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MicroRNA-143 Regulates Proliferative and apoptotic potential of pituitary tumor cell via direct targeting on MAPK7 gene

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Abstract

Purpose: To study the influence of microRNA-143 (miR-143) on pituitary tumor cell proliferation and apoptosis, and the underlying mechanism.

Methods: Tissue samples collected from pituitary tumor patients (n = 36), as well as pituitary tumor cell lines (RC-4B/C and GH3) were used in this study. MicroRNA-143 (miR-143) mimics were transfected into the cells. Transfection efficiency was determined with qRT-PCR. Cell proliferation and apoptosis were measured with 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method and flow cytometric analysis, respectively. The protein target of miR-143 was predicted via bioinformatics, and the protein expressions of bax, bcl-2, mcl-1 and MAPK7 were measured using Western blotting.

Results: The concentration of miR-143 was markedly reduced in pituitary tumor cells, relative to paracancerous normal cells, and overexpression of miR-143 markedly reduced pituitary tumor cell proliferation and increased its apoptosis ($p<0.05$). Moreover, miR-143 markedly upregulated the protein expression of bax, but it significantly downregulated the protein expressions of mcl-1 and bcl-2 ($p<0.05$). MicroRNA-143 (miR-143) directly targeted MAPK7 and significantly downregulated its mRNA and protein expressions ($p<0.05$).

Conclusion: Thus, miR-143 functions in a manner similar to that of a tumor suppressor gene. It regulates proliferative potential and apoptosis of pituitary cancer cells through direct targeting of MAPK7 gene.

Keywords: Apoptosis, MicroRNA-143, Mitogen-activated protein kinase 7, Pituitary tumor, Proliferation.

Introduction

Pituitary tumor, the most prevalent intracranial neoplasm, accounts for 10 to 15 % of all intracranial malignancies. Although pituitary tumor starts slowly, it may proliferate and compress the surrounding cerebral nerves or other intracranial structures, thereby resulting in various complications. However, pituitary tumor may produce profound physiological upset via secretion of supra-physiological levels of hormones. Today, pituitary tumor is more readily diagnosed, and the accompanying hormonal activity is assessed more accurately than at any other time in the past. The pathogenesis of pituitary tumor is complex and yet to be fully understood (1).

The miRNAs are small nucleic acids that do not code for amino acids but are involved in control of gene expression through base-pairing with complementary sequences in mRNAs. The miRNA is highly conserved, and it participates in the regulation of expression of target gene via complete or incomplete binding to the 3'untranslated (3'-UTR) region of the gene (2). An imbalance in miRNA/mRNA ratio affects several biological processes, including tumor formation (2, 3). Several studies have reported that miRNAs are abnormally expressed in tumor tissues. The

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expression levels of such miRNAs are correlated with the degree of tumor adhesion and metastasis. MicroRNA-143 (miR-143) expression is regulated epigenetically through the heart beat (4). The expression of miR-143 has been shown to be downregulated in lung cancer, acute leukemia, colorectal cancer (CRC) and prostate cancer (6-8).

MicroRNA-143 (miR-143) inhibits cancer cell proliferative, invasive and metastatic potential, while promoting cell apoptosis. Its role in tumor suppression is similar to that of a tumor suppressor gene or oncogene. MicroRNA-143 (miR-143) increases the sensitivity of anti-tumor drugs by acting in synergy with multiple target genes such as KRAS, c-Myc and ERK5. Currently, the effect of miR-143 in the pathogenesis of pituitary tumor is not clear. An investigation into the regulatory mechanism of miR-143 in pituitary tumor initiation and progression will provide significant insights into the diagnosis and treatment of the disease. The present investigation was focused on elucidation of the influence of miR-143 on proliferative and apoptotic potential of pituitary tumor cells.

Materials and methods

Patients' general information

Patients with pituitary tumor (n = 36) were recruited for this study. The included patients did not receive chemotherapy or radiotherapy before surgery, and they were diagnosed via pathological examination. Tumor and nearby paracancerous normal tissues were analyzed. The patients signed written informed consent with their family members, and their clinicopathological characteristics such as sex and age were comparable.

Cell lines and culture

Pituitary tumor cell lines (RC-4B/C and GH3) were provided by ATCC, USA. The cells were maintained in DMEM containing 10 % FBS and 1 % antibiotics at 37 °C for 24 h in 5 % CO2 and 95 % air until the cells attained 80 % confluency. The medium was replaced with fresh one every 48 hours. One week later, adherent confluent cells were trypsinized with 0.25 % trypsin-EDTA (2 mL), cultured again, and passaged for later use. Cells in logarithmic growth were employed in subsequent investigations.

