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MicroRNA-374b inhibits migration and invasion of glioma cells by targeting EGFR

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Abstract. – OBJECTIVE: To investigate the expression level of microRNA-374b in glioma tissues and its influence on the invasive ability of glioma cells. Meanwhile, the regulatory mechanism of microRNA-374b in glioma was also explored.

PATIENTS AND METHODS: The expression level of microRNA-374b in 32 glioma tissues and para-cancerous tissues were detected by quantitative Real-time polymerase chain reaction (qRT-PCR). The relationship between microRNA-374b expression and clinical indicators of glioma was analyzed. Meanwhile, the expression of microRNA-374b in glioma cells was verified by qRT-PCR as well. Subsequently, microRNA-374b over-expression model was constructed in glioma cell lines, including U251 and U87. Next, the effect of microRNA-374b on cellular biological functions was analyzed using cell counting kit-8 (CCK-8) assay, Wound healing test and transwell invasion assay, respectively. Finally, the relationship between miRNA and epidermal growth factor receptor (EGFR) was explored.

RESULTS: QRT-PCR results showed that the expression level of microRNA-374b in glioma was significantly lower than that of adjacent tissues, and the difference was statistically significant. Compared with patients with higher expression of microRNA-374b, the occurrence rate of lymph node or distant metastasis was significantly higher in those with lower microRNA-374b expression. In addition, compared with NC group, the proliferation, invasion and migration abilities of cells in microRNA-374b mimics group was significantly decreased. Subsequently, results demonstrated that the expression of EGFR was significantly increased in glioma cells and tissues, which was negatively correlated with microRNA-374b expression. Subsequent cell recovery experiment indicated that microRNA-374b and EGFR had a mutual regulation and could affect the malignant progression of glioma all together.

CONCLUSIONS: MicroRNA-374b could inhibit the invasion and migration of glioma by regulating EGFR. Moreover, the expression of microRNA-374b was significantly associated with lymph node metastasis, distant metastasis and poor prognosis.

Key Words:
MicroRNA- 374b, EGFR, Glioma, Metastasis.

Introduction

Glioma is the most common primary tumor of the central nervous system[1,2]. Despite the continuous advancement of medical procedures such as neurosurgery and radiotherapy and chemotherapy, the prognosis and survival rate of patients with glioma have not been significantly improved. Meanwhile, glioma still seriously affects the normal life and health of patients[1-3]. Currently, great development has been achieved in molecular genetics, epigenetics, as well as new disciplines such as tumor molecular epidemiology and molecular pathology. Furthermore, molecular targeted therapy has shown encouraging prospects in the treatment of glioma[3-6]. Therefore, to explore the molecular mechanisms involved in the proliferation, apoptosis, invasion and metastasis of glioma cells, and to elucidate the mechanism of glioma development are the theoretical basis for establishing molecular diagnostic criteria and molecular targeted therapy[4-7]. MicroRNA (miRNA) is a class of non-coding RNA with about 20-23 nucleotides in length. MiRNA can regulate gene expression by partially or completely complementary to the 3'-untranslated regions (3'-UTR) of downstream target genes, thereby inducing degradation of target mRNAs. Eventually, they may participate in cellular physiological and pathological changes[8-10]. Existing studies[11-14] have shown that miRNAs are closely related to the occurrence and development of glioma. For example, miR-21 is highly expressed in malignant glioma tissues and various glioma cell lines. Meanwhile, inhibition of miR-21 expression can induce cell apoptosis[15, 16]. Besides, miR-221/222 is highly expressed in glioma, which can extend the survival rate of tumor cells by directly targeting pro-apoptotic gene PUMA. Inhibiting the expression of miR-221/222 up-reg-

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ulates the protein expression of p27 and suppresses the proliferation of colloid tumor cells[17,18]. In addition, miR-34a can inhibit the proliferation and invasion of glioma cells and promote cell apoptosis by targeting multiple oncogenes, such as c-Met, Noah-1 and Notch-2[18,19]. Previous studies have also demonstrated that miR-7 is lowly expressed in glioma tissues. However, it can inhibit the proliferation and invasion of glioma cells through regulating epidermal growth factor receptor (EGFR) and Akt pathways[20]. Accumulating findings[11-20] have indicated that increasing the expression of tumor suppressor miRNA or inhibiting the expression of cancer-promoting miRNA exerts a good anti-tumor effect. Therefore, miRNA can be used as a potential therapeutic target and a new choice for glioma treatment in the future[11-14]. Epidermal growth factor receptor (EGFR), a receptor tyrosine kinase, is one of the hotspots in molecular biology research currently[21]. EGFR-mediated signal transduction is closely related to cell proliferation, differentiation and regulation[21,22]. Researches have shown that EGFR over-expression and activation lead to malignant transformation of cells as well as the occurrence and development of tumors. Therefore, the signal transduction pathway involved in EGFR has become a research hotspot for targeted therapy of malignant glioma[23,24]. However, no authors have investigated the mechanism of microRNA-374b on EGFR in glioma cells. In this study, we explored the possible role of microRNA-374b and EGFR in the development of glioma and the underlying molecular regulation mechanism. Our findings might bring new ideas for the diagnosis and treatment of glioma.

