ORIGINAL RESEARCH

MicroRNA-145 inhibits the activation of the mTOR signaling pathway to suppress the proliferation and invasion of invasive pituitary adenoma cells by targeting AKT3 in vivo and in vitro



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K-145 re lates the mTOR signaling re how **Purpose:** This study was designed to exp) by targetin, pathway in invasive pituitary adenoma Methods: A total of 71 cases of IP., tissue. nd 66 cases of non-IPA tissues were obtained in this study. In vitro, the IPA cells were assigned to blank control, empty plasmid, miR-145 mimic, miR-145 inhibitor, n K-145 mimic + rapany cin, miR-145 inhibitor + rapamycin and rapamycin groups. Quantitative real-time lymerase chain reaction (qRT-PCR) and Western tect the prote blotting were performed to expressions of PI3K, AKT3, mTOR mRNA and the mRNA expression of miR- both vivo and in vitro. Additionally, the S6K and RPS6 mRNA and prote ions as well as the relative phosphorylation levels were determined exp in vitro. MTT assa flow flow y and transwell assay were used to testify the cell proliferasion ability, respectively. s and i tion. a

its: Th PA tiss. s exhibited significantly lower expression of miR-145 but higher PI3K, Re T3 and **R** A and protein expressions when compared with the non-IPA tissues. d with the blank control and empty plasmid groups, the miR-145 mimic group showed Con significa v decreased PI3K, AKT3, mTOR, S6K and RPS6 mRNA and protein expressions as well as prosphorylation levels; besides, the IPA cell proliferation, migration and invasion ity were strongly inhibited, accompanied with the increased number of apoptotic cells. In the miR-145 inhibitor group, the PI3K, AKT3, mTOR, S6K and RPS6 mRNA and protein expressions as well as the phosphorylation levels were significantly increased; cell proliferation, migration and invasion ability were remarkably elevated, accompanied with reduced apoptotic cell number.

Conclusion: The study demonstrates that miR-145 inhibits the mTOR signaling pathway to suppress the IPA cell proliferation and invasion and promotes its apoptosis by targeting AKT3. **Keywords:** invasive pituitary adenoma, microRNA-145, mTOR signaling pathway, proliferation, invasion, AKT3

Introduction

Pituitary adenomas are common benign neoplasms and account for 15%–20% of primary brain tumors. Although they do not metastasize in most cases, tumor invasion into adjacent tissue is detected at initial diagnosis,¹ which is invasive pituitary adenoma (IPA). IPA infiltrates adjacent tissues and grows in several directions: superiorly, permeating the diaphragma sellae and reaching the suprasellar cistern; laterally, disrupting the medial wall of the cavernous sinus, obliterating the venous structures or even encasing the

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internal carotid artery; inferiorly, extending into the sphenoid sinus and eroding the sellar floor; posteriorly, destroying the clivus and reaching the brain stem.² Notoriously, IPA is difficult to manage because of its size, speed of growth, invasiveness and high frequency of recurrence.3 Despite multimodal therapeutic options, including radiotherapy, alternative medical therapies and repeated surgeries, a large number of cases are refractory to the conventional therapies and tend to quickly recur after initial treatment.⁴ Therefore, these IPAs are associated with poor prognosis and can even be fatal.5 Although several factors, such as gene mutations, locally produced growth factors and hypothalamic dysregulation, are controversial and appear to be involved in the transformation of pituitary cells,⁶ the identification of molecular pathway mechanism resulting in pituitary tumorigenesis is one of the major challenges in endocrine oncology.7

