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Original Research Article

MicroRNA-197-3p induces apoptosis in human prolactinoma cells via ERK/EGR1 signaling pathway

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Abstract

Purpose: *To determine the effect and therapeutic implications of miR-1973p in human prolactinoma cells.*
Methods: *The expression of miRNA-197-3p was assayed by quantitative real-time polymerase chain reaction (qRT-PCR). Cell counting kit 8 (CCK-8), 5-ethynyl-2'-deoxyuridine (EdU), and annexin V/PI staining assays were used for the determination of cell viability, proliferation and apoptosis, respectively. Protein expressions were assessed by western blotting.*
Results: *Overexpression of miR-197-3p caused significant ($p < 0.05$) inhibition of the viability and proliferation of GH3 prolactinoma cells. Furthermore, the colony-forming potential of GH3 cells was significantly reduced by miR-197-3p overexpression. The tumor-suppressive effects of miR-197-3p occurred via initiation of apoptosis which, in turn, was accompanied by up-regulation of Bax and downregulation of Bcl-2 ($p < 0.05$). Overexpression of miR-197-3p also inhibited ERK/EGR1 signaling pathway in prolactinoma cells.*
Conclusion: *MiR-197-3p exhibits a tumor suppressive effect in prolactinoma cells. Therefore, it may have therapeutic potential for the management of prolactinoma.*

Keywords: *Prolactinoma, adenoma, micro-RNA, miR-197-3p, apoptosis, ERK/EGR1 pathway*

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INTRODUCTION

Adenomas of pituitary gland make up to 15 percent of all intra-cranial tumors, half of which are prolactinomas [1]. The latter are among the leading causes of hyperprolactinemia which are responsible for clinical disorders such as galactorrhea and menstrual malfunctions in females, and sexual disorders such as erectile dysfunction and gynecomastia in males [2]. Although the morbidity associated with prolactinomas is not a serious concern, the disease may, in rare cases, exhibit aggressiveness and become resistant to therapeutic procedures [3]. Moreover, there are no standard molecular markers for predicting the aggressive behaviour of pituitary adenomas [4].

Malignant prolactinomas have very poor prognosis, and the malignancy becomes apparent only at the stage of metastasis [5]. Several research reports have revealed that micro-RNAs (miRNAs) play vital roles in regulating the physiological functioning of the

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pituitary gland, in addition to their prominent involvement in controlling the pathogenesis of pituitary adenomas [6,7]. Recent findings have shown that miRNAs target specific cell signaling pathways in pituitary adenoma cells, thereby inhibiting their growth and proliferation. For instance, miR-16 suppressed pituitary tumor cell growth by targeting the ERK/MAPK signaling pathway [8]. Likewise, miR-137 acted as suppressor of pituitary tumors via post-transcriptional targeting of AKT2 [9].

With this background, the present study focused on investigation of the regulatory role of miR197-3p in prolactinoma, with the primary aim of elucidating the mechanism involved. Studies have revealed that miR-197-3p was suppressed in a number of human malignancies, and its re-expression suppressed the growth of malignant cells via diverse mechanisms [10,11]. Furthermore, the overexpression of miR-197-3p in prolactinoma cells inhibited their proliferation *in vitro,* and reduced their proliferation. Prolactinoma cells that over-expressed miR-197-3p showed significantly lower colony-forming potential than their respective negative control cells. The anti-proliferative effect of miR-197-3p on prolactinoma cells was shown to occur through induction of apoptosis. At the molecular level, miR-19-3p exerted its tumor-suppressive effect on prolactinoma cells *in vitro* by targeting the ERK/EGR1 signaling pathway. The present study was carried out to determine the therapeutic effectiveness of miR-197-3p against prolactinoma cells, as well as the associated mechanism.

EXPERIMENTAL

In vitro cell culture and transfection

The GH3 cells were obtained from Cell Culture Centre, Chinese Academy of Medical Sciences, China. The cells were propagated in F12 medium containing 10 % fetal bovine serum and 1 % of penicillin and 1 % streptomycin at 37 °C in a 5-% CO_2 atmosphere. Single-stranded miR-97-3p mimic oligos and negative control (NC) were purchased from Ribobio, Guangzhou, China. The constructs were transfected into GH3 prolactinoma cells using Lipofectamine 3000 (Invitrogen), as per the manufacturer's guidelines.

