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# MiR-128 promotes the apoptosis of glioma cells via binding to NEK2

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**Abstract. – OBJECTIVE: The aim of this study was to explore whether miR-128 could promote the apoptosis of glioma cells by targeting NIMA related kinase-2 (NEK2), thus participating in the occurrence and progression of glioma.**

**PATIENTS AND METHODS: Expression levels of miR-128 and NEK2 in glioma tissues and normal brain tissues were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The relationship between miR-128 expression, tumor size and stage of glioma was analyzed. The effect of miR-128 on the apoptosis of glioma cells was detected by flow cytometry and Western blot, respectively. Dual-luciferase reporter gene assay was applied to verify the binding condition of miR-128 and NEK2. Meanwhile, rescue experiments were conducted to determine whether miR-128 could promote the apoptosis of glioma cells by targeting NEK2.**

**RESULTS: The expression level of miR-128 in glioma tissues was significantly lower than that of normal brain tissues. However, NEK2 was highly expressed in glioma tissues. MiR-128 expression was correlated to tumor size and malignant level of glioma, whereas not related to age and gender of glioma patients. Meanwhile, overexpression of miR-128 promoted the apoptosis of U87 cells, upregulated protein levels of cleaved Caspase-3 and BCL2-associated X (Bax), and downregulated B-cell lymphoma-2 (Bcl-2). Dual-luciferase reporter gene assay indicated that miR-128 directly bound to NEK2. Further rescue experiments suggested that NEK2 overexpression partially reversed the effect of miR-128 on the apoptosis of glioma cells.**

**CONCLUSIONS: Downregulated miR-128 inhibited the apoptosis of glioma cells via targeting NEK2.**

*Key Words:*
MicroRNA-128 (MiR-128), Glioma, Apoptosis, NEK2.

## Introduction

Glioma is the most common intracranial tumor, accounting for over 50% of brain tumors. According to the differentiation degree and proliferation potential of tumor cells, gliomas are classified into four pathological grades by the WHO classification system. Low-grade gliomas may develop local relapse and progress to high-grade gliomas[1,2]. Although huge progress has been achieved in surgical procedures and other adjuvant therapies for glioma, the mortality of these patients remains high. Even managed with the most aggressive treatments including surgery, radiotherapy, and chemotherapy, the two-year survival rate of low-grade astrocytoma, degenerative astrocytoma, and polygenic glioblastoma is only 66%, 45%, and 9%, respectively[3].

MicroRNA (MiRNA) is a type of endogenous, single-stranded, non-coding RNA discovered in recent years, with 22 nucleotides in length. It's reported that miRNA can recognize target mRNA to promote its degradation and/or inhibit translation at the post-transcriptional level[4]. Many studies have shown that miRNAs participate in the development of malignant tumors by regulating the expression of target genes[5]. For example, miR-21 promotes the invasion and metastasis of colorectal cancer by downregulating programmed cell death gene 4[6]. In non-metastatic and non-invasive breast cancer, miR-10b promotes invasion and metastasis by acting on the homologous gene DIO[7]. Meanwhile, miR-143 and miR-21 are served as tumor biomarkers for cervical cancer[8,9]. A large number of studies have demonstrated that miR-128 is lowly expressed in medulloblastoma[9]. However, miR-128 is highly expressed in acute lymphoblastic leukemia[10] and letrozole-resistant breast cancer[11]. These results suggest that differentially expressed miR-128 may exert different roles in tumor development.

In the present work, we aimed to explore the specific role of miR-128 in glioma, which might provide new directions for further treatment of glioma.

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## Patients and Methods

### Sample Collection

From July 2013 to August 2017, fresh glioma tissues and normal brain tissues of 40 gliomas patients undergoing surgery in the Third Affiliated Hospital of Soochow University were collected. All patients enrolled in this study were pathologically diagnosed as glioma. The clinic-pathological data of all patients were collected, including sex, age, tumor size, and the number of tumors. Follow-up data were also collected in each patient. All patients did not receive any preoperative treatment, and no one had the family history of glioma. Signed written informed consent was obtained from each patient. The Ethics Committee of the Third Affiliated Hospital of Soochow University approved this investigation. All specimens were immediately stored in liquid nitrogen.

