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Effects of microRNA-21 targeting PITX2 on proliferation and apoptosis of pituitary tumor cells

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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 t Ki-67 expression and apoptosis. Expres miR-21 and PITX2 were compared, along wit ir targeted relationship by dual-luciferase rep assay. Cultured HP75 cells were transfected v miR-21 mimic and/or si-PITX2. Caspase-3 activ was further quantified, followe cytome try for apoptosis. MiR-21, cle ca e-3 and PITX2 expressions were te

RESULTS: Invasive ary ad sues had significantly hig Ki- and lower miR-21 ex sion optosis than non-invasive tumo iR-21 ta d 3'-UTR of PITX2 gene to i its expres Elevated miR-21 and/or PITX2 signi ntly depressed PITX xpres in HP75 cells, potentiating cas e-3 activity reasing cell proliferation acilitating apo

CON SIONS: MiR-21 wa down-regulated whil X2 wa -regulated in pituitary adenoma es. 21 can inhibit pituitary adenoma ce roliferat and facilitate apoptosis via i ng PIT expression.

Key ds:
M RNA-21, uitary homeobox 2, Pituitary tum tosis.

Introduction

Pituitary adenoma (PA) is a group of endocrine tumors derived from anterior/posterior pituitary and craniopharyngeal canal epithelial cells[1]. As a common intracranial tumor, PA occupies about 10% pituitary tumors, next to glioma and meningioma. The averaged incidence of PA is about 7.5-10 per 100 people. It was reported that more than 20% of people in head MRI scan were found to have PA[3]. Most PA cases are of benign nature without metastasis. However, it is common to observe series of endocrine symptoms including acral growth and hypo-sexual function, plus and site occupancy effects. About one third showed malignant behavior such as invasive growth, showing infiltration towards peri tissues and vascular walls of sellar region, destroying cavernous sinus and adjacent brain tissues, thus causing severe clinical symptoms and affecting patient's growth and working capacity, threatening life and health[4]. 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%[5]. Proliferative activity and growth velocity are closely correlated with clinical symptoms, disease course and prognosis[6]. PA cells had high proliferative activity and growth speed, shown as rapid and invasive growth of tumors[7]. These criteria were theoretical basis for discriminating non-invasive and invasive PA, judging the invasiveness of tumors[8]. 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 between development of brain, liver, optic nerve, teeth, heart and spleen[9,10]. PITX2 is one downstream gene in Wnt signal pathway, and is under the regulation of

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Wnt/Dvl/β-catenin[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[12,13]. Abnormal expression of PITX2 is related with multiple tumors such as prostate cancer[14], esophageal squamous carcinoma[15], papillary thyroid cancer[16] and ovary cancer[11]. Moreno et al[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[18,19]. 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[20]. MiR can regulate more than one third of human genes' expression and affect multiple biological processes including cell proliferation, apoptosis and differentiation[21]. Previous researches showed the modulation of miR-21 on proliferation, apoptosis and invasion of multiple tumor cells including gastric carcin[22] and liver cancer[23]. Acunzo et al[24] found t correlation between the role of PITX2 in its anti-apoptotic effects. Amaral et al[25] rev significantly depressed miR-21 expression in tumor tissues, suggesting its po role as t mor suppressor gene in PA. B cs study revealed satisfactory target omplem tary relationship between miR-2 3'-UT TX2. This study investigated f dy of and PITX2 played a n pro on and apoptosis of PA cells a thogenesis.

Patients and Methods

Major Reagent and Materials

Human pituitary adenoma cell line HP75 was id y ATC cell bank (Manassas, VA, U ulbecc modified eagle medium (DM) m se serum and fetal bovine ser (FBS) urchased from Gibco (Grand Isla NY, USA). RNA extraction reagent Trizol a ine 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. ab135 was purchased from Cell Signaling Inc. (Danvers, MA, USA). Horsera peroxidase (HRP)-conjugated goat anti-mous Catalogue No. 115-035-003; 1: 2000) a HR ugated goat anti-rabbit IgG (Catal No. 11 003) were purchased from Ja n ImmunoR (West Grove, PA, USA ouse a uman K with FITC labels (C ue N 011-5699) was purchased from e iago, C USA). Annexin V/PI tosis k pur d from Yusheng Bio ghai, China -3 activity assay kit w d from Bey e (Shanghai, China). D -luciferase reporter assay system and pGL3-promoter plasm e purchased from Promega, Madison, WI, USA.

