

Original Article

MicroRNA-137 suppresses cell migration and invasion in renal cell carcinoma by targeting PIK3R3

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Abstract: Our recently study had showed that miR-137 could inhibit renal cell carcinoma (RCC) growth *in vitro* and *in vivo*, and function as tumor suppressor in RCC. However, the mechanism leading to suppressive effect of the miR-137 remains largely unclear in RCC due to lack of molecular target information. In this study, we investigated underlying molecular mechanism of miR-137 regulating invasion and migration in RCC cells. It was found that miR-137 over-express significantly in cell migration and invasion of RCC cells. A bioinformatics analysis identified a putative miR-137 binding site within the 3'-UTR of PIK3R3. Luciferase reporter assay revealed that PIK3R3 was a direct target of miR-137 in RCC cells. Quantitative RT-PCR and western blot assay showed that overexpression of miR-137 could decrease the expression of PIK3R3 on mRNA level and protein level. Importantly, we found that downregulation of PIK3R3 by siRNA performed similar effects with miR-154 overexpression on NSCLC cells in migration and invasion, while overexpression of PIK3R3 in RCC cells attenuated the suppressive effects of miR-137 on cell migration and invasion. Taken together, these results showed that miR-137 inhibited migration and invasion of RCC cells by repressing PIK3R3 expression, suggesting that miR-137 might be a novel target for the treatment of RCC.

Keywords: Renal cell carcinoma, miR-137, migration, invasion, PIK3R3

Introduction

Renal cell carcinoma (RCC) is the sixth most common cancer, and the third most common urological cancer with the highest mortality rate at over 40% [1]. Clear-cell renal cell carcinoma is the most common renal parenchymal carcinoma (ccRCC), accounting for approximately 75% of all RCC cases [2]. Despite increased early detection of RCC and more frequent surgery, the mortality rate has not changed significantly [3]. Especially, patients with metastatic RCC have a very poor prognosis after surgery, with a 5-year survival rate under 10%, due to the refractory nature of RCC to current treatment strategies [4]. Therefore, a better understanding of the biological basis of RCC progression might provide important insights for disease treatment.

MicroRNAs are a group of small non-coding RNAs that regulate the expression of protein-

coding genes via inhibiting translation or inducing messenger RNA (mRNA) degradation by binding to the 3'-untranslated region (3'-UTR) of target mRNA [5]. MicroRNAs have been reported to involve in various biology procession, such as cell proliferation, differentiation, cycle, migration, invasion and apoptosis [6-8]. Accumulating evidence has indicated that microRNAs may act as both classical oncogenes and tumor suppressor genes to regulate the progression and metastasis of different malignancies [9]. For RCC, it has been showed that some miRNAs play essential roles in RCC procession and metastasis [10]. Therefore, microRNAs may be useful as potential biomarkers for the diagnosis and prognosis of RCC as well as its therapy [10].

miR-137, located on chromosome 1p22 and has been found to be downregulated and function as tumor suppressors in several cancers including non-small lung cancer [11], gastric

cancer [12], gastrointestinal stromal tumor [13] and breast cancer [14]. For RCC, Our recently study showed that restoration of miR-137 in RCC cells significantly inhibited tumor growth *in vitro* and *in vivo*. However, the underlying molecular of miR-137 inhibition effect in RCC remains unknown due to lack of molecular target information. Therefore, in the present study, we investigate the potential mechanism of miR-137 in RCC by serial of molecular experiments.

Materials and method

Cell culture and transfection

Human RCC cell line, 786-O cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), and were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD, USA) with 1% penicillin/streptomycin at 37°C in 5% CO₂.

MiR-137 mimic and corresponding negative control miRNA (miR-NC), siRNA against PIK3R3 (si-PIK3R3) and siRNA against negative control (si-NC) plasmid were purchased from Shanghai GenePharma (Shanghai, China), Overexpression PIK3R3 plasmid (pVAX1-PIK3R3) was granted from Doctor Xue Wang (Shanghai, China). These molecular productions were transiently transfected into were transiently transfected into 786-O cells in 6-well plates using Oligofectamine™ Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA extraction, reverse transcription and quantitative real-time PCR

Total RNA of tissues and cells was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Then miR-137 expression level was quantified as previously described by Bi et al. [15] on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). PIK3R3 mRNA was measured as previously described [16]. The comparative 2^{-ΔΔCt} method was used for relative quantification and statistical analysis.

