MicroRNA-200b inhibits pituitary tumor cell proliferation and invasion by targeting PKCα

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Abstract. The present study aimed to investigate the expression of miR-200b and protein kinase Cα (PKCα) in pituitary tumors and to determine whether miR-200b may inhibit proliferation and invasion of pituitary tumor cells. The regulation of PKCα expression was targeted in order to find novel targets for the treatment of pituitary tumors. In total, 53 pituitary tumor tissue samples were collected; these included 28 cases of invasive pituitary tumors and 25 cases of non-invasive tumors, in addition to 5 normal pituitaries. The expression level of miR-200b in the pituitary tumor tissue was detected by quantitative polymerase chain reaction (qPCR) and the expression of PKCα protein was detected by immunohistochemistry. A PKCα 3'untranslated region (UTR) luciferase vector was constructed and a dual luciferase reporter gene assay was employed in order to examine the effect of miR-200b on the PKCα 3'UTR luciferase activity. AtT-20 cells were transfected with miR-200b mimics, PKCα siRNA and miR-200b mimics + PKCα, and the changes in cellular proliferation, invasion and apoptosis were observed via MTT, Transwell assay and flow cytometric analysis. Furthermore, PKCα mRNA expression was determined by qPCR, and Western blotting was performed to detect the expression of PKCα protein. miR-200b revealed downregulation in invasive pituitary tumor tissue, and the expression level was significantly down-regulated compared with normal and non-invasive pituitary tumor tissue ($P<0.01$). In addition, the positive rate of PKCα protein expression in invasive pituitary tumor tissues was significantly higher than in normal and non-invasive tissues ($P<0.01$). PKCα protein levels are inversely correlated with miR-200b levels in invasive pituitary tumor tissues ($r=-0.436$, $P=0.021$). The dual luciferase reporter gene assay revealed that miR-200b could specifically bind to the 3'UTR of PKCα and significantly inhibit the luciferase activity by 39% ($P<0.01$). Upregulation of miR-200b or downregulation of PKCα could suppress cell proliferation and invasion, and increase apoptosis of AtT-20 cells. It was revealed that PKCα siRNA could suppress both proliferation and invasion of AtT-20 cells and partially simulate the function of miR-200b. Expression of PKCα mRNA and protein decreased significantly in AtT-20 cells overexpressing miR-200b. Additionally, miR-200b was significantly down-regulated in invasive pituitary tumor tissue and inversely correlated with PKCα protein levels. In conclusion, miR-200b inhibited proliferation and invasiveness and promoted the apoptosis of pituitary tumor cells by targeting PKCα. The observations of the present study indicate that miR-200b and PKCα may serve as promising therapeutic targets for invasive pituitary tumors.

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Introduction

Pituitary adenomas generally account for 25% of intracranial tumors and can be divided into functioning and non-functioning pituitary adenomas. Non-functioning pituitary adenomas are not associated with sufficient hormone secretion, whereas functioning pituitary tumors are associated with excess pituitary hormones (prolactin, growth hormone, adrenocorticotropic hormone and thyroid-stimulating hormone) (1,2). The majority of pituitary tumors are benign; however, 5 to 35% of tumors are locally invasive and are therefore difficult to treat (1). Several genetic syndromes, including Carney complex and McCune-Albright syndrome, are known to be associated with the development of pituitary adenomas, but for sporadic adenomas the molecular pathology remains poorly understood (3-5). Invasive pituitary tumors have a high mortality rate because they are not easily cured by surgical, medical and radiation therapies, which tend to be partially effective or wholly ineffective (6). Therefore, there is an urgent requirement to develop early diagnostic biomarkers and to demonstrate the molecular mechanism of invasive pituitary tumors in order to improve disease management.

MicroRNAs (miRNAs) are a large family of short non-protein-coding RNA molecules of ~22 nucleotides in length, functioning as key regulators of gene expression by binding to the 3'untranslated region (3'UTR) of their target

mRNAs (7). In total, ~50% of miRNAs are located in chromosomal fragile genomic sites (8), emphasizing a key role of miRNAs in tumor pathogenesis. Emerging evidence demonstrates that aberrant miRNA expression is linked to cancer, including pituitary tumors (9-12). A previous study of treatment with the dopamine agonist, miR-200a, demonstrated a significantly different expression between treated and untreated nonfunctioning pituitary adenomas (13). The human miR-200 family includes five members divided into two groups: The miR-200b/-200a/-429 group located on chromosome 1 and the miR-200c/-141 group located on chromosome 12 (14). miR-200 are potential inhibitors of epithelial-to-mesenchymal transition, which is associated with promoting tumor cell invasion and proliferation (15-18). However, the potential roles of miR-200b in pituitary tumorigenesis are less known. The present study therefore aimed to demonstrate the potential function and regulatory mechanism of miR-200b in pituitary tumorigenesis.

