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MicroRNA-424 inhibits cell migration, invasion and epithelial-mesenchymal transition in human glioma by targeting KIF23 and functions as a novel prognostic predictor

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Abstract. – OBJECTIVE: To investigate the expressions, biological effects and potential mechanism of miR-424 in glioma.

METHODS AND METHODS: A total of 54 glioma tissues and 12 normal brain tissues were collected. Human glioma cells (A172, SHG-44, T98, LN18, and LN229) and normal human astrocytes (NHAs) were cultured. Cell invasion and migration capacities were detected by transwell assay. KIF23 was predicted and confirmed as a direct target of miR-424 by TargetScan prediction and Dual-luciferase reporter assay. Six-week-old female nude mice were used for Xenograft tumor formation assay.

RESULTS: Results of this study demonstrated a significant decrease of miR-424 expressions both in glioma cells and tissues. Moreover, the declined miR-424 expressions were observed to be correlated with the poor OS and worse clinicopathological parameters of glioma patients. Functional assays indicated that miR-424 restoration could inhibit the glioma cell epithelial-to-mesenchymal transition (EMT) and metastasis, as well as the tumor growth rate and tumor size of glioma mice. Additionally, kinesin family member 23 (KIF23) expressions were found to be significantly enhanced in glioma specimens, and KIF23 was considered to be a functional target for miR-424 in glioma.

CONCLUSIONS: MiR-424, considered as a tumor-suppressor, inhibited cell metastasis and EMT by targeting KIF23 in glioma, which may provide a novel insight into tumorigenesis and the basis for the development of miRNA-targeting therapies against glioma.

Key Words:
MiR-424, Glioma, Migration, Invasion, Epithelial-mesenchymal transition, KIF23.

Introduction

As one aggressive central nervous system (CNS) malignancy, glioma poses a great health threat to human all over the world[1]. It's hard to cure glioma because of its rapid growth, metastasis, and angiogenesis[2,3]. Also, despite improvement in the existing therapies for glioma, such as immunotherapy, gene therapy, chemotherapy, radiotherapy surgery, and other novel biological therapies, they fail to improve the prognosis of glioma patients[4], which is mainly due to the metastasis and recurrence[5]. Hence, fully understanding the cellular and mechanism of glioma is vitally important to develop more effective therapies. As a physiological process, epithelial-to-mesenchymal transition (EMT) participated in tumor invasion and metastasis *via* transforming polarized and adherent epithelial cells into mesenchymal cells[6]. Although prior research[7,8] has noted that EMT is increasingly significant in glioma progression, the mechanism that regulates EMT still needs full elucidation.

Prior studies that have noted that microRNAs (miRNAs) may function as crucial modulators of gene expressions *via* interacting with their target genes through their 3'-UTRs, thereby enhancing the mRNA degradation or inhibiting translation[9]. Based on the mechanisms, miRNA has been found to play vital roles in regulating pathological and biological processes, including metabolism, apoptosis, growth, and proliferation[10-12]. Aberrant expressions of

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miRNAs have been found in different kinds of tumors, playing tumor-suppressive functions or oncogenic roles by directly regulating their target genes. For example, Li et al[13] indicated that downregulated miR-1269a decreased the susceptibility to gastric carcinoma via regulating ZNF70. Li et al[14] demonstrated that miR-146b-5p exerted anti-tumor functions in non-small cell lung cancer. Feng et al[15] reported that miR-556-3p promoted cell proliferation, invasion, and migration in bladder carcinoma *via* targeting DAB2IP. Therefore, the expressions, functions, and associated mechanism of miRNAs in glioma need to be investigated for the identification of novel and efficient therapies for gliomas. However, the regulatory mechanisms of miR-424 in glioma still remain unclear.

