Abstract. - OBJECTIVE: Expression of pituitary homeobox 2 (PITX2) is significantly elevated in pituitary adenoma tissues, which also has lower microRNA (miR)-21 expression, indicating possible tumor-suppression role of miR-21. Bio-informatics analysis revealed targeting onto 3'-UTR of PITX2 by miR-21. This study aims to investigate the role of miR-21/PITX2 expression in proliferation and apoptosis of pituitary adenoma cells and pathogenesis.

PATIENTS AND METHODS: A total of 48 pituitary adenoma samples were collected in parallel with 12 normal brain tissues and were recruited in this study. Flow cytometry was employed to test Ki-67 expression and apoptosis. Expressions of miR-21 and PITX2 were compared, along with their targeted relationship by dual-luciferase reporter assay. Cultured HP75 cells were transfected with miR-21 mimic and/or si-PITX2. Caspase-3 activity was further quantified, followed by flow cytometry for apoptosis. MiR-21, cleaved caspase-3 and PITX2 expressions were tested.

RESULTS: Invasive pituitary adenoma tissues had significantly higher Ki-67 and PITX2, and lower miR-21 expressions or apoptosis than non-invasive tumors. MiR-21 targeted 3'-UTR of PITX2 gene to inhibit its expression. Elevated miR-21 and/or silencing PITX2 significantly depressed PITX2 expression in HP75 cells, potentiating caspase-3 activity, decreasing cell proliferation and facilitating apoptosis.

CONCLUSIONS: MiR-21 was down-regulated while PITX2 was up-regulated in pituitary adenoma tissues. MiR-21 can inhibit pituitary adenoma cell HP75 proliferation and facilitate apoptosis via inhibiting PITX2 expression.

Key Words: MicroRNA-21, Pituitary homeobox 2, Pituitary tumor, Cell apoptosis.

Introduction

Pituitary adenoma (PA) is a group of endocrine tumors derived from anterior/posterior pituitary and craniopharyngeal canal residual epithelial cells. As a common intracranial tumor, PA occupies about 10% of all primary tumors, only next to glioma and meningioma. Averaged incidence of PA is about 7.5-10 per 100 000 people. It was reported that more than 20% of people in head MRI scan were found to have PA. Most PA cases are of benign nature without metastasis. However, it is common to observe a series of endocrine symptoms including acral growth and hypo-sexual function, plus compression and site occupancy effects. About one third of PA showed malignant behavior such as invasive growth, showing infiltration towards peripheral tissues and vascular walls of sellar region, destructing cavernous sinus and adjacent brain tissues, thus causing severe clinical symptoms and affecting patient’s growth and working capacity, threatening life and health. Invasive pituitary adenoma (IPA) is difficult for radical treatment or complete resection in clinics. Radio- or chemo-therapy is a necessary post-surgery, with high recurrent frequency at about 21-86%. Proliferative activity and growth velocity are closely correlated with clinical symptoms, disease course and prognosis. PA cells had high proliferative activity and growth speed, shown as rapid and invasive growth of tumors. These criteria were theoretical basis for discriminating non-invasive and invasive PA, judging the invasiveness of tumors. During pathogenesis of tumors, cell apoptosis and proliferation are two perspectives, as apoptosis of tumor cells is correlated with growth velocity and biological behavior, and affects pathogenesis, progression and prognosis of tumors. Pituitary homeobox 2 (PITX2) is one member of homeobox gene bicoid related family, and is named initially by its role in pituitary development in human chromosome 4q25. Later studies found its close correlation with development of brain, liver, optic nerve, teeth, heart and spleen. PITX2 is one downstream gene in Wnt signal pathway, and is under the regulation of...
Wnt/Dvl/β-catenin\textsuperscript{11}. Over-activation of Wnt signal pathway potentiates transcription of downstream target gene PITX2, thus facilitating expression of downstream genes such as c-myc, CyclinD1, and Cyclin D2, and potentiating abnormal proliferation and tumor pathogenesis\textsuperscript{12,13}. Abnormal expression of PITX2 is related with multiple tumors such as prostate cancer\textsuperscript{4}, esophageal squamous carcinoma\textsuperscript{15}, papillary thyroid cancer\textsuperscript{6} and ovary cancer\textsuperscript{11}. Moreno et al\textsuperscript{17} found significantly elevated PITX2 expression in tumor tissues of PA patients, indicating its tumor-facilitating role of PA. In addition to cell proliferation, PITX2 also participates in regulating cell apoptosis via multiple pathways and mechanisms\textsuperscript{8,9}. MicroRNA (miR) is a group of non-coding single stranded small molecule RNA with 22-25 nucleic acids in eukaryotic cells, and can modulate target gene expression via inhibiting translation or degrading mRNA\textsuperscript{20}. MiR can regulate more than one third of human genes’ expression and affect multiple biological processes including cell proliferation, apoptosis and differentiation\textsuperscript{21}. Previous researches showed the modulation of miR-21 on proliferation, apoptosis and invasion of multiple tumor cells including gastric carcinoma\textsuperscript{22} and liver cancer\textsuperscript{23}. Acunzo et al\textsuperscript{24} found the close correlation between the role of PITX2 in PA and its anti-apoptotic effects. Amaral et al\textsuperscript{25} revealed significantly depressed miR-21 expression in PA tumor tissues, suggesting its potential role as tumor suppressor gene in PA. Bio-informatics study revealed satisfactory targeted complementary relationship between miR-21 and 3'-UTR of PITX2. This study investigated if dysregulation of miR-21 and PITX2 played a role in proliferation and apoptosis of PA cells and pathogenesis.