Clinical Information

A total of 48 tary adenoma patients in the 5 ffiliated H tal of Zhengzhou University fro to September 2016, were recruited study. There were 26 males and 22 females in the patient cohort, aging between 28 ears old (average age: 34.8 years). A total ients were classified as non-invasive pituitary tumor while the other 30 belonged to invasive adenoma based on Hardy-Wilson grading criteria[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_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 transfect 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 stop[illegible] in 100 μL Stop & Glo, followed by quantific[illegible] fluorescent value II. The relative expressi[illegible]e-vel of reporter gene was calculated as the rat[illegible] fluorescent value I/ fluorescent value II.

Cell Transfection

Using human PITX2 [illegible] as th[illegible]mplate, the small interference se[illegible]se targ[illegible]ITX2 gene was synthesized [illegible]equ[illegible] si[illegible]2 sense: 5'-CAGCC U[illegible]AU A[illegible] GAACT T -3'; si-PITX2 an[illegible]se: 5'-GU[illegible] AGUUA UUCAG GCUC[illegible]i-NC sense[illegible]UCUC CGAAC GU[illegible] AC[illegible]T-3'; si-NC anti-sense: 5'-ACGUC[illegible]CACG UUC[illegible]AGAAT T-3'. Experiments [illegible]e performed in [illegible]arallel groups, namel[illegible]imic N[illegible] miR-21 mimic, si-NC, si-PITX[illegible] miR[illegible]imic+si-PITX2 groups. After 72 h, [illegible]w[illegible] collecte[illegible]r further experiments.

PCR[illegible]Ge[illegible]Expression

[illegible] RNA[illegible]xtracted from cells by Trizol me[illegible]. In brief, cells were lysed by 1 mL Trizol, a[illegible] extracted by 200 μL chloroform. [illegible] supernatant was saved. RNA was precipitated by 1 mL isopropanol, followed by twice rin[illegible] 1 ml 70% ethanol in centrifugation. RNA pre[illegible]itation 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 ddH_2O. The reaction conditions were listed as the followings: 37°C for 15 min, followed [illegible] min. cDNA products were kept at [illegible] frid[illegible] Using cDNA as the template, PCR [illegible]plification was performed under the direct[illegible] TaqDNA polymerase using primers (miR-21P_F[illegible]ACCC GUAGA UCUUG GAUC[illegible]G-3'; [illegible]1P_R: 5'-CAAGA UCAUC U[illegible]G UUUGG U6P_F: 5'-ATTGG A[illegible]A TA[illegible]G AGA[illegible] ATT-3'; U6P_R: 5'-[illegible]AC [illegible]TC ACGAA TTTG-3'; PITX2[illegible]5 [illegible] ACCA[illegible]CCT-TA CGGAA G[illegible]ITX2[illegible]ATG[illegible]CCCAT TGAAC TGG[illegible]3'; β-actinP_F[illegible]ACC CTA-AG GCCA[illegible]actinP_R: 5'-[illegible]CA CGCAC GATTT C[illegible]3'; In[illegible]R system with 10 μL total volume, we added[illegible]μL 2 × SYBR Green Mi[illegible]0 μL of forwa[illegible]verse primer (at 2.5 μ[illegible]), 1 μL cDNA, and 3.0 μL ddH2O. PCR conditions were: 95°C[illegible]or 15 s, 60°C for 30 s and 74°C [illegible]0 s. The rea[illegible]n was performed on Bio-Rad C[illegible]6 fluoresc[illegible]quantitative PCR cycler for 40 cyc[illegible]ll[illegible]orescent data.

Western Blotting

[illegible]immunoprecipitation assay solution [illegible]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 (Amersham 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 for both anti-mouse and anti-rabbit) 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. The membrane was then exposure in the dark. 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-

lues 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 × 10^6 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 loadi flow cytometry assay.

Flow Cytometry for Cell Apoptosis

Cells were digested with tryp were co lected. Cells were then wash old PBS twice by centrifugation. 10 1 × b ng buffer was used to re-suspen s. The added with 5 µL Anne in 5 µ pidium iodide (PI) s ng so The mixture was incubated in for 10 mi the addition of 400 µL buffer, an immediately loade onli ting.

Statisti Analysis

SP 0 soft are was used for data analysis (SP nc., C go, IL, USA). Measurement data ted as n ± standard deviation (SD). S -test used to compare measurement da groups. Statistical significa was de hen $p<0.05$.