Patients and Methods

Patients and Glioma Samples

Tumor tissues and para-cancerous tissues were collected from 32 patients (aged 38-79 years old) who underwent glioma radical resection surgery in our hospital. All patients did not receive any radiotherapy or chemotherapy before surgery. Pathological classification and staging criteria of glioma were performed according to the international collateral cancer staging criteria (international union against cancer, UICC). Informed consents were obtained from patients and their families before the study. Our study was approved by the Ethics Oversight Committee.

Cell Lines and Reagents

Human glioma cell lines (U251, U87, T98-G, A172) and human brain normal glial cell line (HEB) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). High glucose Dulbecco's Modified Eagle's Medium (DMEM) medium and fetal bovine serum (FBS) were purchased from Life Technologies (Gaithersburg, MD, USA). All cells were cultured in high glucose DMEM medium containing 10% FBS, and maintained in an incubator at 37°C with 5% CO_2.

Cell Transfection

U251 and U87 glioma cells were first seeded into 6-well plates and cultured to the density of about 50% to 70%. In NC group and mimics group, 10 μL of miRNA-NC and miRNA-mimics were added for cell transfection, respectively. Subsequently, cells in mimics+NC group or mimics+EGFR group were further transfected with NC or EGFR according to corresponding instructions. Next, cells in above groups were further cultured for 48 hours, followed by collection for quantitative Real-time polymerase chain reaction (qRT-PCR) analysis and cell function experiments.

Cell Proliferation Assay

The proliferation of cells was examined in strict accordance with cell counting kit-8 (CCK-8) kit (Dojindo, Kumamoto, Japan). The main steps were as follows. First, 100 μL of cell suspension (including 2000 cells) was added in each well, followed by the addition of 10 μL of CCK-8 solution. Afterwards, cells were continued to incubate for 1 h in a culture incubator in dark. Lastly, the absorbance of each well at 450 nm was detected by a microplate reader. Wells with the corresponding amount of culture medium and CCK-8 solution but without cells were used as blank controls.

Cell Wound Healing

After transfection for 48 hours, the cells were digested, centrifuged and re-suspended in FBS-free culture. The concentration of cells was adjusted to 5 x 10^5 cells/mL. The density of seeded cells was determined according to the size of cells (the majority of the number of cells plated was set to 50,000 cells/well), and the confluency of the cells reached 90% or more the next day. After scraping a line in the middle of culture wells, cells were rinsed gently with phosphate-buff-

ered saline (PBS) for 2-3 times. 24 hours later, low-concentration serum medium was added in, and the cells were observed. Scratch pre-experiment was used to judge whether the cells had healing ability according to the migration area. Meanwhile, scratch test was performed to evaluate the difference in cell healing ability according to the migration area.

Transwell Migration and Invasion Assay

Cells were seeded into 6-well plates at the concentration of 3×10^5 / well. Liposomal transfection experiments were performed when cell density reached 80%. Positive clones were selected for expanded culture and subsequent transwell experiments. The specific steps were as follows. First, matrigel and serum-free medium were diluted at a ratio of 1:100, and the dilution was used to soak the invasion chamber. 100 μL of diluted matrigel was then added in the chamber. The whole device was placed on a clean bench and sterilized by ultraviolet light overnight before proceeding to the next experiment. Afterwards, 200 μL of serum-free medium was added to the upper chamber; meanwhile, 600 μL was added to the lower chamber to balance the pressure in the chamber. Subsequently, the invading chamber was taken out, and cells on the membrane were fixed with absolute ethanol, followed by staining with crystal violet. Tumor cells that did not invade the stroma were gently wiped off with a cotton swab, and those that had successfully invaded the matrigel were retained. Cells were then counted under a high power microscope (x 200). 10 fields were randomly selected for each sample, and the number of invading cells was counted. Finally, the average value was taken for statistical analysis.

Real-Time Quantitative PCR (QRT-PCR)

Real-time quantitative PCR was used to detect the expressions of EGFR, β-actin, microRNA-374b and U6 in glioma tissues and cells. Total RNA was extracted according to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNA was then reverse transcribed into complementary deoxyribose nucleic acid (cDNA) using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). Primers were designed using Primer 5.0 software. QRT-PCR reaction was performed in strict accordance with SYBR® Premix Ex TaqTM (TaKaRa, Otsu, Shiga, Japan) and StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The following primers were used for qRT-PCR reaction: microRNA-374b: forward: 5'-ATTTTAGAGGAGGGGATT-3', reverse: 5'-TCACTTTAGCAGGCACAC-3'; U6: forward: 5'-CGCAAGGATGACACGCAAATTC-3'', reverse: 5' -TATATCACTCTTGCTTCA-3'; EGFR: forward: 5'-AACGTCTTCTCCCTTCTCTCTCTGTCA-3', reverse: 5'-CCACAGCAGCAGAAACT-3'; β-actin: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-GCTGATCCACATCTGCTGGAA-3'.