miRs are small, noncoding RNA molecules regulating the protein-coding gene expression at the translational expression.8 miR-145 is highly expressed in mesodermderived tissues and germ line, such as ovary, uterus, spleen, heart, testis and prostate,9 and it plays important roles in the differentiation of vascular smooth muscle cells and stem cells.^{10,11} As a well-known tumor suppressor (miR), miR-145 is downregulated in several human cancers, including brea cancer, lung cancer, bladder cancer and ovarian cancer.¹²⁻ mTOR is a key player to integrate different big **b**emical and growth factor signals, including gluco , adei sine triphosphate (ATP), amino acids and instant.¹⁶ Re input from multiple signals, such as ground fact *Autrients*, hormones and other mitogens or mulants, the mTOR pathway stimulates protein synthesis by posphorylating key translation regulators.¹⁷ The athway regular s cell growth, and increasing studies b e demonstrated its dysregulation in many cancers, including by ast cancer, prostate cancer ^{18–20} h. dition peractivation of the and renal cell cap d in IPA, especially the nway AKT/mTOR1 also n form of AKT, AKT3 is proved to invasive ty ²¹ As be increased in. ors.²² Moreover, Boufragech et al²³ have pressed miR-145 may inhibit the PI3K/ reported that over AKT pathway and target AKT3 directly in suppressing the development and metastasis of thyroid cancer. However, there are few studies or reports discussing the relationship of miR-145, AKT3 and the mTOR pathway in IPA. In this study, the functions and mechanism of miR-145 regulating the mTOR signaling pathway in proliferation and invasion by targeting AKT3 are discussed to figure out the relevant molecular mechanism and to lay a theoretical foundation for molecular targeted therapy for IPA.

Materials and methods Ethics statement

This study was approved by the regional committees for medical and health research ethics of the First Affiliated Hospital of Xinjiang Medical University. All participants provided and signed the informed consent.

Study subjects

The pituitary tissues were collected from 137 patients with pituitary adenomas treated in the Department of Neurosurgery at the First Affiliated of Xinjiang Medical University from March 2 3 to September 2015, among whom 53 were male and were fema with the average age of 41.6±0.4 v is. The gnosti criteria of IPA were as follows:²⁴ arst, according Ilson-Hardy classification standard, uita adenomas at III-IV grades es were assifie into the IPA group; or C, D and E st second, dame es h adjacent es including cavernous sinus, parasellar and pothalamus were observed through imaging expination, computed tomography preop and magnetic resonance imaging (MRI); third, the (CT bon at the bottom of sellar and adjacent endocranium uring the surgery were verified with the invasion taken f tumor cons on the basis of pathology; fourth, it was during the surgery that the bones at the bottom ob f sellar and endocranium were invaded and destroyed, nd tumors protruded into sphenoid sinus cavity or nerve ind vessels around parasellar. Conforming to any one of the abovementioned criteria, the patients were assigned into the IPA group; otherwise, they were classified into the non-IPA group. Based on the abovementioned criteria, there were 71 cases in the IPA group and 66 cases in the non-IPA group, which was further definitely diagnosed according to pathology.

In vivo and in vitro grouping

In the in vivo experiment, all the 137 patients were divided into the non-IPA group and the IPA group. In the in vitro experiment, samples were assigned into the blank control group (without treatment), the empty plasmid group, the miR-145 mimic group, the miR-145 inhibitor group, the miR-145 mimic + rapamycin group, the miR-145 inhibitor + rapamycin group and the rapamycin group.

Cell culture

After rinsing with normal saline, the tissues were placed into serum-free Dulbecco's Minimum Essential Medium (DMEM) under sterile conditions and immediately sent to the laboratory. Then, the tissues were rinsed with phosphatebuffered saline (PBS) on a clean bench under sterile conditions to remove connective tissues, necrotic tissues and blood clots. Afterward, the tissues were cut into tissue blocks with a size of 1 mm³ using ophthalmic scissors. After preheating to 37°C, 2.5 g/L trypsase (5–6 times the volume of the tissues) was added, and then the tissues were digested for 30-50 min at 37°C, during which they were dispersed using straw every 5 min until being well dispersed. Then, as much as five times of DMEM culture solution (containing 105 U/L penicillin, 0.1 g/L streptomycin, 2.2 g/L NaHCO₂, 2.0 g/L HEPES and 15% fetal calf serum) was added to terminate the digestion for 5 min. After being filtered by a 100-mesh cell strainer, the tissues were centrifuged at 1,000 rpm (r=15 cm) for 10 min and the supernatant was aspirated. DMEM culture solution, containing 105 U/L penicillin, 0.1 g/L streptomycin, 2.2 g/L NaHCO₂, 2.0 g/L HEPES and 15% fetal calf serum, was added to resuspend and disperse the cells. The number of cells was counted using Japan Olympus microscope, and the concentration of cells was adjusted to 1.0×106/mL. Trypan blue staining was performed to detect cell viability. The cells were inoculated in a 24-well plate, which was pre-surrounded by polylysine with 1 mL/well, and the culture solution was changed every 2 days.

