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Original Article

The effects of miR-34a on the apoptosis, proliferation, and invasion of pituitary tumor cells and on the regulation of the nerve growth factor and prolactin

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Abstract: Objective: This study aimed to investigate the effects of miR-34a on the apoptosis, proliferation, and invasion of pituitary tumor cells and its regulatory mechanism on the expressions of nerve growth factor (NGF) and prolactin (PRL). Methods: Pituitary tumor cell lines were divided into three groups: the miR-34a group, the pituitary tumor group (the PT group), and the idling group (the ID group). Cell proliferation was measured using CCK-8 assays, and cell invasion was analyzed using Transwell assays. Results: Compared with the ID and PT groups, the expression of miR-34a and the apoptotic rate were increased, the content of the NGF and PRL proteins were decreased, and proliferation and invasion were inhibited in the miR-34a group (all $P<0.05$). Conclusion: The overexpression of miR-34a inhibits the activity of NGF and PRL, promotes apoptosis, and inhibits the proliferation and invasion of tumor cells.

Keywords: Nerve growth factor, miR-34a, pituitary tumors, apoptosis, prolactin

Introduction

Pituitary tumors are clinically treated by eliminating cancer cells through surgery, drugs, radiation, and other methods. Drugs and radiation can promote apoptosis in pituitary tumor cells and, thereby, reduce cancer cell proliferation and invasion [1, 2]. Studies have shown that the expression of nerve growth factor (NGF) and prolactin (PRL) in the body correlates with apoptosis in pituitary tumor cells [3, 4]. A previous study showed that abnormal expressions of NGF and PRL have been detected in patients with breast cancer, and that these proteins are also involved in the development of tumor cells [5]. miRNA exhibits a myriad of biological characteristics and endogenous regulatory functions. It regulates the expression of about half of the total protein genes in humans by impeding the mRNA transcription of target genes while promoting the degradation of mRNA [6]. miR-34a, is a regulator of the development of various cancers and may regulate the growth and metastasis of cancer cells [7-9]. miRNA-34a can participate in the regulation of the growth processes of a variety of cancer cells [10, 11]. Therefore, it was hypothesized that miR-34a may regulate the apoptosis and proliferation of pituitary tumor cells by regulating NGF and PRL. Therefore, in the present study we have analyzed the mRNA expression of miR-34a and the apoptosis, proliferation, and invasiveness, along with the NGF and PRL protein expressions, in pituitary tumor cells.

Materials and methods

General data

Pituitary tumor cell lines were purchased from ATCC and randomly divided into three groups. (a) The miR-34a group: Pituitary tumor cells transfected with miR-34a mimics. (b) The pituitary tumor group (the PT group): Pituitary tumor cell lines cultured without any treatment. (c) The idling group (the ID group): Pituitary tumor cells transfected with an miR-34a nega-

Table 1. Primer sequences

Protein	Gene	Primer sequence
miR-34a	F	5'-TGCGGTAGCAGCACATAATGG-3'
	R	5'-CCAGTGCAGGGTCCGAGGT-3'
U6	F	5'-CTCGCTTCGGCAGCACA-3'
	R	5'-AACGCTTCACGAATTTGCGT-3'

tive control. Meanwhile, we also purchased healthy pituitary cells from ATCC to evaluate the differences in the expressions of miR-34a between normal pituitary cells and pituitary tumor cells.

Laboratory instruments and reagents

The miR-34a mimics and the negative control were purchased from RIBOBIO, Guangzhou Co., Ltd., China. The fetal bovine serum (FBS) and the DMEM medium were purchased from KeyGEN BioTECH, Nanjing Co., Ltd., China. The fluorescent quantitative PCR kits and the transfection reagent Lipofectamine were purchased from Invitrogen, USA. The TUNEL Apoptosis Detection Kits were purchased from the Yeasen Biotechnology Shanghai Co., Ltd., China. The Cell Invasion Analysis Kits were purchased from AmyJet Scientific, Wuhan Co., Ltd., China, and the Trypsin was purchased from Life Technologies, USA. A Constant Temperature Shaker (Sujing Equipment Chengdu Co., Ltd., China) was used for the shaking process.