Each sample was subjected to a three-well repeated experiment and repeated twice. Bio-Rad PCR instrument (Hercules, CA, USA) was used to analyze and process data. The β-actin and U6 were used as internal parameters. The expression level of relative genes was calculated by the $2^{-\Delta\Delta Ct}$ method.

Western Blot Assay

Pre-cooled protein lysate on ice was added to cells or tissues. After shaking vigorously, they were placed on ice for lysis for 30 min. Total protein in tissue or cells was collected, and the concentration of protein was determined by the Bradford method. Subsequently, protein sample was denatured in a water bath at 100°C for 5 min, with an appropriate amount of loading buffer applied (Beyotime, Shanghai, China). Extracted proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Western blot analysis was performed according to standard procedures. Primary antibodies were EGFR (1:l500), CTGF (1:l500), AKT (1:1000), Erk (1:1000), and corresponding secondary antibodies were anti-mouse and anti-rabbit. X-ray film was pressed in dark. Finally, the proteins were detected by enhanced chemiluminescence (ECL), followed by expose about 60 s for scanning and measuring.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA) was used for all statistical analysis. *t*-test was used to compare the difference between measurement data. Categorical variables were analyzed by χ^2-test or Fisher's exact probability method. Survival analysis was performed using Kaplan-Meier method, and survival curves were plotted. $p<0.05$ was considered statistically significant.

Results

MicroRNA- 374b was Lowly Expressed in Glioma Tissues and Cell Lines

The expression level of microRNA-374b in glioma tissues was significantly lower than that of adjacent tissues, and the difference was statistically significant (Figure 1A and 1C). In addition, compared with HEB cells, microRNA-374b was significantly down-regulated in glioma cells, especially in U251 and U87 cells. Therefore, we chose these two cell lines for subsequent experiments (Figure 1B).

MicroRNA-374b Expression was C orrelated with Lymph Node and Distance Metastasis in Glioma Patients

According to 32 pairs of glioma tumor tissues and para-cancerous tissues, the relationship between microRNA- 374b expression and age, sex, clinical stage, lymph node metastasis and distant metastasis of glioma patients was analyzed. As shown in Table I, lowly expressed microRNA-374b was positively correlated with glioma lymph node or distant metastasis, whereas was not correlated with age and gender. In addition, to explore the relationship between the expression of microRNA-374b and the prognosis of patients with glioma, relevant follow-up data was collected. Kaplan-Meier survival curves showed that low expression of microRNA-374b was significantly associated with poor prognosis of glioma (p<0.05; Figure 1D).

Over-Expression of microRNA-374b Inhibited the Proliferation, Migration and Invasion of Glioma Cells

To explore the effects of microRNA-374b on glioma cell functions, we first successfully constructed microRNA-374b over-expression mod-