Wound-healing assays

To measure cell motility, wound healing assay was performed in 786-O cells. In briefly, 5 × 10⁴

transfected cells were seeded in six-well plates to near confluence. A linear wound was carefully created by a sterile pipette tip across the confluent cell monolayer, and the cell debris was removed and incubated with DMEM medium containing 1% FBS for 24 h. The wounded monolayers were then photographed at 0 and 24 after wounding.

Transwell invasion assays

Cell invasion assays were analyzed using a 24-well transwell plate with 8-μm pore polycarbonate membrane inserts (Corning, New York, USA), according to the manufacturer's protocol. In briefly, 5 × 10⁴ cells in 200 μl serum-free media were plated in the upper chambers coated with the 60 μl Matrigel (200 ng/ml) (BD Biosciences, Bedford, MA). The lower chambers were filled with 600 μl DMEM containing with 10% FBS. After 48 h of incubation, cells that migrated to the underside of the membrane were fixed in 75% methanol, stained with 0.05% crystal violet and quantified by counting them in five randomly selected fields.

Prediction of putative targets

Two online softwares were applied: TargetScan (<http://www.targetscan.org/>), miRanda (<http://www.microrna.org/>), to predict the putative targets of miR-137.

Luciferase reporter assay

To construct a luciferase reporter vector, the PIK3R3 3'-UTR fragment containing putative binding sites for miR-137 was amplified using PCR and inserted into downstream of the luciferase gene in the pGL3-luciferase reporter plasmid (Promega, Madison, WI, USA) at the NheI and XhoI sites, designated WT-PIK3R3-3'UTR. A mutant 3'-UTR of PIK3R3 containing a mutation in the complementary seed region of miR-137 was synthesized and inserted into pGL3-control vector at the NheI and XhoI sites, designate MUT-PIK3R3-3'UTR. Constructs were verified by sequencing. For luciferase assay, miR-137 expressing or miR-NC cells were cultured in 24-well plates and transfected with 100 ng luciferase reporter plasmid (WT/Mut PIK3R3 3'UTR) and 5 ng pRL-TK vector expressing the Renilla luciferase (Promega, Madison, WI, USA) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. After 48 h of

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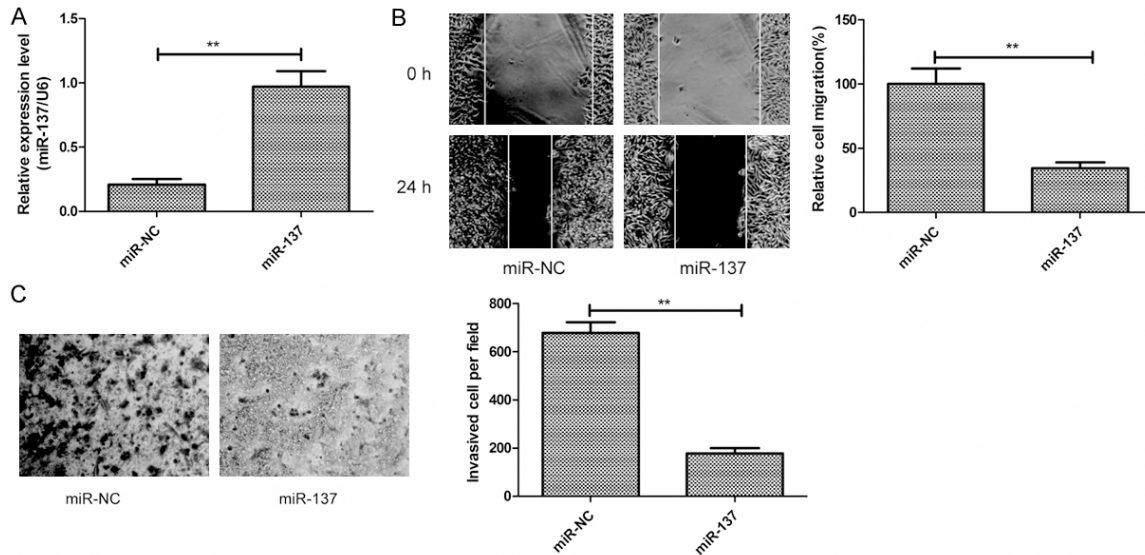