Results

n-Regulation of miR-21 and Up egulation 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 differentiating IPA and non-IPA tu s. The results also indicated the involv nt of active cell proliferation and decrea optosis in PA pathogenesis and its inv sive Further test showed significantly r miR- IPA tissues compared to no A ones, whi lower miR-21 expres han co l group (gure 1C). PITX2 ex ion A tiss s was also remarkably i rea high pression level in cases red on-IPA patients (Fig C and 1D). sults suggested tha lly elevate TX2 in PA tissues w possib ult of depressed miR-21 expression, which m a role in PA pathoge ng with eleva PITX2 expression.

gulation of TX2 expression by miR-21

Bioinformatic nalysis showed satisfactory ta d compl ntary relationship between mi R of PITX2 (Figure 2A). Luciferase er gene assay showed that the transfection of miR-21 mimics and hence, force d miR-21 expression, significantly lowe- ve 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 increasing[27], as lots of patients

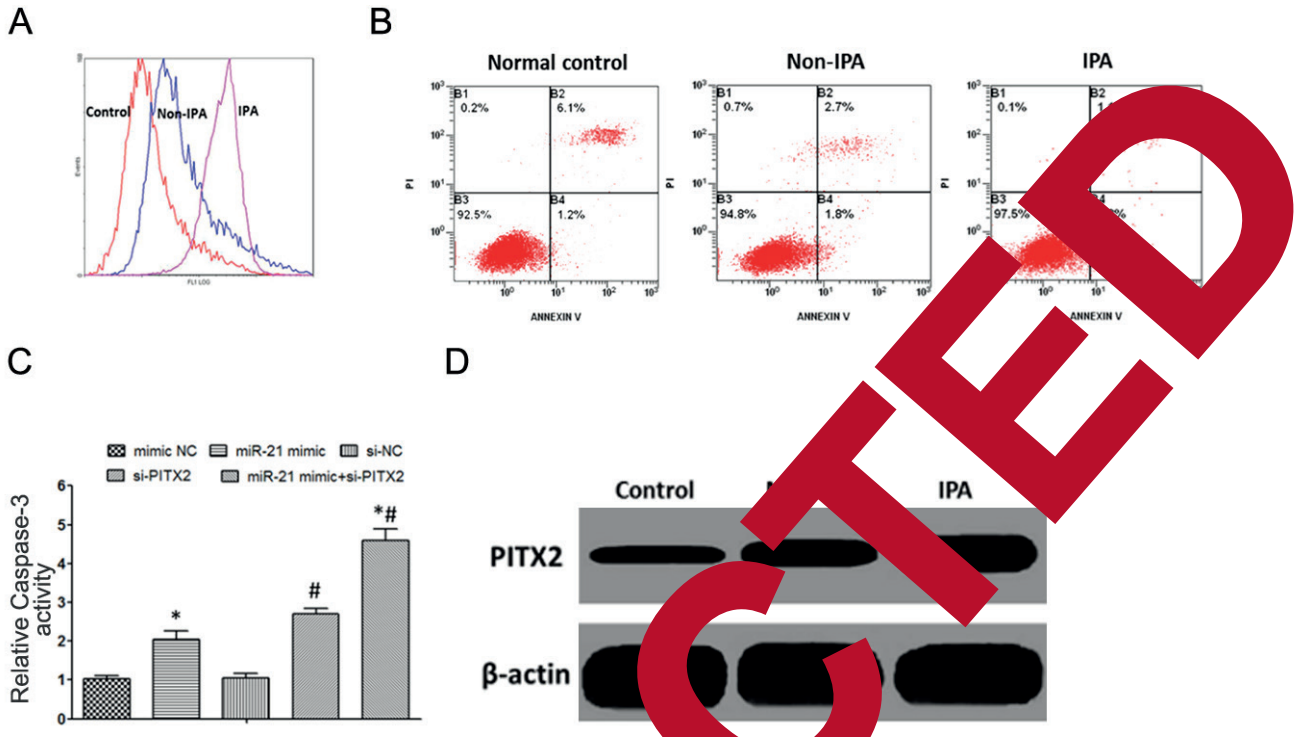


Figure 1. Lower miR-21 and higher PITX2 expression in PA tissues. ***(A)*** ...try of Ki-67 expression; ***(B)*** Flow cytometry for cell apoptosis; ***(C)*** qRT-PCR for gene ex... ***(D)*** Western blotting for BIM protein expression. *, $p<0.05$ compared to normal group. BIM: Bcl-2 interacting me... PITX2: pituitary homeobox 2, PA: pituitary adenoma.