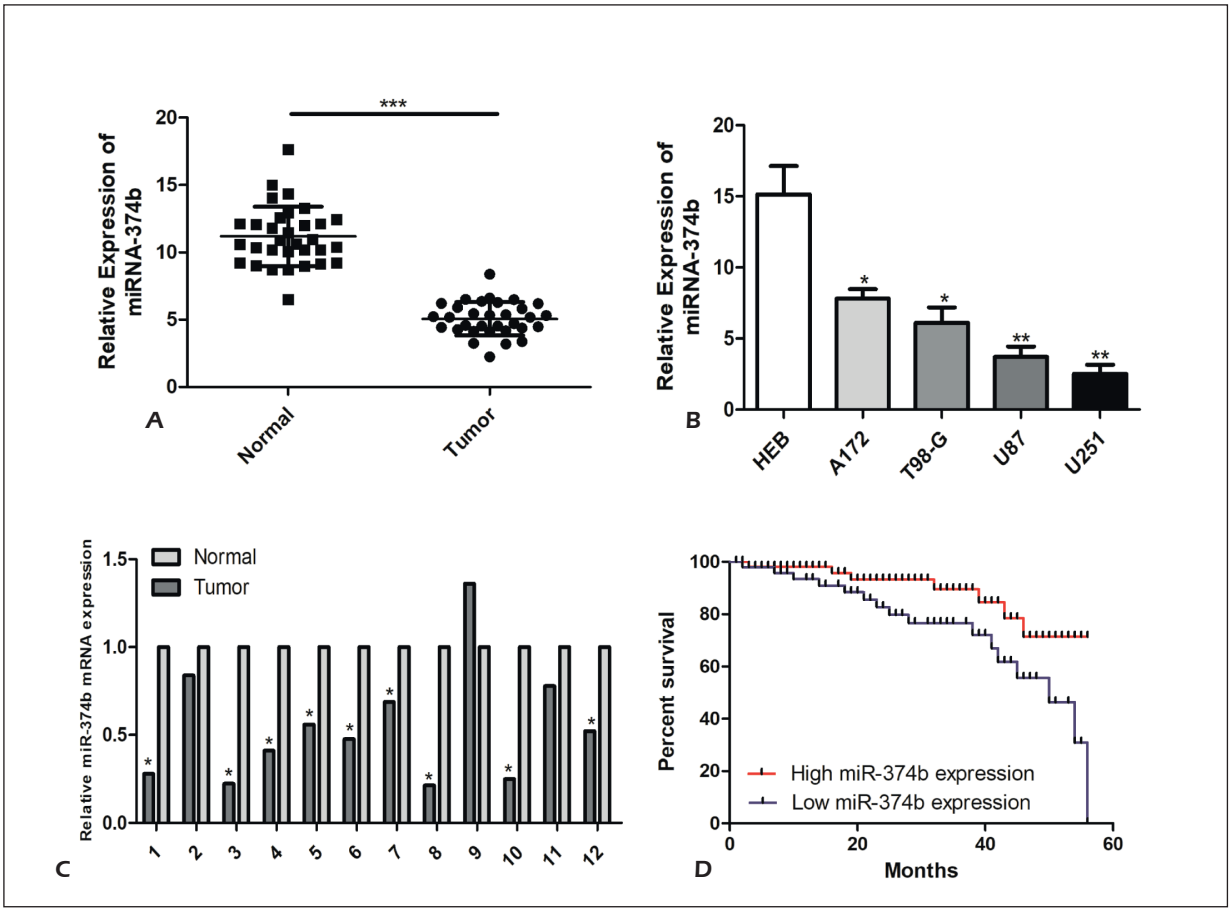

Figure 1. MiRNA-374b was lowly expressed in glioma tissues and cell lines. **A**, QRT-PCR detection of miRNA-374b expression in glioma tumor tissues and para-cancerous tissues; **B**, QRT-PCR detection of miRNA-374b expression level in glioma cell lines; **C**, QRT-PCR detection of 12 differential expression of miRNA-374b in glioma tumor tissues and para-cancerous tissues; **D**, Kaplan Meier survival curve of glioma patients based on miRNA-374b expression. Patients with low expression of miRNA-374b exhibited significantly worse prognosis when compared with patients in high expression group. Data were expressed by mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

Table I. Association of miRNA-374b expression with clinicopathologic characteristics of glioma.

Parameters	No. of cases	miRNA-374b expression		*p*-value
		High (%)	Low (%)	
Age (years)				0.492
< 60	17	10	7	
≥ 60	15	7	8	
Gender				0.492
Male	15	7	8	
Female	17	10	7	
T stage				0.907
T1-T2	21	11	10	
T3-T4	11	6	5	
Lymph node metastasis				0.028
No	23	15	8	
Yes	9	2	7	
Distance metastasis				0.011
No	22	15	7	
Yes	10	2	8	

According to the Union for International Cancer Control.

el. Transfection efficiency was verified by qRT-PCR (Figure 2A). Cell proliferation, invasion and migration experiments were then performed in U251 and U87 cell lines, respectively. CCK-8 results indicated that the proliferation of cells in microRNA-374b mimics group was significantly decreased, and the difference was statistically significant (Figure 2B). In addition, transwell migration and cell scratch assay were used to explore the effects of microRNA-374b on migration and invasion of glioma cells. The results showed that overexpression of microRNA-374b significantly decreased cell invasive ability when compared with NC group (Figure 2C). In addition, similar results were obtained from transwell migration and invasion assay. The above findings suggested that cell migration and invasion was remarkably inhibited (Figure 2D).

EGFR was Highly Expressed in Glioma Tissues and Cell Lines

Subsequent bioinformatics research predicted that microRNA-374b and EGFR might have some relationship in glioma tissues. QRT-PCR results found that EGFR expression level was significantly increased in glioma tissues when compared with para-cancerous tissues (Figure 3A). In addition, EGFR expression in glioma cells was significantly higher than HEB cells, and the difference was statistically significant (Figure 3B). Therefore, we detected the expressions of microRNA-374b and EGFR in 32 glioma tumor tissues and adjacent tissues by qRT-PCR. Results demonstrated that the expression level of microRNA-374b was negatively correlated with that of EGFR (Figure 3C). Similarly, Western Blot indicated that in glioma cell lines, over-expression of microRNA-374b could significantly reduce the protein expression levels of EGFR, CTGF, AKT and Erk. These results indicated that there might be a close relationship between miRNA-374b and EGFR-related proteins (Figure 3D).

