MicroRNA-16/VEGFR2/p38/NF-κB signaling pathway regulates cell growth of human pituitary neoplasms

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Abstract. The association of microRNA (miRNA) with tumor has gradually become an active medical research field, since its discovery in 1993. The aim of the present study was to clarify how microRNA-16 expression affects the proliferation and survival of pituitary tumor, revealing its potential mechanism. MicroRNA-16 expression of pituitary tumor patients was observably declined, compared with the normal group. A high expression of microRNA-16 showed longer survival in pituitary tumor patients, compared to a low expression of microRNA-16 in pituitary tumor patients. MicroRNA-16 upregulation effectively decreased cell proliferation and induced apoptosis in HP75 cells. MicroRNA-16 overexpression effectively induced p27, Bax protein expression and caspase-3/8 activities, and suppressed phosphorylation-(p)-p38, NF-κB, MMP-9 and VEGFR2 protein expression in HP75 cells. After VEGFR2 suppression, the effects of microRNA-16 overexpression on cell proliferation and apoptosis were significantly inhibited in HP75 cells. Moreover, the effects of microRNA-16 overexpression on p27, Bax protein expression and caspase-3/8 activities were significantly decreased in HP75 cells after p38 suppression. VEGFR2 or NF-κB suppression reduced the effects of microRNA-16 overexpression on p-p38, NF-κB, MMP-9 and VEGFR2 protein expression inhibition in HP75 cells. Our results suggest that microRNA-16 expression affects the proliferation and angiogenesis of pituitary cancer through the VEGFR2/p38/NF-κB signaling pathway.

Introduction

Pituitary tumor is a common neuroendocrine system tumor, the morbidity of which accounts for 10-15% of all central nervous system tumors. Moreover, the morbidity increases annually, as is shown in epidemiological investigations (1). A majority of pituitary tumors are benign tumors that grow slowly, with no typical early symptoms in clinic. However, a small number of these tumors can induce certain central nervous system symptoms since the excessive growth has compressed the intracranial structures (2). Furthermore, some tumors belong to functional pituitary adenomas, which cause endocrine function disorders as a result of the abnormal secretion of hormones. Consequently, this has led to numerous complications (3). Early tumor diagnosis is of vital importance to improve prognosis for cancer patients, while the early diagnosis of pituitary tumor, not only improves patient quality of life, but can also effectively prolong the life of patients (4).

miRNA generally acts on various types of oncogenes (including proto-oncogenes and tumor suppressor genes) through direct or indirect pathways, and thereby participates in tumor pathogenesis. It is indicated in current research that, ~50% of miRNAs are related to tumor pathogenesis. Such miRNAs, which are located in tumor-associated fragile sites or particular tumor growth-associated genome domains, have dual identities (oncogenes or tumor suppressor genes) (5). Cancer miRNAs possess certain properties of oncogenes, which can specifically bind with the mRNA of tumor suppressor genes, and inhibit or degrade the expression of tumor suppressor genes. It can lead to silencing of targeted oncogenes, and thus indirectly promote growth or genesis of some tumors. In addition, such miRNAs are mostly excessively expressed in tumors, which means that they have elevated expression levels in the event of tumor genesis. In other words, their expression levels are upregulated in the event of tumor genesis. Anticancer miRNAs can specifically bind with cancer mRNA, and inhibit or degrade the expression of oncogenes (6). Therefore, they can inhibit transcription of targeted oncogenes and eventually inhibit tumor growth or genesis. Different from the former, the expression levels of such miRNAs are reduced (6). The discovery of cancer miRNA and anticancer miRNA has led to medical workers investigating the association of miRNA with tumor (7). This provides insight into the investigation of the specific pathogenesis, diagnosis and treatment of pituitary tumor (7).

Pituitary tumor is a kind of benign tumor that does not metastasize under general conditions, but ~30% invades the surrounding structures (8). Such tumors can hardly be radically treated, and total resection is difficult, leading to a high postoperative recurrence rate. CD147, which is also referred
to as the extracellular matrix metalloproteinase inducer (EMMPRIN), is a cell surface adhesion molecule. It plays a role in stimulating the production of matrix metalloproteinases (MMPs) and affects tumor invasion and metastasis. In the subfamily of MMPs, MMP-9 is most closely associated with the invasion of pituitary tumor (9). Tumor angiogenesis is closely related to tumor growth and metastasis. Vascular endothelial growth factor (VEGF2) is one of the pro-angiogenic factors known currently to have the strongest effect, and it plays an important role in tumor formation (10).