Cell culture

Pituitary tumor cell lines were cultured in DMEM medium containing FBS. Until reaching an 80%-90% confluence of the cells, the pituitary tumor cells were digested by trypsin, centrifuged at 1,600×g for 5 min, and then collected, suspended, and cultured.

Cell transfection

The pituitary tumor cells were diluted to 2*10^5/mL and seeded to a 16-well plate. When the confluence of pituitary tumor cells reached 60%-80%, miRNA-34a mimics of 30 pmol and a negative control of 30 pmol were prepared into a compound, respectively. The compound was transfected into pituitary tumor cells according to the Lipofectamine 2000 kit manufacturer's instructions. After 24 h of transfection, the supernatant was replaced by fresh medium, and the cells were cultured for another 48 h. The transfections of the miRNA-34a mimics and the negative controls were analyzed using qRT-PCR.

Quantification of the miRNA-34a expressions in the pituitary tumor cells and normal pituitary cells using qRT-PCR

Chloroform (Huaxu Chemical Co., Ltd., Hebei, China) was added to the culture medium of the pituitary tumor cell lines and the normal pituitary cells. The shaken solution was milky white, and then was centrifuged at 4°C and 1,200×g for 5 min. Then isopropanol was added to the solution and 75% ethanol successively added and followed by centrifugation. Finally, the precipitate was dried and stored at -80°C. Total RNA was extracted and reverse transcribed into cDNA according to the manufacturer's instructions with U6 as an internal reference. The protocol was pre-denaturation at 60°C for 10 min, denaturation at 95°C for 30 s, annealing at 72°C for 30 s, and elongation at 95°C for 5 min, for 40 cycles. The experiments were conducted in at least triplicate. The miRNA expressions were calculated using relative quantification $2^{-\Delta\Delta CT}$. The primer sequences are detailed in **Table 1**.

Quantification of the apoptosis in pituitary tumor cells using flow cytometry

The apoptosis was measured using Annexin-V-FITC kits. The ratio of 100 μL dye solution was done according to the Annexin-V-FITC, PI, and HEPES buffer solutions ratio of 1:2:50, respectively. 1*10^6 cells were selected, vibrated and quiesced for 15 min. 1 mL HEPES buffer solution was additionally mixed by concussion. FITC and PI fluorescence were determined at 488 nm to observe the apoptosis of the spinal cord cells.

Quantification of the pituitary tumor cell proliferation using CCK-8 assays

The transfected cells were diluted to 1*10^5/mL and cultured for 12 h. The cultured pituitary tumor cells were again seeded into a 24-well plate and cultured. After 10 μL of CCK-8 was added to the each well, the optical density (OD) was measured using a reader at 450 nm.

Quantification of the NGF and PRL protein contents in the pituitary tumor cells using Western blot

A total of 50 μg of pituitary tumor cells were collected through the routine method. Polyacrylamide gel electrophoresis was performed using a 12% separating gel. Post-electrophoresis, the gel was removed, and membrane transfer was carried out on a PVDF membrane. The membrane was washed with TBST for 10 min and blocked using a blocking buffer in a horizontal shaker for 2 h. The PVDF membrane was then packed into a self-made hybridization bag with a primary antibody (Abcam Company, UK) and incubated overnight at 4°C (primary antibody diluted with hybridization solution, GAPDH: 1:1,000). After incubation overnight, the PVDF membrane was washed three times with 1×TBST (10 min/wash). After washing, the membrane was incubated with the corresponding secondary antibody solution (KPL, USA) at room temperature for 1.5 h, with gentle shaking. After incubation, the membrane was washed three times with TBST (10 min/wash). Some working solution mixed with the electrochemiluminescence color developing solution A and B (Xitang Biotechnology Co., Ltd., Shanghai, China) at a 1:1 ratio, was added to the PVDF membrane. About one minute later, the PVDF membrane was analyzed using an automated chemiluminescence imaging analysis.

