

Original Article

miR-137 functions as a tumor suppressor gene in pituitary adenoma by targeting AKT2

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Abstract: Pituitary adenoma is a common intracranial tumor, but the underlying molecular carcinogenesis mechanisms remain unclear. Accumulative evidence has demonstrated that aberrant expression of microRNAs (miRNAs) is an important feature of cancer. The aim of the current study was to explore the role of miR-137 in pituitary tumor. The expression level of miR-137 in pituitary tumor tissues was measured by quantitative RT-PCR. Then the effects of miR-137 upregulation/downregulation on the proliferation and invasion as well as the potential molecular mechanisms were further investigated. Our results showed that the expression level of miR-137 was significantly reduced in pituitary tumor tissues compared to normal controls. Ectopic expression of miR-137 inhibited the proliferation and invasion of pituitary tumor cells, while miR-137 suppression promoted the proliferation and invasion capacity of cancer cells. Bioinformatic analysis of the downstream targets of miR-137 revealed that many enriched gene ontology functions and pathways were closely associated with carcinogenesis. Mechanically, AKT2 was demonstrated to be a direct downstream target of miR-137. The expression level of miR-137 was negatively correlated with AKT2 in pituitary tumor tissues. Taken together, miR-137 plays a tumor suppressive role in pituitary adenoma through regulating AKT2.

Keywords: Pituitary adenoma, miR-137, AKT2, proliferation, invasion

Introduction

Pituitary adenomas are common neoplasms, accounting for approximately 25% of all intracranial tumors [1]. Although the majority of pituitary adenomas are benign and slow-growing malignancy, patients might suffer from severe headache, visual problems, and hormone-associated symptoms [2]. Some pituitary adenomas are aggressive, leading to serious morbidity or even mortality. The etiology for the tumorigenesis of pituitary adenoma remains poorly understood. Therefore, elucidating the underlying molecular mechanisms of pituitary adenoma contributes to its early detection, treatment and prognosis.

MicroRNAs (miRNAs) are a class of small, endogenous non-coding RNA with 22-nt in length. They can regulate gene expression by binding the 3'-untranslated region (UTR) of their target mRNAs, leading to the translation inhibition or mRNA degradation [3]. miRNAs have been shown to be involved in numerous biological processes such as proliferation, differentia-

tion, apoptosis and development [4]. Deviant levels of miRNAs have been closely linked to various human disorders including cancer [5]. miRNA might be either a tumor suppressor or oncogenic promoter during tumorigenesis of cancer. For instance, the expression level of miR-16 was downregulated in pituitary tumor tissues, and reduced miR-16 was associated with unfavorable clinical outcome. In addition, overexpression of miR-16 inhibited cancer cell proliferation and promoted apoptosis [6], indicating that miR-16 acts as a tumor suppressor gene in pituitary tumor. The miR-106b levels were remarkably increased in pituitary adenoma. Downregulation of miR-106b significantly reduced the proliferation and invasion capacity of pituitary adenoma cells [7], suggesting that miR-106b plays an oncogenic role in the progression of pituitary adenoma.

miR-137, a nervous system enriched miRNA, is located within a long non-coding host gene named MIR137HG. The downstream targets of miR-137 are closely linked with cell cycle, proliferation, and differentiation [8]. Deregulation of

The role of miR-137-AKT2 axis in pituitary adenoma

miR-137 has been reported in various tumor types such as pancreatic cancer, melanoma, and lung cancer [9-11]. However, its role in pituitary tumor is poorly known. The current study aimed to elucidate the expression pattern and the regulatory mechanisms of miR-137 in pituitary tumor.

Materials and methods

Patient samples and cell culture

A total of forty patients with pituitary tumors were enrolled in this study. Another 20 normal tissue samples obtained from head trauma surgery were recruited as the control group. There were 27 patients with non-invasive pituitary tumors and 13 patients with invasive pituitary tumors respectively. The study was approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University, and all the patients gave written informed consent.

The human pituitary adenoma cell line HP-75 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HP-75 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. They were incubated at 37°C in a humidified atmosphere with 5% CO₂.

