

MiR-130b functions as a tumor promoter in glioma via regulation of ERK/MAPK pathway

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Abstract. – OBJECTIVE: To investigate the miR-130b expression in patients with glioma and to analyze its role and underlying molecular mechanism on the carcinogenesis.

PATIENTS AND METHODS: The expression levels of miR-130b were detected with quantitative Real-time PCR. The relationship between miR-130b expression and clinicopathologic characteristics were analyzed. MiR-130b inhibitor was transfected into glioma cell lines to investigate its role in HCC. MTT assays were conducted to explore the impact of miR-130b down-expression on the proliferation of human glioma cells. Cell cycle and cell apoptosis assays were performed using flow cytometry. Levels of ERK/MAPK pathway related proteins were evaluated by Western blotting. Data were analyzed using the 2- $\Delta\Delta$ CT method through student's t-test via the GraphPad Prism software (La Jolla, CA, USA).

RESULTS: The expression of miR-130b was markedly upregulated in glioma cell lines and tissues, and high miR-130b expression was significantly associated with advanced WHO grade ($p = 0.022$) and low Karnofsky performance score ($p = 0.001$). In addition, downregulation of miR-130b inhibited the proliferation of glioma cells and induced cell-cycle arrest and cells apoptosis in vivo. Importantly, ERK/MAPK pathway was found to be inactivated in the glioma cell lines after miR-130b knockout experiment.

CONCLUSIONS: The current data indicated that miR-130b may play a critical role in the progression of glioma via ERK/MAPK signaling cascades, suggesting that it may be a useful therapeutic agent in glioma patients.

Key Words:

miR-130b, Glioma, ERK/MAPK, Proliferation, Apoptosis.

system^{1,2}. In clinical treatment, with the advanced in surgery combined with chemotherapy, the 5-year survival rate of patients with glioma has been raised to 60%^{3,4}. However, the median survival time for patients with high-grade glioma is around 14-15 months⁵. Although previous studies have achieved markedly advances in understanding of the tumorigenesis of glioma, elucidating the detailed mechanisms of glioma has been a challenging task for researchers. MicroRNAs (miRNAs) are evolutionarily conserved, endogenous non-coding RNAs of about 22 nucleotides⁶. It has been known to us that miRNAs could modulate gene expression at the post-transcriptional level by mainly binding to 3'-UTR of target messenger RNAs (mRNAs)⁷. In human glioma, miRNAs are broadly involved in many aspects of cancer development, such as proliferation, metastasis, or apoptosis^{8,9}. Growing evidence showed that miRNAs can act as either oncogenes or tumor suppressors, through different mechanisms that finally contribute to tumor formation and progression¹⁰⁻¹². Previous researches^{13,14} showed that miR-130b played a critical role in various tumors, including glioma. However, the relationship between glioma and the expression of miR-130b has not been elucidated yet. In the present work, we determined the expression levels of miR-130b in glioma tissues and cells by PCR. We further explored the especially effect of miR-130b in the glioma cells proliferation and apoptosis. Then, we investigated the molecular mechanisms of how miR-130b regulates the proliferation and apoptosis of glioma.

Introduction

Glioma is one of the most common types of primary brain tumors in adults, and accounts for a majority of malignances of the central nervous

Patients and Methods

Clinical Specimens

Human glioma specimens were obtained from primary patients diagnosed with glioma who

underwent gross total tumor resection at the Department of Linyi People's Hospital, from April 2011 to April 2013. All the tissues were obtained at the time of surgery and immediately stored in liquid nitrogen until use. Clinicopathologic information about the patient samples used in this study is summarized in Table I. The study was approved by the Research Ethics Committee of Linyi People's Hospital and all participants provided written informed consent before enrolment.