Quantification of the cancer cell invasion using Transwell assays

The transfected cells were diluted to $1*10^5$/mL and added to the Transwell chamber containing 500 μL culture medium containing 10% FBS while the upper chamber was covered with an extracellular matrix gel. After incubation for 4 h, the liquid was sucked out. A total of 200 μL cultured cells were inoculated in the migration chamber. After incubation for 24 h, the cells were collected, fixed, and stained with crystal violet solution. The cellular invasion was observed under a microscope (×100).

Statistical analysis

The results were analyzed using SPSS 22.0 software. The measurement data were expressed as the mean ± standard deviation ($\bar{x}$ ± sd). The count data were expressed as n (%). The apoptosis, proliferation, invasion, and expression of NGF and PRL in the pituitary tumor cells in the miR-34a group, PT group, and ID group were analyzed using one-way ANOVA. Pairwise comparisons between groups were performed using LSD, t, or Bonferroni tests. A difference was considered significant when $P<0.05$.

Results

Quantification of the miRNA-34a expressions in the pituitary tumor cells and in the normal pituitary cells using qRT-PCR

The qRT-PCR results showed that the expression of miRNA-34a in the normal pituitary cells was significantly higher compared to its expression in the pituitary tumor cells ($P<0.05$). In the pituitary tumor cells, the expression of miRNA-34a was lowest in the PT group and highest in the miRNA-34a group. Compared with the ID and PT groups, the miRNA-34a expression in the miRNA-34a group was significantly higher, indicating that the transfection was successful (both $P<0.05$). The results are shown in **Figure 1**.

Comparison of the apoptosis in the three groups of pituitary tumor cells

The TUNEL assay results showed that the apoptosis rates of the pituitary tumor cells in the PT group, the ID group, and miR-34a group were 0.23±0.16, 0.29±0.12, and 0.67±0.23, respectively. The apoptotic number of pituitary tumor cells was the lowest in the PT group and highest in miR-34a group. Compared with the PT and ID groups, the apoptotic rate in the pituitary tumor cells in the miR-34a group was significantly higher (both $P<0.05$). There was no significant difference in the apoptotic rate between the PT and ID groups ($P>0.05$). The results are shown in **Figure 2**.

Measurement of the pituitary tumor cell proliferation using CCK-8 assays

The CCK-8 assays showed that at 24 h-60 h, the cell proliferation in the ID and PT groups was significantly higher than it was in the miR-34a group (both $P<0.05$), and it was highest in the PT group. There was no significant difference in the proliferation of pituitary tumor cells