### Cell Culture and Transfection

One human fetal glial cell line (SVGP12) and three glioma cell lines (U87, U373, and T98) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 1000 U/mL penicillin and 100 μg/mL streptomycin. The cells were maintained in a 37°C, 5% $CO_2$ incubator. For cell transfection, glioma cells were first seeded in 6-well plates for overnight culture. After cell density reached 70-80%, the cells were transfected with corresponding plasmid in accordance with the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

### RNA Extraction

1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and chloroform were added into cells for gentle mixture. After centrifugation (12000 rpm/min) at 4°C for 10 min and isopropanol incubation, the precipitant was washed with 75% ethanol, followed by air dry at room temperature. Total RNA was finally dissolved in diethyl pyrocarbonate (DEPC) water. The concentration of RNA was determined by a spectrophotometer, and those with A260/A280 of 1.8-2.1 were considered as high purity. All RNA samples were stored at -80°C for subsequent experiments.

### Quantitative Real-Time Fluorescence Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from the tissues and cells using the TRIzol kit (Invitrogen, Carlsbad, CA, USA), respectively, followed by measurement of RNA concentration using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). The complementary Deoxyribose Nucleic Acid (cDNA) was synthesized according to the instructions of the PrimeScriptTM RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. The relative expression level of the target gene was expressed by $2^{-\Delta\Delta Ct}$. Primers used in the study were as follows: MiR-128, F: TCACAGTGAACCGGTCTCTTT, R: GAGCCATAGTCAAGTTCTCCA; U6, F: CTCGCTTCGGCAGCAGCACATATA, R: AAATATGGAACGCTTCACGA; NEK2 (NIMA Related Kinase 2), F: TGCTTCGTGAACTGAAACATCC, R: CCAGAGTCAACTGAGTCATCACT; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), F: AGCCACATCGCTCAGACAC, R: GCCCAATACGACCAAATCC.

### Dual-Luciferase Reporter Gene Assay

The 3'-UTR sequence of NEK2 was amplified by PCR, followed by primer insertion in the restriction sites of Xho I and Not I. The amplified primers were ligated to psiCHECK-2 vector for wild-type NEK2 (NEK2-WT) and mutant-type NEK2 (NEK2-MUT) construction. Luciferase activity of glioma cells was detected after cell transfection.

### Western Blot

Transfected cells were lysed with cell lysis buffer, followed by shaken on ice for 30 min and centrifugation at 14,000×g, at 4°C for 15 min. The concentration of total proteins was determined by the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Extracted proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skim milk at room temperature for 2 h, the membranes were incubated with primary antibodies at 4°C overnight. After washing three times with Tris-Buffered Saline with Tween 20 (TBST) (Beyotime, Shanghai, China), the membranes were incubated with

corresponding secondary antibody at room temperature for 1-2 h. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method (Thermo Fisher Scientific, Waltham, MA, USA).

### Cell Apoptosis

Transfected cells were collected and washed twice with phosphate-buffered saline (PBS; Beyotime, Shanghai, China). Then, the cells were res-uspended in 240 μL l × binding buffer, and the density of cells was adjusted to $2 \times 10^5$/mL. Subsequently, the cells were incubated with 5 μL Annexin V-APC and 10 μL 7-AAD for 30 min in the dark. Transfected cells were further diluted in 260 μL l × binding buffer. Finally, cell apoptosis was detected by flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA), and FACS express version 3 software (BD Biosciences, Franklin Lakes, NJ, USA) was used for analysis.

### Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 Software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Data were expressed as mean ± standard deviation. The *t*-test was used to compare the difference between the two groups. $p<0.05$ was considered statistically significant.