RNA isolation and qRT-PCR

Total RNA was extracted from stably transfected prolactinoma cells using TRIzol reagent (Invitrogen) as per the manufacturer's instructions. Following its quantification, 2 µg of total RNA was used for cDNA synthesis with M-MLV reverse transcriptase. Quantitative real-time PCR (qRT-PCR) was then performed on a Quant-Studio Real-Time PCR System using SYBR Green Master Mix (Invitrogen). The relative transcript levels of miR-197-3p were quantified using the $2^{-\Delta\Delta Ct}$ method, with snRNA U6 and GADPH gene as endogenous controls.

CCK-8 viability and EdU incorporation assays

The proliferation of prolactinoma cells transfected with miR-197 mimics or miR-NC was assayed using cell counting kit-8 assay. Stably-transfected cells were seeded in a 96-well plate at a density of 2.5 × 10^5 cells/mL. Following incubation for 0 - 96 h, the prolactinoma cells were inoculated with 10 µL of CCK-8 solution, followed by further incubation for 2 h at 37 °C. Finally, the absorbance of each well was read at 450 nm in a micro-plate reader (Sunrise, Tecan, Austria).

The EdU incorporation assay kit (Invitrogen) was used for assessing the viability of stably-transfected prolactinoma cells. The cells were seeded in a 96-well plate and cultured for 24 h at 37 °C. Each well was then inoculated with 100 µL of 50 µM EdU solution, followed by incubation at 37°C for 2 h, after which the cells were fixed in 4 % paraformaldehyde. The cells were then treated with 100 µL of Apollo® 488 fluorescent staining solution at 37 °C for 30 min, followed by counter-staining of the nuclei with DAPI solution. Thereafter, the cells were visualized and photographed under a fluorescent microscope.

Colony forming assay

Approximately, 10^3 transfected prolactinoma cells were seeded in 1.5 mL of culture medium containing 10 % FBS. The prolactinoma cells were allowed to grow for 18 days, during which period the culture medium was changed every 3 days. Next, the colonies formed were fixed in paraformaldehyde and then stained using 0.2% crystal violet for 25 min at room temperature. The relative colony populations were determined with ImageJ software.

Determination of apoptosis

The EB/AO double staining was performed to assess morphological changes in the transfected prolactinoma cells. To determine apoptosis of 197-3p or miR-NC-overexpressing prolactinoma cells transfected with miR-197-3p or miR-NC, the cells were plated in a 6-well plate at a density of 5 x 10^4 cells per well, and cultured for 24 h at

37°C. Next, the cells were washed thrice with PBS and fixed with 70 % ethanol for 20 min. To determine percentage apoptosis, the cells were stained using 5 μL each of AO and EB (EB, 100 μg/mL), and incubated for 20 min. Finally, the prolactinoma cells were subjected to fluorescent microscopic examination.

Quantification of apoptosis of prolactinoma cells was made using Annexin V-FITC/PI Apoptosis Detection Kit (Keygen, Nanjing, China). In essence, prolactinoma cells were seeded in a 6-well plate at a density of 10^5 cells per well, and incubated at 37°C for 24 h. Then, the cells were washed with PBS and resuspended in binding buffer. Subsequently, the cells were fixed in methanol and stained with Annexin V-FITC/PI solution in line with the manufacturer's guidelines. The percentage of apoptotic cells was determined using a fluorescence-activated cell sorting (FACS) flow cytometer.

Western blotting

The prolactinoma cells transfected with miR-197 mimics or miR-NC were incubated in RIPA buffer containing protease inhibitor. The resultant cell lysates were homogenized in a tissue homogenizer. The extracted proteins were quantified using Bradford protein assay method. Then, 35-μg protein portions were resolved on 10 – 12% SDS-PAGE. The separated proteins were subsequently transferred to PVDF membranes which were incubated with 5 % skimmed milk to block non-specific binding of the blot. Thereafter, the membranes were incubated overnight at 4 °C with the relevant primary antibodies, followed by rinsing three times with TBST. Then, they were incubated with secondary antibodies conjugated with horseradish peroxidase. Finally, the protein signals were detected using enhanced chemiluminescence.

Statistical analysis

The results are presented as mean ± standard deviation (SD) calculated from data obtained from at least three replicates per experiment. Student's *t*-test used to for statistical analysis with SPSS software (Chicago, IL, USA). Values of $p < 0.05$ were taken as indicative of statistically significant differences.