MicroRNA-374b Modulated EGFR Expression in Human Glioma Cells

To further explore the underlying mechanism in which microRNA-374b inhibited the malignant progression of glioma, we overexpressed EGFR in glioma cell lines. Cells were transfected with NC and microRNA- 374b mimics, and the relationship between the two molecules was investigated. QRT-PCR and Western Blot experiments were performed to examine the transfection efficiency of EGFR over-expression (Figures 4A and 4B). Subsequently, transwell migration assay and cell scratch assay showed that overexpression of EGFR significantly increased the number of glioma cells. Meanwhile, cell invasive ability in the transwell chamber of microRNA-374b mimics group was also remarkably increased, thereby offsetting the effect of miRNA-374b over-expression on invasion and metastasis of glioma cells (Figure 4C and 4D).

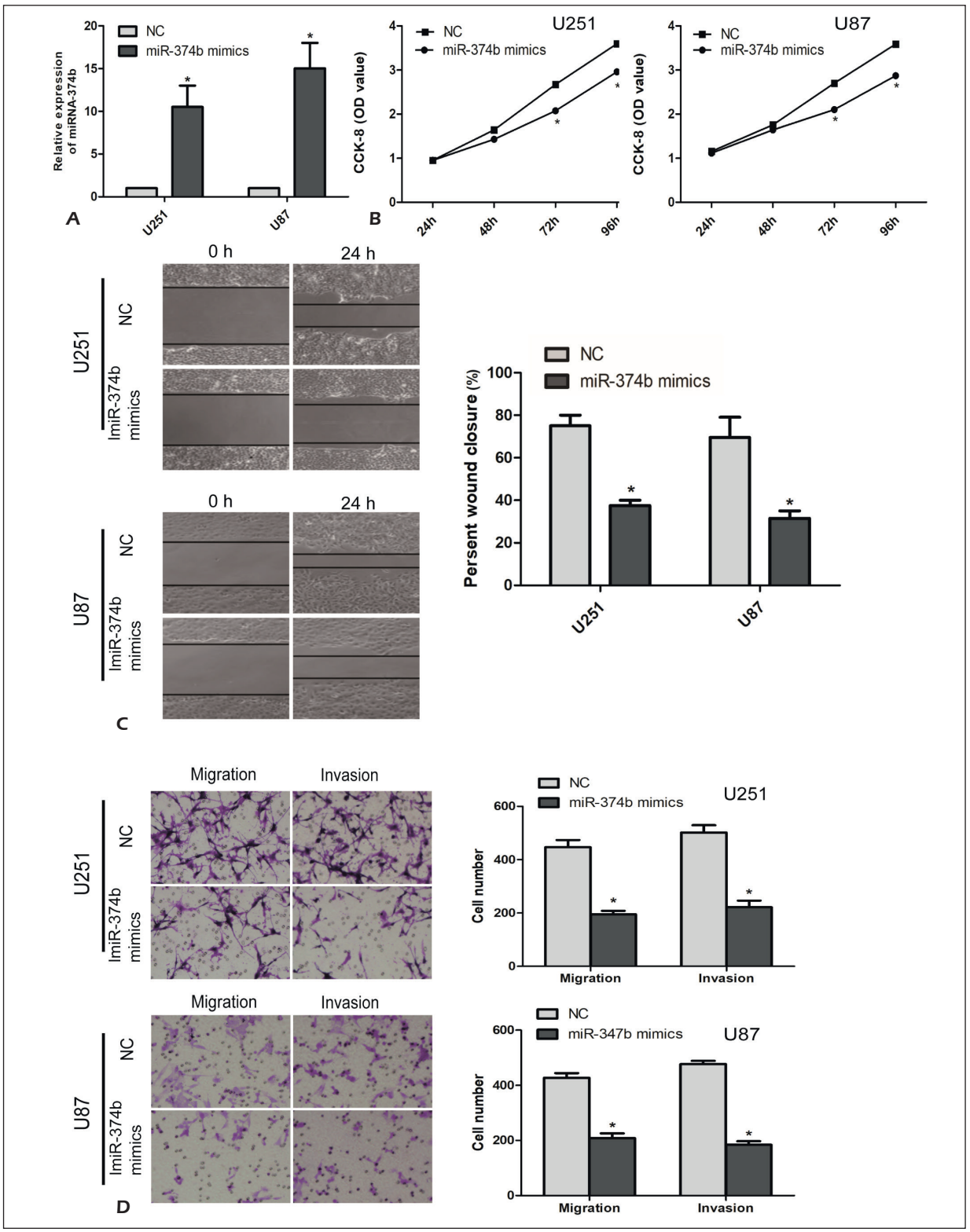

Figure 2. Over-expression of miRNA-374b inhibited glioma cell proliferation, invasion and migration. **A**, QRT-PCR validated the interference efficiency of miRNA-374b in U251 and U87 cell