In the current study, miR-200b was investigated in both invasive pituitary tumor tissues and pituitary tumor cell lines, and cell proliferation and invasion in response to this were also examined. A direct target of miR-200b, protein kinase α (PKCα), is important in regulating cell migration and cancer metastasis (19,20), and it was therefore studied in the present research. The current study may therefore promote the development of an effective therapeutic strategy for pituitary tumors.

Materials and methods

Patients and tissue samples. Specimens were obtained from the Department of Neurosurgery of the Affiliated Hospital of North Sichuan Medical College between November 2013 and December 2014 in patients undergoing surgery. In total, 53 patients with pituitary tumors, including 28 cases of invasive pituitary tumors and 25 non-invasive cases were included. Previous conventional or gamma knife radiotherapy was not performed in any of the patients. The diagnosis of invasive pituitary tumors was based on the Hardy-Wilson and Vezina (21) and Knosp *et al* (22) grading and staging criteria. In total, 5 normal pituitary glands were obtained from autopsy (2 h post mortem) from patients with no history of endocrine abnormality. Their families signed the informed consent. None of these cases had a recognized pituitary disease, and pituitaries exhibited a normal gland appearance. Tissue samples were immediately frozen in liquid nitrogen at the time of surgery and stored at -70˚C until RNA was extracted. A section of the tumor tissue was fixed in 10% formalin, paraffin-embedded and used for immunohistochemistry. Finally, informed consent for the present study was obtained from all patients, and the present study was approved by the Research Ethics Committee of Xiangya Hospital (Xiangya, China).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA was extracted and purified using miRNeasy mini kit (Qiagen, Inc., Valencia, CA, USA) and quantified using a NanoDrop ND-2000 (Nanodrop; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). The OD_{260}/OD_{280} ratio of RNA was between 1.7-2.1, indicating a high purity. The cDNA template was acquired by reverse transcription (RT). RNA was extracted from ~100 mg pituitary gland tissue using the TRI reagent® (Sigma-Aldrich; Merck KGaG, Darmstadt, Germany) following the manufacturer's protocol. RNA was reverse transcribed using AMV Reverse Transcriptase (Promega Corporation, Madison, WI, USA) in a total volume of 20 μl, with 1 μg pituitary RNA, 30 pmol oligo (dT) 15, 4 μl 5xAMV reverse transcriptase buffer, 2 μl deoxynucleotide triphosphate (dNTP) mix, 20 units ribonuclease inhibitor and 15 units of AMV reverse transcriptase. The RT reaction (RNA concentration is 2 μg/ml) was conducted using a Tetrad2 Peltier Thermal Cycler (BioRad Laboratories, Ltd., Hercules, CA, USA) under the following conditions: 16˚C for 30 min, 42˚C for 30 min, 85˚C for 5 min and hold at 4˚C. RT products were stored at -20˚C prior to RT-qPCR. qPCR was performed using 25 μl volumes on 96-well plates with a reaction buffer containing 1xTaqMan Universal PCR Master Mix (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 3 mM $Mn(OAc)_2$, 200 μM dNTPs, 1x25 U AmpliTaq Gold polymerase (#4398823, Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1x25 U AmpErase UNG (#N8080096, Thermo Fisher Scientific, Inc.), 100-200 nmol TaqMan probe (#4316034, Thermo Fisher Scientific, Inc.) and 900 nmol primers (sequences presented in Table I). U6 snRNA served as an endogenous control and was used to normalize the expression levels of miR-200b. The relative expression of miR-200b was calculated using the $2^{-\Delta\Delta Cq}$ method and the experiment was repeated 3 times (23). qPCR was performed using an ABI7500 qPCR instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.) under the following conditions: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec, 60˚C for 1 min and hold at 4˚C. Raw data were analysed with the Relative Quantification Software version 2.2.3 (Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Immunohistochemistry to detect the expression of PKCα. The expression of PKCα protein in the pituitary tumor tissue was detected by the universal PV-9000 two-step method (24,25). Briefly, formalin-fixed paraffin-embedded tissues from 53 pituitary tumors and 5 normal pituitary tissues were cut into 4-μm slices. Deparaffinization, graded ethanol dehydration, antigen retrieval and 3% hydrogen peroxide were used to block endogenous peroxidase. Rabbit anti-human PKCα monoclonal antibody (catalogue no. bs-3531R, 1:200, Bioss, Beijng, China) were added and incubated at 4˚C overnight. Mouse anti-rabbit IgG/horseradish peroxidase (HRP) antibody (catalogue no. bs-0295M-HRP, 1:800, Bioss, Beijng, China) was then applied and incubated for 30 min at room temperature. Staining was performed using diaminobenzidine (DAB) and slices were counterstained using hematoxylin (Sigma-Aldrich; Merck KGaG) before mounting.