RESULTS

miR-197-3p inhibited *in vitro* prolactinoma cell growth and viability

Since miR-197-3p re-expression suppresses the growth of malignant cells, miR197-3p was over-expressed in prolactinoma cells using miR-197 mimics. The over-expression was confirmed with qRT-PCR, with miR-NC transfected prolactinoma cells as negative control (Figure 1 A). Then, CCK-8 assay was done to determine the effect of miR-197-3p over-expression on the prolactinoma cell proliferation. At different culture durations, prolactinoma cells overexpressing miR-197-3p exhibited significantly lower percentage proliferation than the negative control cells ($p < 0.05$; Figure 1 B). Results from EdU assay showed that prolactinoma cells over-expressing miR-197-3p had significantly lower EdU incorporation, when compared to the negative control cells, thereby demonstrating the anti-proliferative effect of miR-197-3p on prolactinoma cells (Figure 1 C). Results from colony-forming assay indicated that miR-197-3p significantly reduced the colony-forming potential of prolactinoma cells, with more than 70 % decrease in the number of colonies (Figure 1 C). These results reveal the growth-inhibitory molecular effect of miR-197-3p on prolactinoma cells.

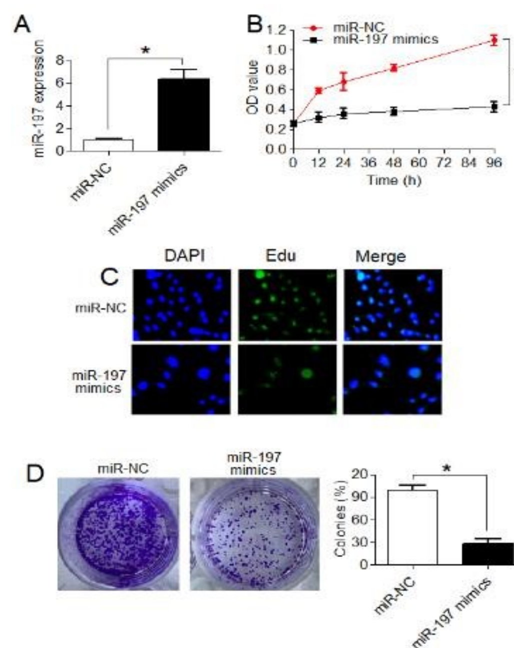

Figure 1: Effect of miR-197-3p on the proliferation of prolactinoma cells. (A) Expression level of miR-197-3p in miR-197 mimics or miR-NC-transfected GH3 prolactinoma. (B) CCK-8 proliferation analysis of GH3 prolactinoma cells transfected with miR-197 mimics or miR-NC at indicated culture durations (C) EdU incorporation assay of GH3 prolactinoma cells transfected with miR-197 mimics or miR-NC. (D) Colony-forming assay of GH3 prolactinoma cells transfected with miR-197 mimics or miR-NC. Results are shown as mean ± standard deviation (SD); $*p < 0.05$

MiR-197-3p induced apoptosis of prolactinoma cells

To unravel the basis of the anti-proliferative action of miR-197-3p on prolactinoma cells, the apoptosis of prolactinoma cells over-expressing miR-197-3p, and apoptosis of the respective negative control cells were studied using AO/EB dual staining method. The proportion of acridine-orange prolactinoma cells was markedly higher under miR-197-3p than in negative control transfection (miR-NC; Figure 2 A). Moreover, apoptosis analysis with flow cytometry showed that miR-197-3p over-expression increased the relative number of apoptotic prolactinoma cells from 9.6 ± 0.34 to 33 ± 1.74 % (Figure 2 B). Western blotting revealed that Bax expression was markedly up-regulated, while that of Bcl-2 was down-regulated when miR-197-3p was overexpressed (Figure 2 C). Taken together, these results show that miR-197-3p exerted anti-proliferative effect in prolactinoma cells via induction of apoptosis.

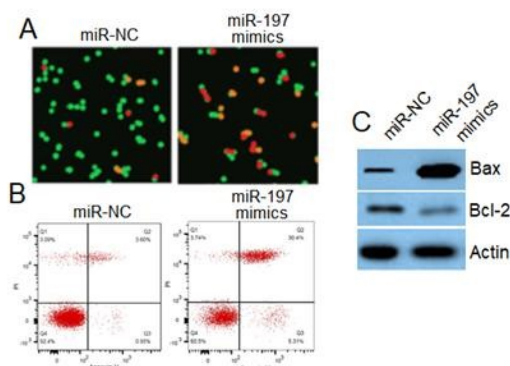

Figure 2: miR-197-3p induced apoptosis in prolactinoma cells**.** (A) AO/EB staining analysis of prolactinoma cells transfected with miR-197 mimics or miR-NC. (B) Flow cytometric apoptosis study of prolactinoma cells transfected with miR-197 mimics or miR-NC. (C) Protein expression levels of Bax and Bcl-2 in prolactinoma cells transfected with miR-197 mimics or miR-NC, as determined using Western blotting. Results are shown as mean ± SD; $^{*}p < 0.05$

