MicroRNA-18a regulates invasive meningiomas via hypoxia-inducible factor-1α

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Received January 6, 2015; Accepted June 24, 2015

DOI: 10.3892/etm.2015.2630

Abstract. The aim of the present study was to investigate the effects of microRNA-18a (miR-18a) on the invasiveness and metastasis of invasive meningiomas and the underlying mechanism. A total of 69 patients with meningiomas (30 patients in the invasive meningioma group and 39 patients in the non-invasive meningioma group) and 48 cases in the control group were enrolled. Samples of meningioma tissues, serum and cerebrospinal fluid were collected. Reverse transcription-quantitative polymerase chain reaction was performed to quantify the expression levels of hypoxia-inducible factor-1α (HIF-1α) mRNA and miR-18a. Western blot analysis was used to determine protein expression levels of HIF-1α. The expression levels of HIF-1α mRNA and protein in all three types of sample from the invasive meningioma group were significantly higher compared with those in the control and non-invasive meningioma groups ($P<0.05$), and the expression levels of HIF-1α mRNA in the serum and cerebrospinal fluid of the non-invasive meningioma group were significantly higher compared with those in the control group ($P<0.05$). The expression levels of miR-18a in the invasive meningioma group were significantly reduced compared with those in the control and non-invasive meningioma groups ($P<0.05$), whereas the levels of miR-18a in the non-invasive meningioma group were significantly lower compared with those in the control group ($P<0.05$). The expression of HIF-1α is significantly upregulated in patients with invasive meningiomas, possibly due to the downregulation of miR-18a expression. Therefore, miR-18a may regulate invasive meningiomas via HIF-1α.

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Key words: microRNA-18a, hypoxia-inducible factor-1α, meningioma, invasiveness

Introduction

Meningioma is among the most prevalent intracranial tumors, and has the second highest incidence among all intracranial tumors (~19.2%) (1). Although meningiomas are usually classified as benign at early stages, previous studies have identified malignant meningioma types that are fast-growing, invasive and prone to recurrence and metastasis (2,3). According to the standards of the World Health Organization (WHO), meningiomas may be classified into 3 grades and 15 subtypes based on their malignancy. Grade I meningiomas are benign with slow growth and grade III meningiomas are malignant, invasive and prone to recurrence, while the severity of grade II meningiomas is between that of grades I and III (4).

In studies investigating tumorigenesis and tumor treatments, angiogenesis is frequently a key concern (5). Rapid proliferation of tumor tissues and irregular angiogenesis may cause hypoxia within tumor tissues, leading to differences in the expression of regulatory factors, such as hypoxia-inducible factor-1α (HIF-1α) (6). A number of studies have focused on regulatory factors that target HIF-1α. For example, microRNA-18a (miR-18a) has been observed to regulate the expression of HIF-1α in certain tumor tissues (7-9). However, to the best of our knowledge, the effects of miR-18a on HIF-1α expression in invasive meningiomas has not previously been reported. In this study, the expression of miR-18a and HIF-1α was evaluated in patients with invasive meningiomas, with the aim of elucidating the association between them.

Materials and methods

Patients. Between February 2011 and June 2014, 69 patients were confirmed to have meningiomas by pathology, clinical manifestations and radiology in Laiwu City People's Hospital (Laiwu, China). The study population included 38 males and 31 females, who were classified into the invasive meningioma group (30 cases) or non-invasive meningioma group (39 cases) according to-surgical observation and pathological section following surgery. In the control group, 48 healthy subjects were included. Among the 69 cases, 18 cases were WHO grade I meningiomas, including 6 meningothelial cases, 4 fibrous cases, 4 transitional cases, 3 psammomatous cases and 1 angioblastic case; 21 cases were WHO grade II meningiomas, including 9 atypical cases, 7 chordoid cases, and 5 clear cell cases; 30 cases

were WHO grade III meningiomas, including 16 anaplastic cases, 8 papillary cases and 6 rhabdoid cases. The treatment durations ranged between 2 and 56 months, with an average duration of 26.1 months. No patients had received previous treatment for meningioma. Prior to surgery, no patient had received hormones or traditional Chinese medicine, or had any history of radio-chemotherapy. All procedures were approved by the Ethics Committee of Laiwu City People's Hospital and written informed consent was obtained from all patients or their families.