From the point of view of cell apoptosis, out of control growth and excessive proliferation of tumor cells occurs owing to the fact that that death cells cannot be eliminated normally since the tumor apoptotic mechanism is inhibited (11). p38MAPK pathway is an important pathway involved in the initiation of cell apoptosis, which can exert biological effects after being activated by the extracellular stimulus, thus regulating various cell functions (12). It mainly mediates physiological functions such as differentiation, proliferation and apoptosis (11). Therefore, clarifying its mechanism of action during tumor genesis, development and treatment outcome is necessary.

NF-κB is a family of transcription factors existing in eukaryotic cells with extensive distribution and multiple effects. It is one of the most important intracellular nuclear transcription factors that participate in the expression and regulation of multiple genes, which is the symbol of activated cells (13). NF-κB is verified in research to participate in inflammatory reaction and immune response of the body. In addition, it is involved in pathophysiological processes, such as cell proliferation, differentiation and apoptosis (14). Furthermore, the nuclear factor NF-κB is associated with tumor invasion and metastasis. NF-κB controls DNA transcription and the binding of the fixed nucleotide sequence in the promoter region of the gene. It is involved in pathophysiological processes including immune reaction, inflammatory reaction, and cell apoptosis. Thus, it promotes a series of important vital activities, such as cell proliferation and differentiation, tumor formation and metastasis (15). The aim of the present study was to clarify how microRNA-16 expression affects the proliferation and survival of pituitary tumor and reveal its potential mechanism.

Materials and methods

Human tissue samples and reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). Thirty-six patients with pituitary tumor and 8 healthy volunteers were selected, and the peripheral blood was collected and centrifuged at 2,000 x g for 20 min. Serum was collected and saved at -80˚C. Total RNA was extracted using RNAiso Plus according to the manufacturer’s instructions (Takara, Japan). cDNA was reverse-transcribed using the One Step PrimeScript® miRNA cDNA Synthesis kit (Takara) and PrimeScript® RT Master Mix Perfect Real Time (Takara), according to the manufacturer’s instructions. RT-qPCR reactions were performed using SYBR® Premix Ex Taq™ II (Perfect Real Time; Takara) and 50 nM of miR-16 mimics (miR-16) and negative control RNA mimics (Ribobio, Guangzhou, China) were transfected using Lipofectamine 3000 (Invitrogen, Guangzhou, China). HP75 cells were treated with 5 µM of PDTC (NF-κB inhibitor) for 24 h after transfection.

Cells and overexpression of miR-16. Human pituitary cancer HP75 cells were incubated with low-glucose Dulbecco’s modified Eagle’s medium complete medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS, Harlan, Madison, WI, USA) in a humidified chamber with 5% CO₂ at 37˚C. HP75 cells were seeded in 6-well plates (~70% confluent), and 50 nM of miR-16 mimics (miR-16) and negative control RNA mimics (Ribobio, Guangzhou, China) were transfected.

Flow cytometry. The anticancer effect of dihydroartemisinin on apoptosis of tumor cells was examined using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). HP75 cells were seeded in a 6-well plate after transfection for 48 h. Then, MTT (5 mg/ml in sodium chloride) was added to the cells for the last 2 h of incubation and cell viability was measured at 570 nm using a microplate reader (Tecan M1000, Invitrogen, Carlsbad, CA, USA) after being dissolved in DMSO.

Western blot assay. The anticancer effect of dihydroartemisinin on apoptosis of tumor cells was examined using a FACSCalibur flow cytometer (BD Biosciences). HP75 cells were seeded in a 6-well plate after transfection for 48 h. After rinsing in PBS three times, HP75 cells were resuspended with Annexin V-FITC (5 µl, KeyGen Biotech Co., Ltd.) in the dark for 30 min followed by PI dye (5 µl, KeyGen Biotech Co., Ltd., Jiangsu, China). Apoptosis rate of HP75 cells was measured by FACSCalibur flow cytometer (BD Biosciences).
Technology, Inc.) and anti-GAPDH (sc-25778, 1:2,000, Santa Cruz Biotechnology) at 4°C. Then, the membrane was incubated with secondary antibody (sc-2004, 1:5,000, Santa Cruz Biotechnology) at 37°C for 1 h and was determined with ImageJ software (open source, http://rsb.info.nih.gov/ij/index.html).