## Results

### MiR-128 Was Lowly Expressed in Glioma

Expression levels of miR-128 and NEK2 in 40 glioma tissues and 18 normal brain tissues were detected by qRT-PCR. Results showed that the expression of miR-128 in glioma tissues was significantly lower than that of normal brain tissues, whereas NEK2 was highly expressed in glioma tissues (Figure 1A and 1B). Subsequently, we analyzed the relationship between clinic-pathological data and miR-128 expression in glioma patients. Results demonstrated that larger tumor size and higher tumor stage were found in glioma patients with lower expression level of miR-128 than those with higher level of miR-128 (Table I).

### Overexpressed MiR-128 Promoted the Apoptosis of Glioma Cells

MiR-128 expression was markedly decreased in U87, U373, and T98 cells than that of SVGP12 cells (Figure 2A). Subsequently, U87 cells were selected for the following *in vitro* experiments. We first verified the transfection efficiency of miR-128 mimics and miR-128 inhibitor (Figure 2B). Cell apoptosis was detected by flow cytometry and Western blot, respectively. Flow cytometry results demonstrated that overexpression promoted the apoptosis

**Table I.** The correlation between microRNA-128 expression and pathological characteristics in glioma patients with glioma (n = 40).

| Clinicopathologic features | Number of cases | lncRNA NBAT1 expression | | *p*-value |
|---|---|---|---|---|
| | | Low (n=20) | High (n=20) | |
| Age (years) | | | | 0.7491 |
| <50 | 23 | 11 | 12 | |
| ≥50 | 17 | 9 | 8 | |
| Gender | | | | 0.7515 |
| Male | 19 | 10 | 9 | |
| Female | 21 | 10 | 11 | |
| Tumor size | | | | 0.0267* |
| <5 cm | 21 | 7 | 14 | |
| ≥ 5 cm | 19 | 13 | 6 | |
| WHO stage | | | | 0.0004* |
| I-II | 21 | 6 | 15 | |
| III-IV | 19 | 14 | 5 | |

*$p<0.05$

of U87 cells (Figure 2C and 2D). Meanwhile, miR-128 overexpression upregulated cleaved Caspase-3 and BCL2-associated X (Bax), and downregulated B-cell lymphoma-2 (Bcl-2) in U87 cells (Figure 2E and 2F).

### *MiR-128 Directly Regulated NEK2 Expression*

According to TargetScan, miRDB, and microRNA websites, NEK2 was predicted as the target gene of miR-128 (Figure 3A). Luciferase Reporter Assays was used to further verify the binding condition of miR-128 and NEK2. The results of Luciferase Reporter Assays indicated that miRNA-128 mimics transfection remarkably downregulated luciferase activity of NEK2-WT. However, no significant change in luciferase activity of NEK2-MUT was found, indicating that miR-128 directly bound to NEK2 (Figure 3B). To further explore the interaction between miR-128 and NEK2, we detected their expression levels by Western blot, respectively. Results confirmed that NEK2 expression was negatively regulated by miR-128 (Figure 3C). Moreover, the effect of miR-128 on cell apoptosis could be reversed by NEK2 overexpression (Figure 3D).

## Discussion

Glioma is the most common intracranial malignancy with highest incidence among central nervous system diseases. Meanwhile, the therapeutic efficiency of glioma is poor, especially for high-grade glioma with rapid postoperative recurrence[12]. Therefore, it is of great significance to investigate the potential mechanism of glioma, thereby improving clinical outcomes. Apoptosis is an active process controlled by multiple gene expression programs. It's known to all that apoptosis is greatly involved in the evolution of organisms, stability of internal environment, and development of multiple systems. Abnormal cell apoptosis may lead to the occurrence and progression of malignant tumors. MiRNAs participate in the process of tumorigenesis *via* regulating anti-apoptotic genes. For example, miR-29 regulates the anti-apoptotic gene Mcl-1[13]. MiR-34[14], miR-15, and miR-16 served as tumor-suppressor genes *via* acting on Bcl-2[15]. P53 mutation or 13q14 deletion inhibits the expression of anti-apoptotic genes and apoptosis of tumor cells. MiR-128 is highly expressed in benign tumors, which is also reported to be involved in the development of different stages of prostate cancer *via* promoting cell adhesion and reducing invasive ability[16]. In this study, we found that miR-128 was significantly lowly expressed in glioma tissues. Flow cytometry results showed that miR-128 overexpression could promote the apoptosis of U87 cells. Moreover, Western blot indicated that the protein expression levels of cleaved Caspase-3, Bcl-2, and Bax could be regulated by miR-128.