**Patients and Methods**

**Major Reagent and Materials**

Human pituitary adenoma cell line HP75 was provided by ATCC cell bank (Manassas, VA, USA). Dulbecco’s modified eagle medium (DMEM) medium, horse serum and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). RNA extraction reagent Trizol and Lipofectamine 2000 were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Reverse transcription and SYBR dye were purchased from TaKaRa (Dalian, China). Oligonucleotide fragment of miRNA and PCR primers were designed and synthesized by Ruibo Bio (Shanghai, China). Rabbit anti-human PITX2 polyclonal antibody (Catalogue No. ab32832; 1:2000) was purchased from Abcam Biotechnology (Cambridge, MA, USA). Mouse anti-human cleaved caspase-3 monoclonal antibody (Catalogue No. ab13585; 1:2000) was purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Catalogue No. 115-035-003; 1: 2000) and HRP-conjugated goat anti-rabbit IgG (Catalogue No. 111-035-003) were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Mouse anti-human Ki-67 with FITC labels (Catalogue No. 8011-5699) was purchased from eBioscience (Santiago, CA, USA). Annexin V/PI apoptosis kit was purchased from Yusheng Bio (Shanghai, China). Caspase-3 activity assay kit was purchased from Beyotime (Shanghai, China). Dual-luciferase reporter assay system and pGL3-promoter plasmid were purchased from Promega (Madison, WI, USA).

**Clinical Information**

A total of 48 pituitary adenoma patients in the 5th Affiliated Hospital of Zhengzhou University from October 2014 to September 2016, were recruited in this study. There were 26 males and 22 females in the patient cohort, aging between 28 and 59 years old (average age: 34.8 years). A total of 18 patients were classified as non-invasive pituitary tumor while the other 30 belonged to invasive adenoma based on Hardy-Wilson grading criteria\textsuperscript{26}. Another cohort of 12 normal brain tissues collected from head trauma surgery was recruited as the control group, in which there were 7 males and 5 females, aging between 31 and 58 years (average age: 35.7 years). All sample collections have obtained consents from patients, along with approval by the Ethical Committee of the 5th Affiliated Hospital of Zhengzhou University.

**Cell Culture**

Human pituitary adenoma cell line HP75 was incubated in Dulbecco’s Modified Eagle Medium (DMEM) containing 12.5% horse serum, 2.5% fetal bovine serum (FBS) and 1% penicillin-streptomycin, in a 37°C chamber with 5% CO\textsubscript{2}. Culture medium was changed every three days. Further experiments were performed when reaching 60-80% confluence.