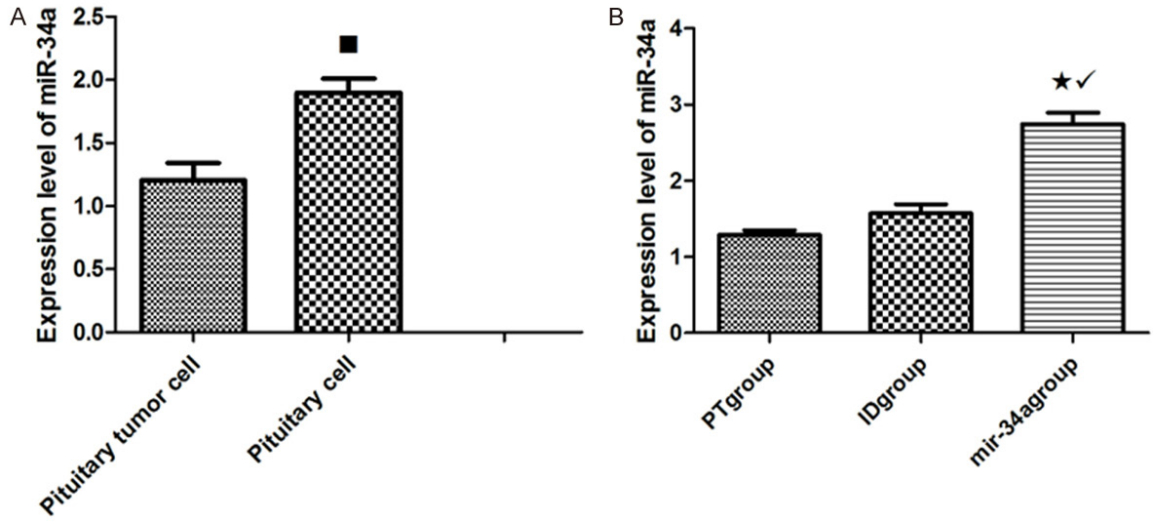

Figure 1. The expression of miR-34a mRNA. A: The expressions of miR-34a in normal pituitary cells and in pituitary tumor cells. B: The expressions of miR-34a in the pituitary tumor cells of the three groups. Compared with the PT group, ★P<0.05; compared with the ID group, √P<0.05; compared with the pituitary adenoma cells, ▪P<0.05.

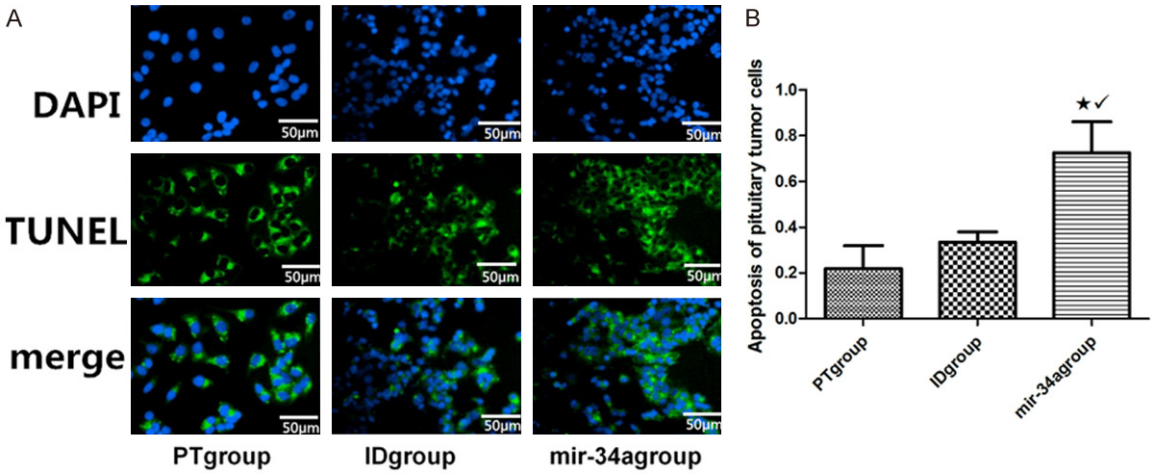

Figure 2. Apoptosis of the pituitary tumor cells. A: The apoptosis of the pituitary tumor cells in the three groups. B: The apoptosis rate of the pituitary tumor cells in the three groups. Compared with the PT group, ★P<0.05; compared with the ID group, √P<0.05. PT: pituitary tumor; ID: idling.

between the ID and PT groups (P>0.05). The results are shown in **Figure 3**.

Determination of the NGF and PRL protein contents in pituitary tumor cells using Western blot

Western blot was used to determine the NGF and PRL protein expressions in the pituitary tumor cells of the miR-34a, PT, and ID groups. According to gray-scale analysis, the miR-34a group showed the lowest protein content of NGF and PRL compared to the PT and ID groups (both P<0.05), and the PT group showed the highest content. The results are shown in **Figure 4**.