Figure 1. miR-137 inhibited RCC cells migration and invasion. A. miR-137 expression was determined in 786-O cells after transfected with miR-137 mimic or miR-NC. B. Cell migration was determined in 786-O cells transfected with miR-137 mimic or miR-NC by wound healing. C. Cell invasion was analyzed in 786-O cells transfected with miR-137 mimic or miR-NC by transwell chamber. * $P < 0.05$, ** $P < 0.01$ versus NC.

transfection, cells were harvested and lysed and luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Renilla luciferase was used for control.

Western blot

Cultured cell protein was isolated using RIPA buffer (Beyotime Institute of Biotechnology, Jiangsu, China) supplemented with a protease inhibitor mixture stock solution (Roche Molecular Biochemicals, Mannheim, Germany). Concentrations of total cellular protein were determined using a BCA assay kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Equivalent amounts of protein (30 μ g) were resolved by 10% sodium dodecylsulfate-polyacrylamide gels (SDS-PAGE) and then electrotransferred to the nitrocellulose membrane (Bio-Rad, Munich, Germany). The membrane then were blocked with 5% (w/v) non-fat dried milk and incubated with the indicated primary antibody in Tris buffered saline overnight at 4°C. Primary antibodies used in this study were: anti-PIK3R3, anti-AKT, anti-phosphor-AKT (ser473), anti-GAPDH. All antibodies were brought from Santa Cruz Biotechnology (Santa Cruz, CA). GAPDH was used as the internal control. The blots then were stained with the appropriate horseradish

peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) at room temperature for 2 h. Bands were visualized by enhanced chemiluminescence (Thermo Fisher Scientific Inc., Waltham, MA) using the Chemidoc XRS + system (Bio-Rad, Hercules, CA). Blots were stripped and reprobed with anti-GAPDH to control for loading variations.

Statistical analysis

All results were expressed as mean \pm SD (standard deviation) from at least three independent experiments. Data were analyzed using Student's two tailed t test or ANOVA. All data were analyzed using the GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

miR-137 inhibited cell migration and invasion in RCC cells

To explore the biological effect of miR-137 on metastasis, we stably overexpressed miR-137 in 786-O cells by transfection with miR-137 mimic and its control (miR-NC). The efficacy of transfection was tested using quantitative RT-PCR (qRT-PCR). The results showed that