**Construction of Luciferase Reporter Assay Plasmid**

Using HEK293 genome as the template, full-length fragment of 3'-UTR of PITX2 gene was amplified. PCR products were purified from agarose gel,
and were ligated into pGL-3M luciferase reporter plasmid after XbaI/NotI dual digestion. Recombinant plasmid was then used to transform DH5α competent cells. Positive clones with primary screening were selected for sequencing. Those plasmids with correct sequence were used for further cell transfection and following experiments.

Luciferase Reporter Assay

Lipofectamine 2000 was used to transfet HEK293 cells with 400 ng pGL3-PITX2-3’UTR plasmid, 25 nmol miR-21 mimic (or miR-21 negative control), and 25 ng controlled plasmid pRL-TK. After 4-6 h transfection, Opti-MEM medium was discarded, with the replacement of normal DMEM medium containing 10% FBS and 1% streptomycin-penicillin. After 48 h continuous incubation, dual-luciferase assay was performed. In brief, cells were washed twice in PBS, with the addition of 100 μL PLB lysis buffer. With vortex at room temperature for 20 min, the mixture was centrifuged at 300 r/min for 5 min. A total of 20 μL cell lysate was mixed with 100 μL LAR II. Fluorescent value I was measured in a microplate reader. The enzymatic reaction was stopped in 100 μL Stop & Glo, followed by quantification of fluorescent value II. The relative expression level of reporter gene was calculated as the ratio of fluorescent value I/ fluorescent value II.

Cell Transfection

Using human PITX2 gene as the template, the small interference sequence targeting PITX2 gene was synthesized. Sequences were: si-PITX2 sense: 5’-CAGCC UGAAU AACUU GAACT T-3’; si-PITX2 anti-sense: 5’-GUUCA AGUUA UUCAG GCUGT T-3’; si-NC sense: 5’-UUCUGC AACGA GUGUC ACGUT T-3’; si-NC anti-sense: 5’-ACGUG ACACG UUCGG AGAAT T-3’. Experiments were performed in five parallel groups, namely, mimic NC, miR-21 mimic, si-NC, si-PITX2 and miR-21 mimic+si-PITX2 groups. After 72 h, cells were collected for further experiments.

qRT-PCR for Gene Expression

Total RNA was extracted from cells by Trizol method. In brief, cells were lysed by 1 mL Trizol, and RNA was extracted by 200 μL chloroform. The supernatant was saved. RNA was precipitated by 1 mL isopropanol, followed by twice rinsing in 1 ml 70% ethanol in centrifugation. RNA precipitation was solved in diethyl pyrocarbonate (DEPC) treated water. cDNA was synthesized in a 10 μL system including 1 μg total RNA, 2 μL RT buffer (5×), 0.5 μL oligo dT + random primer mix, 0.5 μL RT enzyme mix, 0.5 μL RNase, and ddH2O. The reaction conditions were listed as the followings: 37°C for 15 min, followed by 98°C 5 min. cDNA products were kept at -20°C fridge. Using cDNA as the template, PCR amplification was performed under the direction of TaqDNA polymerase using primers (miR-21PF: 5’-AACCC GUAGA UCUUG GAUCC UG-3’; miR-21PR: 5’-CAAGA UCAUC UACGG UUUGG GU-3’; U6PF: 5’-ATTGG AACGA TACAG AGAGA ATT-3’; U6PR: 5’-GGAAC GCTTC ACGAA TTTG-3’; PITX2PF: 5’-TGTCA AGUCG UGTCG CTTA TGGA AA-3’; PITX2PR: 5’-ATGAG CCCAT TGAAC TGCGG-3’; β-actinPF: 5’-GAAAC CTAAG GCCAA C-3’; β-actinPR: 5’-TGTCA GGCGA GCCTT CC-3’). In a PCR system with 10 μL total volume, we added 4.5 μL 2 × SYBR Green Mixture, 1.0 μL of forward/reverse primer (at 2.5 μm/L), 1 μL cDNA, and 3.0 μL ddH2O. PCR conditions were: 95°C for 15 s, 60°C for 30 s and 74°C for 30 s. The reaction was performed on Bio-Rad CFX96 fluorescent quantitative PCR cycler for 40 cycles to collect fluorescent data.