Cell transfection

All RNA oligonucleotides were synthesized by Shanghai GenePharma Co., Ltd (Shanghai, China). The HP-75 cells were transfected with either miR-137 mimic, miR-137 inhibitor, and their respective controls (negative control mimic/negative control inhibitor) with Lipofectamine[®] RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA).

Cell count

The transfected cells were harvested with 0.5% trypsin and suspended in complete culture medium. They were seeded at a density of 1×10^5 cells per well in 12-well plated. Cell counts were performed following 24, 48, 72 and 96 h of culture using a hemocytometer under a light microscope. The trypan blue was used to exclude the dead cells.

CCK-8 assay

The transfected cells were harvested and seeded at a density of 3×10^3 cells per well in 96-

well plates. Briefly, 10 μ l CCK-8 reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to culture medium and incubated at 37°C for 4 h. Absorbance was measured at a wavelength of 450 nm following 24, 48, 72 and 96 h of culture with a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Transwell invasion assay

The effects of miR-137 upregulation/downregulation were assessed using Matrigel-coated transwell chamber with a pore size of 8 μ m (Corning Inc., Corning, NY, USA). Briefly, 2×10^4 transfected cells in 200 μ l of DMEM were seeded into the upper chamber. Then 800 μ l complete culture medium was added to the lower chamber. After 36 h of incubation, the cells remaining in the upper chamber were removed with cotton swab. Those have invaded to the lower chamber were fixed with 100% cold methanol and stained with crystal violet. The results were visualized and counted under a microscope.

Quantitative RT-PCR

Total RNA was isolated from cells and tissues using the TRIzol reagent (Takara, Dalian, China) according to the manufacturer's instruction. The SYBR PrimeScript RT-PCR kit (Takara) was used for reverse-transcribing the RNA to cDNA. Quantitative RT-PCR was conducted using SYBR[®] Premix Ex Taq[™] II (Takara) on an ABI 7500 fast real-time PCR system (Applied Biosystems, Waltham, MA, USA). U6 and β -actin were used as the endogenous control for miR-137 and AKT2 respectively. Relative fold changes were calculated using the 2^{- $\Delta\Delta$ CT} method. The primers used were as follows: miR-137-forward, 5'-TTATTGCTTAAGAATACGCG-3'; miR-137-reverse, 5'-TCGTATCCAGTGCAGGGTC-3'; U6-forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3'; U6-reverse, 5'-CGCTTACGAA-TTTGCGTGTGCAT-3'; AKT2-forward, 5'-TCACATGGATTTGGAGGCACGCC-3'; AKT2-reverse, 5'-TACTGCTTTTGTGCCCCAGGTGG-3'; ACTB-forward 5'-AAGGCCAACC-GTGAAAAGAT-3'; ACTB-reverse 5'-GTGGTACGACCA GAGGCATAC-3'.

Luciferase reporter assay

Cells were co-transfected with pMIR-AKT2-3'-UTR wild-type vector or pMIR-AKT2-3'-UTR mutant vector and miR-137 mimic or NC mimic using Lipofectamine[®] 2000 (Invitrogen). Follow-

The role of miR-137-AKT2 axis in pituitary adenoma

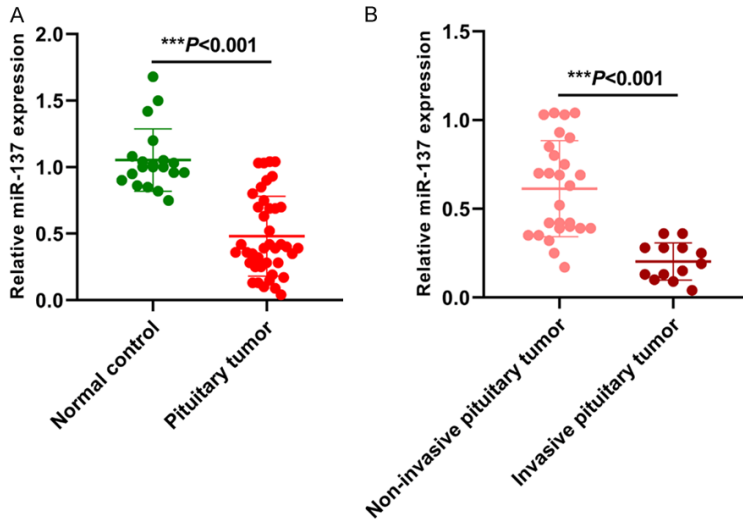


Figure 1. Expression pattern of miR-137 in pituitary tumor tissues.