Cell Culture and Transfection

Human glioma cell lines U251, U87, U118 and LN18 were purchased from the Cell Bank of Chinese Academy of Sciences (Pudong, Shanghai, China). All glioma cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 100 μ L fetal bovine serum (FBS), 10 μ L penicillin, and 10 μ L streptomycin per mL medium at 37°C with 5% CO₂. A small interfering RNA (siRNA) designed to target miR-130b (Qiagen, Hilden, Germany) and a matched negative control oligonucleotide were purchased from Invitrogen (Carlsbad, CA, USA). U251, U118 and NHA cells were seeded into 6-well culture plates and transfected at 70-80% confluence with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA Extraction and Quantitative Real-time (qRT)-PCR

The total RNAs were extracted from cells and tissues with Trizol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA template was amplified by

Real-time PCR using the SYBR Premix Dimmer Eraser kit (TaKaRa, Dalian, China). Fluorescence quantitative PCR reactions were performed with a miScript SYBR Green PCR Kit (Tiangen, Beijing, China). Cycle passing threshold (Ct) was recorded and normalized to RNU6B expression. The primers for miR-130b and RNU6B were designed and purchased from Biosystems (Foster City, CA, USA). All experiments were done in triplicate.

Cell Proliferation and Colony Formation Assays

Cell viability was detected using the MTT assay. Cells were seeded in 96-well plates at a density of 2×10^3 cells per well and incubated for 1, 2, 3, 4, and 5 days. The absorbance wavelength was measured at 570 nm, and 620 nm as the reference wavelength. For colony formation, transfected U251 and U118 cells were re-suspended and seeded onto six-well plates at a density of 1000 cells/well and cultured for 2 weeks and then were stained with 0.5% crystal violet for 30 min. The percentage of colony formation was calculated by adjusting control cells to 100%.

Cell Cycle and Apoptosis Analysis

Cells were collected by trypsinization, fixed in ice-cold 75% ethanol in PBS, counted, and treated with 20 mg/mL RNase. Cells were analyzed by flow cytometry (FACS Calibur; Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). A total of 20,000 events were collected for each sample. For the apoptosis analysis, the stained cells were analyzed by flow cytometry

Table I. Relationship between miR-130b expression with clinicopathological features.

Clinicopathological features	Patients (n)	miR-130b expression		p
		Low	High	
Age (years)				0.790
< 50	41	21	20	
≥ 50	72	35	37	
Sex				0.371
Male	43	19	24	
Female	70	37	33	
WHO grade				0.022
Low, I-II	73	42	31	
High, III-IV	40	14	27	
KPS score				0.001
< 90	51	18	33	
≥ 90	62	38	24	
Tumor size				0.644
< 5 cm	48	25	23	
≥ 5 cm	65	31	34	

BD Biosciences (Franklin Lakes, NJ, USA). Data analysis was performed using CellQuest software BD Biosciences.

Western Blotting

Protein extracted from cells using PIPA lysis buffer (Beyotime, Jiangsu, China) was measured with the bicinchoninic acid method (Pierce, Rockford, IL, USA). Membranes were blocked by 5% non-fat milk and incubated with anti-ERK/MAPK-related protein antibody (Abcam, Cambridge, MA, USA) or anti- β -actin antibody (Abcam, Cambridge, MA, USA). After three washes with tris-buffered saline with Tween-20 (TBST-20), horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000; Bioss Inc., Beijing, China) were added and incubated for 1 h. Results were detected by the chemiluminescent detection system. All transfection experiments were repeated three times independently.

Statistical Analysis

Data were imaged with GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Groups were compared with Student's *t*-test. The correlation between expression levels of miR-130b and clinicopathological parameters was analyzed with Fisher's exact test. All statistical tests were two-sided. $p < 0.05$ was considered statistically significant.

Results

miR-130b Expression in Glioma Tissues and Cell Lines

Firstly, we explored miR-130b levels in clinical samples by comparing miR-130b expression in glioma tissues with normal brain tissues. The

results of PCR showed that miR-130b exhibited a higher expression level in glioma tissues compared with normal brain tissues ($p < 0.01$, Figure 1A). In addition, higher expression of miR-130b was observed in glioma patients with high-grade compared with those with low-grade grade ($p < 0.01$, Figure 1B). Moreover, miR-130b expression was also significantly increased in four-glioma cell lines compared with that of NHA cells ($p < 0.01$, Figure 1C).