**Caspase-3 and -9 activity assay.** HP75 cells were seeded in a 6-well plate after transfection for 48 h. The cells were then collected and lysed in Laemmli buffer for the extraction of total proteins. Protein content was measured using the bicinchoninic acid protein assay kit method (Beyotime Institute of Biotechnology). Proteins (20 µg) were used to measure caspase-3 and -9 activity with Ac-DEVD-pNA for caspase-3 or Ac-LEHD-pNA for caspase-9. Caspase-3 and -9 activity was measured using a microplate reader (Tecan M1000) at 405 nm.

**Statistical analysis.** Statistical significance was calculated employing analysis of variance (one-way ANOVA, Tukey’s multiple comparison test). Data were presented as mean ± standard deviation (SD). A statistical significance was defined as P<0.05.

**Results**

**MicroRNA-16 expression of pituitary tumor patients.** Firstly, 36 patients with pituitary tumor and 8 healthy volunteers were selected, and serum was collected and used to analyze microRNA-16 expression of pituitary tumor patients. As shown in Fig. 1, microRNA-16 expression of pituitary tumor patients was observably declined, compared with the normal group. The differences were statistically significant (P<0.05).

**Survival rate of pituitary tumor patients with microRNA-16 expression.** The high expression of microRNA-16 shows longer survival (overall survival, OS and disease-free survival, DFS) in pituitary tumor patients, compared to a low expression microRNA-16 in pituitary tumor patients (Fig. 2).

**MicroRNA-16 upregulation affects cell proliferation and apoptosis of HP75 cells.** In order to predict the upstream microRNA-16 effects on cell proliferation and apoptosis of HP75 cells, miR-16 mimics and mimics-NC were transiently transfected into HP75 cells. microRNA-16 mimics decreased cell proliferation and induced apoptosis of HP75 cells in a dose-dependent manner, compared to the mimics-NC group (Fig. 3).

**MicroRNA-16 upregulation affects caspase-3/8 activities in HP75 cells.** To investigate the role of microRNA-16 in the regulation of caspase-3/8 activities of HP75 cells, caspase-3/8 activities were measured using caspase-3 and -8 activity kits. Additionally, Fig. 4A shows that microRNA-16 mimics...
effectively increased caspase-3/8 activities in HP75 cells, compared to the mimics-NC group.

**MicroRNA-16 upregulation affects p27 and Bax protein expression in HP75 cell.** To confirm the prediction of microRNA-16 mechanism on pituitary tumor, we measured p27, Bax protein expression in HP75 cells. Our results indicated a significant increase of p27, Bax protein expression in HP75 cells after microRNA-16 upregulation, compared to the mimics-NC group (Fig. 4B-D).
MicroRNA-16 upregulation affects p38MAPK, NF-κB, MMP-9 and VEGFR2 protein expression in HP75 cells. To confirm the mechanism of microRNA-16 on pituitary tumor, we measured p-p38MAPK, NF-κB, MMP-9 and VEGFR2 protein expression in HP75 cells. Our results indicated significant reduction of p-p38MAPK, NF-κB, MMP-9 and VEGFR2 protein expression in HP75 cells after microRNA-16 upregulation, compared to the mimics-NC group (Fig. 4E-I). We used immunohistochemistry to observe VEGFR2 protein expression in HP75 cells after microRNA-16 upregulation. MicroRNA-16 upregulation significantly suppressed VEGFR2 protein expression in HP75 cells, compared to the mimics-NC group (Fig. 4E-I).

Effects of microRNA-16 overexpression on cell proliferation and apoptosis of HP75 cells following VEGFR2 suppression. Our results indicated that VEGFR2 inhibitor significantly suppressed the effects of microRNA-16 overexpression on cell proliferation reduction and apoptosis induction in HP75 cells, compared to the microRNA-16 mimics group (Fig. 6F and G).

Effects of microRNA-16 overexpression on caspase-3/8 activities in HP75 cells following VEGFR2 suppression. To investigate whether VEGFR2 participated in the effects of microRNA-16 overexpression on caspase-3/8 activities in HP75 cells, caspase-3/8 activities were also measured. As shown in Fig. 6I-K, NF-κB inhibitor significantly increased the effects of microRNA-16 overexpression on caspase-3/8 activities in HP75 cell, compared to the microRNA-16 mimics group (Fig. 6H).