were diagnosed by magnetic ...nce ima... ging (MRI) or computed ...y (CT) scan for nasal sinus dise... head ...uma or other brain diseases, wi... erall f... as high as 20%[3]. Altho...h ...g... re, certain PA tum... lowed ...ive growth, and presented ...nant feat... as it can invade adjace... including ..., dura, para-sellar r...n, sp...idal sinus and cavernous sinu... nd encirc... els/nerves[28]. The invasive ... of PA may ... se occurrence of cer... spinal fluid (CSF) ...akage, injury of intr... nial n... and cavernous sinus internal ...id ...ry du... surgery, and is correlated ...umor ...rence and unfavorable ...osis. ...n ...showed invasive growth, re...ely lar... mor usually existed, accompan... with foc... infiltration and invasion of a... s, thus severely affecting neurological function. These reasons make it hard ...completely remove tumors during surgery, ...high level of recurrence, relatively unfavo...e 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,

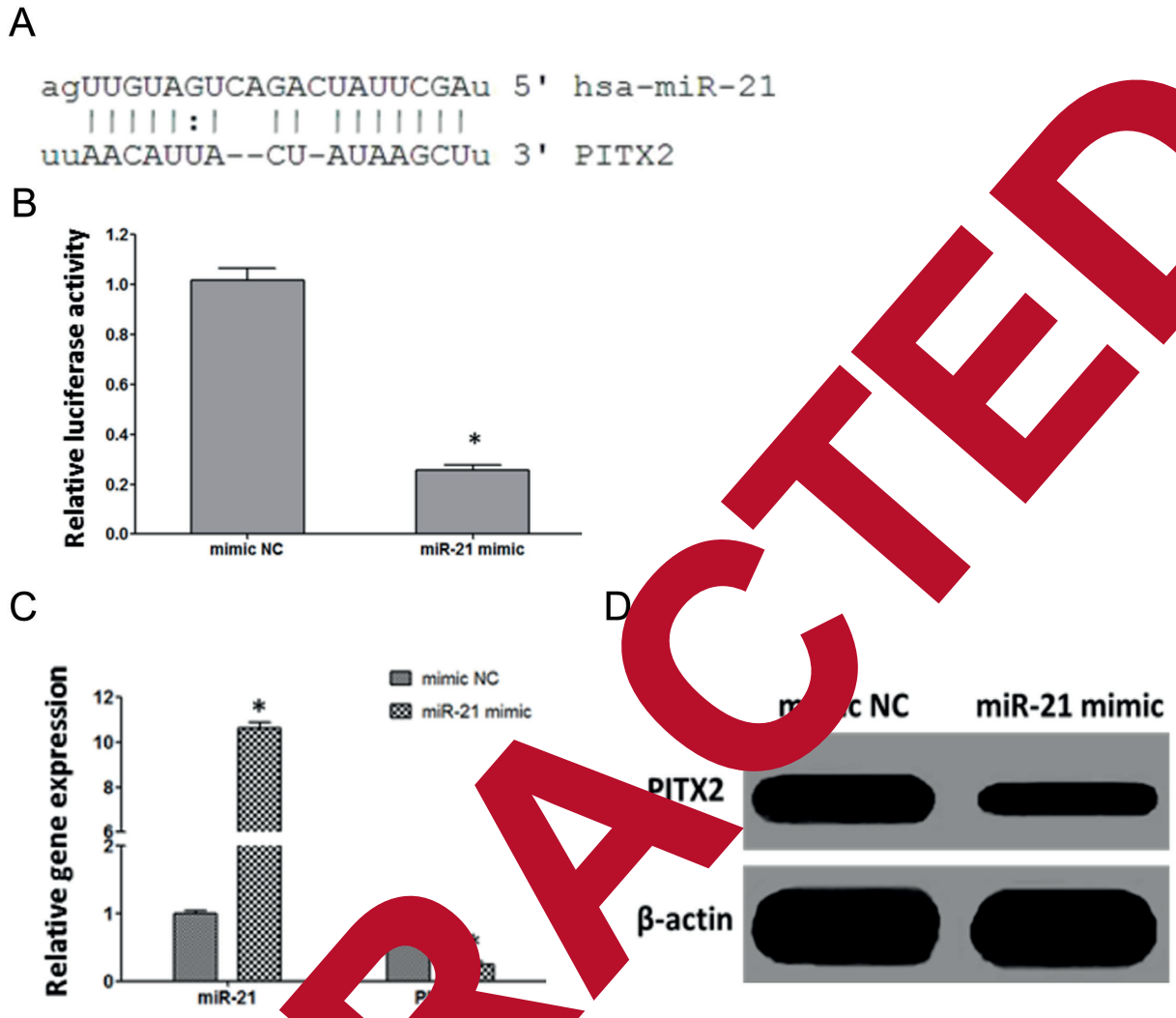


Figure 2. Targeted regula[illegible]n o[illegible]2 [illegible]ssio[illegible]R-21. ***(A)*** MiR-21 targeted 3'-UTR of PITX2 mRNA; ***(B)*** Dual luciferase reporter gene as[illegible] ***(C)*** Mi[illegible] PITX2 gene expression by qRT-PCR; (D) PITX2 protein expression by Western blotting. *, $p<0.05$ co[illegible]d to mimic [illegible]TX2: pituitary homeobox 2, MiR-21: miRNA-21, NC: normal control.