Cell transfection

Pituitary tumor cells (RC-4B/C and GH3) were cultured until they attained 70 % fusion. The cells were subsequently cultured with equivalent volumes of miR-143 mimic or mimic-NC (negative control group) in a medium without serum. Incubation was carried out at room temperature for 6 h. Lipofectamine 2000 was dissolved in serum-free medium and kept at laboratory temperature for 10 min to form a mixture. The mixture was then added to cells in each group, and cultured at 37 oC under 5 % CO2 and 95 % air for 48 h. Normal cell culture without miR-143 mimic or mimic-NC served as normal control group. Transfection efficiency was determined using qRT-PCR.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

After 48 h of cell transfection, miR-143 expression was determined using qRT-PCR. Total RNA was extracted using TRIzol, and reverse-transcribed to cDNA using cDNA synthesis kit. The concentration and quality of extracted RNA were measured spectrophotometrically. The expression levels of miR-143 were measured with Light Cycler 1536 RT-PCR equipment, with GAPDH as reference gene. The qRT-PCR reaction conditions were: pre-denaturation at 95 oC for 5 min, PCR reaction at 95 oC for 5 sec and 60 oC for 30 sec, and 40 cycles in all. The 2-ΔΔCt procedure was employed for calculation of expression levels of miR-143. Table 1 presents the primers used.

Table 1. **Primer sequences used for qRT-PCR**

Gene	Sequence
MicroRNA-143	Forward: 5'-TGT AGT CGA CGT AAA GCT ATT GCT GC-'3
	Reverse: 5'-CCT ATG AAA CGA GTA CGA TTC GTG GT-3'
GAPDH	Forward: 5'-GTT TGA ACA CAT GAC CAA AAG ACG CATA-3'
	Reverse: 5'-GTC AAC GTT AGG GCT CAT TGC GGC GAAA-3'

Cell proliferation assay

The proliferative capacity of RC-4B/C and GH3 cells was determined using MTT assay. The cells were cultured in DMEM for 48 h in 96-well plates. This was followed by incubation of the plates with MTT

solution (5 g/L) for 4 h, and replacement of medium with 0.1 % of DMSO. The absorbance of the sample was read at 490 nm. All assays were performed in triplicate.

Apoptosis assay

Following culturing of the cells in 6-well plates (2.5 □ 106 cells/well) for 24 h, they were washed twice with phosphate-buffered saline, and added to binding buffer prior to Annexin V FITC and PI staining for 25 min in the dark. Apoptosis was analysed flow cytometrically at 485 nm.

Luciferase reporter gene assay

The 3'URT region or the mutated 3' URT region was cloned into the psiCHECK-2 vector to construct 3'UTR WT and MUT plasmids. Pituitary tumor cells (RC-4B/C) were cultured for 24 h before transfection. Then, miR-143 mimics was co-transfected with the plasmid into the cells, and after 48 h, luciferase activity was determined using luciferase reporter assay system.

Western blotting

The RC-4B/C cells were rinsed in PBS and lysed with ice-cold RIPA buffer containing protease inhibitor. The protein concentration of the lysate was measured with bicinchoninic acid protein kit. The protein (20 µg) was subjected to 10 SDS-PAGE prior to transfer to PVDF membranes. Then, the membranes were blocked by incubation with 5 % de-fatted milk. Thereafter, incubation was done overnight at 4 ◦C with primary antibodies for bax, bcl-2, mcl-1, MAPK7 and G6PDH, each at a dilution of 1 to 1000. Thereafter, incubation was done with HRP-linked 2o antibody for 120 min at laboratory temperature. X-ray film was used for blot development, while Grayscale analysis was done with ImageJ Launcher software, with G6PDH as standard.

Statistical analysis

Results are indicated as mean ± SD, and statistically analyzed with SPSS (22.0). Two-group comparisons were performed with t-test. Values of $p < 0.05$ were taken as indicative of significant differences.

Results

Levels of expression of miR-143 in normal and pituitary tumor tissues

The miR-143 level was markedly reduced in pituitary tumor tissues, relative to paracancerous healthy cells ($p<0.05$; Figure 1).