Cell transfection

Cells at the logarithmic growth phase w inoci ated in id and 96-well plate (100 µL/well). Corresponding 1 lentiviral packaging were co-transferred in A cells, 24 h he plate for before which cells were laid onventional incubation. One hour before the transfection, cell culture fluid was changed. The transfection mixture as produced using Lipofectamine 2000 nvitrogon Inc., Cansbad, CA, USA). Serum-free and double-approved by codies-free medium (Invitrogen pligon, eotide f gment (with the final con-Inc.), containi edded in liposome, was added centration .300 p ol/weh restected cells were cultured with serumto each pup. Th a 5% CO₂ incubator at 37°C for 4 h, after which free mediu. 10% fetal box serum (FBS) was added for further culture in incubator with CO₂ at 37°C. After 24 h transfection, the cells with miR-145 mimic plasmid, miR-145 inhibitor plasmid and blank plasmid were collected. In each group, cells were diluted at 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} and 10^{-8} /mL and incubated in a 96-well plate, with three repeated wells in each group. After being cultured in 5% CO₂ incubator for 18 h at 37°C, the cells were counted under inverted fluorescence microscope (Nikon Corporation, Tokyo, Japan), and viral titer was calculated using computer. Viral titer $(pfu/mL) = (10 \times mean fluorescence)/$

corresponding dilution degree. After 48 h, the cells were collected to extract the total RNA by RNA extraction kit (Invitrogen Inc.). Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the mRNA expressions of PI3K, AKT3, mTOR and miR-145. In Dual-luciferase reporter gene assay system, the IPA cells at the logarithmic growth phase were incubated into a 96-well plate and the cells were transfected using Lipofectamine 2000 when the cell density reached 70%. AKT3-3' untranslated region (UTR)-wild-type (WT) plasmid and miR-145 mimic plasmid were fully mixed to transfect the 293T cells. Meanwhith the control groups (AKT3-3'UTR-WT + negative Atrol [NC] nd AKT3-3'UT R-mutant type [MUT] + miR 15 mimic) we established. After culturing for 6 h, 16 cultur medium as replaced by 10% FBS, which was plowed by converse culture for 48 h. Telomerase activity as de cted by chemiluminescence.

qRT-PC

According to the pecification of Trizol kit, the total RNA of IP/ tes and cells, was extracted and preserved at -80° C. he content of extracted RNA was determined by the optical ensity (OD) 260 nm measured by ultraviolet spectrophoneter (typ 752), and the RNA yield was defined in the Ingle OD260 equaling to 40 µg RNA, with OD260 way o ing from 1.8 to 2.0 deemed as high RNA purity. In the experiment, a two-step qRT-PCR was conducted. First, the extracted RNA was reverse transcribed to cDNA with RT kit (Takara Biotechnology Ltd., Dalian, People's Republic of China; the specific surgery was performed according to the specification). The conditions for the amplification of reverse transcription were as follows: at 37°C for 15 min and at 85°C for 5 s. The cDNA products were amplified by PCR in the following conditions: pre-denaturation for 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C; extension for 10 min at 72°C. Reaction system included 2 µL PCR upstream primer, 2 µL PCR downstream primer, 4 µL template DNA, 1 µL ROX Reference Dye (50×), 25 µL SYBR Premix Ex TaqII and 16 µL dH₂O. With GAPDH as the internal reference, mRNA expressions of PI3K, AKT3, mTOR and miR-145 were calculated by $2^{-\Delta\Delta Ct}$ (Table 1).

Western blotting

After digesting with pancreatin, the cells were collected and rinsed with PBS precooled to 4°C for three times. Radioimmunoprecipitation assay (RIPA) lysis buffer was added to extracted total protein of the transfected cells, and the protein quantification was measured by bicinchoninic acid (BCA). Approximately 20 µg of total protein of the