Western Blotting

Radioimmunoprecipitation assay solution (RIPA) buffer was used to lyse cells, which were incubated on ice for 20 min, followed by 12000 × g centrifugation for 20 min. A total of 50 μg protein samples was separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (40 V for 330 min), and were transferred to polyvinylidene fluoride (PVDF) membrane (Amerham Biosciences, Little Chalfont, UK) (250 mA for 120 min). The membrane was blocked in 5% defatted milk powder for 1 h, followed by primary antibody (anti-PITX2 at 1:300, anti-cleaved caspase-3 at 1:400, or anti-beta-actin at 1:500) incubation at 4°C overnight. By PBST washing (3 min × 5 times), HRP-labeled secondary antibody (1:10000 dilution) was added for 1 h incubation. After PBST rinsing for three times (5 min each), enhanced chemiluminescence (ECL), Amersham Biosciences (Little Chalfont, UK) reagent, was added for 2 min of dark incubation. Quantity One image analysis software (Bio-Rad Laboratories, Hercules, CA, USA) was used to analyze relative grey density of bands.

Caspase-3 Activity Assay

Standard dilutions of 0, 10, 20, 50, 100 and 200 μM pNA were prepared. Absorbance va-
values at 405 nm wavelength were measured by a microplate reader to plot a standard curve with pNA concentration against A405 value. Attached cells were digested in trypsin, and were collected into culture medium for 4°C centrifugation for 5 min at 600 × g. Supernatant was carefully removed and washed out by PBS. 100 μL lysis buffer was added for every 2 × 10⁶ cells. Cells were lysed at 4°C for 15 min, and were centrifuged at 18000 × g with 4°C for 10 min. Supernatants were saved for further use. 2 mM Ac-DEVD-pNA was placed on ice, mixed with buffer and test samples, with 10 μL Ac-DEVD-pNA. The mixture was incubated at 37°C for 2 h. A405 value was measured when color changed significantly.

**Flow Cytometry for Ki-67 Expression**

Phosphate-buffered saline (PBS) containing 2% FBS was used to rinse cells, which were fixed in 4% paraformaldehyde for 30 min at 4°C. PBS containing 0.1% triton X-100 was used to rupture the membrane in 30 min incubation. FITC-labeled Ki-67 antibody (1:40) was used for 4°C dark incubation for 40 min, followed by twice PBS (containing 2% FBS) rinsing and loading onto flow cytometry assay.

**Flow Cytometry for Cell Apoptosis**

Cells were digested with trypsin and were collected. Cells were then washed in pre-cold PBS twice by centrifugation. 100 μL 1× binding buffer was used to re-suspend cells. The mixture was added with 5 μL Annexin V-FITC and 5 μL propidium iodide (PI) staining solution. The mixture was incubated in dark for 10 min, with the addition of 400 μL 1× binding buffer, and was immediately loaded for online testing.

**Statistical Analysis**

SPSS18.0 software was used for data analysis (SPSS Inc., Chicago, IL, USA). Measurement data were presented as mean ± standard deviation (SD). Student t-test was used to compare measurement data between groups. Statistical significance was defined when p<0.05.

**Results**

**Down-Regulation of miR-21 and Up-Regulation of PITX2 in PA Tissues**

Flow cytometry results showed significantly elevated Ki-67 expression in IPA tissues compared to non-IPA or normal brain tissues (Figure 1A). Cell apoptosis, however, was significantly depressed in IPA tumors (Figure 1B), thus indicating the reliability of Ki-67 in differentiating IPA and non-IPA tumors. These results also indicated the involvement of active cell proliferation and decreased apoptosis in PA pathogenesis and its invasiveness. Further test showed significantly lower miR-21 in IPA tissues compared to non-IPA ones, which had lower miR-21 expression than control group (Figure 1C). PITX2 expression in PA tissues was also remarkably increased, with higher expression level in IPA cases compared to non-IPA patients (Figure 1C and 1D). These results suggested that abnormally elevated PITX2 in PA tissues was possible result of depressed miR-21 expression, which may play a role in PA pathogenesis along with elevated PITX2 expression.