Kinesin family member 23 (KIF23), also known as CHO1/MKLP, is a nuclear protein and essential for spindle midbody formation, being identified as a key regulator of cytokinesis[16]. Disruption of the KIF23 functions resulted in the formation of binucleated or multinucleated cell and incomplete cytokinesis, which were considered as the characteristic of tumor cells[17,18]. Recently, there has been some research on the KIF23 functions in cancer cells. For example, Kato et al[19] found that KIF23 overexpression acted as a clinical predictor of primary lung cancer patients. Sun et al[20] identified KIF23 variant 1 as a novel prognostic factor for hepatocellular carcinoma patients. Kato et al[21] found that KIF23 was a potential therapeutic biomarker for malignant pleural mesothelioma patients. Although dysregulations of KIF23 have been confirmed in multiple human malignancies, the biological significance and mechanism in glioma still need to be fully elucidated.

Patients and Methods

Human Tissue Samples

Clinical specimens of glioma tissues were collected from 54 glioma patients at The Affiliated Hospital of Qingdao University between May 2015 and April 2017. Twelve cases of normal brain tissues were obtained from patients with traumatic brain injury as controls. All the patients received no radiotherapy or chemotherapy before surgery and provided the written informed consent. Collected tissue samples were immediately frozen in liquid nitrogen and subsequently stored at -80°C for further analysis. The Ethics Committee of The Affiliated Hospital of Qingdao University approved this investigation.

Cell Lines

Human glioma cells (A172, SHG-44, T98, LN18, and LN229) and normal human astrocytes (NHAs) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) in a humidified atmosphere of 5% CO_2 at 37°C.

Cell Transfections

MiR-424 mimics, inhibitor, and scrambled miRNA controls (NC) were obtained from RiboBio (Guangzhou, China). Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was performed to transfected the miRNAs into glioma cells following the manufacturer's instructions. Efficiencies of transfections were confirmed by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

qRT-PCR

Glioma cells and tissues were subjected to total RNA isolation using TRIzol reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA). Then, for miR-424 detection, a TaqMan miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) was utilized to carry out the reverse transcription, and a TaqMan miRNA PCR kit (Applied Biosystems, Foster City, CA, USA) was used for RT-PCR, with U6 as an internal reference. Meantime, to detect the mRNA expressions of related genes, a PrimeScript RT reagent kit (TaKaRa Bio, Inc., Otsu, Shiga, Japan) was applied to synthesize the cDNA, and qRT-PCR reactions were conducted with an ABI 7900 Real-Time PCR Detection System (Applied Biosystems, Foster City, CA, USA) by SYBR Green (TaKaRa Bio, Inc., Otsu, Japan), with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. Relative expressions were measured by the $2^{-\Delta\Delta Ct}$ method. The sequences of the primers were listed in Table I.

Transwell Assay

The invasion capacities of glioma cells were detected utilizing transwell filters (8.0 μm pore size, Corning, Corning, NY, USA) pre-treated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). After the transfections, glioma cells were collected and resuspended in serum-free medium and plated into the top chambers, whereas medium containing 10% FBS was added into

Table I. Primer sequences for qRT-PCR.

Primer	Sequence
miR-424 forward	5'-GCTACGCTAA CTGGGTAACCTTG-3'
miR-424 reverse	5'; GTCGAGGGGCATCGGTT-3'
U6 forward	5'-GCTTCGGCAGCACATATACTAAAAT-3'
U6 reverse	5'-CGCTTCACGAATTTGCGTGTCAT-3'
KIF23 forward	5'-CTGACCCAGAGCAAAGCTTTC-3'
KIF23 reverse	5'-GTTCTAAAGTGCATTCTTGCAGC-3'
GAPDH forward	5'-GCAGGGGGGAGCCAAAAGGGT-3'
GAPDH reverse	5'-TGGGTGGCAGTGATGGCATGG-3'
E-cadherin forward	5'-CACCTGGAGAGAGGCCATGT-3'
E-cadherin reverse	
N-Cadherin forward	
N-Cadherin reverse	5'-TGGGAAACATGAGCAGCTCT-3'
5′-GAAGGAGGTGGGGAGGAAGATA-3′	
5′-GGTGGTCTCTGACGAGGTAAACA-3′	
vimentin forward	5'-CTTGAACGGAAAGTGGAATCCT-3'
vimentin reverse	5'-GTCAGGCTTGGAAACGTCC-3'