Samples. Three types of sample were collected from the patients. First, tumor tissues (invasive and non-invasive) and normal tissues (control) were collected and stored in liquid nitrogen. Second, fasting peripheral blood was collected from all patients on the morning of the day of surgery, followed by storage in ethylene diamine tetraacetic acid tubes at -20˚C. Third, 2 ml cerebrospinal fluid was collected from all patients during surgeries, followed by centrifugation and storage at -20˚C.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using RNAqueous® Total RNA Isolation Kit (AM1912; Invitrogen Life Technologies). RNA purity was determined using ultraviolet spectrophotometry (NanoDrop 1000; Thermo Fisher Scientific, Waltham, MA, USA) by determination of the A260/A280 ratio. cDNA was obtained by RT; qPCR was performed using a iQ5 real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

The primers for HIF-1α were as follows: Upstream, 5'-GACAAGCCACCTGAGGAGAG-3' (381 bp) and downstream, 5'-GTTCGCATCTTGATAAGGCC-3'. The primers for β-actin were: Upstream, 5'-GGCATGGGTCAGAAGGATTCC-3' (316 bp) and downstream, 5'-ATGTCACGCACGATTTCCCGC-3'. PCR amplification conditions were as follows: Initial denaturation at 94˚C for 2 min; 45 cycles of denaturation at 94˚C for 30 sec, annealing at 60˚C for 1 min, and elongation at 68˚C for 2 min; and final extension at 68˚C for 7 min. The $2^{-\Delta\Delta Ct}$ method was used to calculate HIF-1α/β-actin levels.

The upstream primers for miR-18a and U6 small nuclear RNA (internal control) were 5'-GATAGCAGCACAGAAATATTGGC-3' and 5'-GCGCGTCGTGAAGCGTTC-3', respectively. The common downstream primer for miR-18a and U6 was 5'-GTGCAGGGTCCGAGGT-3'. PCR amplification conditions were as follows: Initial denaturation at 95˚C for 10 min; 40 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 1 min, and elongation at 72˚C for 2 min; and final extension at 72˚C for 7 min. The $2^{-\Delta\Delta Ct}$ method was used to calculate miR-18a/U6 levels.

Western blot analysis. Proteins were extracted and protein concentration was determined using a bicinchoninic acid protein concentration determination kit (RTP7102; Real-Times Biotechnology Co., Ltd., Beijing, China). Protein samples (20 μg) were then subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Subsequently, the resolved proteins were transferred to polyvinylidene difluoride membranes on ice (100 V, 2 h) and blocked with 5% skimmed milk at room temperature for 1 h. Then, the membranes were incubated with polyclonal mouse anti-human HIF-1α (1:2,000; ab6489) and monoclonal mouse anti-human β-actin antibodies (1:5,000; ab6276; Abcam, Cambridge, MA, USA) at 4˚C overnight. After extensive washing, the membranes were incubated with polyclonal anti-mouse IgG-horseradish peroxidase-conjugated antibody (1:3,000; ab34961; Abcam) for 1 h at room temperature. Then, the membrane was developed using an enhanced chemiluminescence detection kit (Sigma-Aldrich, St. Louis, MO, USA) for imaging. Image Lab software, version 3.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to acquire and analyze imaging signals. The relative content of HIF-1α protein was expressed as the HIF-1α/β-actin ratio.

Statistical analysis. All statistical analyses were performed using SPSS software for Windows, version 18.0 (SPSS Inc., Chicago, IL, USA). Results are expressed as the mean ± standard deviation for test of normality. Multi-group measurements were subjected to one-way analysis of variance. In cases of homogeneity of variance, least significant difference and Student-Newman-Keuls methods were used, while in cases of heterogeneity of variance, Tamhane's T2 or Dunnett's T3 method was used. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical data of patients with meningioma. In order to evaluate the clinical parameters of the patients, classification of patients was conducted according to preoperative symptoms and signs, edema band widths, tumor invasion and WHO grades. Preoperative symptoms and manifestations were used to allocate patients into 4 grades (I-IV; Table I). Edema band widths were allocated to 3 grades (I-III; Table II). In addition, tumor invasion was graded as A or B (Table III). Clinical pathological characteristics of tumor patients and control group subjects are shown in Table IV.