The following integral calculation method for PKCα was used: 5 high-power fields of view (magnification, x400) were randomly selected in each slice with 100 cells per field, and the scores were calculated as the product of staining intensity (3, brown; 2, yellow; 1, light yellow and 0, colorless) and the percentage of positive cells (4, >75%; 3, 51-75%; 2, 26-50%; 1, 6-25% and 0, <5%). Evaluation values are represented by a product of two types of scores according to the integral level: Negative (-), 0 points; weakly positive (+), 1-4 points;

Table I. The sequences of primers used for reverse transcription-quantitative polymerase chain reaction.

cDNA	Forward Primer (5'-3')	Reverse Primer (5'-3')
MiR-200b	TAATACTGCCTGGTAATGATGA	ATCATTACCAGGCAGTATAAAT
PKCα	TGCTGACTTTGGGATGTG	TGTTTGTTCTCGCTGGTG
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

PKCα, protein kinase Cα; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

and strongly positive (++), >4 points. The slides were evaluated independently by two pathologists blinded to treatment groups.

Luciferase assay. DNA extraction was performed using the TIANamp Genomic DNA kit (Tiangen Biotech, Co. Ltd., Beijjng, China) according to the manufacturer's instructions. Wild-type and mutant-type PKCα 3'UTR luciferase reporter vectors (Shanghai GenePharma Co., Ltd., Shanghai, China) were constructed. MiR-200b mimics (catalogue no. B01001, Shanghai GenePharma Co., Ltd.) or negative controls (NC, catalogue no. B04001, Shanghai GenePharma Co., Ltd.) were co-transfected with a constructed wild- or mutant-type luciferase reporter vector into mouse pituitary tumor AtT-20 cells (purchased from the Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China), as described in the subsequent section. A pRL-TK control vector (catalogue no. E2241, Promega Corporation) served as a control. Following 48 h of transfection, the activity of luciferase was measured using a Dual-Luciferase Reporter Assay System (E1910; Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Furthermore, the *Renilla* luciferase activity was used for normalization. The cell transfection was conducted using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Inc.).

Cell culture and cell transfection. All cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, Inc.), and incubated at 37˚C in a humidified atmosphere of 5% CO_2. Cultured cells were grown in monolayers and the cell confluency was expected to reach 90% when passaged. The old media were discarded during passaging; PBS was used to wash the cells, 0.25% trypsin digestion was performed and trypsin was neutralized with RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.) upon detachment of the monolayer. Next, cells were suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) to obtain single cell suspensions, and the cells were routinely passaged.

For transfection, the treatment groups were as follows: No transfection (control), NC of miR-200b mimics negative control (NC), miR-200b mimics (mimics), PKCα siRNA (siRNA) and miR-200b mimics + PKCα (mix) groups. The cells were inoculated into a 50-ml culture bottle, and grown in complete medium (RPMI 1640 medium supplemented with 10% FBS; Gibco; Thermo Fisher Scientific, Inc.) to a density of 30-50%. The AtT-20 cells were transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in serum-free RPMI 1640 medium. Transfected cells were incubated in RPMI 1640 medium supplemented with 10% FBS at 37˚C in a humidified atmosphere of 5% CO_2. The RPMI 1640 medium supplemented with 10% FBS was replaced after 6-8 h and the culture was continued.

MTT assay. MTT assays were used to measure cell proliferation. PBS was used for two washes, and transfected cells were cultured to a confluency of 80%. Cells were subjected to trypsin digestion and resuspended into single cell suspensions and counted using a hemocytometer. Transfected AtT-20 cells were seeded in 96-well plates at a density of 3-6x10^3 cells/well and cultured in 200 μl of RPMI 1640 medium supplemented with 10% FBS. The culture was repeated in six wells and incubated at 37˚C in a humidified atmosphere of 5% CO_2. RPMI 1640 medium was added to unseeded cell wells and was determined to represent '0'. The cells were examined following incubation for 24, 48 and 72 h. A volume of 20 μl MTT (5 μg/ml; Sigma-Aldrich; Merck KGaA) was added to each well and the cells were incubated at 37˚C in a humidified atmosphere of 5% CO_2 for 4 h. The cell culture was terminated by replacing the medium with new RPMI 1640 medium. Dimethyl sulfoxide (150 μl) was added to each well and shaken for 10 min in order to dissolve the formazan crystals. The absorbance was then measured using a microplate reader (SpectraMAX Plus, Molecular Devices, LLC, Sunnyvale, CA, USA) at a wavelength of 490 nm. An MTT curve was drawn using SPSS 19.0 software (IBM SPSS, Armonk, NY, USA) with an absorbance value set to the vertical axis and the interval was set to abscissa. This experiment was repeated three times.

Transwell cell invasion assay. Matrigel (BD Biosciences, San Jose, CA, USA) was diluted in serum-free RPMI 1640 medium into a 1:3 ratio, and this was followed by an overnight incubation at 4˚C. Diluted Matrigel was added to each Transwell chamber (BD Biosciences, Franklin Lakes, NJ, USA), ensuring appropriate spreading of Matrigel. At 48 h after transfection, cells were plated in Transwell chambers and 0.5 ml RPMI 1640 medium supplemented with 10% fetal bovine serum was added in the lower chamber as a chemoattractant. The membrane was then stained using 0.1% crystal violate staining solution. The number of cells traversing the Matrigel in each group was used as an evaluation index of their invasive ability. This experiment was repeated 3 times.