MiR-197-3p targeted ERK/EGR1 pathway in prolactinoma cells

As per a previous report which demonstrated that blockage of ERK/EGR1 signaling pathway decreased the proliferation of prolactinoma cells [16], the regulatory effect of miR-197-3p on ERK/EGR1 pathway was assessed in this study. Prolactinoma cells over-expressing miR-197-3p showed marked decrease in protein expression level of phosphorylated ERK (p-ERK; Figure 3 A). However, the protein expression level of non-phosphorylated ERK was unaffected. Similarly, miR-197-3p up-regulation in prolactinoma cells significantly reduced the protein expression of EGR1 (Figure 3 B). Thus, miR-1973p suppressed the growth of prolactinoma cells *in vitro* by inhibiting the ERK/EGR1 signaling pathway.

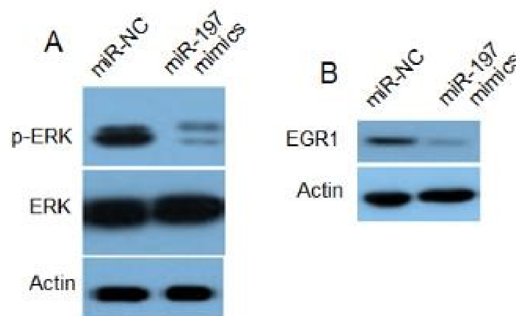

Figure 3: miR-197-3p blocked the ERK/EGR1 signaling pathway in prolactinoma cells**. (A)** Protein expression levels of p-ERK and ERK in prolactinoma cells transfected with miR-197 mimics or miR-NC, as determined using Western blotting assay. (B) EGR1 protein expression levels. Results are shown as mean ± SD; $p < 0.05$

DISCUSSION

Pituitary disorders are often linked to serious and chronic morbidities involving multiple-organs. Pituitary disorders are treated using multi-disciplinary approach through life-long drug administration. In particular, pituitary adenomas and their associated compressive symptoms and clinical manifestations constitute major burdens to the affected patients [12]. Prolactinomas, the most common pituitary adenomas, are yet to be studied at the molecular level, with respect to the pre-disposing factors so as to generate information for therapeutic and curative purposes [13]. Growing evidence suggest that micro-RNAs (miRs) are involved in regulating the growth and development of prolactinomas [6-10].

The current study has shown that over-expression of miR-197-3p inhibited the growth and proliferation of prolactinoma cells *in vitro*. The involvement of miR-197-3p in endocrine tumors has been previously reported [14]. It was demonstrated through *in vitro* experiments that miR-197-3p inhibited the growth of prolactinoma cells through induction of apoptosis. The signal for induction of apoptosis was mediated through the Bax/Bcl-2 pathway. In prolactinoma cells, increases in Bax/Bcl-2 ratio induced programmed cell death under miR-197-3p overexpression. The pro-apoptotic regulatory action of miR-197-3p is also reflected in previous reports which also suggested its potential therapeutic benefits

against human malignancies [15]. Prolactinoma cells express significantly higher protein levels of ERK and EGR1, and the ERK/EGR1 signaling pathway has been shown to present a crucial therapeutic target against these cells [16].

Thus, the effect of miR-197-3p on ERK/EGR1 signaling pathway was investigated in this study. It was found that over-expression of miR-197-3p suppressed the protein expression of phosphorylated ERK, and also down-regulated EGR1 protein expression, both of which have been reported to profoundly affect the growth and proliferation of malignant human cells [17]. Thus, the results of this study revealed that miR-197-3p exerted its anti-proliferative effect on prolactinoma cells through a mechanism that involved blockage of the ERK/EGR1 signaling pathway. This finding indicates the therapeutic potential of miR-197-3p, as well as ERK/EGR1 pathway crosstalk against prolactinoma cells.

CONCLUSION

The present study has demonstrated that miR-197-3p exerts anti-proliferative and anti-growth effects against prolactinoma cells via induction of apoptosis. At the molecular level, ERK/EGR1 signaling pathway has been identified as the regulatory target of miR-197-3p in prolactinoma cells. These results suggest that miR-197-3p may have therapeutic potential in the management of prolactinoma.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Binbin Zhang and KaiXue contributed equally to this work. Binbin Zhang and KaiXue performed all the experiments. The whole study was designed by Yanming Zhang.

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