Levels of HIF-1α mRNA in tumor tissues, serum and cerebrospinal fluid are markedly elevated in patients with invasive meningiomas. To investigate the levels of HIF-1α mRNA, RT-qPCR was used to measure the HIF-1α mRNA expression levels in tumor tissues, serum and cerebrospinal fluid. The results indicate that the mRNA expression levels of HIF-1α in the tumor tissues of the invasive meningioma group were significantly elevated compared with those in the control and non-invasive meningioma groups ($P<0.01$). In addition, the mRNA expression levels of HIF-1α in the tumor tissues of the non-invasive meningioma group were not significantly different compared with those in the control group ($P>0.05$) (Fig. 1A). Similarly, the mRNA expression levels of HIF-1α in the serum of the invasive meningioma group were significantly increased compared with those in the control and non-invasive meningioma groups ($P<0.01$). Notably, the mRNA expression levels of HIF-1α in the serum of the non-invasive meningioma group were significantly higher compared with those in control group ($P<0.05$; Fig. 1B). Furthermore, the mRNA expression levels of HIF-1α in the cerebrospinal fluid of the invasive meningioma group were significantly higher compared with those in the control and non-invasive meningioma groups ($P<0.01$). The mRNA expression levels of HIF-1α in the cerebrospinal fluid of the non-invasive meningioma group

Table I. Preoperative symptom grading of patients.

Grade	Symptoms
I	No neurological symptoms or focal neurological signs
II	Mild neurological deficits
III	Neurological deficits and difficulty in daily life
IV	Disturbance of consciousness

Table II. Grading of edema surrounding tumor tissues.

Grade	Symptoms
I	Edema band <2 cm
II	Edema band ≥2 cm that is restricted within the hemisphere
III	Edema band crosses hemispheres

Table III. Classification of tumor invasion.

Classification	Symptoms
A	No invasion into tissues surrounding the tumor
B	Invasion into brain tissues, arachnoid, extracranial soft tissues, and intracranial venous sinus

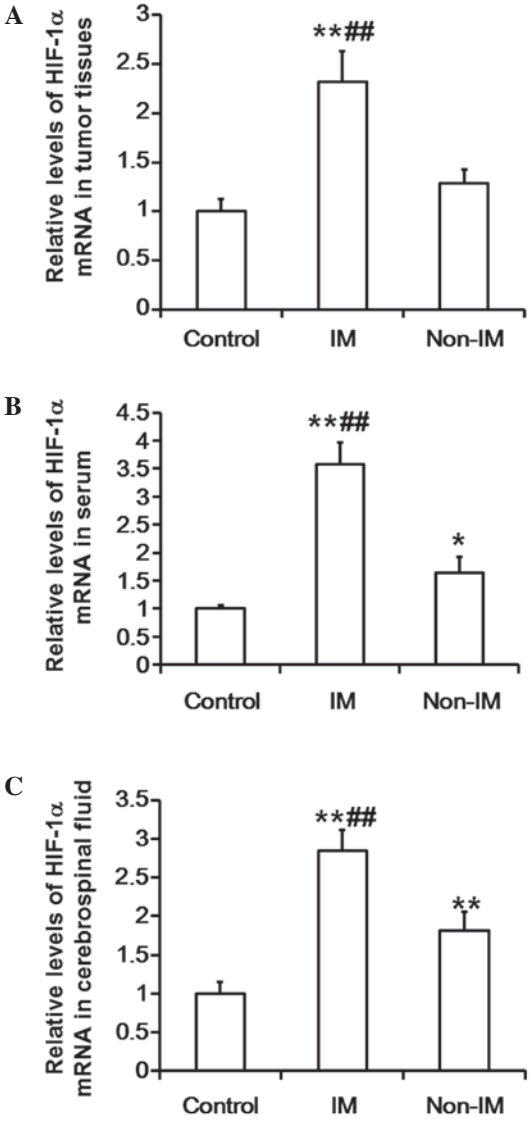

Figure 1. Relative levels of HIF-1α mRNA in (A) tumor tissues, (B) serum, and (C) cerebrospinal fluid. Reverse transcription-quantitative polymerase chain reaction was used to measure mRNA levels, which were then normalized against β-actin levels. All data are expressed as the mean ± standard deviation and presented as bar graphs with error bars. *P<0.05 vs. control group; **P<0.01 vs. control group; $^{\#\#}$P<0.01 vs. non-invasive meningioma group. HIF-1α, hypoxia-inducible factor-1α; IM, invasive meningioma.

were significantly elevated compared with those in the control group (P<0.01; Fig. 1C). These results suggest that the levels of HIF-1α mRNA in the tumor tissues, serum and cerebrospinal fluid of patients with invasive meningioma are markedly increased.