U6: small nuclear RNA, snRNA; KIF23: Kinesin family member 23; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

the bottom chamber, being used as a chemo-attractant. Followed by the incubation at 37°C in a 5% CO_2 atmosphere for 48 h, cells remaining on the upper surface were removed with cotton swabs. On the other hand, cells adhered to the lower surface of the insert were fixed and stained with paraformaldehyde and crystal violet. Finally, the cells were counted under a microscope (Olympus, Tokyo, Japan) in five independent fields. The difference between invasion and migration assays was that the transwell chambers for migration assays were not coated with Matrigel.

Western Blot

Cells were lysed with ice-cold lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA), which contained protease inhibitors. Then, a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) was utilized to assess the protein concentrations. Followed by being separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), which were blocked with 5% non-fat milk at room temperature for 2 h in Tris-Buffered Saline-Tween (TBST). After that, the proteins were incubated overnight at 4°C with specific primary antibodies which were a rabbit antibody against KIF23 (1:1000, 70R-5564, Fitzgerald Industries International, Acton, MA, USA), a rabbit antibody against E-cadherin (1:1000, ab15148, Abcam, Cambridge, MA, USA), a rabbit antibody against Vimentin (1:1000, ab137321, Abcam, Cambridge, MA, USA) and a rabbit antibody against GAPDH (1:2000, ab128915, Abcam, Cambridge, MA, USA). Subsequently, the membranes were washed 3 times with TBST and incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000, ab7090, Abcam, Cambridge, MA, USA) at room temperature for 2h. Finally, an enhanced chemiluminescence Western blotting kit (Pierce; Thermo Fisher Scientifc, Inc., Waltham, MA, USA) was utilized to visualize the protein bands. GAPDH was used as an internal control.

Dual-Luciferase Reporter Assay

The mutant or wild-type KIF23 3'UTR was synthesized and inserted into the pGL3 luciferase reporter vector (Invitrogen, Carlsbad, CA, USA) named KIF23-3'UTR-Mut or KIF23-3'UTR-WT, followed by the transfection into glioma cells along with miR-424 mimics. The relative luciferase activities were detected by Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) 48 h after the transfections.

Xenograft Tumor Formation Assay

Six-week-old female nude mice were used for the *in vivo* assays. Briefly, they were randomly divided into two groups and subcutaneously injected with glioma cells which had been stably transfected with lentiviral miR-424 (lenti-miR-424) or the negative lentiviral miR-control (lenti-control) into the right flank. The tu-

mor volume for each mouse was measured using an external caliper. Tumor volumes were measured with the equation (L × W2)/2. The mice were sacrificed 30 days after injection, and the tumors were dissected and trimmed. The Animal Ethics Committee of Qingdao University Animal Center approved this investigation.

Statistical Analysis

All experiments were performed at least three times. Statistical Product and Service Solutions (SPSS) software version 17.0 (SPSS Inc., Chicago, IL, USA) was applied to carry out the statistical analysis. The Student's *t*-test, ANOVA, and Scheffe's post-hoc analysis were applied, where appropriate. The Kaplan-Meier method and log-rank test were utilized to determine the survival rates and compare the survival curves respectively. $p<0.05$ was regarded as statistically significant.

Results

MiR-424 Expressions Were Prominently Decreased in Glioma Tissues

To explore the potential functions of miR-424 in glioma, miR-424 expressions in 12 normal brain tissue samples and 54 human glioma specimens were examined by qRT-PCR. qRT-PCR analysis revealed that miR-424 was remarkably down-regulated in glioma tissue samples in comparison with the normal brain tissue samples (Figure 1A). Moreover, we further studied the clinical significance of miR-424 in patients with glioma who had been divided into two groups on the basis of the mean miR-424 expression level. Clinical association analysis indicated that the low miR-424 expressions were notably related to advanced WHO grade ($p=0.0065$) and low KPS ($p=0.0171$) (Table II). Additionally, the Kaplan-Meier method demonstrated that patients with relatively lower miR-424 expressions had significantly shorter OS (Figure 1B).