lines; **B**, CCK-8 assay detected the proliferation of U251 and U87 cell lines after miRNA-374b mimics transfection; **C**, Transwell migration and invasion assay were used to detect the invasion and migration of U251 and U87 cells after miRNA-374b mimics transfection (Magnification: 40×); **D**, Cell scratch test in U251 and U87 cell lines indicated that miRNA-374b mimics transfection could inhibit glioma cell crawling ability (Magnification: 40×). Data were expressed by mean ± SD, *p<0.05.

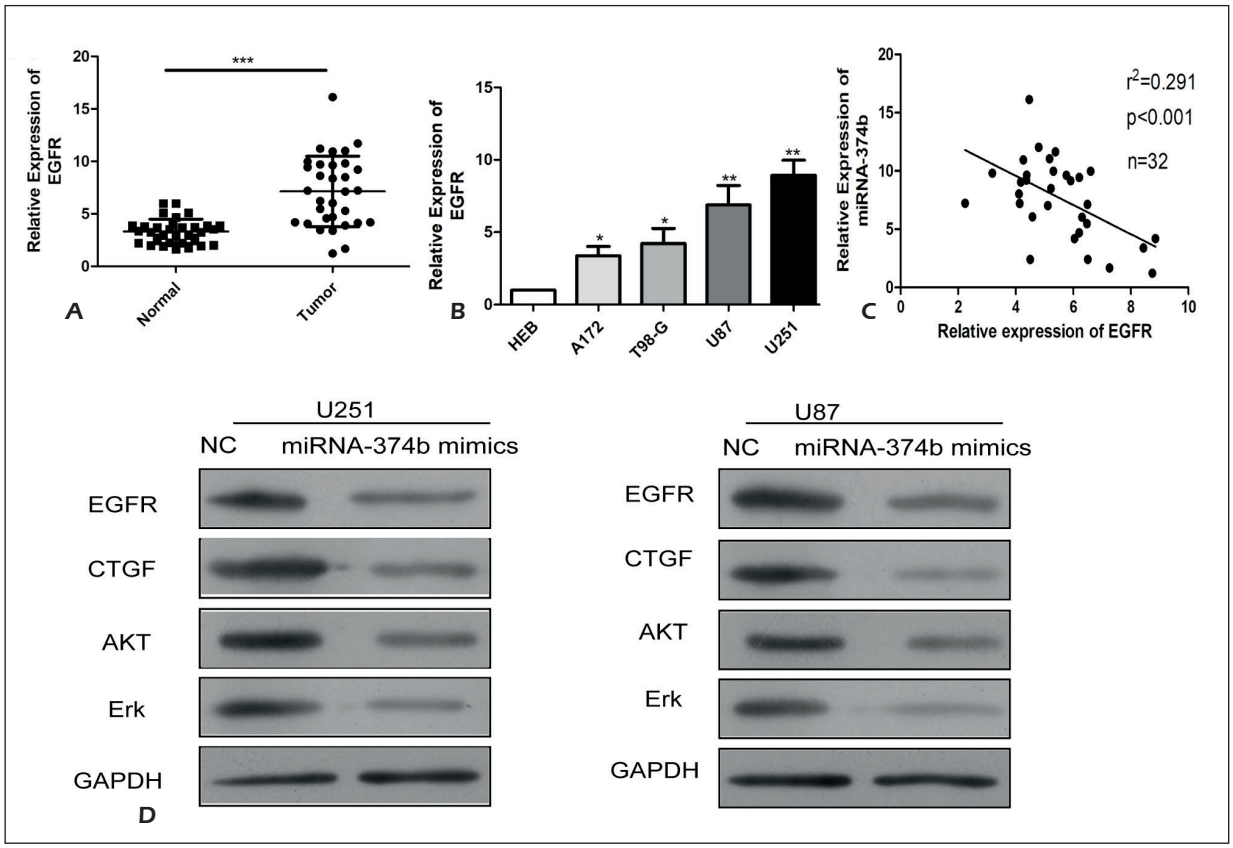

Figure 3. EGFR was highly expressed in glioma tissues and cell lines. **A**, QRT-PCR detection of EGFR expression in glioma tumor tissues and para-cancerous tissues; **B**, QRT-PCR detection of EGFR expression level in glioma cell lines; **C**, Significant negative correlation was found between miRNA-374b and EGFR expression levels; **D**, Western Blot verified the protein expression levels of EGFR, CTGF, AKT and Erk after transfection of miRNA-374b mimics in U251 and U87 cell lines. Data were expressed by mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