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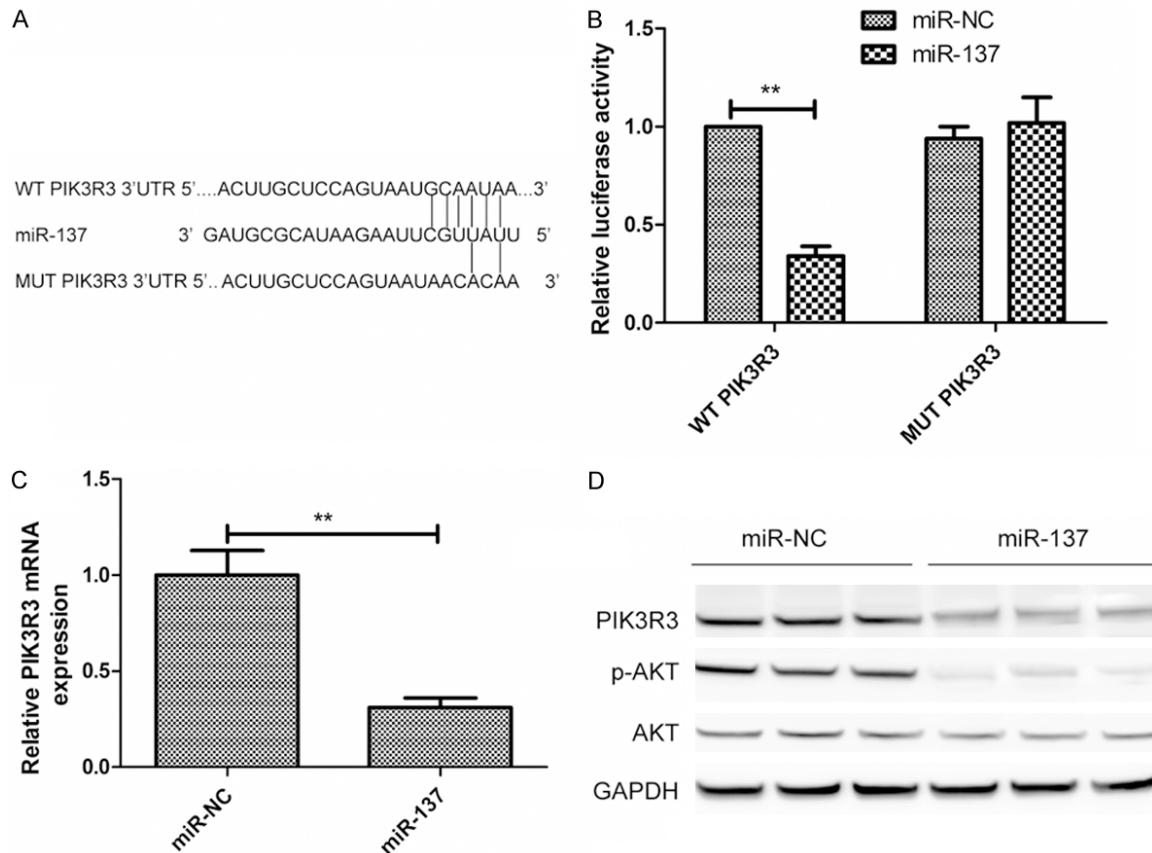


Figure 2. PIK3R3 is a target gene of miR-137. **A.** The predicted binding sites for miR-137 in the 3'UTR of PIK3R3 and the mutations in the binding sites are shown. **B.** The relative luciferase activities was determined in 786-O cells stable expression miR-137/miR-NC after transfected with WT/MUT-PIK3R3-3'UTR report plasmids. **C.** PIK3R3 mRNA expression level was determined in 786-O cells transfected with miR-137 mimic or miR-NC by qRT-PCR. The GAPDH was used for as an internal control. **D.** PIK3R3, AKT and p-AKT protein expression were detected in 786-O cells transfected with miR-137 mimic or miR-NC by western blot analysis. GAPDH was used as an internal control. * $P < 0.05$, ** $P < 0.01$ versus miR-NC.

miR-137 expression was increased in 786-O cells after transfected with miR-137 mimic compared to cells transfected with miR-NC (**Figure 1A**). Next, we tested the role of miR-137 in migration and invasion of RCC cells by wound healing assay and transwell assay, respectively. It was found that miR-137 overexpression in 786-O cells significantly inhibited cell migration and invasion capabilities (**Figure 1B** and **1C**).

PIK3R3 is a target gene of miR-137

To elucidate the underlying mechanisms by which miR-137 exerts its function, we predict targets of miR-137 using the TargetScan bioinformatics algorithm and miRanda software. Our analysis found that PIK3R3 was a potential target of miR-137 based on putative target sequences at position 3058-3514 of the PIK3R3-3'UTR (**Figure 2A**). To confirm PIK3R3 as a direct target of miR-137, luciferase report-

er assays was performed and found that miR-137 significantly decreased the luciferase activity of the WT-PIK3R3-3'UTR but not that of the MUT-PIK3R3-3'UTR in 786-O cells (**Figure 2B**). qRT-PCR and western blot analyses showed that overexpression of miR-137 significantly inhibited PIK3R3 expression on the messenger RNA (mRNA) and protein levels in 786-O cells (**Figure 2C** and **2D**). In addition, we also found that overexpression of miR-137 obviously downregulated PIK3R3 downstream protein expression, PIK3R3 and p-AKT expression (**Figure 2D**). These results suggested that PIK3R3 is a target gene of miR-137.