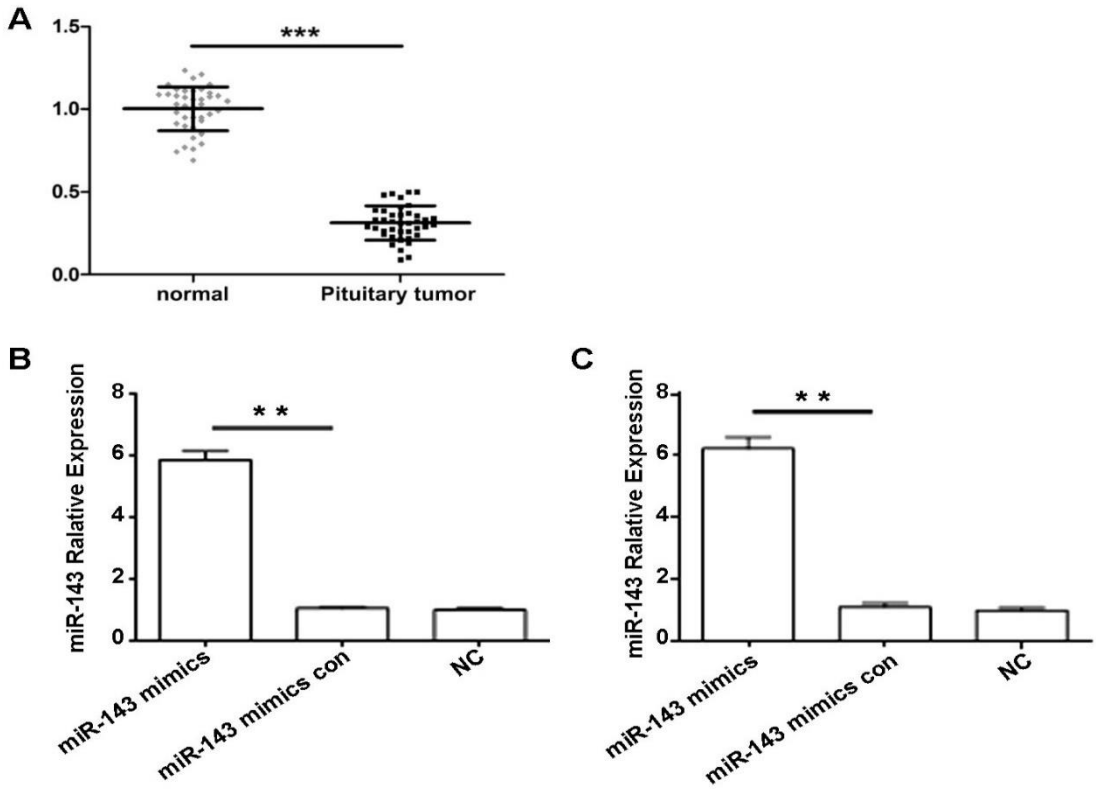

Figure 1. **Amounts of miR-143 in healthy and pituitary tumor cells.**

A: miR-143 levels in normal and pituitary tumor cells; B: miR-143 levels in miR-143 mimic-transfected RC-4B/C cells; C: miR-143 levels in miR-143 mimic-transfected GH3 cells.

Influence of miR-143 on pituitary tumor cell proliferation

Overexpression of miR-143 significantly reduced the proliferation of RC-4B/C and GH3 cells ($p<0.05$). There were few cell clones in miR-143 overexpression group, relative to the negative control group ($p<0.05$). These results are shown in Figure 2.

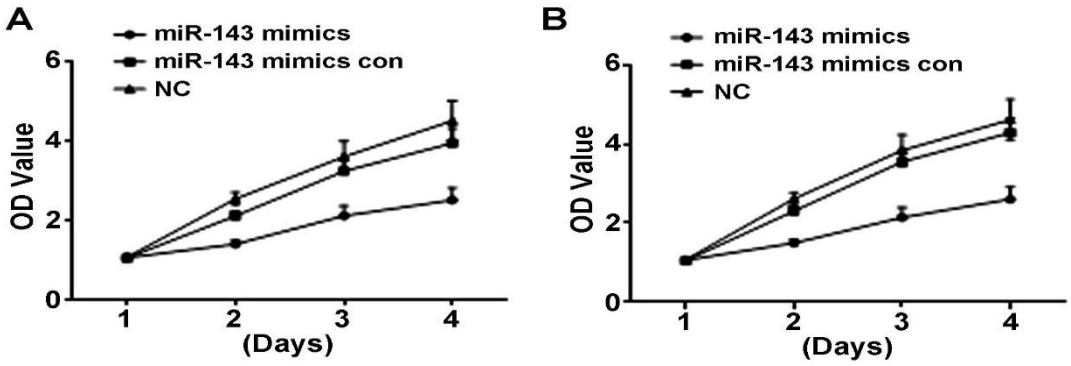

Figure 2. **Influence of miR-143 on pituitary tumor cell proliferation.**

A: Proliferation of RC-4B/C cells in the three groups; B: Proliferation of GH3 cells in the three groups.