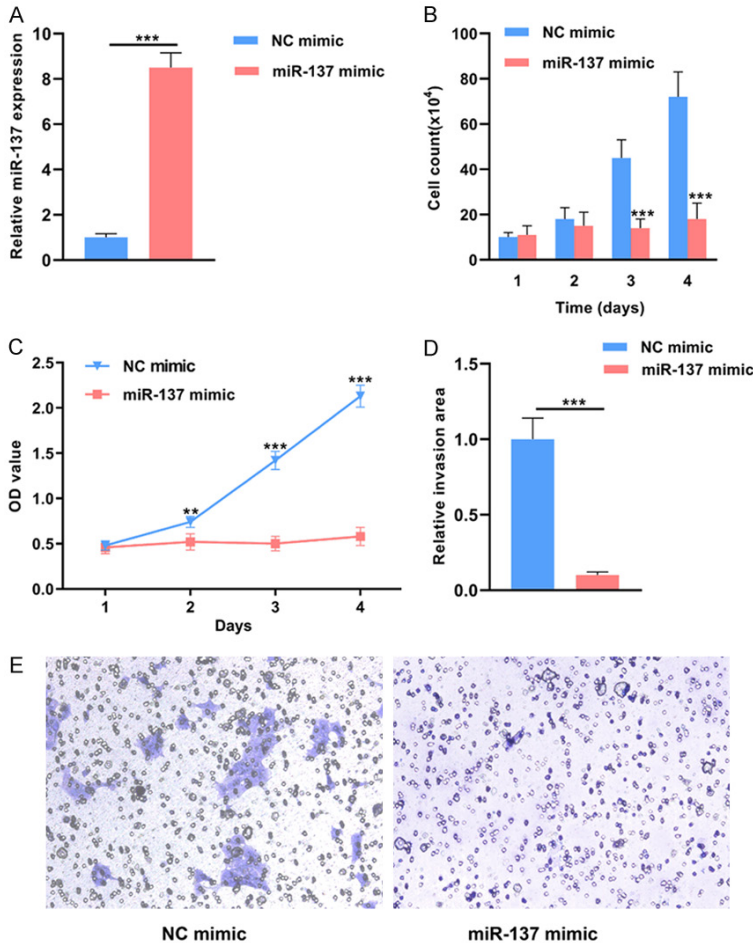


Figure 2. Effects of miR-137 overexpression on the proliferation and invasion of pituitary tumor cells.

ferase Reporter Assay Kit (Promega, Madison, WI, USA) based on the manufacturer's instructions. Renilla luciferase activity was normalized to Firefly luciferase expression.

Statistical analysis

All data were expressed as the mean \pm standard deviation. All statistical analyses were performed using Graphpad Prism version 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). The differences between two groups were analyzed with the Student's t-test. Correlation analysis was performed to determine the association between miR-137 and AKT2 expression level in the pituitary tumor tissues. The Database for Annotation, Visualization and Integrated Discovery was used for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. P values < 0.05 were considered significant.

Results

miR-137 was downregulated in pituitary tumor tissues

We first compared the miR-137 level between pituitary tumor tissues and the normal control tissues. Our results showed that the expression level of miR-137 was dramatically reduced in pituitary tumor tissues compared to the normal control tissues ($***P < 0.001$) (**Figure 1A**). Interestingly, miR-137 levels were significantly lower in invasive pituitary tumor than non-invasive pituitary tumor ($***P < 0.001$) (**Figure 1B**).

Effects of miR-137 upregulation on the proliferation and invasion of pituitary tumor cells

ing incubation for 48 h, the luciferase reporter activity was determined using a Dual Luci-

Quantitative RT-PCR was performed to evaluate the transfection efficiency of miR-137 mimic.