Association between miR-130b Upregulation and Clinicopathological Parameters of Patients With Glioma

To further analyze the clinicopathological significance of miR-130b in glioma, we divided patients into two groups: high-miR-130b expression group and low-miR-130b expression group, based on the median value of miR-130b. As shown in Table I, we found that high miR-130b expression was significantly associated with advanced WHO grade ($p = 0.022$) and low Karnofsky performance score (KPS) ($p = 0.001$). However, there was no significant association between miR-130b expression level and age, sex, and tumor size (all $p > 0.05$).

Knockdown of miR-130b Expression Inhibited Cancer Cell Proliferation

To characterize the function of miR-130b cancer cell proliferation, miR-130b inhibitor or negative control were transiently transfected into human glioma cells lines. Successful decrease of miR-130b expression in U251 and U118 cells was confirmed by qRT-PCR (Figure 2A). Then, as shown in Figure 2B-C, U251 and U118 cells transfected with miR-130b inhibitor displayed a significant growth inhibition com-

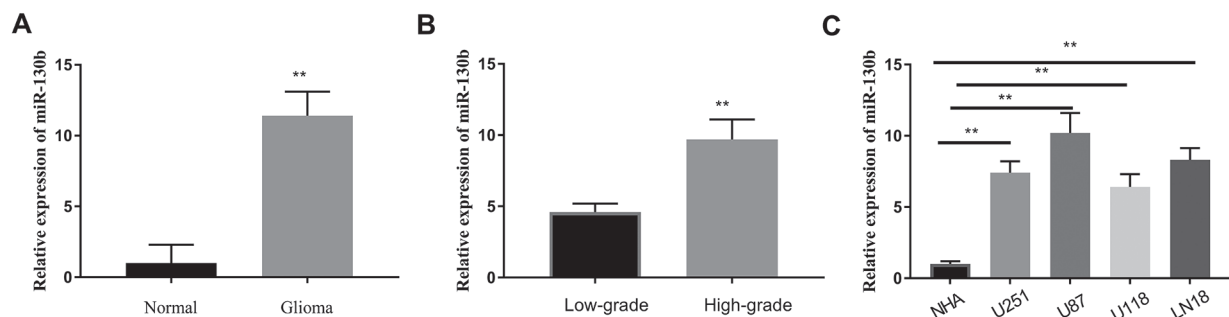


Figure 1. The expression of miR-130b was upregulated in glioma tissues and cell lines. **(A)** miR-130b expression in glioma tissues and normal brain tissues. **(B)** qRT-PCR analysis of miR-130b expression in low-grade glioma and high-grade glioma. **(C)** The relative expression levels of miR-130b were determined by qRT-PCR in the human glioma cell lines (U251, U87, U118 and LN18) as well as in normal human astrocytes (NHA). * $p < 0.05$, ** $p < 0.01$.