Table I Primer sequence of qRT-PCR

Name	Sequence
miR-145	Upstream 5'-GGTCCAGTTTTCCCAGG-3'
	Downstream 5'-CAGTGCGTGTCGTGGAGT-3'
PI3K	Upstream 5'-CAAAGCCGAGAACCTATTGCGAG-3'
	Downstream 5'-GTTTGACTTCGCCATCTACCAC-3'
AKT3	Upstream 5'-GAAGATCTGATGAGCGATGTTACCA
	TTGTG-3′
	Downstream 5'-CCGCTCGAGTTCTCGTCCACTTG
	CAGAGTAG-3′
mTOR	Upstream 5'-GACCGTCTGAGTGGGAAG-3'
	Downstream 5'-TTACCAGAAAGGGCACCAG-3'
GAPDH	Upstream 5'-AGAAGGCTGGGGCTCATTTG-3'
	Downstream 5'-AGGGGCCATCCACAGTCTTC-3'

Abbreviation: qRT-PCR, quantitative real-time polymerase chain reaction.

sample was collected and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); after which the upper sample was transferred onto polyvinylidene fluoride (PVDF) membrane and blocked for 1 h by blocking buffer with the addition of corresponding antibodies (diluted 1:1,000) for incubation at 4°C overnight, including p-PI3K (ab182651), PI3K (ab123752), p-mTOR (ab84400), mTOR (ab2732), p-AKT3 (ab138365), AKT3 (ab112002), p-S6K (ab126818), S6K (ab139438), p-RPS6 (ab13153 and RPS6 (ab59261). All the antibodies were purchased from Abcam Inc. (Cambridge, MA, USA). After being ri ed with TBST, goat anti-rabbit Immunoglobulin G (Ig labele with 1100 horseradish peroxidase and diluted at 1:5 00 was to the protein sample and incubated or 45 at room temperature. Afterward, the sample as rinsed w TBST again, colored and exposed by Jectro, emiluminescence (ECL). Gray value was explaned, with the gray value of β -actin as internal refere e. The experiment was repeated for three times.

Dual-lucify ase epoint resene assay

miR-145 matric was a othesized according to the mature miR-145 sequence of ABase accession no: MIMAT0000437). miR-145 inhibitor has the reverse complementary sequence of mature miR-145. AKT3 was silenced according to the siRNA design principle, and homological analysis was conducted for alternative target sequence. NC sequence of double-stranded RNA had no homology with mammalian genome. When both ends of oligonucleotide were synthesized, restriction enzyme sites, Bam HI and Xho I, were added. The entire DNA sequence synthesis was performed by Shanghai GenePharma, Co., Ltd (Shanghai, People's Republic of China; Table 2). DNA fragments were cloned into pRNA-Lenti-enhanced green fluorescent protein (EGFP) lentivirus vector to construct corresponding expression vectors pRNA-Lenti-miR-145 mimic-EGFP (miR-145 mimic plasmid), pRNA-Lenti-miR-145 inhibitor-EGFP (miR-145 inhibitor plasmid) and pRNA-Lenti-vector-EGFP (empty plasmid). Meanwhile, a fragment of targeted gene was inserted into AKT3-3'UTR-WT plasmid and AKT3-3'UTR-MUT plasmid to construct dual-luciferase reporter gene plasmid of AKT3. All the abovementioned plasmids were identified by PCR, double enzyme instion and DNA sequencing technique.

Bioinformatics software on ·//www.tar tscan.org was adopted to predict the argeted orrelation between miR-145 and AKT3, which was further ied by dualluciferase reporter gen assar system. According to the specification of ly derase way kit, P S was used to rinse the 96-well place with 100 µ. sive lysis buffer (PLB) d the 96-well plate was shaken at a added to each well, n of cell lysis buffer. In addition, the collect low sp *L* cell lysis buffer and 100 μL luciferase detection 20 nt II were well mixed, followed by luminometer readreas ing (20/20; Toner Designs, Sunnyvale, CA, USA), and erase activity was obtained. Afterward, 100 the renilia. Stop&Glo reagents was added and fully mixed to μD otain renilla luciferase activity value. The expression of eporter gene was presented by the activity ratio of firefly ciferase and renilla luciferase.

MTT assay

Cells in each group were collected and incubated in a 96-well plate with 5×10^3 cells per well, and three repeated wells were set up. When the cells adhered to the wall, $15 \,\mu\text{L}$ MTT (5 g/L, G3582; Promega Corporation, Madison, WI, USA) was added to culture the cells for 4 h in incubator with 5% CO₂ at 37°C. In addition, 150 μ L dimethyl sulfoxide (DMSO) was added and the 96-well plate was shaken for 10 min.