Effects of microRNA-16 overexpression on p27 and Bax protein expression in HP75 cells following VEGFR2 suppression. After VEGFR2 suppression, we also observed p27 and Bax protein expression in HP75 cells following microRNA-16 overexpression. p27 and Bax protein expression in HP75 cells following microRNA-16 overexpression was higher than those of microRNA-16 mimics group (Fig. 6I-K).

Effects of microRNA-16 overexpression on NF-κB and MMP-9 protein expression in HP75 cells following NF-κB suppression. Next, we explored the function of NF-κB and the effects of microRNA-16 overexpression on NF-κB, and MMP-9 protein expression in HP75 cells. As shown in Fig. 7A-C, NF-κB
Figure 6. MicroRNA-16 upregulation affects p38MAPK/NF-κB/MMP-9/VEGFR2 protein expression, cell proliferation and apoptosis, caspase-3/8 activities, and p27/Bax protein expression in HP75 cells. MicroRNA-16 upregulation affects (A) p-p38MAPK, NF-κB, MMP-9 and VEGFR2 protein expression, determined using western blot analysis and statistical analysis of (B) MMP-9, (C) VEGFR2, (D) p-p38MAPK, and (E) NF-κB protein expression, (F) cell proliferation and (G) apoptosis, (H) caspase-3/8 activities, (I) p27 and Bax protein expression, determined using western blot analysis, and statistical analysis of (J) Bax, (K) p27 protein expression in HP75 cells. Control, control group. Negative, negative control group. MicroRNA-16, microRNA-16 upregulation group. Anti-VEGFR2, VEGFR2 inhibitor + microRNA-16 upregulation group. Repeat times (n=3). ***P<0.01 versus normal group, **p<0.01 versus microRNA-16 upregulation group.
inhibitor suppressed NF-κB and MMP-9 protein expression in the effects of microRNA-16 overexpression.

**Effects of microRNA-16 overexpression on cell proliferation and apoptosis of HP75 cells following NF-κB suppression.**

Then, we found that the effects of microRNA-16 overexpression on the inhibition of cell proliferation and the induction of apoptosis in HP75 cells by NF-κB inhibitor were effectively accelerated, compared with the microRNA-16 mimics group (Fig. 7D and E).
Effects of microRNA-16 overexpression on caspase-3/8 activities in HP75 cells following NF-κB suppression. However, the caspase-3/8 activities in HP75 cells following microRNA-16 overexpression in NF-κB inhibitor were higher than those of the microRNA-16 mimics group (Fig. 7F).

Effects of microRNA-16 overexpression on p27 and Bax protein expression in HP75 cells following NF-κB suppression. NF-κB inhibitor facilitated the effects of microRNA-16 overexpression on p27 and Bax protein expression in HP75 cells, compared with the microRNA-16 mimics group (Fig. 7G-I).

Discussion

Pituitary tumors constitute a fairly common intracranial tumor, most of which are benign. However, some secretory adenomas frequently result in endocrine diseases, including Cushing syndrome, amenorrhea and gigantism (16). Some large adenomas grow in the brain, which may compress brain tissues as a result of increased tumor volume, leading to symptoms such as visual impairment or headache. In addition, some tumors manifest certain characteristics of malignancy. For instance, some pituitary tumors grow across the sella turcica in an infiltration manner and invade the peripheral cranial structures, which may not be completely removed through surgery, and are susceptible to postoperative recurrence (17). Therefore, early diagnosis and clinical treatment of pituitary tumor is essential (3,17). Our current results show that microRNA-16 expression of pituitary tumor patients was observably declined, compared with the normal group, and the high expression of microRNA-16 has longer OS and DFS in pituitary tumor patients, compared to a low microRNA-16 expression in pituitary tumor patients.

LIN-4 was identified in *caenorhabditis elegans* in 1993, and a large number of similar miRNAs were found in succession subsequently (18). Research regarding the relationship of miRNA with pituitary tumor has been carried out for several years, but relatively few substantial achievements have been attained. Nonetheless, the results definitely demonstrate that the pathogenesis of pituitary tumor is closely associated with the abnormal expression of miRNA (19). In the present study, the upstream microRNA-16 decreased cell proliferation and induced apoptosis of HP75 cells in a dose-dependent manner. MicroRNA-16 has been shown to sensitize breast cancer (20), ovarian cancer (21) and hepatocellular carcinoma (22) cells.