and ent[illegible]g nucleus to b[illegible]with transcriptional[illegible]or T-[illegible]ll factor/lymphoid enhancing fac[illegible]TCF/L[illegible], to activate transcription of dow[illegible]n[illegible]rget g[illegible] PITX2[11]. Cell cycle regulat[illegible]ctors i[illegible]uding cyclinD1, cyclin [illegible]nd c[illegible]n[illegible] target genes of PITX2, as[illegible] can [illegible] cell proliferation or facilita[illegible]umor for[illegible]ation via modulation at G0/G[illegible]. Elevated expression of PITX2 was [illegible]erved [illegible]n various tumor tissues including [illegible] suggesting its potential tumor-facilitating [illegible][15,17]. Acuzo et al[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 acquirement of invasiveness. Thapar et al[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 acquirement 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[29]. Flow cytometry results showed lower apoptosis level in IPA than non-IPA, as opposed to

elevated PITX2 expression, further indicating potential role of PITX2 in affecting IPA apoptosis. Amaral et al[25] 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 c elevated caspase-3 enzymatic a potentiated cell apoptosis, sugg the targeted inhibition of PITX2 exp PA cell proliferation and facilitating apopt miR-21. Currently it is widely pted th plays an oncogene rol tumor path sis. For example, Li sho signifi inhibition of drug-i d a sis of astric carcinoma cells l mi jafi et ound that the elevat f miR pres remarkably inhibi ver cancer ptosis and played a cer pathog sis. We, for the first t e, rev the potential tumor suppressor gene by miR PA pathogenesis, via fac tumor cell a osis.