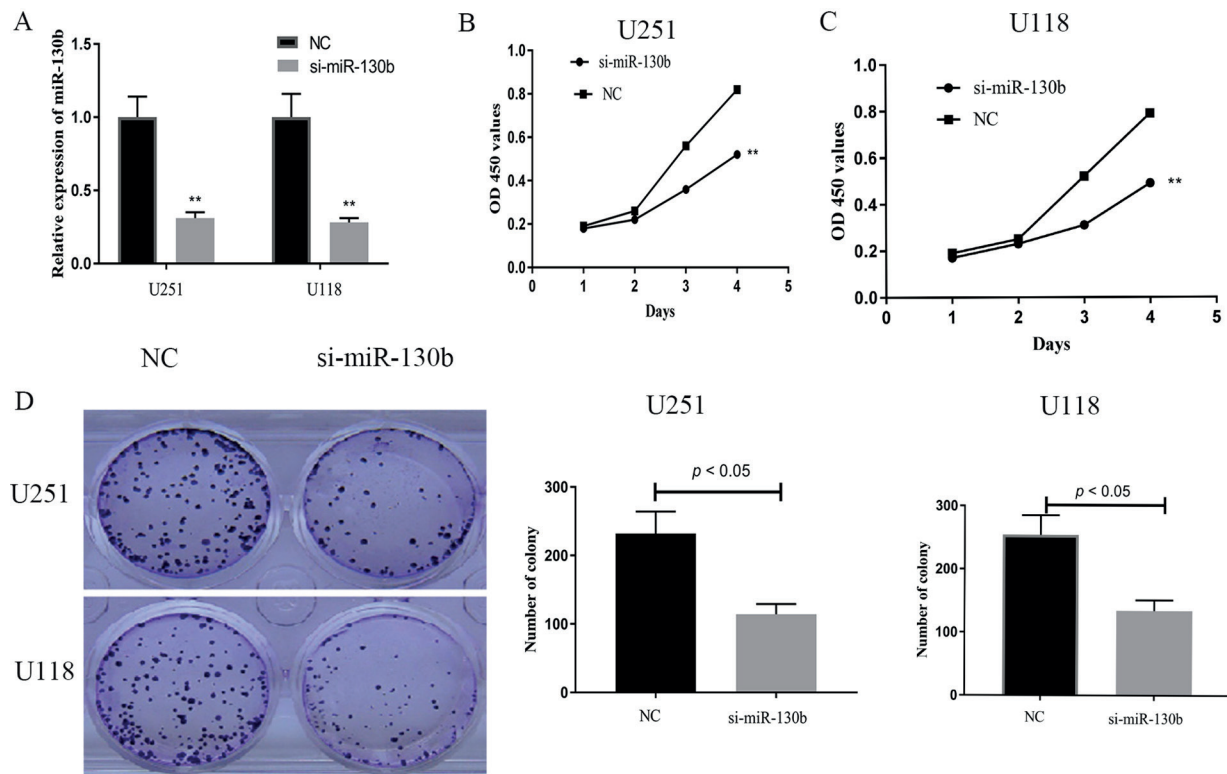


Figure 2. Knockdown of miR-130b inhibited proliferation of glioma cell lines. **(A)** Expression of miR-130b was determined by qRT-PCR after transfection of miR-130b inhibitors or scramble. **(B, C)** MTT assay was performed to examine proliferation of U251 and U118 cells at different time points. **(D)** Colony formation assay of U251 and U118 cells. * $p < 0.05$, ** $p < 0.01$.

pared to those transfected with miR control. In addition, the colony forming experiment revealed that inhibition of miR-130b also significantly suppressed colony formation ability (Figure 2C).

Cell Cycle and Apoptosis

Based on that miR-130b knockdown can suppressed cell proliferation, we further detected its impact on cell cycle. The results showed that si-miR-130b of U251 and U118 had an obvious cell-cycle arrest at the G1-G0 phase and had a decreased at G2-S phase (Figure 3A). Moreover, we investigated the role of miR-130b on cell apoptosis. The result of flow cytometry showed that transfection with anti-miR-130b promoted apoptosis in glioma cell lines.

Deregulation of ERK/MAPK Pathway is Associated with Inhibition of miR-130b

ERK/MAPK signaling has been proved to be associated with various disease pathologies. We

performed Western blot to detect the expression levels of the ERK/MAPK pathway related proteins in U251 cell lines. As shown in Figure 4, the level of phosphorylated MEK1/2, ERK1/2, MAPK and JNK1/2/3 decreased significantly in U118 cells transfected miR-130b inhibitor compared with control cells. These data revealed that miR-130b may play a role in glioma via the ERK/MAPK pathway.

Discussion

It has been frequently reported that the functions of miRNAs are related to tumorigenesis¹⁵. Previous studies indicated that miR-130b functioned as a tumor promoter in various tumors, and some potential mechanism has been identified. For instance, Leone et al¹⁶ found that up-regulation of miR-130b promoted thyroid adenomas proliferation by targeting CCDC6 gene. Egawa et al¹⁷ reported that miR-130 promoted cell migration and invasion in bladder cancer through FAK and Akt phosphorylation