Comparison of three groups in pituitary tumor cell invasiveness

The transwell assay results showed that the invasiveness of the pituitary tumor cells in the PT group was strong, but in the miR-34a group it was weak. Compared with miR-34a group, the more invasive pituitary tumor cells in the ID and PT groups indicated the significantly

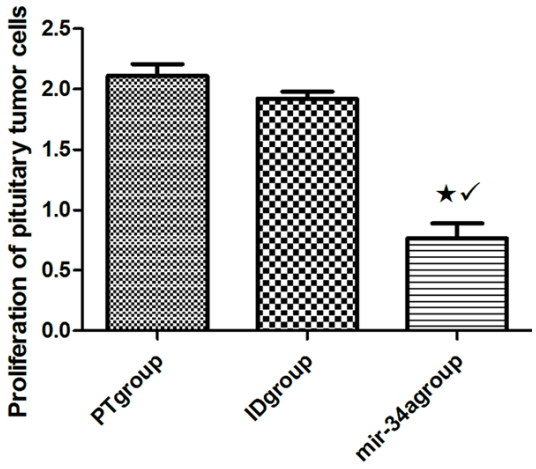

Figure 3. Proliferation of the pituitary tumor cells in the three groups. Compared with the PT group, ★P<0.05; compared with the ID group, √P<0.05. PT: pituitary tumor; ID: idling.

enhanced overall invasiveness (P<0.05). The results are shown in **Figure 5**.

Discussion

The results from the present study showed that the expression of miR-34a in the miR-34a group was higher than it was in the PT and the ID groups. Compared to the PT and the ID groups, the total number of apoptotic pituitary tumor cells in the miR-34a group was significantly higher, but the cell proliferation and invasiveness were significantly lower. Li et al. reported that miR-34a can inhibit the invasion and migration of tumor cells by targeting Bcl-2 and SIRT1 [12]. What's more, in gastric cancer cell lines, the transient transfection of miR-34a can inhibit the migration and invasion of gastric cancer cells, in addition to the proliferation of cancer cells [13]. These results suggest that miR-34a is an inhibitory factor for tumor cell migration, invasion, and proliferation, which is consistent with previous studies on other malignant tumors such as breast and colorectal cancer [14]. In recent years, the importance of miR-34a in the overall biological behavior of cancer cells has gradually emerged, especially in terms of metabolism, metastasis, etc. [15]. The expression of miR-34a in hepatocellular carcinoma cell lines with strong migration and invasiveness is significantly lower compared to normal hepatocellular carcinoma cell lines, suggesting a negative correlation [16]. A previous study found that the expression of miR-34a is low in many types of cancer cells, a finding similar to the results observed in the present study. By increasing the expression of miR-34a in pituitary tumor cells, the apoptosis of these cells can be increased and the proliferation and invasiveness can be reduced [17].

The expressions of the NGF and PRL proteins in the pituitary tumor cells of the miR-34a group were significantly lower compared to the pituitary tumor and idling groups. A large number of studies have confirmed the expression imbalance in the epidermal growth factor receptor in most tumors [18]. The occurrence and enlargement of cholangiocarcinoma is correlated with the abnormal expressions of NGF and PRL, especially in bile duct cancer cells [19]. Also, blocking the expression of NGF in pancreatic cancer cells can inhibit the proliferation and invasiveness of pancreatic cancer cells, and significantly reduce the expression of PRL, indicating that the growth of pancreatic cancer cells is positively correlated with NGF and PRL expressions [20, 21]. Zhang et al. found that there exists an abnormal expression of nerve growth factor receptors in most functional adenomas, indicating that tumor proliferation involves an autocrine stimulation of the epidermal growth factor [22]. What's more, in the post-transfection with miR-34a in the adenoid cystic cancer cells of the salivary glands, the expressions of NGF and PRL are reduced, which means that miR-34a regulates apoptosis in cancer cells by affecting the expressions of NGF and PRL [23]. All these previous observations are consistent with our experimental results.