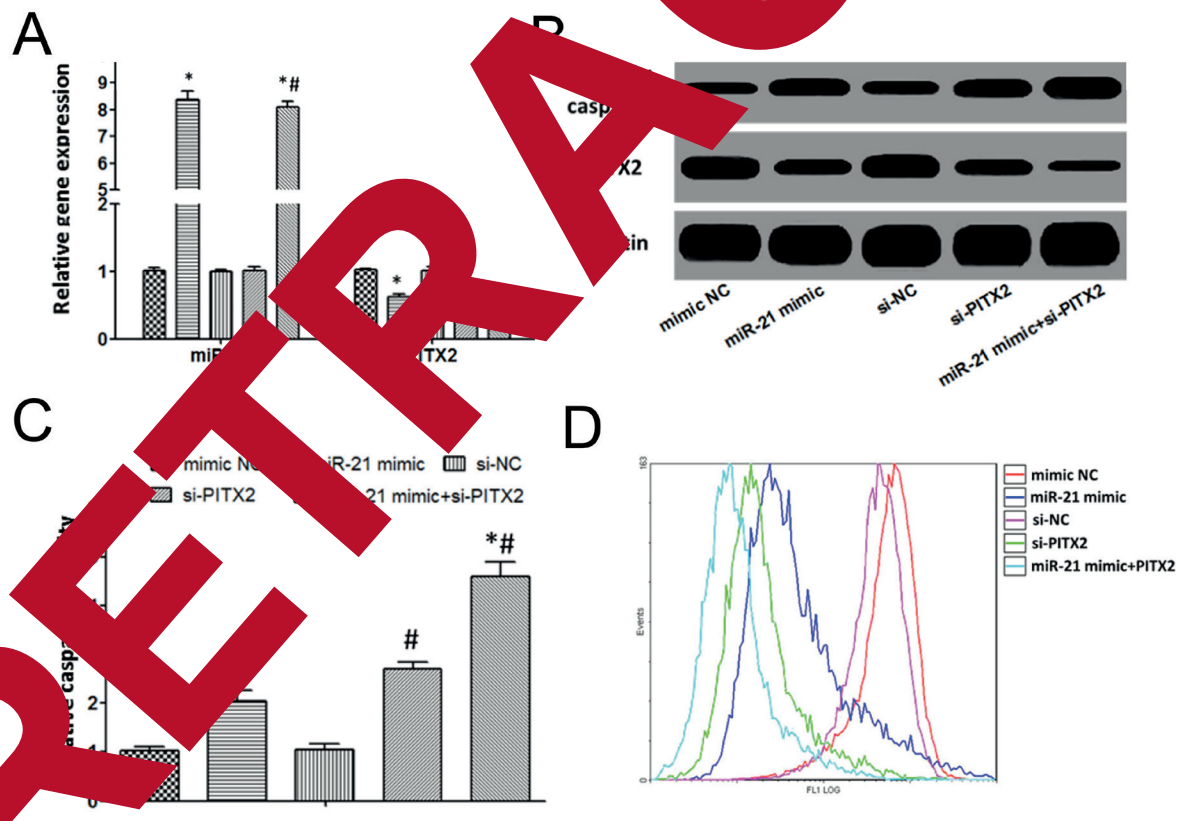
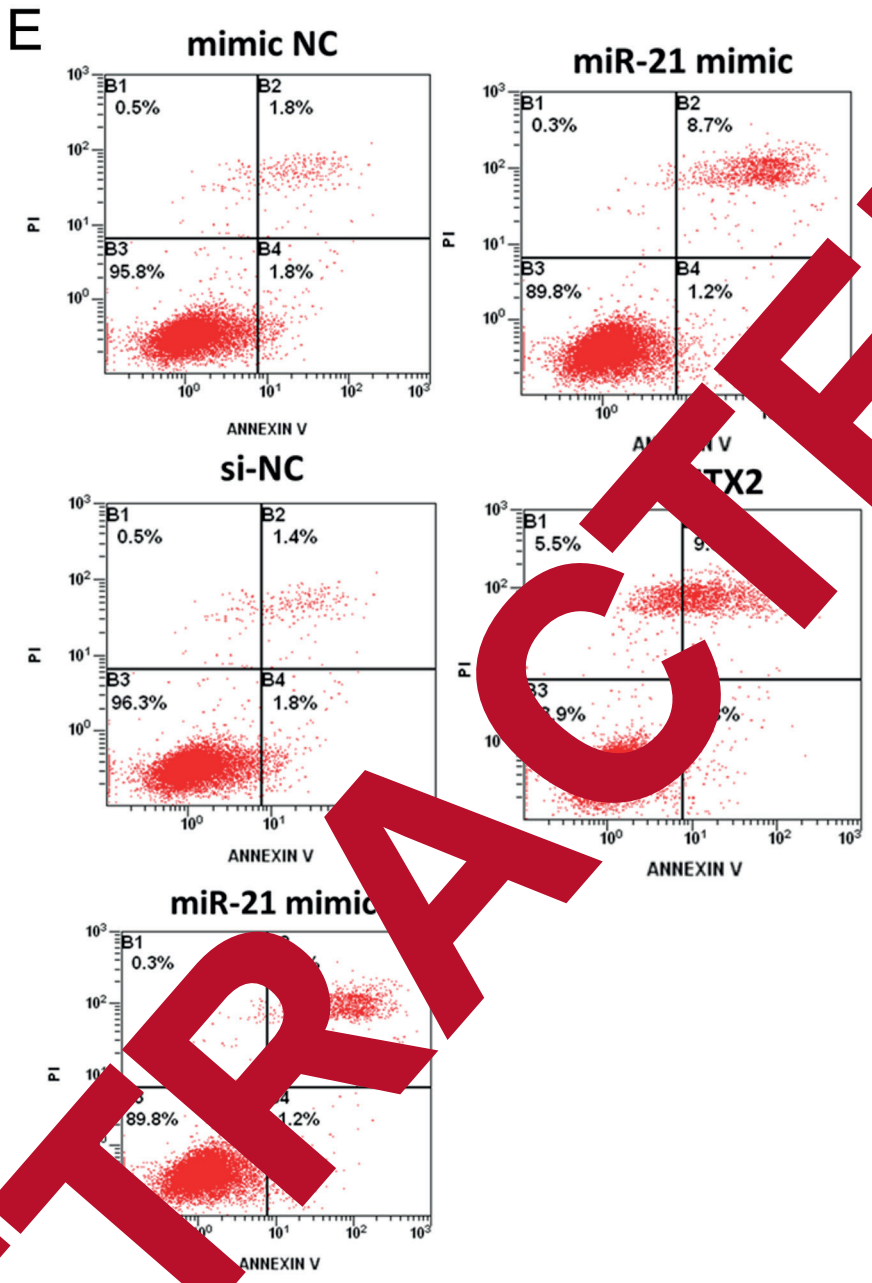


