell lines, Figure 3f). However, YWHAZ overexpression could not rescue the anti-migratory or anti-invasive effect of miR-375 in the Transwell assay (data not shown). Above findings reinforced that YWHAZ was a functional target of miR-375.

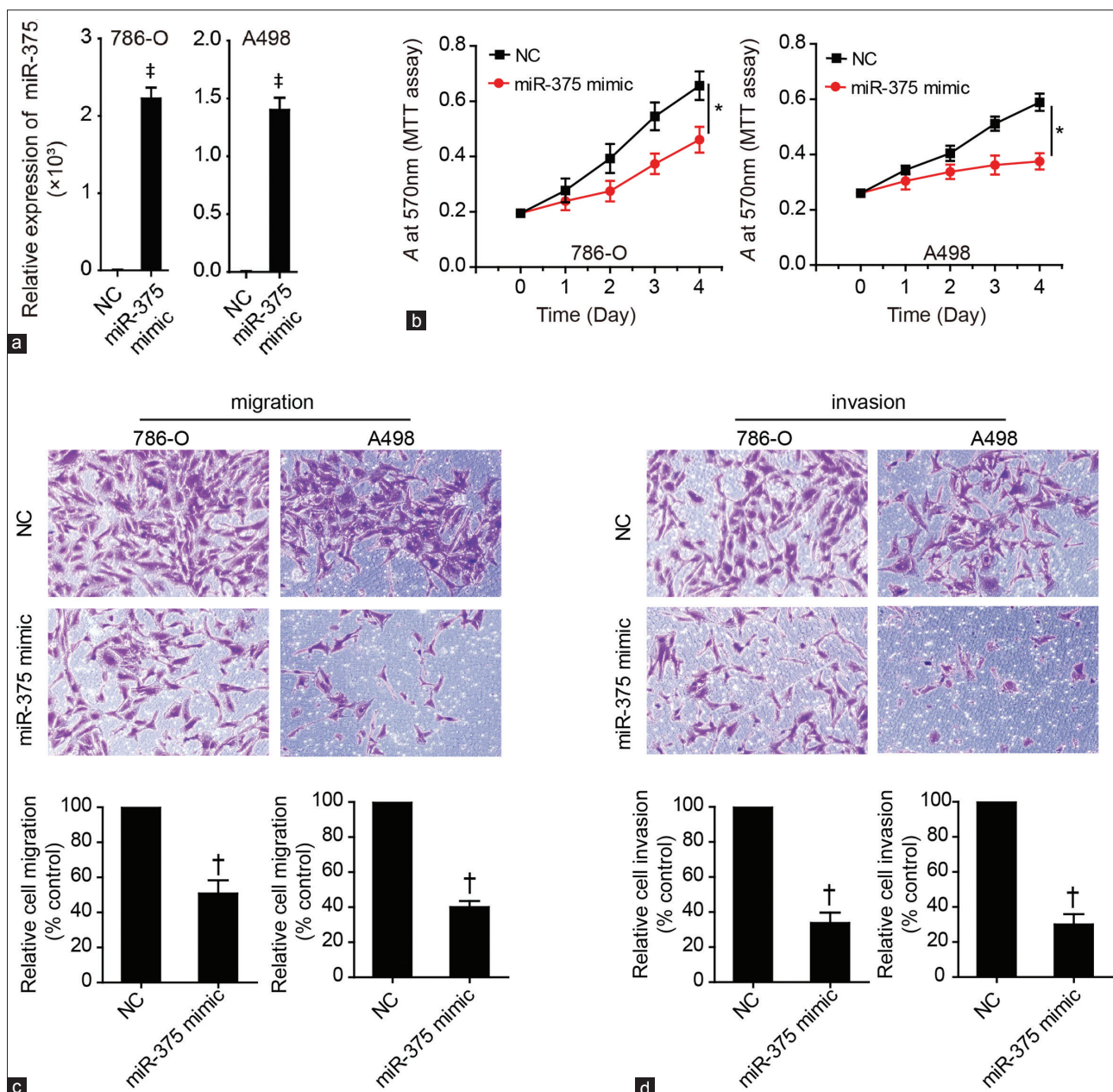
## DISCUSSION

Recently, a number of studies have demonstrated that miRNAs play a crucial role in tumor initiation and progression. miR-375, locating on chromosome 2, was first described from the murine pancreatic  $\beta$ -cell line MIN6 as a pancreatic islet-specific miRNA able to regulate insulin secretion.<sup>[19]</sup> Recent studies<sup>[20-22]</sup> found that miR-375 is frequently deleted or expressed at a reduced level in several types of cancer originated from different organs and functions as a tumor suppressor including liver cancer, gastric cancer, colorectal cancer, pancreatic cancer, and prostate cancer. Xu