HIF-1α protein expression levels in tumor tissues, serum and cerebrospinal fluid are markedly increased in patients with invasive meningiomas, but not in patients with non-invasive meningiomas. Western blot analysis was employed to determine HIF-1α protein expression levels in tumor tissue, serum and cerebrospinal fluid samples. The results showed that the protein expression levels of HIF-1α in the tumor tissues of the invasive meningioma group were significantly higher compared with those in the control and non-invasive meningioma groups (P<0.05). However, the protein expression levels of HIF-1α in the tumor tissues of the non-invasive meningioma group were not significantly different compared with those in the control group (P>0.05; Fig. 2A). Similarly, the protein expression levels of HIF-1α in the serum of the invasive meningioma group were significantly higher compared with those in the control and non-invasive meningioma groups (P<0.05); however, the protein expression levels of HIF-1α in the serum of the non-invasive meningioma group were not significantly different compared with those in the control group (P>0.05; Fig. 2B). Furthermore, the protein expression levels of HIF-1α in the cerebrospinal fluid of the invasive meningioma group were significantly higher compared with those in the control and non-invasive meningioma groups (P<0.01). However, the protein expression levels of HIF-1α in the cerebrospinal fluid of the non-invasive meningioma group were not significantly different compared with those in the control group (P>0.05; Fig. 2C). These results suggest that HIF-1α protein expression in tumor tissues, serum and cerebrospinal fluid is markedly enhanced in patients with invasive meningiomas, but not in patients with non-invasive meningiomas.

Levels of miR-18a in tumor tissues, serum and cerebrospinal fluid of patients with invasive meningiomas and patients with non-invasive meningiomas are notably reduced. RT-qPCR was used to evaluate the levels of miR-18a in tumor tissue,

Table IV. Clinical pathological characteristics of menangioma patient and control groups.

Patient characteristic	Control group (n=48)	Invasive meningioma group (n=30)	Non-invasive meningioma group (n=39)
Age (years)			
<60	22	19	21
≥60	26	11	18
Gender			
Male	20	20	19
Female	28	10	20
Invasion classification			
A	48	26	28
B	0	4	11
WHO pathological grade			
I	-	0	18
II	-	0	21
III	-	30	0
Tumor diameter (cm)			
<5	-	24	30
≥5	-	6	9
Edema grade			
I	-	5	16
II	-	10	19
III	-	15	4
Preoperative symptom grade			
I	-	4	11
II	-	7	8
III	-	13	15
IV	-	6	5

WHO, World Health Organization.

serum and cerebrospinal fluid samples. The results show that the levels of miR-18a in the tumor tissues of the invasive meningioma group were significantly reduced compared with those in the control and non-invasive meningioma groups ($P<0.01$). In addition, the miR-18a expression levels in the tumor tissues of the non-invasive meningioma group were significantly lower compared with those in the control group ($P<0.05$; Fig. 3A). Furthermore, miR-18a expression levels in the serum of the invasive meningioma group were significantly reduced compared with those in the control group ($P<0.05$), but were not different from those in the non-invasive meningioma group ($P>0.05$). The levels of miR-18a in the serum of the non-invasive meningioma group were significantly lower compared with those in the control group ($P<0.05$; Fig. 3B). The levels of miR-18a in the cerebrospinal fluid of the invasive meningioma group were significantly reduced compared with those in the control and non-invasive meningioma groups ($P<0.01$), and the miR-18a expression in the cerebrospinal fluid of the non-invasive meningioma group was significantly lower compared with that in the control group ($P<0.05$; Fig. 3C). These results indicate that the levels of miR-18a in the tumor tissues, serum and cerebrospinal fluid of patients with invasive meningiomas and patients with non-invasive meningiomas are markedly reduced.

Discussion

In the present study the expression of miR-18a in healthy subjects and patients with invasive meningiomas or non-invasive meningiomas was investigated. In addition, the mRNA and protein expression levels of HIF-1α, a downstream target gene of miR-18a, were evaluated.