MiR-424 Inhibited Glioma Cell Invasion and Migration Capacities

The mechanisms of decreased miR-424 in glioma were further studied. Firstly, miR-424 expressions in glioma cells and NHAs were examined. The qRT-PCR analysis revealed that, compared to NHAs, the miR-424 expressions in all glioma cells were markedly downregulated (Figure 2A). Then, miR-424 was over-expressed or inhibited by transfecting its mimics or inhibitor into LN229 and A172 cells, which have low and high endogenous miR-424 expressions respectively. qRT-PCR was conducted to confirm the overexpression and downregulation of miR-424 in LN229 and A172 cells (Figure 2B and 2C). Subsequently, transwell assays were utilized to determine the functions of miR-424 in glioma invasion and migration. As presented in Figure 2D, miR-424 overexpression significantly repressed the invasion and migration capacities of LN229 cells, whereas miR-424 inhibition in A172 cells prominently promoted the invasion and migration abilities (Figure 2E). These data

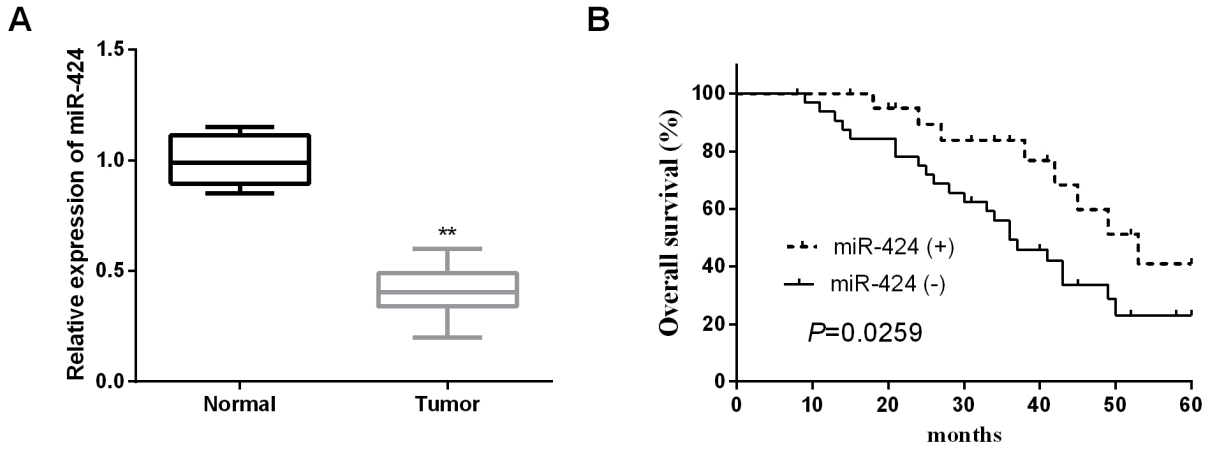

Figure 1. MiR-424 expressions were decreased in glioma. ***A,*** qRT-PCR was utilized to analyze the miR-424 expression in glioma tissues and normal brain tissue samples. ***B,*** Overall survival (OS) of glioma patients with low or low miR-424 expressions. $^{**}p<0.01$.