NEK2 is a member of the NI-MA-related serine/threonine protein kinase family, which is closely related to cell mitosis. The expression level of NEK2 reaches the peak in the S-G2 phase[17].

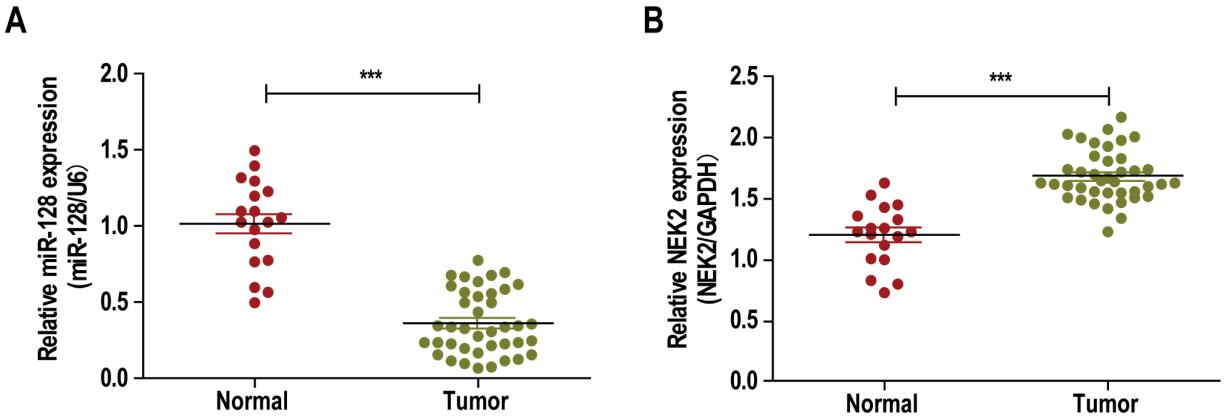


**Figure 1.** MiR-128 was lowly expressed in glioma. Expression levels of miR-128 (***A***) and NEK2 (***B***) in 40 glioma tissues and 18 normal brain tissues.