The role of miR-137-AKT2 axis in pituitary adenoma

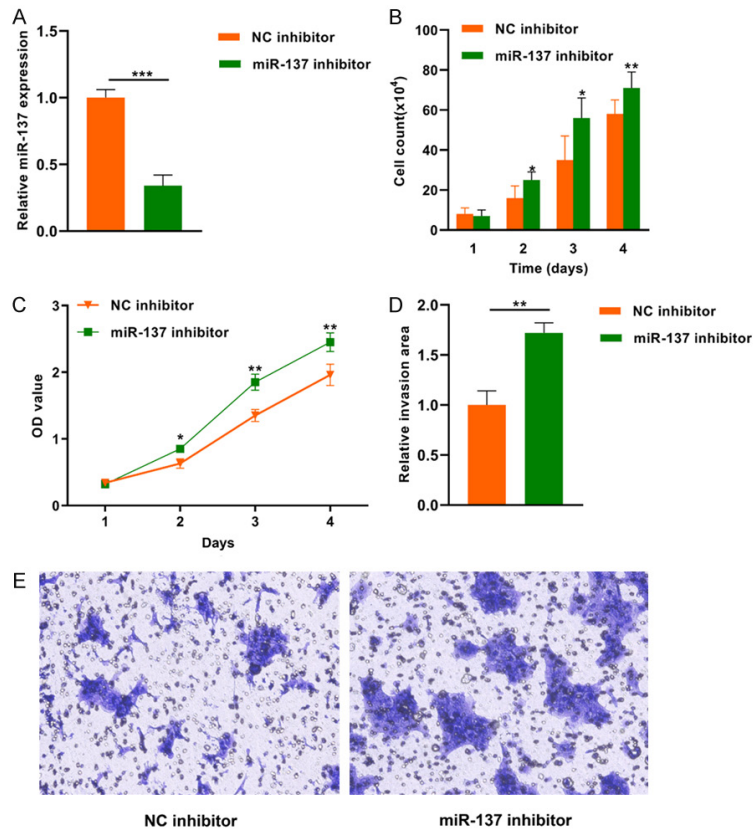


Figure 3. Effects of miR-137 downregulation on the proliferation and invasion of pituitary tumor cells.

The expression level of miR-137 was significantly higher in the miR-137 mimic transfected cancer cells compared with the NC mimic transfected cells ($***P < 0.001$) (**Figure 2A**). The cell count assay showed that the number of cells was markedly lower in the miR-137 mimic group than the control group at day 3 and 4 ($***P < 0.001$) (**Figure 2B**). Similarly, the OD values were lower in the miR-137 mimic group than the control group at day 2, 3 and 4 ($**P < 0.01$; $***P < 0.001$) (**Figure 2C**). The matrigel invasion assay revealed that miR-137 mimic transfected cancer cells had lower invasive capacity than the NC mimic transfected cells ($***P < 0.001$) (**Figure 2D, 2E**).

The effects of miR-137 downregulation on the proliferation and invasion of pituitary tumor cells

miR-137 level was significantly lower in the miR-137 inhibitor group compared to the NC inhibitor group ($***P < 0.001$) (**Figure 3A**). The cell count assay demonstrated that the number

of cells was higher in the miR-137 inhibitor group than the control group at day 2, 3 and 4 ($*P < 0.05$, $**P < 0.01$) (**Figure 3B**). The OD values were higher in miR-137 inhibitor group than the control group at day 2, 3 and 4 ($*P < 0.05$, $**P < 0.01$) (**Figure 3C**). The matrigel invasion assay showed that miR-137 inhibitor transfected cancer cells had higher invasive capacity than the NC inhibitor transfected cells ($**P < 0.01$) (**Figure 3D, 3E**).