*et al.*<sup>[23]</sup> demonstrated that miR-375 could directly act upon FZD8 to suppress colorectal cancer metastasis. Meanwhile, the same group found that miR-375 could inhibit colorectal cancer cells' proliferation by downregulating JAK2/STAT3 and MAP3K8/ERK signaling pathways.<sup>[24]</sup> Moreover, Selth *et al.*<sup>[25]</sup> demonstrated that miR-375 is upregulated in prostate cancer and significantly correlated with an epithelial phenotype. Multiple independent studies support a tumor-suppressive role of miR-375 in different types of cancer. As far as we know, there are few studies focused on the role of miR-375 in kidney cancer.

We proved that miR-375 was significantly downregulated in ccRCC tissues and cell lines, and its downregulation significantly associated with advanced Fuhrman nuclear grades. Functional experiments demonstrated that exogenous overexpression of miR-375 remarkably suppressed ccRCC cell proliferation, migration, and invasion, suggesting a tumor-suppressive role of miR-375 in ccRCC cells.

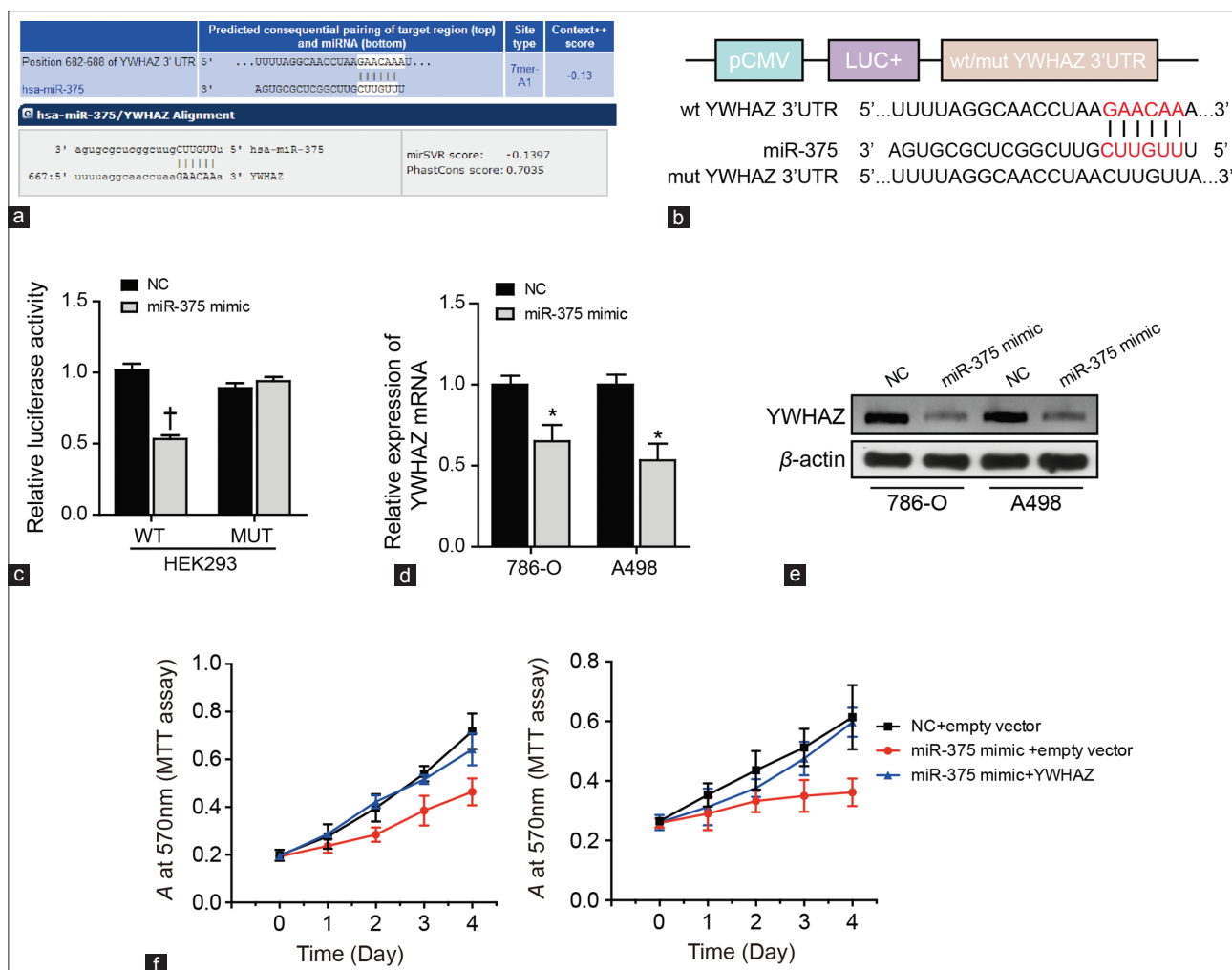
To explore the molecular mechanism underlying the antitumor effect of miR-375, we used three miRNA target prediction algorithms (TargetScan, miRanda, and miRWalk 2.0) to primarily explore the putative targets