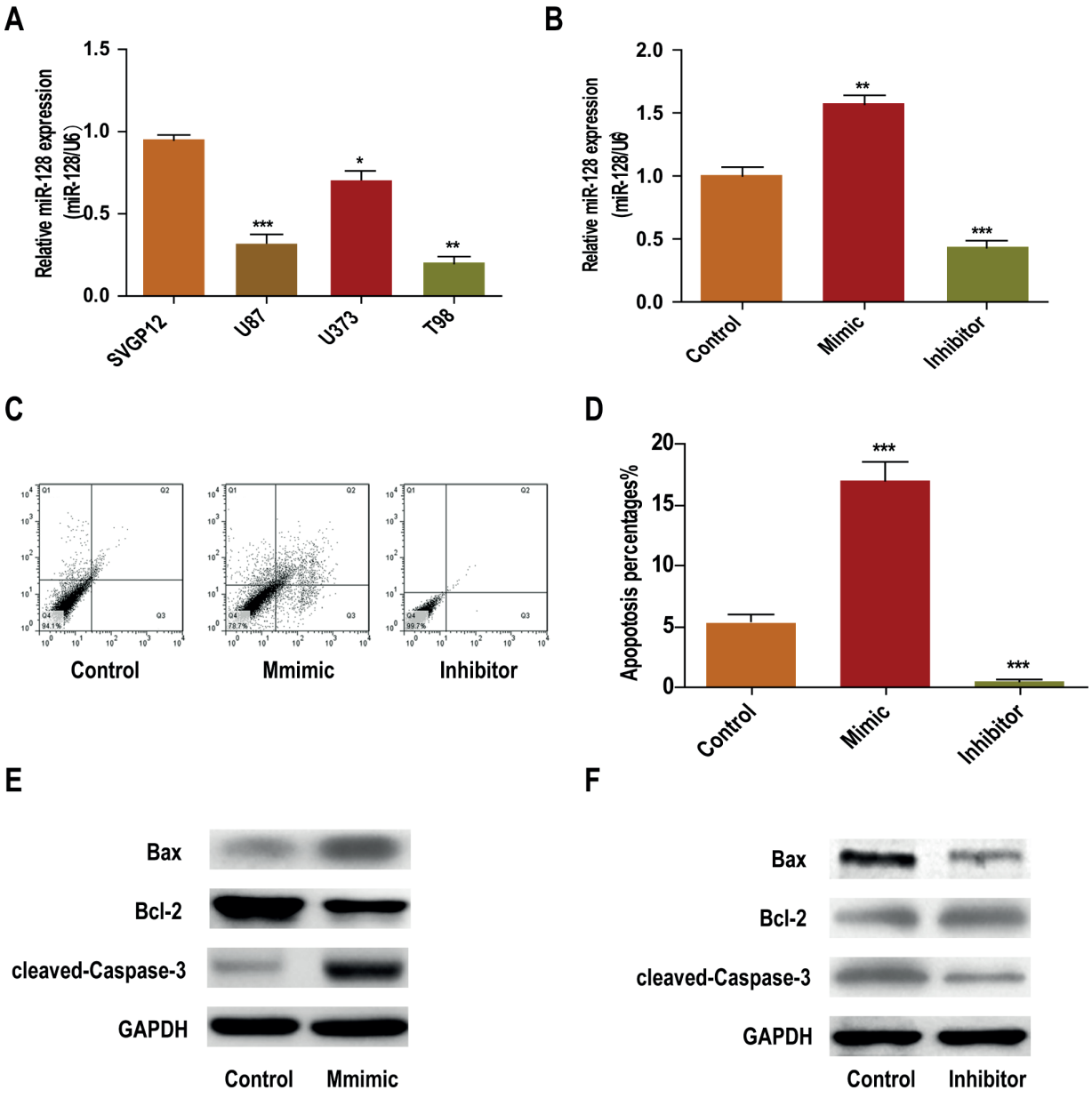


**Figure 2.** Overexpressed miR-128 promoted the apoptosis of glioma cells. ***A,*** MiR-128 was downregulated in U87, U373, and T98 cells than that of SVGP12 cells. ***B,*** Transfection efficiency of miR-128 mimics and miR-128 inhibitor was first verified. ***C, D,*** Flow cytometry demonstrated that overexpression of miR-128 promoted the apoptosis of U87 cells. ***E, F,*** MiR-128 overexpression upregulated cleaved Caspase-3 and Bax, and downregulated Bcl-2.

Moreover, NEK2 dysregulation may lead to abnormal formation and/or dysfunction of the mitotic metaphase, formation of abnormal centrosome, unipolar spindle, and aneuploid cells[18]. These pathological alterations have been showed to be closely related to tumorigenesis. Some studies[19-22] have found that NEK2 expression is abnormally elevated in Ewing's sarcoma, diffuse large B lymphoma, breast cancer, prostate cancer, cholangiocarcinoma, and testicular seminoma. However, no reports have clarified whether NEK2 is differentially expressed in glioma. In this study, we found that NEK2 was a target gene of miR-128 by dual-luciferase reporter gene assay. QRT-PCR results indicated that NEK2 was highly expressed in glioma. In addition, rescue experiments