Discussion

Glioma is the most common primary intracranial tumor with multiple invasive growth and unclear borders. Tumor cells usually grow in brain tissue within 2 cm of the tumor, and the pathological grade is progressively worse[1-3]. Studies on the unique biological characteristics and cell dynamics around the tumor have shown that tumor cells in the sub-clinical region of normal brain tissue around the tumor exhibit proliferative capacity[2,3]. Traditional treatment of glioma is mainly based on surgical treatment, which is combined with radiation therapy and chemical therapy. However, other characteristics of glioma, such as special location, invasive growth, poor differentiation, and progressive deterioration of pathological grade, make it difficult to be totally treated[3]. Due to high incidence, high recurrence rate and poor therapeutic effect of glioma, neurosurgical scholars' enthusiasm for glioma research has never diminished[1-3]. Molecular genetics and epigenetics have always been the hotspots of tumor research. The establishment of new disciplines, including tumor molecular epidemiology and molecular pathology, has further revealed the occurrence, development, proliferation and invasion of central nervous system tumors. Meanwhile, the molecular mechanism of neovascularization is demonstrated, which may uphold the molecular targeted therapy for glioma[4-6]. Currently, molecular targeted therapy is developing from the initial single target inhibition to multi-target therapy. Furthermore, the interaction between multi-target cell signaling pathways and bypass activation has become a research hotspot of targeted therapy[5,6]. MiRNAs are a class of endogenous, non-coding, small-molecule single-stranded RNAs with about 20-25 nt in length. They are one of the important epigenetic regulatory molecular[7-9]. The maturation process of miRNA is sim-