**Figure 2:** miR-375 overexpression inhibits the aggressive behaviors of ccRCC cells. (a) The expression of miR-375 was determined by qRT-PCR. (b) Cell proliferation was significantly inhibited in 786-O and A498 cells transfected with miR-375 mimics compared with NC determined by MTT assay ( $P = 0.029$  in both cell lines). Transwell assay revealed that 786-O and A498 cells transfected with miR-375 mimics had significantly lower migratory (c) and invasive (d) potentials. Representative images of crystal violet staining are shown on the upper panel (original magnification,  $\times 400$ ), with statistical results on the bottom panel. Percentage of negative controls (NC) was recorded to minimize variations.  $*P < 0.05$ ,  $^{\dagger}P < 0.01$ ,  $^{\ddagger}P < 0.001$ . ccRCC: Clear cell renal cell carcinoma; qRT-PCR: Quantitative real-time polymerase chain reaction; miR-375: MicroRNA-375.

of miR375. All three prediction algorithms revealed a direct binding of miR-375 to the 3'UTR of YWHAZ, with the sequence of the binding site highly evolutionarily conserved, which was further confirmed by luciferase reporter assay. Consistently, a downregulation of YWHAZ by miR-375 overexpression was also identified by qRT-PCR and Western blot. YWHAZ is one of the seven members of the 14-3-3 protein family, which encodes tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (14-3-3 $\zeta$ ).<sup>[26]</sup> 14-3-3 protein family is highly conserved in plants as well as in mammals and are involved

in a wide-range cellular processes including cell cycle regulation, apoptosis, metabolism, and transcription.<sup>[27,28]</sup> In particular, 14-3-3 $\zeta$  is a major regulator of cell survival, and it interacts with a number of apoptosis-associated proteins including Raf kinase, BAD, and BAX and plays an important role as an oncogene in a number of cancers including the kidney cancer.<sup>[29]</sup> For the most cases, 14-3-3 $\zeta$  could sequester BAX and BAD, which preventing the activation of pro-apoptotic Bcl-XL and Bcl-2, to inhibit cell apoptosis.<sup>[29]</sup> According to the Human Protein Atlas (<https://www.proteinatlas.org>), YWHAZ protein expression