Downregulation of PIK3R3 has similar effect with miR-137 overexpression in RCC cells

To explore the biological role in RCC cells, endogenous expression of PIK3R3 was knocked down in 786-O cells with specific siRNA against

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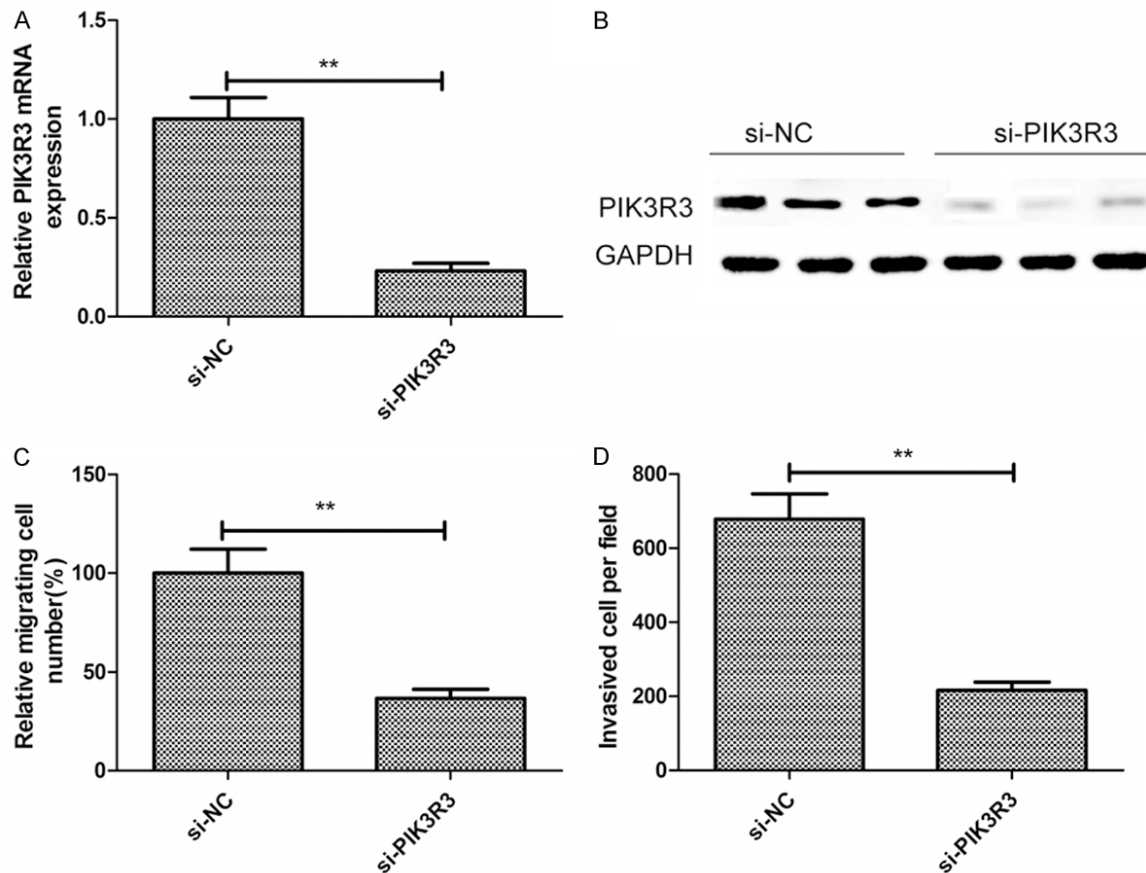


Figure 3. Downregulation of PIK3R3 mimicked effect with miR-137 overexpression in RCC cells. A and B. PIK3R3 on mRNA level and protein level were determined in 786-O cells transfected with si-PIK3R3 or si-NC by qRT-PCR or western blot, respectively. GAPDH was used as an internal control. C and D. Cell migration and invasion were determined in 786-O cells transfected with si-PIK3R3 or si-NC by wound healing and transwell invasion assays, respectively. * $P < 0.05$, ** $P < 0.01$ compared to si-NC.

PIK3R3 (si-PIK3R3). The qRT-PCR and western blot assays confirmed that si-PIK3R3 could significantly decreased PIK3R3 expression on mRNA level and protein level (**Figure 3A** and **3B**) in RCC cells. Furthermore, we also found that downregulation of PIK3R3 expression in 786-O cells significantly inhibited cell migration (**Figure 3C**) and invasion (**Figure 3D**) capabilities, suggesting that inhibition of PIK3R3 had similar effect with miR-137 overexpression in RCC cells.