**Regulation of PITX2 expression by miR-21**

Bioinformatics analysis showed satisfactory targeted complementary relationship between miR-21 and 3’-UTR of PITX2 (Figure 2A). Luciferase reporter gene assay showed that the transfection of miR-21 mimics and hence, force elevated miR-21 expression, significantly lowered relative activity of luciferase in HEK293 cell lysates (Figure 2B), indicating targeted action of miR-21 on 3’-UTR of PITX2 mRNA for expressional regulation. Further assay showed that the elevation of miR-21 expression significantly down-regulated mRNA and protein expression of PITX2 in HP75 cells, demonstrating targeted regulation of PITX2 expression by miR-21 (Figure 2C and 2D).

**MiR-21 Regulated PITX2 Expression and HP75 Cell Apoptosis**

The transfection of miR-21 mimic and/or si-PITX2 significantly down-regulated PITX2 gene and protein expression in HP75 cells (Figure 3A and 3B); also, it potentiated the activity and cleavage of caspase-3 (Figure 3B and 3C), lowered the cell proliferation (Figure 3D), and facilitated the cell apoptosis (Figure 3E).

**Discussion**

PA is derived from anterior pituitary, and consists about 10-15% of all primary intracranial tumors. Recently, the incidence of PA is significantly increasing27, as lots of patients...
were diagnosed by magnetic resonance imaging (MRI) or computed tomography (CT) scan for nasal sinus disease, head trauma or other brain diseases, with overall frequency as high as 20%\(^3\). Although having benign nature, certain PA tumor showed invasive growth, and presented malignant features, as it can invade adjacent tissues including bone, dura, para-sellar region, sphenoidal sinus and cavernous sinus, and encircle vessels/nerves\(^28\). The invasiveness of PA may increase occurrence of cerebrospinal fluid (CSF) leakage, injury of intracranial nerve and cavernous sinus internal carotid artery during surgery, and is correlated with tumor recurrence and unfavorable prognosis. When PA showed invasive growth, relatively larger tumor usually existed, accompanied with focal infiltration and invasion of adjacent tissues, thus severely affecting neurological function. These reasons make it hard to completely remove tumors during surgery, with high level of recurrence, relatively unfavorable prognosis and difficulty in treatment. With progression of molecular biology, various signal molecules involved in cell proliferation, cycle and apoptosis have been found, thus providing new insights for illustrating invasive mechanism and targeted treatment of PA. The proliferation activity and growth speed of PA cells are closely correlated with clinical symptoms, disease course and prognosis\(^6\). PA cells had enhanced proliferation activity and faster growth speed, thus showing rapid increase and invasive growth of tumor lesion\(^7\). This provide evidence for differentiating non-IPA and PA cases by Ki-67\(^8\). Cell apoptosis and proliferation are two major processes in tumor pathogenesis. The invasive growth of PA is the result of common effects regarding active proliferation of adenoma cells and lower apoptosis. As one downstream gene of Wnt signal pathway, PITX2 is under induced expression of Wnt/Dvl/β-catenin\(^11\). After the activating of Wnt signal pathway, Wnt binds with cell surface Fz receptor and co-receptor low density lipoprotein receptor-related protein 5 (LRP5)/LRP6, thus inhibiting the formation of complex between β-catenin and Axin-APC-GSK-3β, inactivating GSK-3β, preventing β-catenin from degradation by GSK-3β phosphorylation and ubiquitin.
and entering nucleus to bind with transcriptional factor T-cell factor/lymphoid enhancing factor (TCF/LEF) to activate transcription of downstream target gene PITX2\textsuperscript{11}. Cell cycle regulatory factors including cyclinD1, cyclin D2 and c-myc are all target genes of PITX2, as they can induce cell proliferation or facilitate tumor formation via modulation at G0/G1 phase\textsuperscript{12}. Elevated expression of PITX2 was observed in various tumor tissues including PA, suggesting its potential tumor-facilitating role\textsuperscript{11,15,17}. Acuzo et al\textsuperscript{24} showed that the interference of PITX2 expression by siRNA significantly facilitated apoptosis of PA cells, suggesting its anti-apoptotic role in PA pathogenesis.