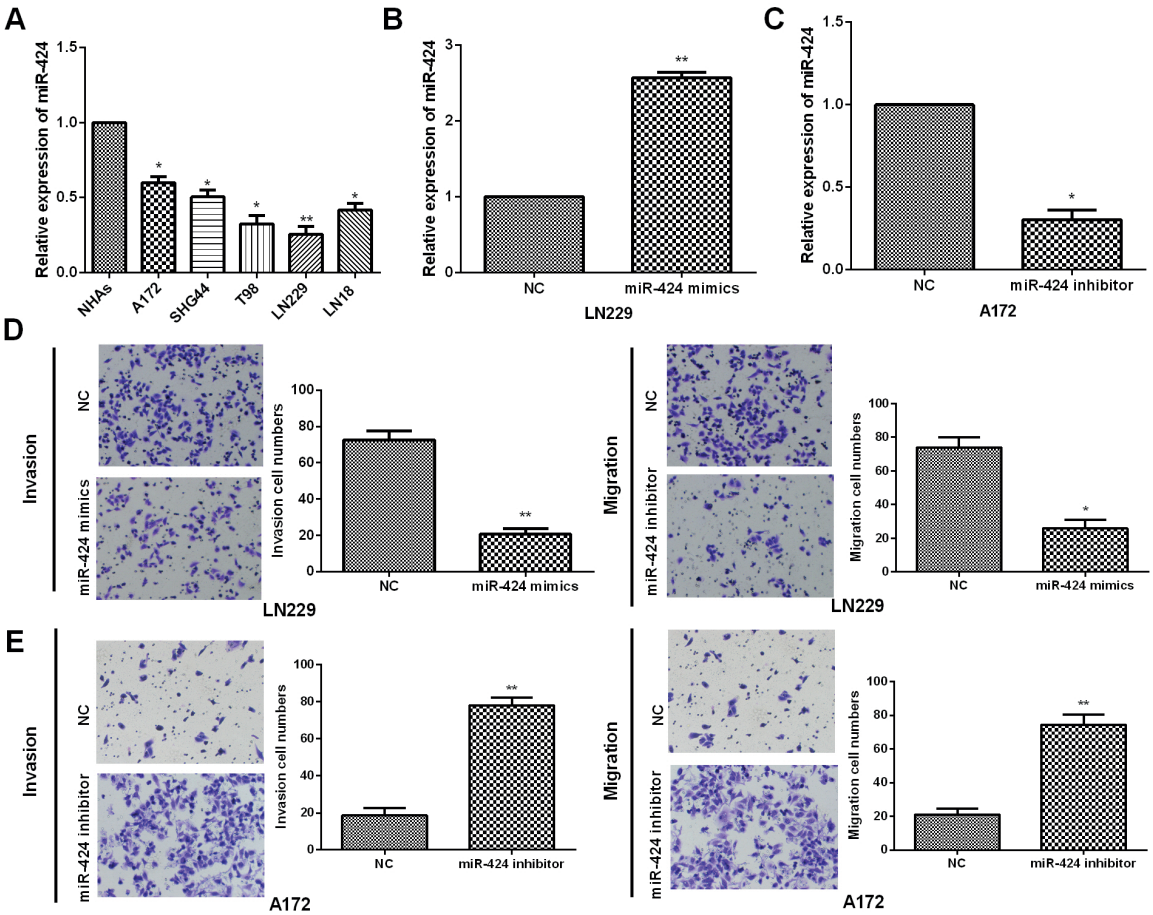

Figure 2. MiR-424 overexpression repressed glioma cell invasion and migration. ***A,*** MiR-424 expressions in glioma cells and NHAs were evaluated using qRT-PCR analysis. ***B, C,*** MiR-424 expressions in LN229 or A172 with transfections of miR-424 mimics or inhibitor. ***D,*** Cell invasion and migration were observed by transwell assays in LN229 treated with miR-424 mimics and ***E,*** A172 treated with miR-424 inhibitor. $^{**}p<0.01$, $^{*}p<0.05$.

Table II. Correlation of miR-424 expression with the clinicopathological characteristics of the glioma patients.