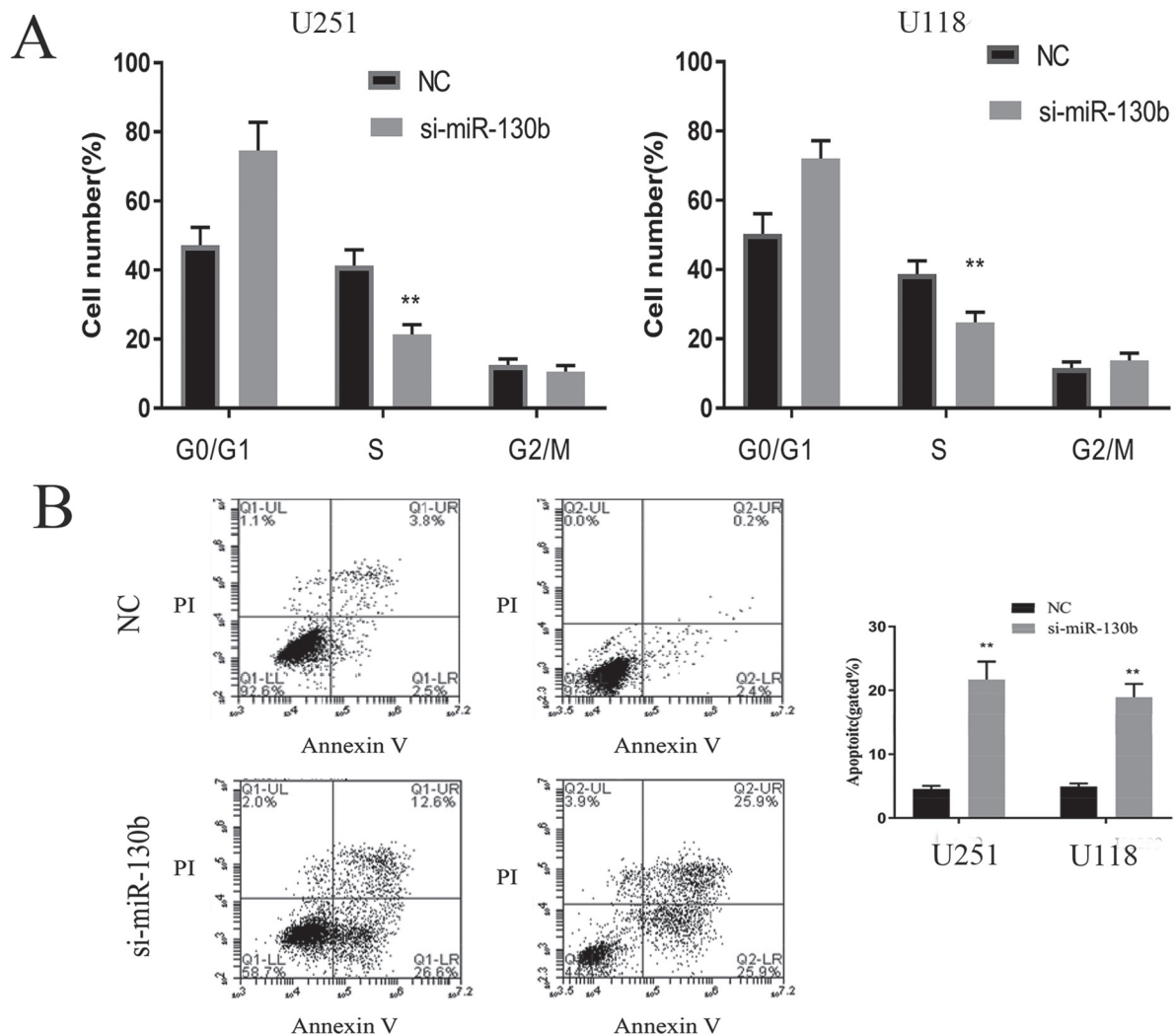


Figure 3. Effects of miR-130b knockdown on cell cycle and apoptosis in U251 and U118 cells. **(A)** Cell cycle was detected by flow cytometry. **(B)** Cell apoptosis was measured by flow cytometric analysis of cells labeled with Annexin V/PI double staining. * $p < 0.05$, ** $p < 0.01$.