PIK3R3 overexpression attenuated the suppressive effect of miR-137 in RCC cells

To further confirm that PIK3R3 is a functional target of miR-137, 786-O cells were transfected with overexpression PIK3R3 plasmids (pVAX1-PIK3R3) after transfection of miR-137 mimic. Our results showed that the expression

of PIK3R3 on mRNA level and protein level were increased in 786-O cells co-transfected with PIK3R3 overexpression plasmid and miR-137 mimic compared to cells transfected with miR-137 alone (**Figure 4A** and **4B**). In addition, overexpression of PIK3R3 partially rescued inhibition effect of miR-137 on migration and invasion in RCC cells (**Figure 4C** and **4D**). These results indicated that miR-137 inhibits RCC cell migration and invasion partially by targeting PIK3R3.

Discussion

With development of molecular and cell technology, the great improvement of cancer therapy, However, major limitations were still existed in treatment RCC due to its recurrence and metastasis. Thus, looking for novel treatment strategies is still the top priority. Increasing evi-

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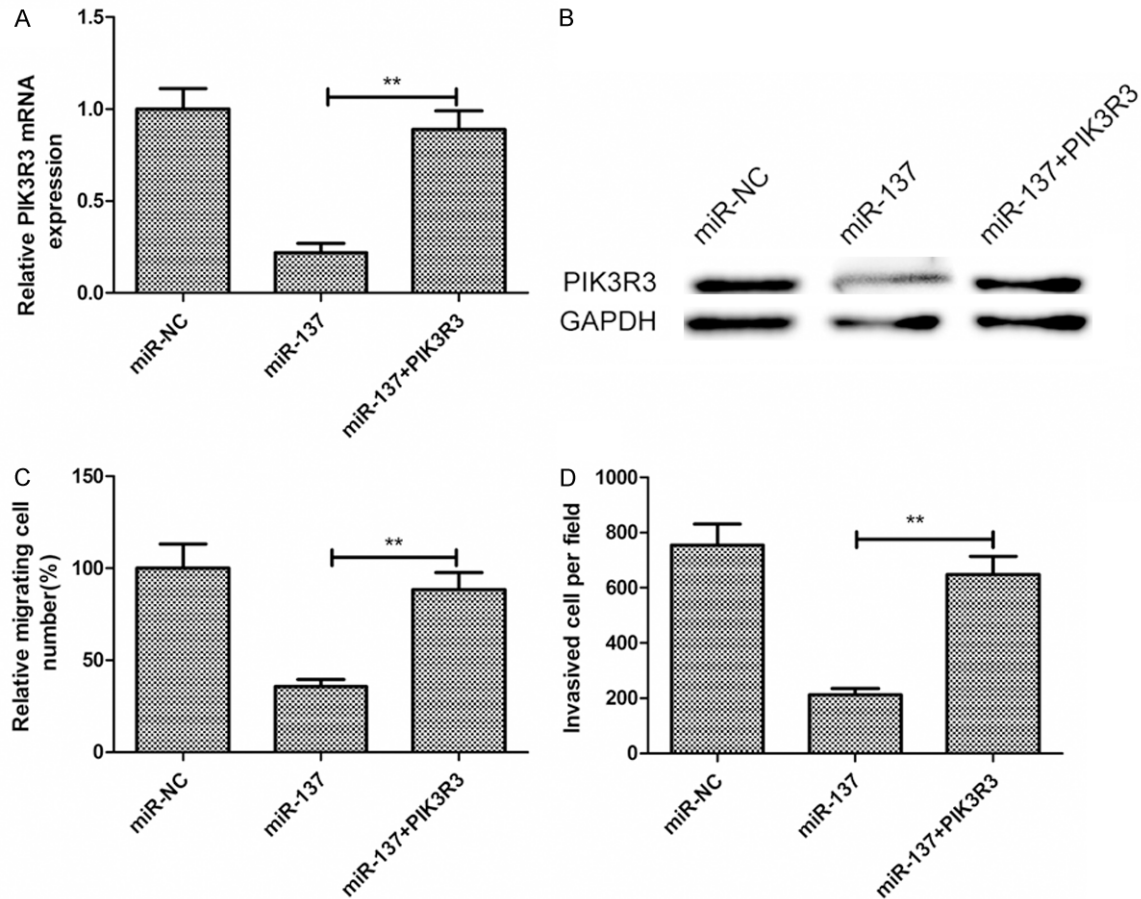