**Figure 3:** Tumor-suppressor effect of miR-375 is mediated by targeting YWHAZ. (a) miRNA target prediction algorithms revealed that YWHAZ was a target of miR-375 with the identical sequence of the binding site. (b) Schematic diagram of the luciferase reporter plasmids containing the 3'UTR sequence with wild-type or mutant miR-375 binding site. (c) The luciferase activity of the HEK293 cells transfected with the pmirGlo-YWHAZ-WT or -MUT together with miR-375 mimic or NC. The expression of YWHAZ mRNA (d) and protein (e) were tested by qPCR and WB. (f) Cell proliferation was tested by MTT assay. \* $P < 0.05$  † $P < 0.01$ . NC: Negative controls; 3'-UTR: 3'-untranslated region; qPCR: Quantitative polymerase chain reaction; WB: Western blotting; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide; miR-375: MicroRNA-375.

is mainly consistent with its RNA expression, and survival analysis using the TCGA renal cancer RNA sequencing data revealed that YWHAZ is an unfavorable prognostic factor of kidney cancer patients in terms of overall Survival. We wonder whether the antitumor effect of miR-375 is mediated by targeting of the oncogene YWHAZ mRNA. Moreover, the rescue experiments showed that exogenous overexpression of YWHAZ could reverse the anti-proliferation effect of miR-375 in ccRCC cell lines. However, the antimigration and anti-invasion effect of miR-375 cannot be reversed by YWHAZ, indicating additional pathways exist to mediate the tumor suppressor effect of miR-375, and high-throughput sequencing techniques could be applied to explore the underlying mechanism, which warrant further studies.

In summary, our study demonstrated that miR-375 was significantly decreased in both primary ccRCC tissues and the ccRCC cell lines. miR-375 could remarkably inhibit ccRCC cell proliferation by directly targeting YWHAZ. Our current study

provides the solid evidence of a tumor-suppressive role of the miR-375/YWHAZ axis in ccRCC, expanding the anti-tumor profiling of miR-375. Further studies should be focused on the more comprehensive molecular mechanism of miR-375 in ccRCC, and the effect of miR-375/YWHAZ axis *in vivo*.

### Financial support and sponsorship

This work was supported by the grants from the National Natural Science Foundation of China (No. 81702521) and Provincial Natural Science Foundation of Shandong (No. ZR2017PH019 and No. ZR2018BH018).

### Conflicts of interest

There are no conflicts of interest.

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# miR-375 通过调控YWHAZ抑制肾透明细胞癌的恶性侵袭性生物学行为

## 摘要

**背景:** 微小RNA(miR-375)在肿瘤的发生与发展中发挥了重要的生物学作用。研究表明miR-375在多种恶性肿瘤发生过程中发挥了重要的抑癌作用,然而,其在肾透明细胞癌(ccRCC)中的生物学作用尚不明确。本研究旨在研究ccRCC中miR-375的生物学作用及其潜在机制。

**方法:** 应用定量PCR(qPCR)检测肾癌临床组织和细胞系中miR-375的表达水平。采取过表达策略应用多种细胞功能学实验研究miR-375在肾癌细胞中的生物学功能。应用生物信息学软件预测miR-375的潜在作用靶点,并应用荧光素酶报告实验、qPCR、蛋白印迹实验及细胞功能学实验验证。

**结果:** 本实验证实miR-375在肾癌临床标本(肾癌组织比正常肾组织,  $0.804 \pm 0.079$  比  $1.784 \pm 0.200$ ,  $P < 0.0001$ )和细胞系中均显著表达下调,且miR-375的低表达与更高的肾癌Fuhrman细胞核分级显著相关(高核分级 vs. 低核分级,  $1.000 \pm 0.099$  比  $1.731 \pm 0.189$ ,  $P = 0.003$ )。功能学实验证实miR-375过表达可显著抑制ccRCC细胞增殖、迁移和侵袭(所有  $P < 0.05$ )。多种miRNA靶点预测软件提示原癌基因YWHAZ是miR-375的靶基因,我们进一步应用荧光素酶报告实验、qPCR和蛋白印迹实验证实miR-375对YWHAZ的调控。并且,挽救实验证实YWHAZ再表达可逆转miR-375抑制细胞增殖的作用。

**结论:** 本实验证实miR-375在ccRCC的恶性进展过程中发挥了重要的抑癌作用,其抑癌作用部分是通过调控YWHAZ来实现的。本实验进一步扩展了miR-375的抑癌谱,为研究其药用价值提供了理论依据。