by regulating PTEN. Yu et al¹⁸ revealed that overexpression of miR-130b increased the proliferation of esophageal squamous cell carcinoma cells and enhanced their ability to migrate and invade by repressing PTEN expression. Of note, Gu et al¹⁹ reported that miR-130b expression was markedly up-regulated in human glioma tissues and cell lines. They also identified that miR-130b exerted its positive oncogene by inhibiting peroxisome proliferator-activated receptor- γ . The above findings strongly suggested that exploring the potential effect of miR-130b in glioma was very important to help us to understand the progression of glioma. In the present work, we found that the expression of miR-130b was markedly upregulated in glioma

cell lines and tissues. Then, we compared the expression levels of miR-130b in different grade of glioma. Our results indicated that patients with high-grade glioma exerted higher miR-130b expression compared with those with low-grade glioma. Moreover, we analyzed clinicopathological associations of miR-130b expression in glioma patients and found that high miR-130b expression was significantly associated with advanced WHO grade and low KPS. These results indicated that miR-130b may contributed to the progression of glioma. To investigate the possible function of miR-130b, we created stable miR-130b knockdown transfectants in two glioma cell lines. Then, cells experiments revealed that downregula-

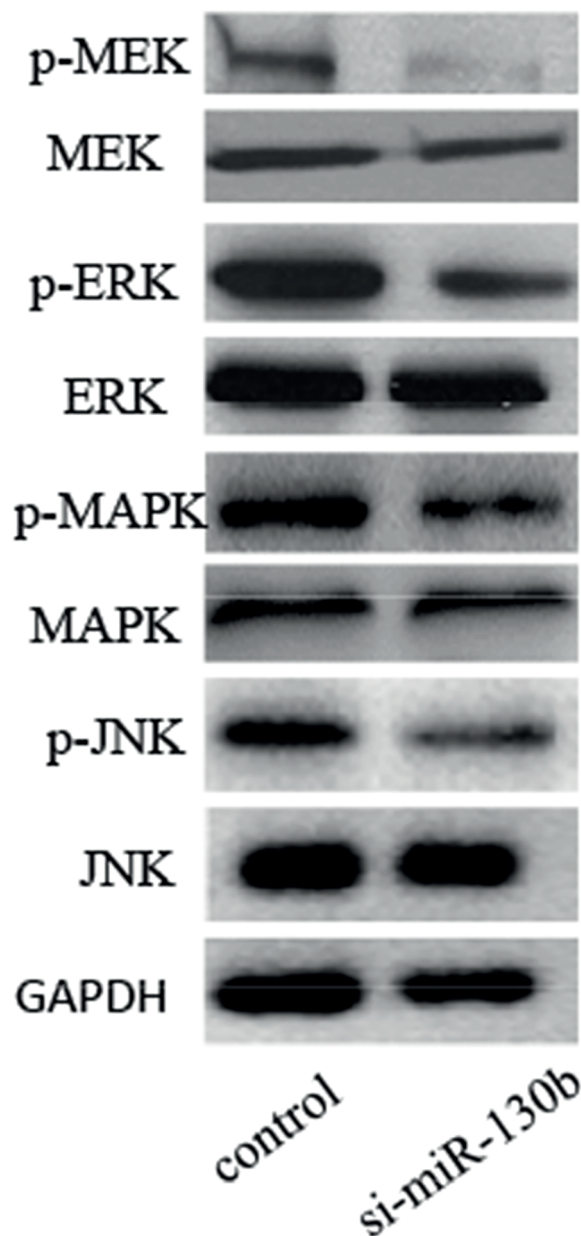


Figure 4. Western blot results of the ERK/MAPK pathway related proteins in glioma cell lines.

tion of miR-130b inhibited the proliferation of glioma cells and induced cell-cycle arrest and cells apoptosis. The above findings were constant with the previous study by Gu et al¹⁹. ERK/MAPK signaling pathway is an essential serine/threonine kinase constituent of the mitogen-activated protein kinase (MAPK) pathway^{20,21}. It has been proved that deregulated ERK/MAPK signaling pathways play critical roles in the pathogenesis of tumors²²⁻²⁴. Previous studies has shown that activation of

ERK/MAPK signaling pathway contributed to glioma cell growth, migration and invasion^{22,25}. Thus, we suggested that miR-130b exerted its oncogene role by affecting ERK/MAPK signaling pathway. In order to identify our assumption we used Western blot to detect the levels of the ERK/MAPK pathway related proteins in glioma cell lines. The results showed that miR-130b can promote the activation of ERK/MAPK signaling pathway.

Conclusions

Our findings enlarged our knowledge about the roles of miR-130b in glioma progression, and miR-130b/ ERK/MAPK signaling pathway might be a novel pathway for glioma treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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