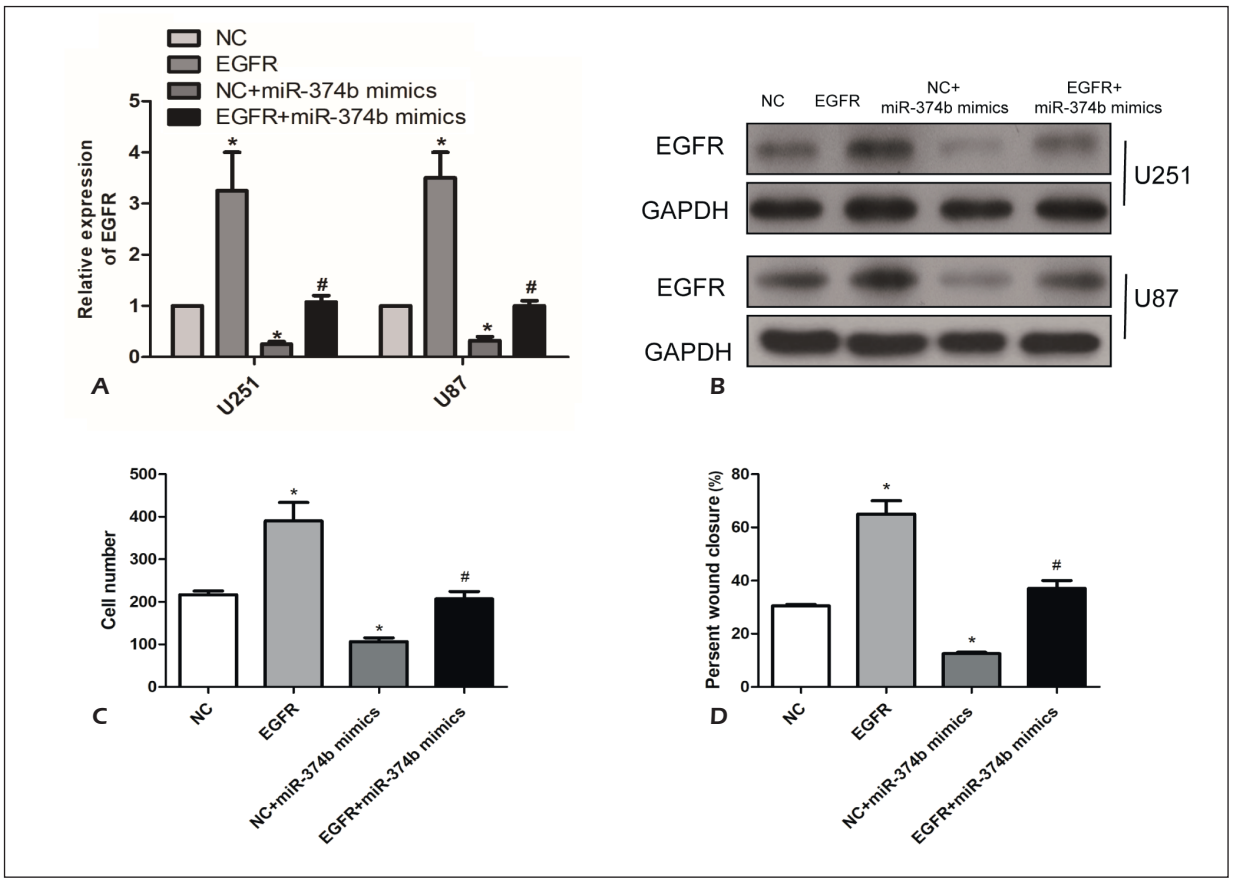

Figure 4. MiRNA-374b regulated the expression of EGFR in glioma tissues and cell lines. **A**, EGFR expression levels in miRNA-374b and EGFR co-transfected cell lines were detected by qRT-PCR; **B**, EGFR expression level in miRNA-374b and EGFR co-transfected cell lines was detected by Western Blot; **C**, Transwell migration assay was used to detect the role of miRNA-374b and EGFR co-transfection in regulating the invasion and migration of glioma cells; **D**, Cell scratch assay was performed to detect the ability of tumor cells to crawling after co-transfection of miRNA-374b and EGFR. Data were expressed by mean ± SD, *#p<0.05.

ilar to that of non-coding small interfering RNA (siRNA). Previous studies[8,10] have demonstrated that it is produced by the cleavage of Dicer by a single-stranded pre-miRNA with a hairpin structure of about 70-90 nt. Mature miRNAs can specifically bind to the 3'-UTR of target mRNAs by base complementary pairing. Eventually, this may promote the degradation of target mRNAs at the post-transcriptional level and/or inhibit translation, playing a role in regulating gene expression[9-11]. Abnormal expression of miRNA is closely related to the occurrence and development of malignant tumors. Meanwhile, this is an important factor for tumor cell immortalization, apoptosis inhibition, invasion, metastasis, tumor cell interstitial remodeling and tumor angiogenesis[11-14]. Therefore, searching for abnormally expressed miRNAs in tumor tissues, determining their target mRNAs, and clarifying the function of target mRNA expression products, are key steps to elucidate the mechanism of tumorigenesis and development. Meanwhile, they are also necessary theoretical basis for the application of miRNA-targeted therapy[7-10]. MiRNAs are classified into tumor-inhibiting miRNAs (TS-miRNAs) and onco-miRNAs (onco-miRNAs). A large number of miRNAs with abnormal expression, such as miRNA-21 and miRNA-128, have been found in human glioma cells. For example, miRNA-146b is a TS-miRNA of glioma, which inhibits the proliferation and metastasis of glioma cells. However, miRNA-26a is an onco-miRNA of glioma, whose overexpression can promote the formation of glioma[10-18]. In this study, we investigated the effects of microRNA-374b on the biology of glioma cells. The results showed that microRNA-374b was significantly down-regulated in glioma tissues and cells, suggesting that microRNA-374b exerted a potential tumor suppressing role in glioma. To

explore the role of microRNA-374b in the development and progression of glioma, the expression of microRNA-374b in 32 paired glioma tissues and para-cancerous tissues was detected by qRT-PCR. The results indicated that microRNA-374b expression in glioma tissues was significantly down-regulated when compared with that of adjacent tissues. Besides, microRNA-374b expression was found negatively correlated with the occurrence rate of lymph node and distant metastasis. Therefore, we believed that microRNA-374b might act as a tumor-suppressing molecular in the development of glioma. Tumor metastasis is a process in which tumor cells are scattered from the *in situ* to the distal target organ and adapt to the new tissue microenvironment. Two conditions that must be met for the smooth implementation of the process are movement and survival. To further explore the effect of microRNA-374b on the biological function of glioma, we constructed microRNA-374b over-expression model using lentivirus transfection. The results of cell wound healing assay and transwell assay showed that microRNA-374b could promote the metastasis of glioma cells. However, its specific molecular mechanism remained unclear. To clarify the biological function of miRNA, we further searched for its target gene. Meanwhile, its influence on tumor development was explored. It was found that the expression of EGFR in tumor tissues was significantly up-regulated when compared with matched adjacent tissues. In addition, the expression level of EGFR was found highest in U251 and U87 cell lines, which laid the foundation for subsequent experiments. Our results further verified that over-expression of microRNA-374b significantly down-regulated mRNA and protein expression of EGFR. Subsequent recovery experiment verified that over-expression of EGFR significantly increased the number of glioma cells and cell invasive ability in the transwell chamber of microRNA-374b mimics group, thereby counteracting the effect of microRNA-374b on glioma cell function. Therefore, our findings demonstrated that microRNA-374b might inhibit the malignant progression of glioma by regulating EGFR.

Conclusions

We found that microRNA-374b could inhibit the invasion and migration of glioma cells by regulating EGFR. Meanwhile, it was significantly associated with lymph node or distant metastasis as well as poor prognosis of glioma patients.

Conflict of Interests

The authors declared no conflict of interest.

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