However, this study also has some shortcomings. Cell migration quantification was not conducted due to time limitations. More experiments need to be carried out to determine the mechanism of miR-34a's role on pituitary tumor cells and on the expressions of NGF and PRL.

In conclusion, the overexpression of miR-34a can inhibit the expression of the NGF and PRL proteins, promote apoptosis, and inhibit the proliferation and invasiveness of tumor cells.

Disclosure of conflict of interest

None.

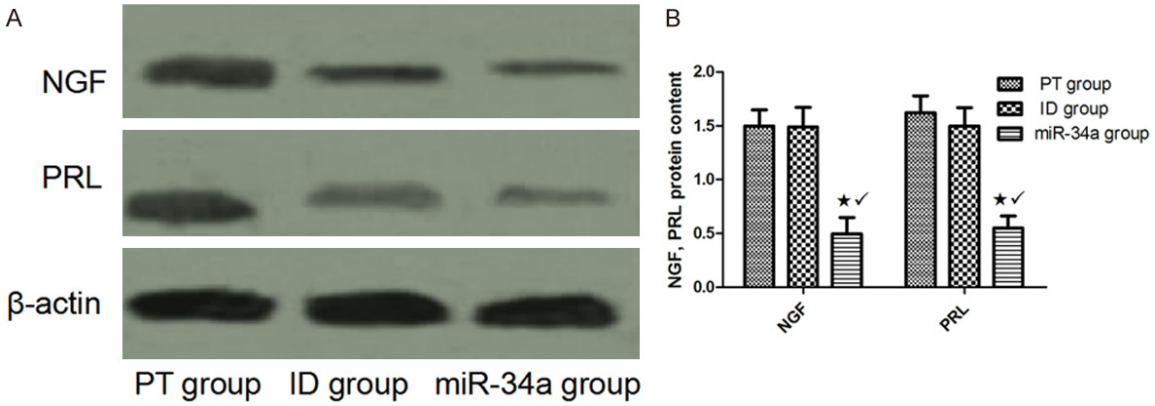

Figure 4. The expressions of NGF and PRL in the pituitary tumor cells of three groups. A: The expressions of NGF and PRL in the pituitary adenoma cells of the three groups using Western blot; B: The expressions of NGF and PRL in the pituitary adenoma cells of three groups. Compared with the PT group, ★P<0.05; compared with the ID group, √P<0.05. PT: pituitary tumor; ID: idling; NGF: nerve growth factor; PRL: prolactin.

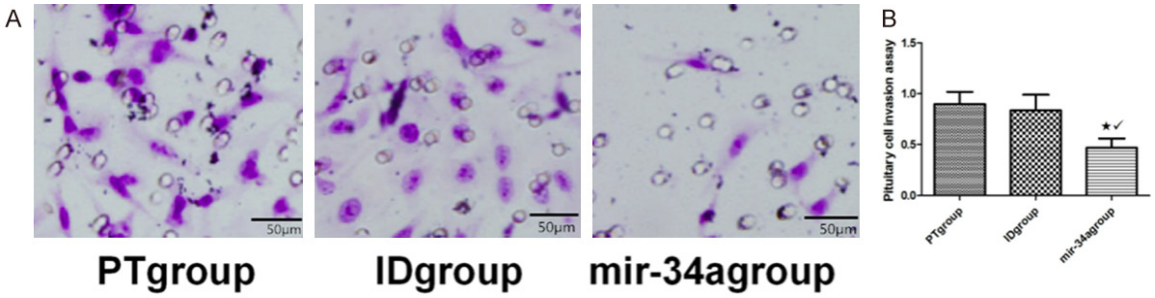

Figure 5. The invasion of the pituitary tumor cells in the three groups (×200). A: The invasion of the pituitary adenoma cells in the three groups; B: The invasion rate of the pituitary adenoma cells in the three groups. Compared with the PT group, ★P<0.05; compared with the ID group, √P<0.05. PT: pituitary tumor; ID: idling.

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