•	
Name	Sequence
miR-145 mimic	5'-GUCCAGUUUUCCCAGGAAUCCCU-3'
miR-145 inhibitor	5'-AGGGAUUCCUGGGAAAACUGGAC-3'
NC	5'-CAGUACUUUUGUGUAGUACAA-3'
AKT3	Upstream 5'-ACCGCACACGTTTCTATGGT-3'
	Downstream 5'-CCCTCCACCAAGGCGTTTAT-3'
GAPDH	Upstream 5'-AGAAGGCTGGGGCTCATTTG-3'
	Downstream 5'-AGGGGCCATCCACAGTCTTC-3'

Abbreviation: NC, negative control.

Microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA, USA) was selected to detect the absorbance (A value) of each well at 490 nm, and cell proliferation in 72 h of each group was also detected.

Detection of IPA cell invasion ability

After 48 h transfection, the cells were digested. In each transwell chamber, 80 μ L 1:8 Matrigel was spread and 1×10⁵ cells were incubated in it, with the addition of 100 μ L serum-free DMEM. Then, the complete medium was added to the lower chamber for 24 h incubation, after cells in the upper chamber were wiped out using cotton bud. Afterward, the cells were fixed with 4% paraformaldehyde for 15 min and stained with crystal violet for 10 min. Under microscope, five fields were randomly selected to be photographed and the cells were thereof counted. In transwell assay, Matrigel spreading was not needed and the incubation lasted for 16 h.

Annexin V-fluorescein isothiocyanate (FITC)-propidium iodide (PI)

Cells in each group were digested with pancreatin, rinsed by PBS and resuspended. In addition, 10 μ L Annexin V-FITC and 5 μ L PI staining solution were added and mixed up with the cells for a 15 min dark incubation at room temperature. With cells resuspended by 300 μ L binding buffer, the tow cytometry was selected to detect cell apoptropole

Statistical analysis

Data were presented as mean \pm sta ard devia. $n(SD; \overline{x} \pm s),$ and the SPSS17.0 software S nc., Chicag IL, USA) was used to process the dam. Comparis between two groups was conducted by independent sample *t*-the while comparisons among three more groups were analyzed by one-way A). The numeration data among analysis of variance JC ² tes 2<0.05 referred to statisti-</p> red b groups wer cal signi cance.

Results

Comparisons of miR-145, PI3K, AKT3 and mTOR mRNA and protein expressions between the IPA and non-IPA tissues

The results of qRT-PCR and Western blotting showed that the miR-145 expression strongly decreased while PI3K, AKT3 and mTOR mRNA and protein expressions significantly increased in the IPA tissues as compared with those in the non-IPA tissues (all P < 0.05; Figures 1–2).



Figure I Comparisons of miR-145, PI3K, A and m between IPA and non-IPA tissues. d 66 cases of no Notes: There were 71 cases of IPA tissue A tissues; *P<0.05 when compared with non-IPAs: the vperim vere repeate hree times. and the mean value was calculated. Abbreviations: AKT3, prot kinase B; IPA, inva ary adenoma; miR-145, nmalian ta microRNA-145; mTOR, of rapam , PI3K, phosphoinosmde-3-kinase.

Comparise is of mix-145, PI3K, AKT3 and mTOR mRNA and protein expressions in A cells among seven groups

ccording to he detection of miR-145, PI3K, AKT3 and OR mRM and protein expressions in each group, no difference was observed between the blank consign and empty plasmid groups (all P > 0.05). Compared with the blank control and empty plasmid groups, the miR-145 mimic group exhibited increased expression of miR-145, but decreased PI3K, AKT3 and mTOR mRNA and protein expressions as well as the phosphorylation levels of p-S6K and p-RPS6 (all P<0.05); in the miR-145 inhibitor group, miR-145 was significantly downregulated but PI3K, AKT3 and mTOR mRNA and protein expressions as well as the phosphorylation levels of p-S6K and p-RPS6 strongly increased (all P < 0.05); the miR-145 mimic + rapamycin group exhibited upregulated miR-145 but significantly decreased PI3K, AKT3 and mTOR mRNA and protein expressions as well as the phosphorylation levels of p-S6K and p-RPS6 (all P<0.05); in the miR-145 inhibitor + rapamycin group, downregulated miR-145 but strongly elevated PI3K and AKT3 mRNA and protein expressions were observed (all P < 0.05), while no difference in the phosphorylation levels of p-S6K and p-RPS6 was noted (all P>0.05); the rapamycin group exhibited significantly decreased mTOR mRNA and protein expression as well as the phosphorylation levels of p-S6K and p-RPS6 (all P < 0.05). There was no significant difference in PI3K and AKT3 mRNA and protein expressions between the miR-145 mimic and miR-145