Flow cytometric analysis of apoptosis. The apoptosis rate of AtT-20 cells was measured via flow cytometric analysis using an Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) apoptosis detection kit (BD Biosciences). Briefly, 48 h after transfection, cells were harvested and were washed twice in cold PBS. The cells were resuspended in binding buffer at a concentration of 0.5-1x10^6 cells/ml. This suspension (100 μl) was then incubated with 5 μl of AnnexinV-FITC and PI for 15 min at room temperature and in the dark. Following the addition of 400 μl binding buffer to each tube, the cells were analyzed with a FC 500 MCL/MPL flow cytometer (Beckman Coulter, Inc., Brea, CA, USA). This experiment was repeated 3 times.

qPCR to detect the expression of PKCα mRNA. The total RNA of cells was extracted using an RNA isolation reagent (TRIzol, catalogue no. 15596026, Ambion®, Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, and was reverse transcribed using the RevertAid First Strand cDNA Synthesis kit (catalogue no. K1622, Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA). In a total volume of 20 μl, with 1 μg PKCα mRNA, 30 pmol oligo (dT) 15, 4 μl 5x reaction buffer, 2 μl deoxynucleotide triphosphate (dNTP) mix, 20 units RiboLock RNase inhibitor and 15 units of RevertAid reverse transcriptase. The RT reaction was conducted using a Tetrad2 Peltier Thermal Cycler (BioRad Laboratories) under the following conditions: 16˚C for 30 min, 42˚C for 30 min, 85˚C for 5 min and hold at 4˚C. The concentration of the RNA sample is 2 μg/μl. RT products were stored at -20˚C prior to RT-qPCR. qPCR was performed according to the manufacturer's instructions of Maxima SYBR Green/Fluorescein qPCR Master Mix (2X) kit (#K0242, Fermentas; Thermo Fisher Scientific, Inc.). The following primers were used for amplification of PKCα: sense, 5'-TGC TGACTTTGGGATGTG-3' and antisense, 5'-TGTTTGTTC TCGCTGGTG-3' (Invitrogen; Thermo Fisher Scientific, Inc.). qPCR was carried out in 20 μl volumes on 96-well plates with a reaction buffer containing 1x Maxima SYBR Green/Fluorescein qPCR Master Mix (2X; #K0242, Thermo Fisher Scientific, Inc.), 200 mM dNTPs, 1x25 U Maxima Hot Start Taq DNA Polymerase (#EP0601, Thermo Fisher Scientific, Inc.), 2-5 mM $MgCl_2$, 100-200 nmol SYBR Green I (#S7563, Thermo Fisher Scientific, Inc.) and 0.5 μM primers (sequences presented in Table I). This reaction included a 30 sec denaturation, followed by 40 cycles of 95˚C for 15 sec, 55˚C annealing for 5 sec and 72˚C extension for 5 sec. U6 snRNA served as an endogenous control for normalization of the expression levels of PKCα. The relative expression quantity of PKCα was calculated using the $2^{-\Delta\Delta Cq}$ method (23). This experiment was repeated three times.

Western blot analysis to detect PKCα protein expression. Cellular proteins were extracted 48 h after transfection using the RIPA Lysis Buffer (#P0013C, Beyotime, Shanghai, China), and protein concentrations were determined with a bicinchoninic acid (BCA) kit (catalogue no. P0011, Beyotime Institute of Biotechnology, Shanghai, China). The cellular protein extraction was completed as follows: RPMI 1640 culture medium supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc.) was carefully discarded and the cells were washed twice with cold PBS. Subsequently 1 ml cold RIPA buffer was added to each flask (75 m^2), and the flask was incubated on ice for 5 min. The lysate was then gathered using a cell scraper, collected and transferred to a centrifuge tube. The lysate was then centrifuged at 14,000 x g at 4˚C for 15 min to pellet the cell debris. The supernatant was then transferred to a new tube. Equal amounts of proteins from each group were loaded and separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene fluoride (PVDF) membranes and blocked with 5% non-fat milk. Membranes were incubated with antibodies against PKCα (catalogue no. ab32376, 1:2,000, Sigma-Aldrich; Merck KGaG) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; catalogue no. ab181602, 1:10,000, Sigma-Aldrich; Merck KGaG) at 4˚C overnight. Membranes were then washed three times with Tris-buffered saline and Tween-20 (TBST; 10 min each) and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Membranes were then washed with TBST three times (10 min each) and signal detection was performed using Super ECL Plus Detection Reagent (Applygen Technologies Inc., Beijing, China). This experiment was performed three times.