The expression of MMP-9 was significantly higher in invasive pituitary tumors than in non-invasive ones, and type IV collagen was markedly reduced in the former (9). Research suggests that in the subclass of MMPs, MMP-9 has the closest association with pituitary tumor invasion (9). The expression level of MMP-9 is notably higher in invasive pituitary tumors than in non-invasive ones, further suggesting the important role of MMP-9 in tumor invasion and metastasis (33). Therefore, MMP-9 can serve as an indicator of tumor invasion (33). Results of the present study indicated microRNA-16 upregulation significantly suppressed MMP-9 protein expression in HP75 cells. Lin et al indicated that osthole suppresses the proliferation of human glioma cells via the upregulation of microRNA-16 and downregulation of MMP-9 (23).

Tumor growth, invasion and metastasis require sufficient nutrition support, and the growth of blood vessels plays an extremely important role during this process (24). Tumor growth depends on angiogenesis, one of the essential conditions for tumor growth, invasion and metastasis (24). VEGF is a highly specific mitogen for vascular endothelial cells, which is a dimer glycoprotein with a molecular weight of...
In-depth research has confirmed that NF-κB degradation of extracellular matrix, and thus promotes tumor progression of MMP transcription. The effect of MMPs allows for the invasive capacities of tumors, and can upregulate the expression of VEGF in vitro demonstrated through experiments (13). In the present study, we found that VEGF2 protein expression in HP75 cells. Yang et al., showed that the downregulation of microRNA-16 significantly impacted the prognosis of colorectal cancer patients targeting VEGF2 (28).

In recent years, great achievements have been attained in research on factors and drugs promoting tumor cell apoptosis, and research on p38MAPK has also achieved considerable progress (29). p38MAPK, which exists in a majority of cells, is a type of important signal system for eukaryotic cells to transfer extracellular signals into cells and thus induce a cell response (30). In addition to enhancing NF-κB expression, activating c-jun and c-fos and participating in other signal transduction, it may mainly influence cell metabolism (31). Furthermore, we found that microRNA-16 upregulation significantly suppressed VEGFR2 protein expression in HP75 cells. Chen et al. reported that microRNA-16 alleviates inflammatory pain through p38 MAPK activation (32).

It is reported that, lowering the incidence of inflammation-associated liver cancer and colon cancer in mouse models results from inhibiting the activation of IKK-β-dependent NF-κB. As previously indicated, the inflammatory response can activate NF-κB, and the continuous activation of NF-κB can further mediate tumorigenesis (33). NF-κB is associated with tumor invasion and metastasis. Furthermore, it can induce tumor cell apoptosis by inhibiting NF-κB activity of tumor cells, and thus inhibits tumor cell growth, as is demonstrated through experiments in vitro and in vivo (15). In-depth research has confirmed that NF-κB is closely related to some malignant solid tumors, such as pancreatic and breast cancer (15). NF-κB activation affects the invasive and metastatic capacities of tumors, and can upregulate the expression of MMP transcription. The effect of MMPs allows for the degradation of extracellular matrix, and thus promotes tumor invasion. NF-κB inhibitor Bay 11-7082 can serve as a new research method to inhibit the NF-κB pathway. Furthermore, it can be used to inhibit MMP expression to achieve the objective of inhibiting the invasive and metastatic capacities of tumor (13). In the present study, we found that VEGF2 suppression reduced the effects of microRNA-16 over-expression on p-p38, NF-κB, MMP-9 and VEGF2 protein expression inhibition in HP75 cells. Yang et al. suggested that microRNA-16 inhibits glioma cell growth and invasion through the NF-κB/MMP-9 signaling pathway.

Taken together, we have demonstrated that microRNA-16 expression suppressed cell proliferation, induced apoptosis and reduced angiogenesis of pituitary cancer through the VEGF2/p38/NF-κB signaling pathway (Fig. 8). Therefore, microRNA-16 may be necessary for pituitary cancer via the VEGF2/p38/NF-κB signaling pathway as a potential therapeutic in clinical application.

References