mimic + rapamycin groups (all P > 0.05). But the miR-145 mimic + rapamycin group had significantly lower mTOR mRNA and protein expression as well as the phosphorylation levels of p-S6K and p-RPS6 (all P < 0.05; Figures 3–4).

miR-145 regulates the mTOR signaling pathway to suppress IPA cell proliferation

Compared with the blank control and empty plasmid groups, the IPA cell proliferation was significantly inhibited in the miR-145 mimic group, but it was significantly elevated in the miR-145 inhibitor group (both P<0.05), indicating that over-expressed miR-145 could inhibit IPA cell proliferation. IPA cell proliferation in the miR-145 inhibitor + randomized



Figure 3 Comparisons of miR-145, PI3K, AKT3 and mTOR mRNA expressions in IPA cells among seven groups.

Notes: A, blank control group; B, empty plasmid group; C, miR-145 mimic group; D, miR-145 inhibitor group; E, miR-145 mimic + rapamycin group; F, miR-145 inhibitor + rapamycin group; G, rapamycin group. *P<0.05 when compared with the blank control group. *P<0.05 when compared with the miR-145 mimic group; the experiments were repeated three times, and the mean value was calculated.

Abbreviations: AKT3, protein kinase B; IPA, invasive pituitary adenoma; miR-145, microRNA-145; mTOR, mammalian target of rapamycin; PI3K, phosphoinosmde-3-kinase.

group was significantly decreated as compared with that in the miR-145 inhoitor group, implying that the inhibited mTOR pathwereby rapamyce occurd reverse the promotion of IPA cell procession caused by miR-145 inhibitor (Figure 5)

min-145 regulates the mTOR signaling pathway to promote IPA cell apoptosis

Compare the blank control and blank empty groups, the threef apoptosis rate significantly increased in the miR-15 mimic group while it strongly decreased in the miR-145 nhibitor group (both P < 0.05), indicating that overexpressed diR-145 could promote IPA cell apoptosis. When compared with the miR-145 inhibitor group, the IPA cell apoptosis rate remarkably elevated in the miR-145 inhibitor + rapamycin group, suggesting that rapamycin could inhibit the mTOR pathway to reverse the inhibition of IPA cell apoptosis caused by miR-145 inhibitor. These results further demonstrated that miR-145 promotes IPA cell apoptosis by targeting the mTOR signaling pathway (Figure 6).

miR-145 regulates the mTOR signaling pathway to suppress IPA cell migration

Compared with the blank control and empty plasmid groups, IPA cell migration ability significantly decreased in the miR-145 mimic group but it remarkably increased in the miR-145 inhibitor group (both P < 0.05), indicating that IPA cell migration ability could be inhibited by overexpressed miR-145. The miR-145 inhibitor + rapamycin group showed significantly decreased IPA cell migration ability when compared with that in the miR-145 inhibitor group, suggesting that the inhibition of the mTOR pathway by rapamycin could reverse the promoted IPA cell migration caused by miR-145.



Figure 4 Comparisons of protein expressions of miR-145, PI3K, AKT3, mTOR, p-S6K and p-RPS6 in IPA cells among seven group. **Notes:** A, blank control group; B, empty plasmid group; C, miR-145 mimic group; D, miR-145 inhibitor group; E, miR-145 mimic group; B, miR-145 inhibitor + rapamycin group; G, rapamycin group, **P*<0.05 when compared with the blank control group. **P*<0.05 when compared with the blank control group. **P*<0.05 when compared with the blank control group. **P*<0.05 when compared with the miRes a mimic of the previous the experiments were repeated three times, and the mean value was calculated. **Abbreviations:** AKT3, protein kinase B; IPA, invasive pituitary adenoma; miR-145, microRNA-145; mTOR, mamment target of the pamycin; Pl3m phosphoinosmde-3-kinase.