Invasive meningioma has emerged as a serious health concern. In contrast with the previously accepted hypothesis that meningioma is a type of benign tumor, invasive meningioma has been demonstrated to be a malignant tumor type due to its invasiveness and metastasis (10,11). The invasiveness of tumors strongly influences the appropriate treatment method. Clinical diagnostic methods for meningioma typically include imaging, puncture biopsy and pathological section biopsy. Imaging inspection depends on the experience of physicians, and is susceptible to subjective misdiagnosis. Puncture biopsy may produce differing diagnostic results due to different sampling sites. Therefore, the gold standard for determining

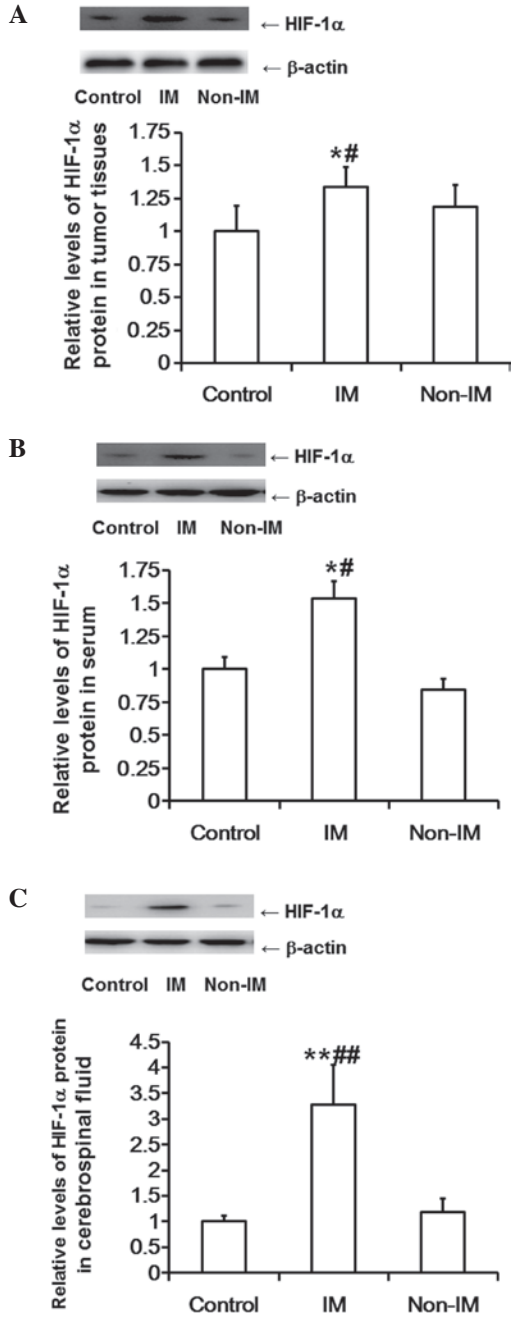

Figure 2. Relative levels of HIF-1α protein in (A) tumor tissues, (B) serum, and (C) cerebrospinal fluid. Western blot analysis was used to measure protein expression, which was then normalized against β-actin levels. All data are expressed as the mean ± standard deviation and presented as bar graphs with error bars. *P<0.05 vs. control group; **P<0.01 vs. control group; #P<0.05 vs. non-invasive meningioma group; ##P<0.01 vs. non-invasive meningioma group. HIF-1α, hypoxia-inducible factor-1α; IM, invasive meningioma.