Figure 3. MiR-21 regulated PITX2 expression and HP75 cell apoptosis. ***(A)*** qRT-PCR for miR-21 and PITX2 gene expression; ***(B)*** Western blotting for protein analysis; ***(C)*** Caspase-3 enzymatic activity by spectrometry; ***(D)*** Flow cytometry for Ki-67 protein expression; ***(E)*** Flow cytometry 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)



Figu *(Conti* ***(E)*** Flow cytometry for apoptosis. *, $p<0.05$ compared to mimic NC group; #, $p<0.05$ compared to si-NC PITX uitary homeobox 2, MiR-21: miRNA-21, NC: normal control.

Conclusions

Significantly down-regulated miR-21 and ulated PITX2 expressions existed in PA tumo ssues. 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.

References

1) Budan RM, Georgescu CE. Multiple pituitary adenomas: a systematic review. Front Endocrinol (Lausanne) 2016; 7: 1.

2) Vasiljevic A, Jouanneau E, Trouillas J, Raverot G. Clinicopathological prognostic and theranostic markers in pituitary tumors. Minerva Endocrinol 2016; 41: 377-389.

3) Kontogeorgos G. Classification and pathology of pituitary tumors. Endocrine 2005; 28: 27-35.

4) Hansen TM, Batra S, Lim M, Gallia GL, Burger PC, Salvatori R, Wand G, Quinones-Hinojosa A, Kleinberg L, Redmond KJ. Invasive adenoma and pituitary carcinoma: a SEER database analysis. Neurosurg Rev 2014; 37: 279-286.

5) Oruckaptan HH, Senmevsim O, Ozcan OE, Ozgen T. Pituitary adenomas: results of 684 surgically treated patients and review of the literature. Surg Neurol 2000; 53: 211-219.

6) Hsu DW, Hakim F, Biller BM, de la Monte S, Zervas NT, Klibanski A, Hedley-Whyte ET. Significance of proliferating cell nuclear antigen index in predicting pituitary adenoma recurrence. J Neurosurg 1993; 78: 753-761.

7) Kulig E, Jin L, Qian X, Horvath E, Kovacs K, Stefaneanu L, Scheithauer BW, Lloyd RV. Apop[illegible] nontumorous and neoplastic human pit[illegible] expression of the Bcl-2 family of proteins[illegible] J Pathol 1999; 154: 767-774.

8) Scheithauer BW, Kurtkaya-Yapicier O, Kovacs [illegible] Young WF, Lloyd RV. Pituitary[illegible]oma: a c[illegible]nicopathological review. M[illegible]y 2005; 56: 1066-1074.

9) L'Honore A, Commere P[illegible]uimette [illegible]arras D, Drouin J, Buckingham[illegible]do[illegible] Pitx2 and Pitx3 is[illegible]cal[illegible]myogenesis. Dev Cell 2014; [illegible]92-405.

10) Chawla B, S[illegible] Williams AL[illegible]sack BL. Retinoic a[illegible]r2 regulate[illegible]y neural crest surv[illegible] and [illegible]on in craniofacial and ocular [illegible]elopment. [illegible]Defects Res B Dev Rep[illegible]oxicol 2016; 10[illegible]135.

11) F[illegible]K, Ch[illegible]DW, Liu VW, Leung TH, Cheung Ngan[illegible] Increased expression of PITX2 [illegible]factor [illegible]tributes to ovarian cance[illegible]ssion. P[illegible]One 2012; 7: e37076.

12) [illegible]e G, [illegible]ng[illegible]ng L, Yi Z, Bo D. Expression [illegible]LRIG[illegible]ary tumor and its clinical significance. Eu[illegible]v Med Pharmacol Sci 2016; 20: [illegible]1973.

13) [illegible] J, Amen M, Lu MF, Amendt BA, Martin JF. Nuclear factor 1 and T-cell factor/LEF recognition elements regulate Pitx2 transcription in [illegible]tuitary development. Mol Cell Biol 2007; 27: [illegible]65-5775.

14) Li JZ, Zhang Y, Wen B, Li M, Wang YJ. Ability of PITX2 methylation to predict survival in patients with prostate cancer. Onco Targets Ther 2015; 8: 3507-3512.