Clinicopathological features	Cases (n=54)	miR-424# expression		*p*-value
		High (n=22)	Low (n=32)	
Age (years)				0.1936
> 60	29	12	17	
≤ 60	25	10	15	
Gender				0.2124
Male	29	11	18	
Female	25	11	14	
Tumor size (cm)				0.0574
≥ 5.0	26	7	19	
< 5.0	28	15	13	
WHO grade				0.0065*
I-II	25	19	6	
III-IV	29	3	26	
KPS				0.0171*
< 80	31	4	27	
≥ 80	23	18	5	

WHO: World Health Organization; KPS: Karnofsky performance scale. # The mean expression level of miR-424 was used as the cutoff. *Statistically significant.

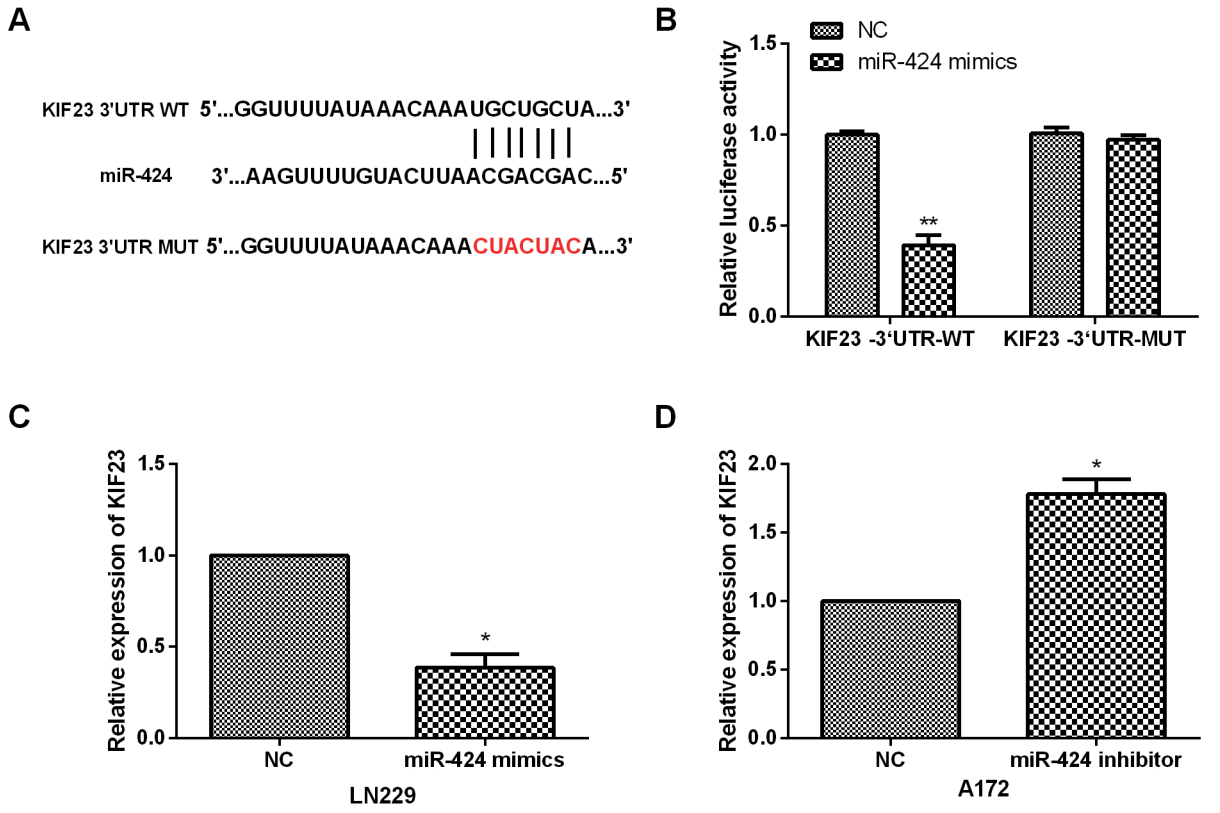

Figure 3. KIF23 was a direct target of miR-424 in glioma. ***A,*** The putative binding sequences of miR-424 in the KIF23 3'-UTR. ***B,*** Luciferase activities in glioma cells cotransfected with luciferase reporters containing KIF23-WT or KIF23-MUT and miR-424 mimics. ***C, D,*** KIF23 expressions in LN229 or A172 treated with miR-424 mimics or inhibitor respectively. $^{**}p<0.01$, $^{*}p<0.05$.

indicated that miR-424 could repress glioma cell invasion and migration capacities.