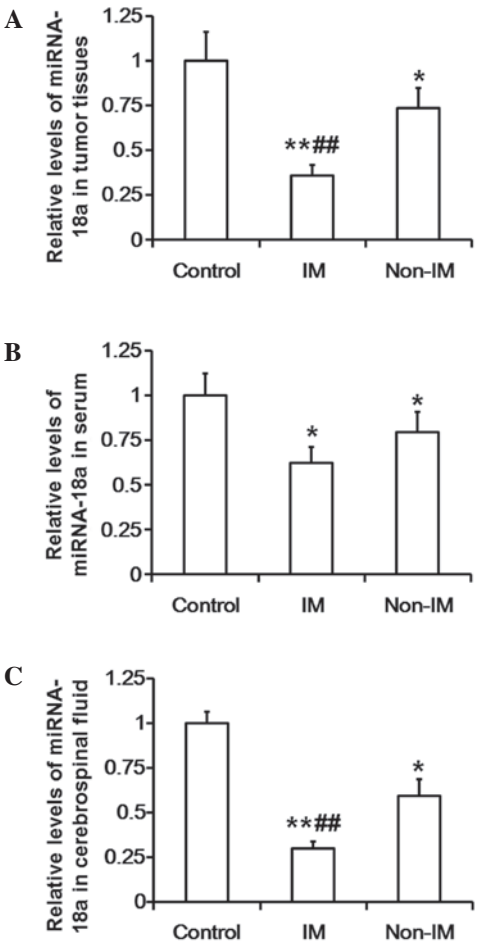

Figure 3. Relative levels of miR-18a in (A) tumor tissues, (B) serum, and (C) cerebrospinal fluid. Reverse transcription-quantitative polymerase chain reaction was used to measure miR-18a levels, which were then normalized against U6 levels. All data are expressed as the mean ± standard deviation and presented as bar graphs with error bars. *P<0.05 vs. control group; **P<0.01 vs. control group; ##P<0.01 vs. non-invasive meningioma group. miR, microRNA; IM, invasive meningioma.

the malignancy of tumors is the pathological section of tumor tissues. However, due to their state of health, or economic circumstances, surgery is not suitable for all patients, which leads to difficulties in determining the appropriate tumor treatment strategy. Therefore, it is necessary to identify more reliable and stable genetic markers for meningioma.

Uncontrolled vascular growth is a marker for tumors, in addition to being a target for tumor treatment. In tissues that are deficient of oxygen, HIF-1α is significantly upregulated, as it is a key transcription factor for the regulation of oxygen homeostasis (12,13). The mechanism underlying this function is the promotion of angiogenesis, which enhances blood supply and ameliorates oxygen deficiency. To a certain extent, differences in HIF-1α expression indicate alterations in angiogenesis that are an auxiliary manifestation of tumor growth.

As tumor blood vessels may be considered to be a potential treatment target, it is necessary to identify the type of mRNA that regulates abnormal vascular growth, and the upstream genes that regulate this type of mRNA. In the current literature regarding gene target therapy for tumors, miRNA regulation pathways are the best understood mechanism. miRNA is a type of non-coding RNA molecule that is able to affect the function of its target genes by inhibiting or enhancing their mRNA and protein expression levels (14,15). miR-18a belongs to the miR17-92 gene cluster, of which HIF-1α is a target gene (16). It has been reported that miR-18a serves a crucial function in the occurrence and development of myocardial ischemia (17), colonic neoplasm (18), liver tumors (19), prostate cancer (20) and malignant gliomas (21). Furthermore, a previous study suggests that miR-18a may be a specific biomarker for the early diagnosis and treatment of tumors (22). Another study observed that miR-18a negatively regulates HIF-1α and inhibits

angiogenesis in tumor tissues (6). Consistent with this finding, the results of the present study indicate that the expression of HIF-1α in patients with invasive meningioma was significantly increased compared with that in control subjects and patients with non-invasive meningioma, while the expression of miR-18a in patients with invasive meningioma was significantly reduced compared with that in control subjects and patients with non-invasive meningioma. Therefore, the present data suggest that miR-18a serves an indicative function in invasive meningiomas, and acts by regulating HIF-1α expression.

As meningiomas occur in the brain, the most complicated region of the human body, it is impractical to obtain samples of meningiomas for the evaluation of HIF-1α expression. However, meningiomas are able to enter the blood circulation and cerebrospinal fluid, which facilitates the indirect determination of the vascular growth and invasiveness of the tumor. In the present study an approximately fixed trend was observed in the association among meningioma grades, HIF-1α expression and miR-18a expression. Therefore, the determination of the expression of HIF-1α and miR-18a in the blood and cerebrospinal fluid may be useful for clinical diagnosis and the evaluation of patient condition.

In contrast with imaging investigation, puncture biopsy and pathological section biopsy, gene markers exhibit unique indicative characteristics, in addition to providing novel targets for the prevention and treatment of diseases. However, the activity and regulatory mechanisms of miR-18a are not fixed across various pathological and physiological conditions, or in different types of tumors, and factors that are associated with invasive meningiomas are not limited to HIF-1α (23-25). In addition, there are abundant miRNAs that may potentially regulate these factors. Therefore, the exact effect and mechanism of action of miR-18a require further investigation in cell, animal and clinical experiments. In conclusion, the results of the present study demonstrate that miR-18a may negatively regulate HIF-1α expression in invasive meningiomas.

Acknowledgements

This study was supported by Dr Quanxiang Wang of Laiwu City People's Hospital.

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