Influence of miR-143 on pituitary tumor cell apoptosis

Overexpression of miR-143 significantly increased pituitary tumor cell apoptosis ($p<0.05$). There were markedly higher population of apoptotic cells in miR-143 mimics group than in the negative control (Figure 3).

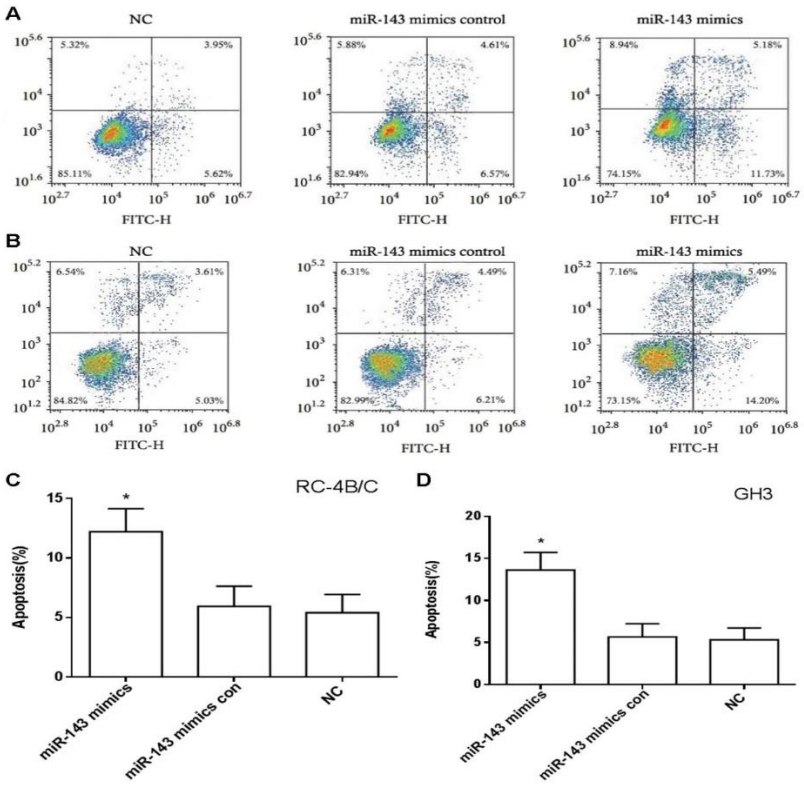

Figure 3. **Influence of miR-143 expression on pituitary tumor cell apoptosis.**

A: Apoptosis of RC-4B/C cells; B: Apoptosis of GH3 cells; C: Quantified apoptosis data involving RC-4B/C cells; D: Quantified apoptosis data involving GH3 cells.

Effect of miR-143 on protein levels of Bax, Bcl-2 and Mcl-1

As shown in Figure 4, miR-143 significantly upregulated the protein expression of Bax, but it significantly downregulated the protein expressions of mcl-1 and bcl-2.

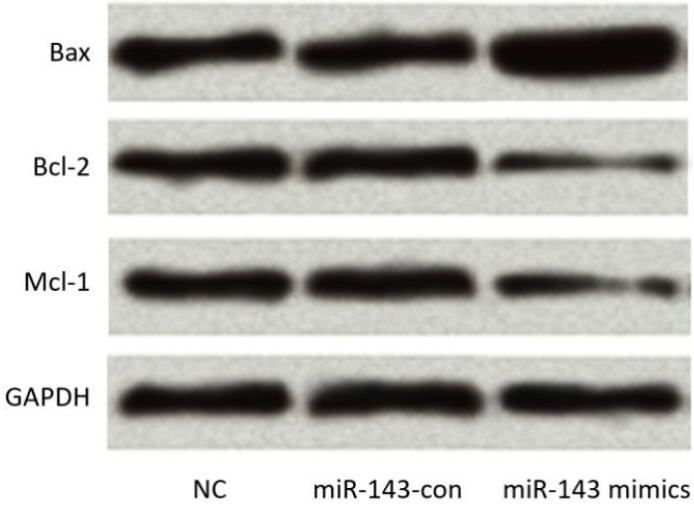

Figure 4. **Influence of miR-143 on levels of apoptosis-related factors**

Gene target of miR-143

As shown in Figure 5, results of TargetScan and miRanda showed that miR-143 was able to bind to the 3'UTR end of MAPK7. The results of luciferase reporter gene assay revealed that 3'UTR luciferase activity of MAPK7 mRNA was significantly reduced ($p<0.05$), but the mutant luciferase activity was not affected ($p>0.05$).