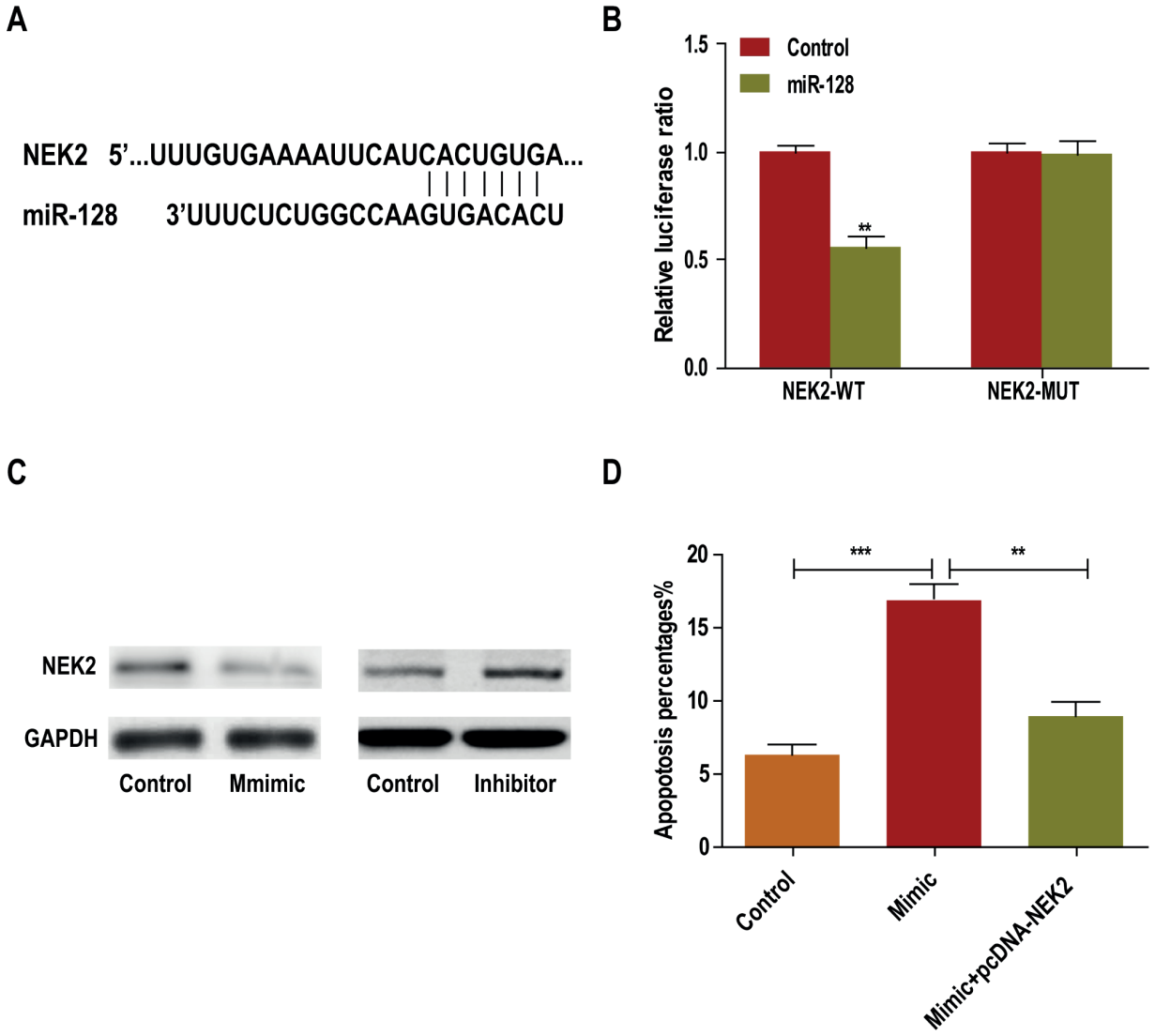


**Figure 3.** MiR-128 directly regulated NEK2 expression. ***A,*** NEK2 was predicted as the target gene of miR-128. ***B,*** MiR-128 mimics remarkably downregulated luciferase activity of NEK2-WT. ***C,*** NEK2 expression was negatively regulated by miR-128. ***D,*** The effect of miR-128 on cell apoptosis could be reversed by NEK2 overexpression.

showed that NEK2 could reverse increased apoptosis caused by miR-128 overexpression.

## Conclusions

We revealed that the ownregulated miR-128 inhibited the apoptosis of glioma cells *via* targeting NEK2, thus participating in the incidence and progression of glioma.

### Conflict of Interest

The Authors declare that they have no conflict of interest.

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