Therefore, miR-145 inhibits IPA cell migration via mTOR signaling pathway (Figure 7).

miR-145 regulates the mTOR signaling pathway to suppress IPA cell invasion ability

The IPA cell invasion ability declined in the miR-145 imic hen group but it elevated in the miR-145 inhibitor group compared with the blank control and empty plasmid gra ps (both P < 0.05). This indicated that overex miR-1 SSL could inhibit IPA cell invasion. The IP cell inv sion abi ity in the miR-145 inhibitor + raparticin 011 лgnin ١Ý in the mine 145 inhibitor decreased as compared with the



Figure 5 miR-145 regulates the mTOR signaling pathway to suppress IPA cell proliferation.

Notes: A, blank control group; B, empty plasmid group; C, miR-145 mimic group; D, miR-145 inhibitor group; E, miR-145 mimic + rapamycin group; F, miR-145 inhibitor + rapamycin group; G, rapamycin group. *P<0.05 when compared with the blank control group. *P<0.05 when compared with the miR-145 inhibitor group; the experiments were repeated three times, and the mean value was calculated. **Abbreviations:** A value, absorbance value; IPA, invasive pituitary adenoma; miR-145, microRNA-145; mTOR, mammalian target of rapamycin.

group, which suggested that in coited mTOR pathway by rapamyon could reverse the promoted IPA cell invasion ability caused by h P-145 inhibitor (Figure 8).

niR-145 nrgets AKT3

WT3 was redicted to be the target gene of miR-145 by commatics software <u>http://www.targetscan.org</u> Figure 9A). After co-transfection of AKT3-3'UTR-WT plasmid and miR-145 mimic plasmid in IPA cells, analyzed by SPASS 21.0 software, luciferase activity was found to be significantly decreased when compared with the AKT3-3'UTR-WT + NC group (P<0.05). However, the co-transfection of AKT3-3'UTR-MUT + miR-145 mimic plasmid showed no significant difference with the AKT3-3'UTR-WT + NC group (P<0.05; Figure 9B).

Discussion

IPA is characterized by rapid growth, postoperative recurrence and poor prognosis.²⁵ At the same time, it is hard to have complete resection surgery of IPA due to the wide range of invasion, as a result of which, it has lower recovery rate and is one of the greatest challenges of clinical treatment.²⁶ In recent years, both tumor suppressor genes and oncogenes have been reported to be potential for genetic therapy of pituitary tumors.²⁷ Therefore, finding the possible gene target of improving treatment and promoting prognosis in IPA, and seeking the regulation mechanism of that, is one of the major subjects in the study.

The study found that miR-145 expression in the IPA tissues was much lower than in the non-IPA tissues, while the PI3K, AKT3 and mTOR mRNA and protein expressions were



Figure 6 miR-145 regulates the mTOR signaling pathway to increase the IPA cell apoptosis rate.

Notes: (**A**) Blank control group; (**B**) empty plasmid group; (**C**) miR-145 mimic group; (**D**) miR-145 inhibitor group; (**E**) -14 mimic + rappycin group; (**F**) miR-145 inhibitor + rappycin group; (**G**) rappycin group; (**F**) miR-145 mimic group; (**F**) miR-145 m

Abbreviations: IPA, invasive pituitary adenoma; miR-145, microRNA-145; mTOR, mammalian target of g



Figure 7 miR-145 regulates the mTOR signaling pathways suppress IPA cell migration ability (hematoxylin staining ×200).

Notes: (**A**) Blank control group; (**B**) every plasmid group, (**b**) miR-145 mimic group; (**D**) miR-145 inhibitor group; (**E**) miR-145 mimic + rapamycin group; (**F**) miR-145 inhibitor + rapamycin group; (**G**) rape with the blank control group. (**H**) comparing of cell migration number in each group. *P<0.05 when compared with the miR-145 mibitor group; the experiments were repeated three times, and the mean value was calculated. **Abbreviations:** IPA, invasive a tary adepted; miR-145, microRNA-145; mTOR, mammalian target of rapamycin.