Statistical analysis. All experiments were repeated at least three times and all statistical analyses were performed using SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). The statistical analyses for the significance of differences in numerical data (mean ± standard deviation) were performed by analysis of variance. Differences between the two groups were analyzed by unpaired t-tests. The results of immunohistochemistry for the PKCα protein were analyzed by the rank-sum test. The association between the expression levels of miR-200b and PKCα protein was analyzed by Spearman rank correlation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

MiR-200b and PKCα protein expression in pituitary tumor clinical specimens. qPCR was used to evaluate the expression level of miR-200b in 28 cases of invasive pituitary tumors, 25 cases of non-invasive and 5 normal pituitaries (Fig. 1A). The results demonstrated that miR-200b expression in invasive pituitary tumor tissues was significantly lower than in non-invasive and normal tissues ($P<0.01$). However, there was no significant difference in miR-200b expression levels between non-invasive and normal tissues ($P>0.05$). In the normal pituitary and non-invasive pituitary tumors, PKCα demonstrated only mild expression in the cell cytoplasm (Fig. 1B); while in the invasive pituitary tumors, PKCα was mainly located in the cell cytoplasm and extracellular matrix. Expression of PKCα was distributed in an uneven manner (Fig. 1B). Expression of PKCα protein in invasive pituitary tumor tissues was significantly higher than in normal and non-invasive tissues ($P<0.01$; Table II). Spearman correlation analysis indicated a negative correlation between the miR-200b level with PKCα protein levels in invasive pituitary tumor tissues ($r=-0.436$; $P=0.021$), but there was no significant

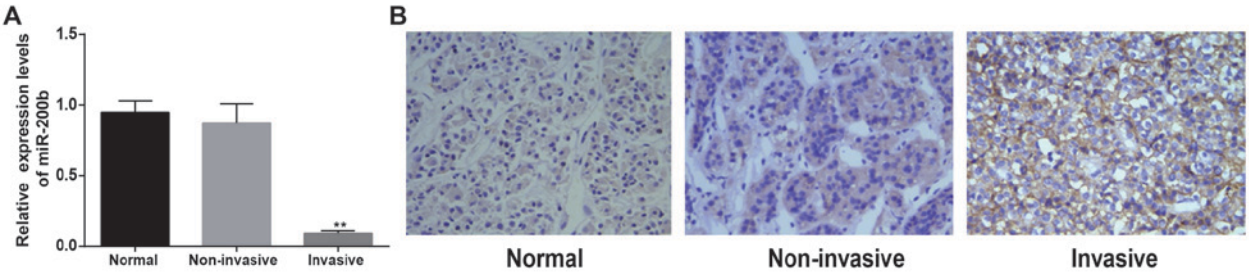

Figure 1. Relative expression of miR-200b and PKCα distribution in pituitaries. (A) Relative expression of miR-200b in invasion pituitaries were significantly lower than that in normal and non-invasive pituitaries. Data were presented as mean ± standard deviation, **P<0.01 vs. normal. (B) Immunohistochemical staining results demonstrating PKCα expression in normal, non-invasive and invasive pituitaries. The brown color indicates the positive staining of PKCα. PKCα expression is significantly overexpressed in invasive pituitaries, and there is no significant difference of PKCα expression between the normal and the non-invasive pituitaries. Staining was performed using diaminobenzidine and slices were counterstained using hematoxylin. Magnification x400. miR, micro RNA; PKCα: protein kinase Cα.

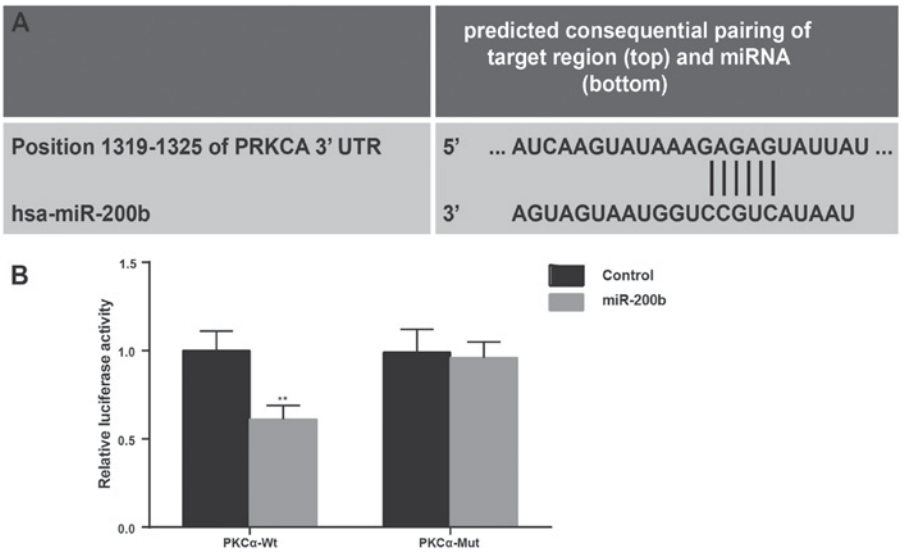

Figure 2. PKCα and miR-200b sequence scheme along with the dual luciferase reporer gene assay results. (A) Base pairing between the seed sequence of mature miR-200b and the 3'UTR of PKCα mRNA. (B) Relative luciferase activities of cells in PKCα-Wt and PKCα-Mut 3'UTR groups in response to miR-200b transfection. Cells transfected with PKCα-Wt and miR-200b mimics demonstrated substantially decreased luciferase activity than those transfected with PKCα-Wt and control. **P<0.01 vs. control. miR, micro RNA; UTR, untranslated region; PKCα, protein kinase Cα; Wt, wild type; Mut, mutated type.