GO and KEGG analysis of the downstream targets of miR-137

GO and KEGG analysis of the downstream targets of miR-137 were performed. Our results showed that regulation of transcription from RNA polymerase II promoter, negative regulation of transcription, negative regulation of RNA metabolic process, negative regulation of gene expression, negative regulation of transcription, DNA-dependent, regulation of

transcription, transcription, negative regulation of nitrogen compound metabolic process, positive regulation of gene expression and positive regulation of transcription were the top enriched biologic processes (**Figure 4A**). Cation channel complex, plasma membrane part, cell junction, nucleoplasm part, ion channel complex, cell projection, nuclear lumen, endomembrane system, potassium channel complex and chromatin were the top enriched cellular components (**Figure 4B**). Transcription factor activity, transcription repressor activity, transcription factor binding, transcription cofactor activity, transcription activator activity, protein kinase activity, DNA binding, protein serine/threonine kinase activity and voltage-gated cation channel activity were the top enriched molecular functions (**Figure 4C**). ErbB signaling pathway, calcium signaling pathway, MAPK signaling pathway, focal adhesion, ubiquitin mediated proteolysis, Wnt signaling pathway, insulin signaling pathway, neurotrophin signaling pathway, pancreatic cancer, and GnRH signaling pa-

The role of miR-137-AKT2 axis in pituitary adenoma

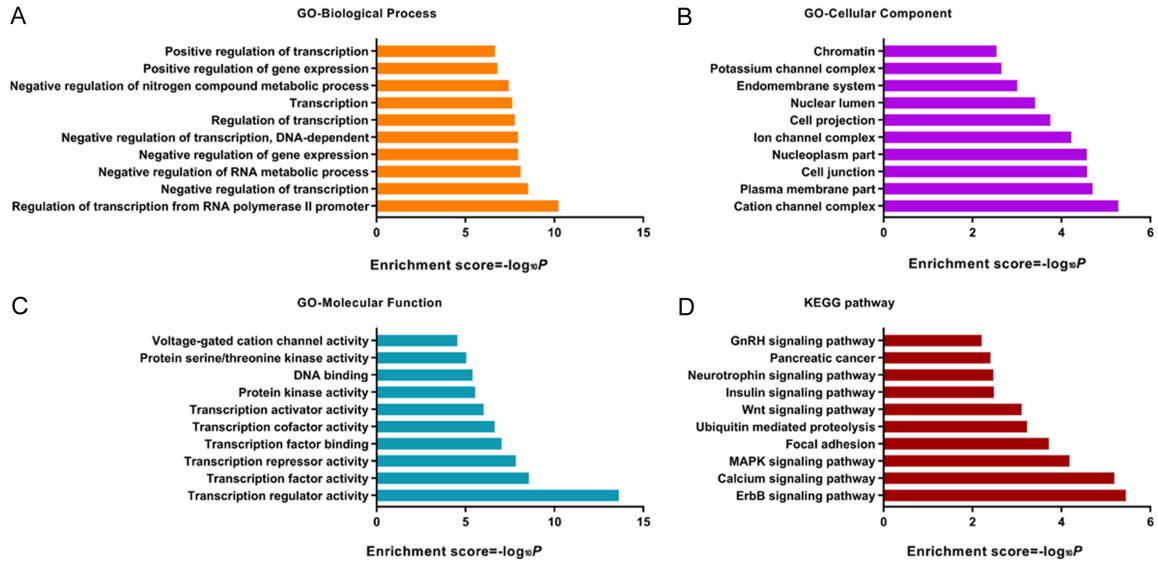


Figure 4. GO and KEGG analysis of the downstream targets of miR-137.