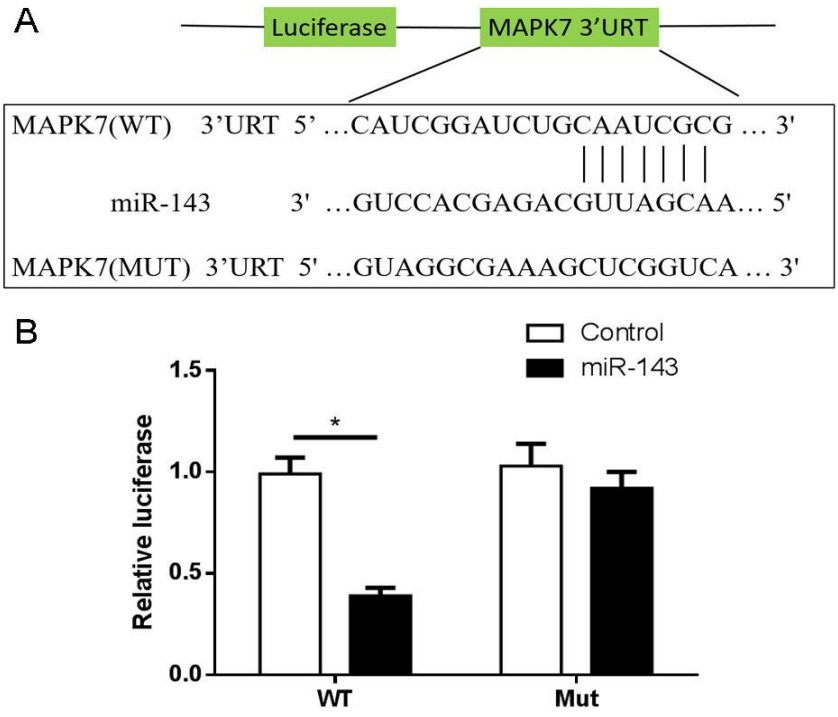

Figure 5. **Gene target of miR-143.**

A: Results of TargetScan and miRanda analysis showing binding interaction between miR-143 and the 3'UTR end of MAPK7; B: Direct targeting of MAPK7 mRNA by miR-143.

Effect of miR-143 on MAPK7 mRNA and protein expressions

Overexpression of miR-143 downregulated the mRNA and protein expressions of MAPK7 ($p<0.05$). These results are presented in Figure 6.

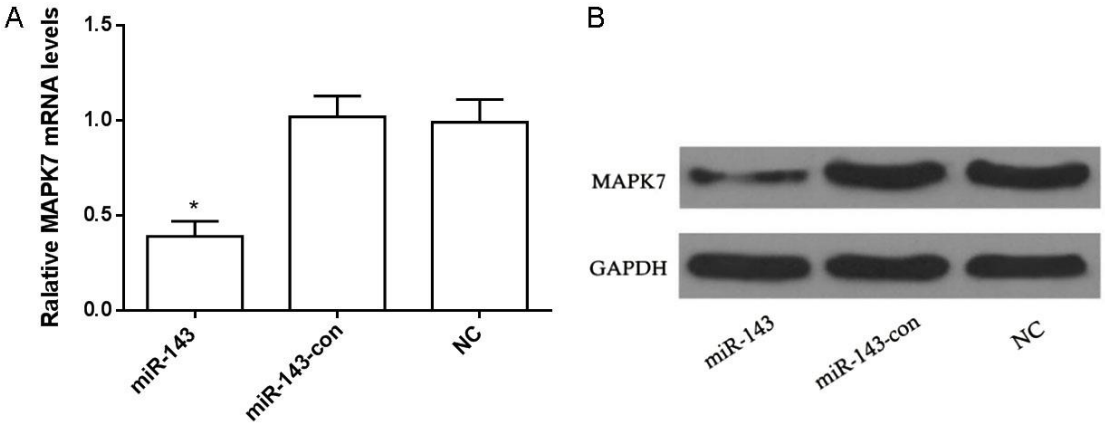

Figure 6. **Influence of miR-143 on MAPK7 mRNA and protein expressions.**

A: Level of expression of MAPK7 mRNA, as measured using qRT-PCR; B: Protein expression of MAPK7, as measured using Western blotting.