Table II. Expression level of PKCα protein in pituitary tissues.

Group	No.	PKCα -	PKCα +	PKCα ++	χ^2	P-value
Control	5	4	1	0	-	-
Non-invasive	25	6	7	12	6.42	<0.01[a]
Invasive	28	0	2	26	27.81	<0.01[a]

PKCα, protein kinase Cα. [a]P<0.01 vs. control.

correlation between miR-200b and PKCα in normal and non-invasive tissues.

Targeting PKCα by miR-200b. A putative conserved binding site for miR-200b at nucleotide position 1319-1325 of human PKCα 3'UTR was predicted using Targetscan (http://www.targetscan.org/vert_71/) and matching base pairing was observed between the seed sequence of mature miR-200b and the 3'UTR of PKCα mRNA (Fig. 2A). Dual luciferase reporter gene assays revealed that re-expressing miR-200b in AtT-20 cells significantly inhibited the luciferase activity of PKCα wild-type 3'UTR following by a downregulation of 39% (P<0.01). However, no significant effect on the PKCα mutant-type 3'UTR luciferase activity was observed (Fig. 2B). Collectively, these observations indicate that PKCα is a target of miR-200b.

Pituitary tumor cell proliferation was detected by the MTT assay. MTT assays were used to assess cell proliferation. As shown in Fig. 3, MTT assays demonstrated decreased proliferation of AtT-20 cells transfected with miR-200b mimics and PKCα siRNA for 48 h compared with the control and NC groups (P<0.05), and the difference is more evident with the extension of time. The number of control and NC group cells were not significantly different at any time point

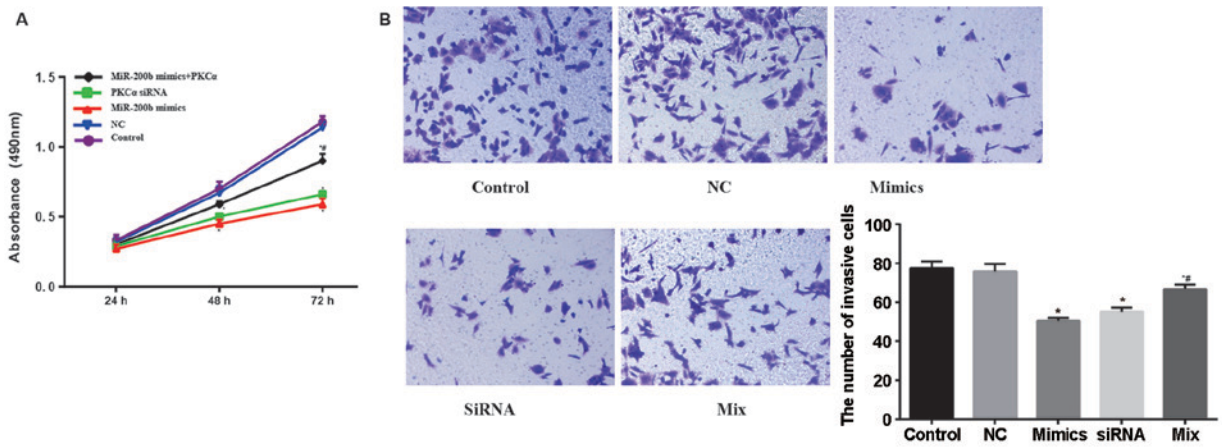

Figure 3. Proliferation rate and invasiveness of AtT-20 cells transfected in different groups. (A) No significant difference was observed between the control and NC groups in terms of cell proliferation rate. There was significant difference of the mimics and siRNA groups in terms of AtT-20 cell proliferation rate from the control and NC groups. Compared with the control and NC groups, the cell proliferation rate of the mix group was also significantly elevated. (B) Transfection of miR-200b mimics or PKCα siRNA led to the decrease of cell invasion, whereas the simultaneous transfection of miR-200b mimics and PKCα-Wt led to significant alteration of cell invasiveness compared with the control. The membrane was then stained using 0.1% crystal violet. Magnification x10. *P<0.05, vs. control and NC groups; #P<0.05, vs. mimics group. Data are presented as mean ± standard deviation. NC, negative control; siRNA, short interfering RNA; miR, micro RNA; PKCα, protein kinase Cα; Wt, wild type; Mut, mutated type.