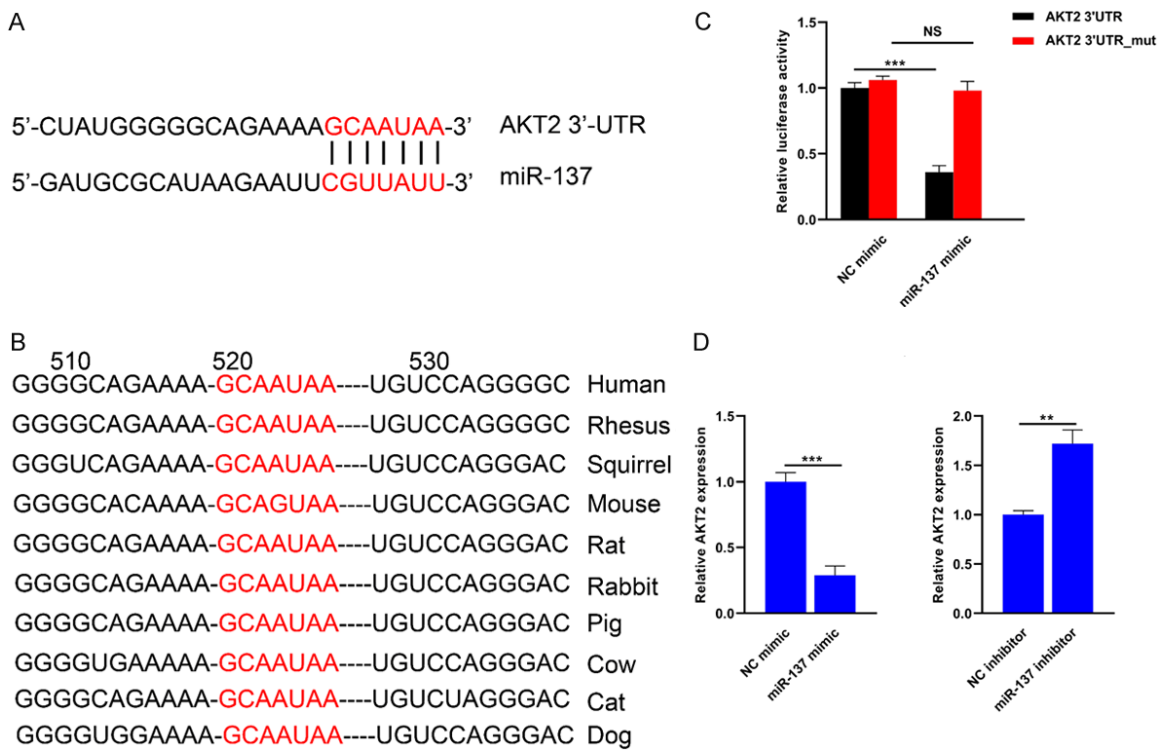


Figure 5. AKT2 is a direct downstream target of miR-137.

thway were the top enriched pathways (**Figure 4D**).

AKT2 is a direct downstream target of miR-137

Figure 5A showed that the 3'-UTR of AKT2 was highly complementary to the seed sequence of

miR-137. In addition, the 3'-UTR of AKT2 that miR-137 targeted was highly conserved across different species (**Figure 5B**). For the wild type AKT2 vector, the luciferase reporter assay revealed that the relative luciferase activity was significantly lower in the miR-137 mimic group compared to that in the NC mimic group.

The role of miR-137-AKT2 axis in pituitary adenoma

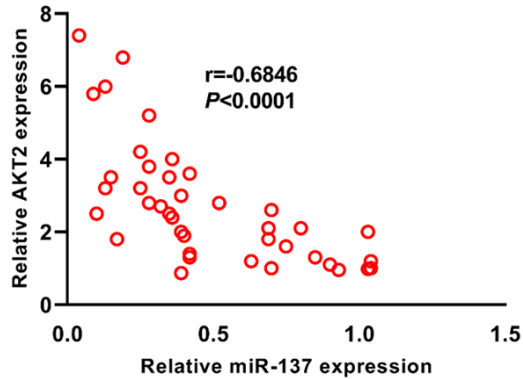


Figure 6. The expression level of miR-137 is negatively associated with AKT2 expression in pituitary tumor tissues.

However, no significant difference was found for the AKT2 mutant vector between groups (**Figure 5C**). The expression level of AKT2 was significantly lower in miR-137 mimic group, while higher in miR-137 inhibitor group compared with their respective controls (**Figure 5D**).

miR-137 level was inversely associated with AKT2 in pituitary tumor tissues

We then explored the association between miR-137 and AKT mRNA in the above forty pituitary tumor tissues. Our results showed that a negative correlation was found between miR-137 and AKT mRNA in the pituitary tumor tissues ($r = -0.6846$, $***P < 0.001$) (**Figure 6**).