Discussion

The role of miRNAs in the pathogenesis of pituitary tumor has received considerable attention. However, only a few of the several miRNAs implicated in pituitary tumor pathogenesis have been studied in detail. Although the pathogenesis of pituitary tumor has not been fully elucidated, it is thought to involve an interplay of several genes/proteins and signaling pathways. MicroRNAs have shown great promise as effective biomarkers for diagnosis of tumors. A single miRNA may regulate multiple downstream target genes. Similarly, a single gene may be regulated by multiple miRNAs (9). It has been demonstrated that miR-106b enhances the proliferative and invasive potential of pituitary tumor cells via control of PI3K/AKT signaling route (10). Similarly, the expression of miR-34a has been shown to be downregulated in pituitary cancer cells. Moreover, the overexpression of miR-493 inhibits the protein expression of SOX7, which in turn suppresses pituitary tumor cell proliferation and metastasis (11).

MicroRNA-143 (miR-143), a recently discovered miRNA, is located on chromosome 5q32. It participates in a number of physiological and pathological processes in humans (12). Downregulation of miR-143 is seen in many malignant tumors. In an earlier investigation, it was shown that miR-143 was markedly reduced in cervical squamous cell carcinoma, and that its overexpression inhibited the proliferation of the cells, while promoting their apoptosis (13). Studies have shown that miR-143 is significantly lower in CRC tissues than in paracancerous healthy cells, and it targets DNMT, thereby regulating DNA methylation (14). The downregulation of miR-143 inhibits the proliferative capacity of osteosarcoma cells, and promotes their apoptosis via regulation of bcl-2 expression (15). The expression pattern of miR-143 in tumor cells makes it a potential molecular marker for tumor diagnosis and prognosis.

The MAPK signaling routes are among the widespread systems of control in eukaryotic cells (16). These pathways are implicated in cell proliferation, growth and synchronization of intercellular functions (16). The MAPK signaling pathways regulate intracellular responses via activation/inactivation of downstream enzymes/proteins. The downstream effect is mediated by tyrosine receptor kinase, G-protein-coupled receptors (GPCRs), cytokine receptors and serine/threonine kinase receptors. The MAPK pathways control varied processes such as gene expressions, cell cycle, apoptosis, and differentiation. The MAPK member i.e. MAPK7 is expressed in a variety of tissues. As a pro-mitotic

and anti-apoptotic protein, MAPK7 contributes to the growth of malignant tumor cells, thereby influencing prognosis of patients (17). On receiving extracellular signals, MAPK7 undergoes nuclear translocation where it regulates target gene expressions via phosphorylation and activation of appropriate transcription factors (18). The upregulation of MAPK7 expression in breast cancer is significantly correlated with lymph node metastasis and prognosis (19). However, the downregulation of MAPK7 expression inhibits cell proliferation in hepatocellular carcinoma (20). In MAPK7 knockout mice, MAPK7 interacted with endothelial cells to reduce tumor vascular density, thereby promoting tumor angiogenesis (21).

This study revealed that miR-143 was markedly decreased in pituitary tumor cells, relative to paracancerous healthy cells. Moreover, its overexpression in pituitary tumor cells markedly reduced their proliferation, while promoting cell apoptosis. These observations suggest that miR-143 may inhibit pituitary tumor cell proliferation via induction of apoptosis, and they are in agreement with results of previous studies (17, 18). In the present study, results from bioinformatics showed that there was a base binding site between the 3'UTR of MAPK7 and miR-143, and that miR-143 overexpression significantly suppressed the mRNA and protein expressions of MAPK7 in RC-4B/C cells. Thus, it appears that miR-143 may suppress the expression of MAPK7 in pituitary tumor cells.

Conclusion

The findings in this investigation indicate that miR-143 plays a role similar to that of a tumor suppressor gene. It regulates the apoptosis and proliferative potential of pituitary cancer cells, with MAPK7 gene as its direct target.

Acknowledgements

None.

Conflicts of interest

There are no conflicts of interest in this study.

Author's contribution

All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Zhihong Li; Zhihong Li, Zhengjiang Zha, Youzhong Yang, Dengxi Xiong, Jian Tang collected and analysed the data; Zhihong Li wrote the text and all authors have read and approved the text prior to publication.

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