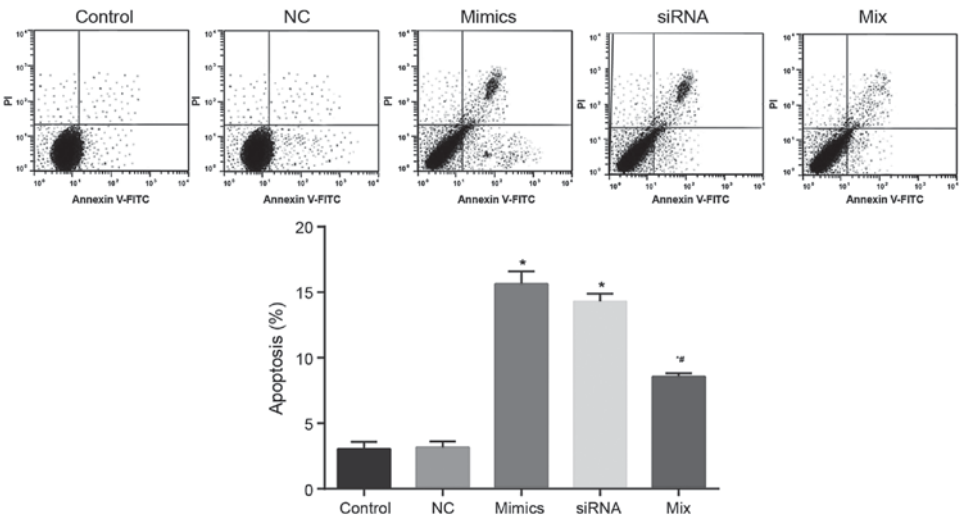

Figure 4. Cell apoptosis following 48 h transfection. The transfection of miR-200b mimics or PKCα siRNA led to the increase of cell death, whereas the simultaneous transfection of miR-200b mimics and PKCα led to significant alteration of cell apoptosis compared with the control, NC, mimics or siRNA group. *P<0.05, vs. control and NC groups; #P<0.05, vs. mimics group. Data are presented as mean ± standard deviation. PKCα, protein kinase Cα; miR, micro RNA; siRNA, short interfering RNA; NC, negative control; FITC, fluorescein isothiocyanate.

recorded (P>0.05). AtT-20 cell proliferation activity decreased following the transfection of miR-200b mimics, but the addition of PKCα appeared to partially reverse the inhibitory effect of miR-200b on AtT-20 cell proliferation (P<0.05).

Transwell assay. As shown in Fig. 3, a Transwell Matrigel invasion assay demonstrated that the ability of miR-200b to inhibit cell invasion is non-significantly better than PKCα siRNA, based on number of cells invading in miR-200b mimics (50.5±1.5) and PKCα siRNA (55.3±2.0) treatment groups (P>0.05). However, a significant difference was observed when comparing these groups to the control (77.5±3.5) and NC (75.8±3.8) groups (P<0.05). Furthermore, there was no significant difference between the control and NC (P>0.05) groups. The number of cells invading the membrane in the mix group was 66.6±2.5, which is significantly different from any other group (P<0.05). These results indicate that upregulation of miR-200b or downregulation of PKCα can inhibit the invasive ability of AtT-20 cells and PKCα can impair the inhibitory effect of miR-200b on AtT-20 cell invasion.

Apoptosis assay. Cell apoptosis 48 h after transfection is reported in Fig. 4. Flow cytometry analysis demonstrated that the apoptosis rate of cells in mimics and siRNA groups were 15.63±0.96 and 14.29±0.61, respectively, and no significant difference was observed between these two groups (P>0.05). However, the apoptosis rate of these two groups was significantly higher compared with the control (3.03±0.54) and NC (3.15±0.46) groups (P<0.05). The apoptosis rate of the mix group was 8.56±0.26, which was significantly different

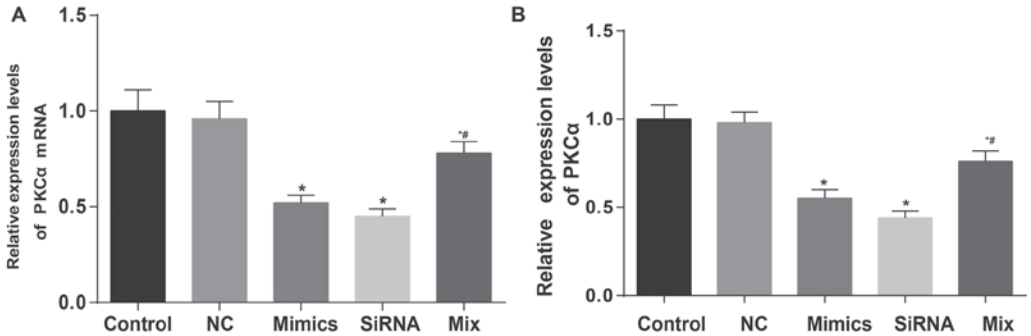

Figure 5. Relative expression level of *PKCα* mRNA and protein. (A) MiR-200b mimics and PKCα siRNA significantly downregulated the expression of PKCα mRNA, whereas the simultaneous transfection of miR-200b mimics and PKCα siRNA led to insignificant alteration of PKCα mRNA expression compared with the mimics or the siRNA group. (B) MiR-200b mimics and PKCα siRNA significantly downregulated the expression of PKCα protein, whereas the simultaneous transfection of miR-200b mimics and PKCα led to significant alteration of PKCα expression compared with the other groups. *P<0.05, vs. control and NC groups; #P<0.05, vs. mimics group. Data were presented as mean ± standard deviation. PKCα, protein kinase Cα; miR, micro RNA; siRNA, short interfering RNA; NC, negative control.

from any other groups (P<0.05). These observations indicate that the upregulation of miR-200b or the downregulation of PKCα can induce the apoptosis of AtT-20 cells and PKCα can partially reverse the effect of miR-200b on AtT-20 cell apoptosis.