15) Zhang JX, Tong ZT, Yang L, Wang F, Chai HP, Zhang F, Xie MR, Zhang AL, Wu LM, Ho[illegible] L, Wang H, Wang HY, Zhao Y. PITX[illegible] sing predictive biomarker of pat[illegible] progno[illegible]sis and chemoradioresistance[illegible]esophageal squamous cell carcinoma. In[illegible]cer 2013; 132: 2567-2577.

16) Liu Y, Huang Y, Zhu GZ. [illegible]n A1 is a[illegible]rip[illegible]tional target of PITX[illegible]d overexpre[illegible] papillary thyroid ca[illegible]oma. M[illegible]Cell Bio[illegible] 2013; 384: 221-2[illegible]

17) Moreno CS, Ev[illegible] C[illegible]Okor M[illegible]derio DM, Oyesiku [illegible]Nove[illegible]ular si[illegible]ng and classificati[illegible] human [illegible]lly [illegible]unctional pituitary [illegible]mas identifi[illegible]he expression p[illegible] proteomic a[illegible]yses. Cancer Res [illegible]; 65:[illegible]1-10222.

18) Acharya M, Lingen[illegible]DJ, Huang L, Gage PJ, [illegible]MA. Human F[illegible]apoptosis WT1 regulator is a novel PITX2-interacting protein that regulates PITX2 transcriptional activity in ocular cells. J B[illegible]hem 2009; 284: 34829-34838.

19) [illegible]hao Y, Mora[illegible]DC, Hermesz E, Lee WK, Pfaff SL, [illegible]tphal H[illegible]uced expression of the LIM-homeobox gene Lhx3 impairs growth and differen[illegible]of Rathke's pouch and increases cell apoptosis during mouse pituitary development. Mech Dev 2006; 123: 605-613.

20) [illegible]YH, Xie ZB, Yue AM, Wei QD, Zhao HF, Yin HD, Mai W, Zhong XG, Huang SR. Expression level of microRNA-195 in the serum of patients with gastric cancer and its relationship with the clinicopathological staging of the cancer. Eur Rev Med Pharmacol Sci 2016; 20: 1283-1287.

21) Jia WZ, Yu T, An Q, Yang H, Zhang Z, Liu X, Xiao G. MicroRNA-190 regulates FOXP2 genes in human gastric cancer. Onco Targets Ther 2016; 9: 3643-3651.

22) Li H, Cheng J, Mao Y, Jiang M, Fan X. miR-21 inhibits the effects of cyclooxygenase-2 inhibitor NS398 on apoptosis and invasion in gastric cancer cells. Onco Targets Ther 2015; 8: 3245-3253.

23) Najafi Z, Sharifi M, Javadi G. Degradation of miR-21 induces apoptosis and inhibits cell proliferation in human hepatocellular carcinoma. Cancer Gene Ther 2015; 22: 530-535.

24) Acunzo J, Roche C, Defilles C, Thirion S, Quentien MH, Figarella-Branger D, Graillon T, Dufour H, Brue T, Pellegrini I, Enjalbert A, Barlier A. Inactivation of PITX2 transcription factor induced apoptosis of gonadotroph tumoral cells. Endocrinology 2011; 152: 3884-3892.

25) Amaral FC, Torres N, Saggioro F, Neder L, Machado HR, Silva WA, Moreira AC, Castro M. MicroRNAs differentially expressed in ACTH-secreting pituitary tumors. J Clin Endocrinol Metab 2009; 94: 320-323.

26) Shin SS, Tormenti MJ, Paluzzi A, Rothfus WE, Chang YF, Zainah H, Fernandez-Miranda JC, Snyderman CH, Challinor SM, Gardner PA. Endoscopic endonasal approach for growth hormone secreting pituitary adenomas: outcomes in 53 patients using 2010 consensus criteria for remission. Pituitary 2013; 16: 435-444.

27) Zhang SX, Shan WX, Yuan LP, Liu YL, Sun LZ. Effects of silencing PTTG expressing by small interference RNA. Eur Rev Med Pharmacol Sci 2016; 20: 2835-2841.

28) Li-Ng M, Sharma M. Invasive pituitary adenoma. J Clin Endocrinol Metab 2008; 93: 328

29) Thapar K, Kovacs K, Scheithauer BW, Ste... L, ... vath E, Pernicone PJ, Murray D, Laws ... roliferative activity and invasiveness among ... y adenomas and carcinomas: an analysis using ... B-1 antibody. Neurosurgery 1996; 38: 99-107.