Figure 4. PIK3R3 overexpression attenuated the suppressive effect of miR-137 in RCC cells. A and B. PIK3R3 on mRNA level and protein level were measured in 786-O cells transfected with miR-137 mimic and with/without PIK3R3 overexpression plasmid by qRT-PCR and western blot, respectively. C and D. Cell migration and invasion were determined in 786-O cells transfected with miR-137 mimic and with/without PIK3R3 overexpression plasmid by wound healing and transwell invasion assays, respectively. *P<0.05, **P<0.01 compared to miR-137.

dence proved that the miRNAs have potential to become molecular biomarkers for the diagnosis and prognosis of cancer as well as therapy agent for treatment cancer, including RCC [10]. For example, Chen *et al.* reported that miR-129-3p can act as a promising diagnostic biomarker for discriminating ccRCC from benign tumors and normal tissues and an independent prognostic biomarker in ccRCC, and that miR-129 can attenuates ccRCC cells migration and invasion via downregulating multiple metastasis-related genes [17]. Wang *et al.* found that miR-335 acts as a novel tumor suppressor to regulate ccRCC cell proliferation and invasion through downregulation of BCL-W expression [18]. Yu *et al.* showed that miR-96 suppresses RCC invasion by modulating Ezrin expression [19]. In the present study, our resulted showed that miR-137 overexpression in RCC cells sig-

nificantly inhibited cell migration and invasion by targeting PIK3R3, which provides a potential novel target for future RCC therapy.

miR-137 is one of the most frequently studied miRNAs in human cancers, including non-small lung cancer [11], gastric cancer [12], gastrointestinal stromal tumor [13] and breast cancer [14]. Downregulation of miR-137 and its functional analysis suggest a tumor-suppressing role for majority types of cancer [11-14]. On the contrary, miR-137 expression was reported to be upregulated in bladder cancer [20] and squamous cells carcinoma of the tongue [21], and it was considered to be an oncomiR. For RCC, Our recently study had showed that miR-137 could inhibit renal cell carcinoma (RCC) growth *in vitro* and *in vivo*, and functioned as tumor suppressor in RCC. However, the mecha-

nism leading to suppressive tumor growth of the miR-137 remains largely unclear in RCC due to lack of molecular target information. In the present study, we found that miR-137 inhibited RCC cell migration and invasion capabilities by targeting PIK3R3.

PIK3R3, a member of the phosphatidylinositol 3-kinase (PI3K) family, has been reported to play crucial roles in cancer development and progression and function as oncogene in several types of cancers, such as ovarian cancer [22], lung cancer [23], gastric cancer [24] and colorectal cancer [25]. In addition, PIK3R3 could involve in regulating AKT/mTOR signal pathway [16, 26]. Of note, PIK3R3 has been identified as direct target of several miRNAs, such as miR-132 [27], miR-511 [16], miR-193a-3p [26] and miR-7 [23]. Here, we identified PIK3R3 was a direct target of miR-137 by luciferase reporter assay. miR-137 overexpression in RCC cells obviously inhibited PIK3R3 expression on mRNA level and protein level, and inhibited its downstream protein expression (p-AKT). In addition, we found that inhibition PIK3R3 expression has similar suppressive effect on migration and invasion of RCC cells, and that reintroduction of PIK3R3 partially rescued the suppression effect induced by miR-137 in RCC cells. These results suggested that miR-137 suppressed cell migration and invasion in RCC cells by repressing PIK3R3 expression.

In summary, the present study provides evidence that miR-137 suppressed cell migration and invasion in RCC cells by directly targeting the PIK3R3, suggesting that miR-137 functions as a tumor suppressor in RCC and that miR-137 can be a potential target for the treatment of RCC.

Disclosure of conflict of interest

None.

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