KIF23 Was a Direct Target of MiR-424 in Glioma Cells

TargetScan was utilized to predict the candidate target genes of miR-424 in order to investigate the underlying mechanism implicated in its functional effect on glioma progression. The data of TargetScan revealed that KIF23 was directly targeted by miR-424 (Figure 3A). Then, dual-luciferase reporter assays were performed to validate the prediction. Results showed that the luciferase activities of glioma cells which were cotransfected with miR-424 mimics and KIF23-3'UTR-Wt were significantly reduced while the cotransfection with miR-424 mimics and KIF23-3'UTR-Mut did not influence the luciferase activities prominently (Figure 3B). To confirm this specificity, the regulation of endogenous KIF23 expressions mediated by miR-424 was further studied. As expected, the KIF23 expressions were markedly declined by miR-424 overexpression while miR-424 inhibition significantly enhanced the expressions of KIF23 (Figure 3C and 3D). Above data indicated that KIF23 was a direct target of miR-424 in glioma.

MiR-424 Suppressed Glioma EMT by Targeting KIF23

To further evaluate the biological functions of KIF23 in glioma progression, the mRNA expressions of KIF23 in glioma tissues and cells were measured using qRT-PCR. The results demonstrated an obvious increase of KIF23 expressions in glioma tissue samples in comparison with the normal tissue samples (Figure 4A). Similarly, the expressions of KIF23 in glioma cells were prominently higher than the NHAs (Figure 4B). Moreover, Kaplan-Meier analysis indicated that the OS of glioma patients with higher KIF23 expressions were shorter than patients with lower KIF23 expressions,

based on the mean expression level of KIF23 as the cutoff (Figure 4C). EMT has been considered to be an important regulator of tumor initiation and metastasis. Therefore, to illustrate the mechanisms about KIF23 in modulating glioma metastasis, the expressions of EMT markers were measured. The results of Western blot demonstrated that the E-cadherin expressions were remarkably increased in miR-424 overexpressed LN229 cells whereas the N-cadherin and vimentin expressions were markedly reduced; also, miR-424 inhibition in A172 cells significantly decreased E-cadherin expressions while enhanced the N-cadherin and vimentin expressions, and additionally, KIF23 expressions were prominently inhibited by miR-424 overexpression and facilitated by miR-424 inhibition (Figure 4D). In conclusion, the findings revealed that miR-424 inhibited the glioma cell EMT *via* targeting KIF23.

Effects of MiR-424 in Tumor Growth of Glioma In vivo

To explore the effects of miR-424 *in vivo*, glioma cells which had been stably transfected with lentiviral miR-424 (lenti-miR-424) or the negative lentiviral miR-control (lenti-control) were implanted subcutaneously into mice. After 4 weeks, the findings revealed that the stable transfection with lenti-miR-424 significantly suppressed the tumor growth rate and tumor size of glioma mice compared to the control group (Figure 5A and 5B).