Discussion

In this study, our results showed that the expression level of miR-137 was significantly downregulated in pituitary tumor tissues, especially those with invasive capacity. In addition, overexpression of miR-137 suppressed the proliferation and invasion of pituitary tumor cells, whereas downregulation of miR-137 promoted malignant behaviors. Bioinformatic analysis of the downstream targets of miR-137 showed that many enriched GO functions and pathways were closely associated with carcinogenesis. Moreover, AKT2 was identified as a direct target of miR-137. Furthermore, the expression level of miR-137 was negatively correlated with AKT2 in pituitary tumor tissues. Collectively, miR-137 acted as a tumor suppressor in pituitary tumor and its downregulation might pro-

mote carcinogenesis through upregulating AKT2.

There are three highly conserved members (AKT1, AKT2 and AKT3) in the AKT family [12]. PI3K-AKT activation is regarded as one of the most important pathways responsible for the oncogenic transformation in various types of cancers [13, 14]. miR-137 was an upstream regulator of AKT2 in pituitary tumor, which might partially explain the importance of miR-137 in the carcinogenesis of pituitary tumor. One limitation of our study was the small sample size. Further studies with larger patient cohort are warranted to validate our findings. In addition, animal studies are needed to explore the role of miR-137 *in vivo*.

Consistent with our findings, miR-137 was hypermethylated in colorectal cancer (CRC), and ectopic expression of miR-137 suppressed the proliferation of CRC cells by regulating LSD-1, indicating that miR-137 functioned as a tumor suppressor gene in CRC [15]. Similarly, Langevin et al. reported that aberrant miR-137 promoter methylation was frequently detected in head and neck squamous cell carcinoma, and the methylation status was associated with prognosis [16]. The expression level of miR-137 was markedly downregulated in lung cancer cell lines. Overexpression of miR-137 restrained the proliferation of lung cancer cells *in vitro* and tumor growth *in vivo*. In addition, *cdc42* and *cdk6* were demonstrated to be downstream targets of miR-137 [17]. Low miR-137 level was strongly correlated with unfavorable clinical outcome of patients with neuroblastoma. Upregulation of miR-137 in neuroblastoma cell lines decreased cell proliferation and viability as well as promoted apoptosis by regulating KDM1A [18]. Cheng et al. reported that the miR-137 level was lower in the gastric cancer tissues and cell lines compared to their respective controls. In addition, miR-137 overexpression suppressed the proliferation, migration, and invasion of gastric cancer cells *in vitro*, and tumor growth *in vivo*. Moreover, ectopic expression of COX2 could partially rescue the tumor suppressive effects of miR-137, indicating that COX2 was a functional downstream target of miR-137 [19]. Interestingly, Li et al. showed that the serum miR-134 level was reduced in patients with glioblastoma. Low serum miR-134 was strongly linked with worse clinical parameters and overall survival [20].

Further studies are warranted to explore the expression pattern of miR-137 in the biofluids of patients with pituitary tumor and its potential clinical significance.

Although most studies found that miR-137 functioned as a tumor suppressor gene in most tumor types, miR-137 might also promote tumor progression. For instance, miR-137 was found to be a target of Slug, a known epithelial mesenchymal transition regulator. Downregulation of miR-137 in lung cancer cells abolished Slug induced invasion and migration, and upregulation of miR-137 promoted invasion by regulating TFAP2C, indicating that miR-137 might function as an oncogene in lung cancer [21]. Ectopic expression of miR-137 promoted the anti-apoptosis and cisplatin resistance capacity of in lung adenocarcinoma cells [22]. The contrary role of miR-137 in different types of cancers or even in the same type of cancer needs further investigation.

In conclusion, this is the first study to explore the role of miR-137 in pituitary tumor. miR-137 is reduced in pituitary tumor tissues and its downregulation is associated with invasiveness. Overexpression of miR-137 suppresses the proliferation and invasion capacity of pituitary tumor cells, and *vice versa*. AKT2 is a direct target of miR-137. Therefore, targeting miR-137-AKT2 axis might provide a novel therapeutic option for treating pituitary tumor.

Acknowledgements

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Disclosure of conflict of interest

None.

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The role of miR-137-AKT2 axis in pituitary adenoma

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