This study showed significantly higher PITX2 expression in IPA tumors compared to non-IPA tumors, which had higher PITX2 level than normal brain tissues, suggesting the potential role of PITX2 in PA pathogenesis and acquisition of invasiveness. Thapar et al\textsuperscript{29} found remarkably higher mitotic index in IPA cells compared to non-IPA cases, suggesting the important role of active mitosis and dysregulated cell growth in the acquisition of invasiveness of PA. This work found significantly elevated Ki-67 expression in IPA tissues than non-IPA individuals, as consistent with Thapar et al\textsuperscript{29}. Flow cytometry results showed lower apoptosis level in IPA than non-IPA, as opposed to

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**Figure 2.** Targeted regulation of PITX2 expression by miR-21. (A) MiR-21 targeted 3’-UTR of PITX2 mRNA; (B) Dual luciferase reporter gene assay; (C) MiR-21 and PITX2 gene expression by qRT-PCR; (D) PITX2 protein expression by Western blotting. *, \(p<0.05\) compared to mimic NC. PITX2: pituitary homeobox 2, MiR-21: miRNA-21, NC: normal control.
elevated PITX2 expression, further indicating potential role of PITX2 in affecting IPA apoptosis. Amaral et al.\(^2\) found significantly decreased miR-21 expression in PA tumor tissues, indicating potential tumor-suppressor role of miR-21 for PA. Online prediction of miR-21 target genes revealed satisfactory targeted complementary relationship between miR-21 and 3'-UTR of PITX2. This research thus investigated if miR-21 played a role in regulating PITX2 expression and affecting PA tumor proliferation/apoptosis and pathogenesis. Our results showed significantly elevated miR-21 expression in IPA tissues compared to non-IPA or normal brain tissues. Dual luciferase reporter gene assay showed that miR-21 mimics significantly depressed luciferase activity in HEK293 cell lysate, and down-regulated PITX2 gene and protein expression, demonstrating targeted regulation of PITX2 by miR-21 targeting. Further assay showed the elevation of miR-21 and/or silencing of PITX2 expression significantly inhibited proliferation of PA tumor cells HP75, elevated caspase-3 enzymatic activity, and potentiated cell apoptosis, suggesting the targeted inhibition of PITX2 expression, PA cell proliferation and facilitating apoptosis by miR-21. Currently it is widely accepted that miR-21 plays an oncogene role in tumor pathogenesis. For example, Li et al.\(^2\) showed significant inhibition of drug-induced apoptosis of gastric carcinoma cells by miR-21. Najafi et al.\(^2\) found that the elevation of miR-21 expression remarkably inhibited liver cancer cell apoptosis and played a role in cancer pathogenesis. We, for the first time, revealed the potential tumor suppressor gene by miR-21 in PA pathogenesis, via facilitating tumor cell apoptosis.

**Figure 3.** MiR-21 regulated PITX2 expression and HP75 cell apoptosis. (A) qRT-PCR for miR-21 and PITX2 gene expression; (B) Western blotting for protein analysis; (C) Caspase-3 enzymatic activity by spectrometry; (D) Flow cytometry for Ki-67 protein expression; (E) Flow cytometry for apoptosis. *, \(p<0.05\) compared to mimic NC group; #, \(p<0.05\) compared to si-NC group. PITX2: pituitary homeobox 2, MiR-21: miRNA-21, NC: normal control. (Figure continued)
Conclusions

Significantly down-regulated miR-21 and up-regulated PITX2 expressions existed in PA tumor tissues. MiR-21 targeted and inhibited PITX2 expression, to suppress PA tumor cell HP75 proliferation and facilitated their apoptosis.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.
MiR-21 regulates tumor proliferation

References