MiR-200b and PKCα protein expression in pituitary tumor cell line. qPCR and western blotting analysis results revealed that the expression of PKCα mRNA and protein was significantly decreased 48 h after transfection with the siRNA and mimics compared with the control and NC groups (P<0.05; Fig. 5). However, there was no significant difference between the control and NC groups (P>0.05). Transfection in the mix group with PKCα could partially recover the expression of PKCα mRNA and protein in AtT-20 cells compared to the miR-200b mimics group. Therefore, miR-200b down-regulated the expression level of PKCα and PKCα siRNA reduced endogenous expression of the PKCα gene. These observations indicated that miR-200b could target the regulation of PKCα gene expression in AtT-20 cells.

Discussion

MiRNAs are involved in diverse biological processes, including cellular differentiation, proliferation and apoptosis (26,27). These small non-coding RNAs function by regulating the target gene expression and influencing the process of translation (28). Furthermore, miRNAs have been suggested to be important in various types of cancer (28). Although the function of multiple miRNAs remains unknown, the miRNAs have demonstrated varying effects in the etiology of cancer (29). A number of miRNAs act as tumor suppressor factors whereas others work in tumorigenesis (30). Among the miRNAs, the miR-200 family is proven to be directly associated with tumor metastasis and invasiveness (30-32). miR-200b, which is one of the members of the miR-200 family, has also been reported to have abnormal expression in various types of cancer, including bladder cancer and gastric carcinoma (33,34). However, the association between miR-200b and pituitary tumors remains unclear. In the present study, the expression of miR-200b in pituitary tumors was analyzed. The results revealed that the downregulation of miR-200b in invasive pituitary tumors was comparable to the normal pituitary gland and non-invasive pituitary tumors, indicating that miR-200b was of note in pituitary tumors and is correlated with their invasiveness. In addition, the correlation analysis implied that miR-200b was negatively correlated with the expression of PKCα, which has a relatively high expression in invasive pituitary tumors. The luciferase reporter assay demonstrated that miR-200b directly targets the PKCα gene by interacting with the 3'UTR in PKCα mRNA. Furthermore, through *in vitro* assays, transfection with PKCα siRNA and miR-200b mimics was shown to be capable of causing reduced invasiveness and proliferation in AtT-20 cells and promoting cell apoptosis. Furthermore, the low expression of miR-200b contributes to an increasing PKCα expression in pituitary tumors. Finally, overexpression of miR-200b in AtT-20 cells downregulated mRNA and protein expression of PKCα, and exogenous PKCα could reverse this inhibitory effect in AtT-20 cells.

PKCα belongs to the protein kinase C family and has been indicated to be involved in tumorigenesis, invasion, metastasis and proliferation (35-37). Previous studies of breast cancer indicated that PKCα could serve as an effective therapeutic target due to its notable mediator role in cancer (38-40). Furthermore, upregulation of PKCα results in cancer cell invasion and proliferation (36,41,42). Therefore, miR-200b interacts with the 3'UTR of PKCα mRNA, affecting the degradation of PKCα mRNA, hindering translation and thus inhibiting pituitary tumor invasion and growth. Notably, the observations of the present study demonstrated that although overexpression of miR-200b and inhibition of PKCα was able to inhibit pituitary tumor cell invasion and growth without a statistically significant difference (P>0.05), the effect of overexpressing miR-200b was slightly higher than that of inhibiting PKCα. However, miR-200b may also target other genes to regulate pituitary tumor growth and metastasis, based on the finding that one miRNA could target multiple target mRNAs (43,26). Additional studies on the details of PKCα function in pituitary tumors are required, including studies on which signaling pathway is involved in promotion of tumor proliferation and invasion. Lastly, miR-200b is an key factor in various cell processes, working via various signaling pathways, including

the cJun/mitogen-activated protein kinase, p27/kip1 and Rho family GTPase 3 pathways (44,45). It is therefore a potentially fruitful area of research to investigate how miR-200b affects in pituitary tumors.

In conclusion, the expression of miR-200b demonstrated downregulation in pituitary tumors and a negative correlation with PKCα expression. In addition, miR-200b could inhibit pituitary tumor proliferation and invasion and promote tumor cell apoptosis by targeting PKCα. PKCα and miR-200b may therefore serve as effective therapeutic targets for pituitary tumor treatment in the future.

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