Figure 8 miR-145 regulates the mTOR signaling pathway to suppress IPA cell invasion ability (hematoxylin staining $\times 200$). **Notes:** (**A**) Blank control group; (**B**) empty plasmid group; (**C**) miR-145 mimic group; (**D**) miR-145 inhibitor group; (**E**) miR-145 mimic + rapamycin group; (**F**) miR-145 inhibitor + rapamycin group; (**G**) rapamycin group; (**H**) comparison of cell invasion number in each group. *P<0.05 when compared with the blank control group, the experiments were repeated three times, and the mean value was calculated. **Abbreviations:** IPA, invasive pituitary adenoma; miR-145, microRNA-145; mTOR, mammalian target of rapamycin. Α



Figure 9 miR-145 targets AKT3.

Notes: (**A**) 3'UTR binding site of miR-145 and AKT3. (**B**) After 293T cells were transfected with AKT3-3'UTR T + miR-145 mimics/NC, AKT3-3'UTR-MUT + miR-145 mimics/NC for 48 h, the luciferase activity of each group was detected. **Difference was signated (P<0.05); the operiments were repeated three times, and the mean value was calculated.

Abbreviations: AKT3, protein kinase B; IPA, invasive pituitary adenoma; miR-145, mooRNA-145; MUT, mutant type; NC, negative control; UTR, untranslated region; WT, wild type.

higher in IPA tissues than that in non-IPA tissues. Based results, miR-145 played an inhibitor role of PI3K and A T3 in IPA. miR-145, as one of the suppressor mP 🔺 s, has b found to be downregulated in many cancer ells.28 ecreas vitro expression of miR-145 is observed both in vivo which is important for the development a rogression of prostate cancer.²⁹ A previous v has den strated that the AKT pathway is overexpressed and activated in pituitary tumors.³⁰ AKT3, identifia as a kinase hich might activate may be involved in the target molecule anti-apoptotic protein of cell metabolism ance PI3K, a heterodimer, includes bunit (0) and gulatory subunit (p85).³² both catalytic The AKT ed to be oncogenic, which 13K 1 thway influen cell p Constion and cell apoptosis in cancers, and to be downregulated by overexpressed miRhas been i growth and promote cell apoptosis in tumor 145 to inhibit development.³³ This study found that with increased miR-145 in IPA, there were decreased mRNA and protein expressions of PI3K and AKT3, where IPA cell proliferation, migration and invasion were inhibited while cell apoptosis was promoted. Besides, AKT3 was demonstrated to be a target gene of miR-145 by dual luciferase report gene assay system. Therefore, miR-145 might inhibit proliferation, migration and invasion of IPA by inhibiting PI3K and AKT3.

In addition, the results of this study showed that miR-145 downregulates the mTOR pathway to inhibit the

on, migration and invasion of IPA. The mTOR, proli rine/threonine kinase, could regulate cell functions, growth and transcription, and was also considered as one of the significant signaling pathways of tumorigenesis.³⁴ The PI3K/AKT/mTOR pathway is involved in human malignancies, where AKT might regulate PI3K-inhibited signaling and mTOR phosphorylation; thus, mTOR inhibitor is considered to be a potential therapy of IPA treatment.³⁵ Hahn-Windgassen et al³⁶ reported that Akt regulates the activation of mTOR through growth factors and it plays as a positive modulator of mTOR. Moreover, PI3K/AKT pathway is indicated to be another upstream stimulating factor of mTOR pathway.³⁷ Mitogenic signaling caused by receptor tyrosine kinases could elevate the activity of PI3K, resulting in the activation of Akt; thereof, it stimulates thyroid tumor cell proliferation and invasion.³⁰ Few studies mentioned the relationship between miR-145 and mTOR pathway, but this study has found that the mTOR pathway might be the mediator of miR-145 to suppress IPA. In this study, with increased miR-145 in IPA, there were decreased mRNA and protein expressions of mTOR. Furthermore, the inhibited mTOR pathway by rapamycin could reverse the function of miR-145 inhibitor in IPA. Therefore, it was demonstrated that overexpressed miR-145 can inactivate mTOR pathway to inhibit the proliferation, migration and invasion of IPA.

Conclusion

miR-145 can not only suppress IPA cell proliferation, migration and invasion but also promote IPA cell apoptosis by mTOR pathway. This finding would be beneficial for further clinical treatment of IPA patients in the field of molecular biology. However, further analysis and larger samples are worth doing for IPA theoretical study.

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Disclosure

The authors report no conflicts of interest in this work.

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