Discussion

Malignant gliomas are the most deadly form of intracranial tumors. They have a high recurrence rate and high risk of brain cancer-associated morbidity and mortality[22]. At present, the mechanism that drives the progression of glio-

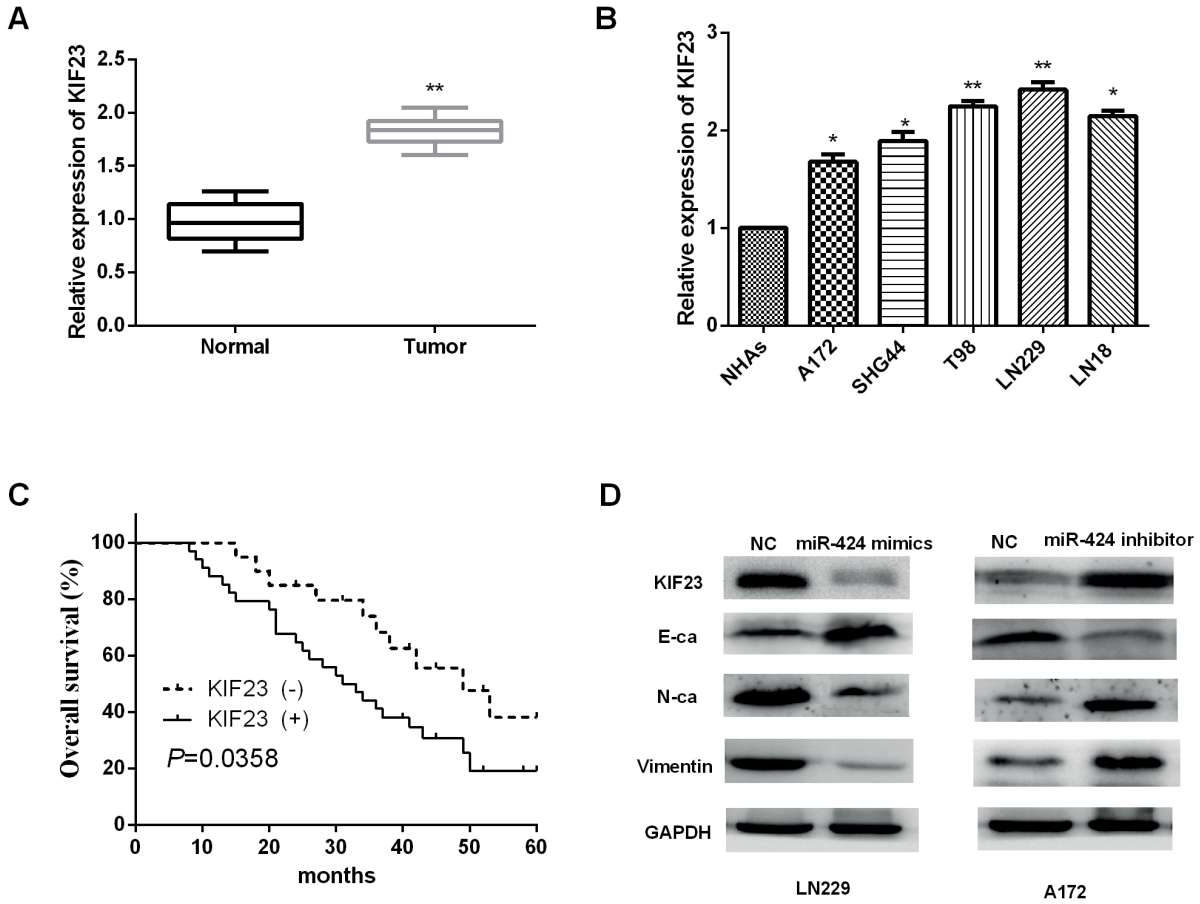

Figure 4. MiR-424 repressed glioma cell EMT by targeting KIF23. ***A,*** KIF23 expressions in glioma tissues and normal brain tissue samples were detected using qRT-PCR. ***B,*** qRT-PCR analysis of KIF23 expression levels in glioma cell lines compared with the NHAs. ***C,*** Kaplan-Meier survival curve analysis showed that glioma patients with high KIF23 expression had significantly shorter overall survival (OS). ***D,*** The expressions of EMT-related markers in LN229 or A172 treated with miR-424 mimics or inhibitor were detected using Western blot analysis. $^{**}p<0.01$, $^{*}p<0.05$.

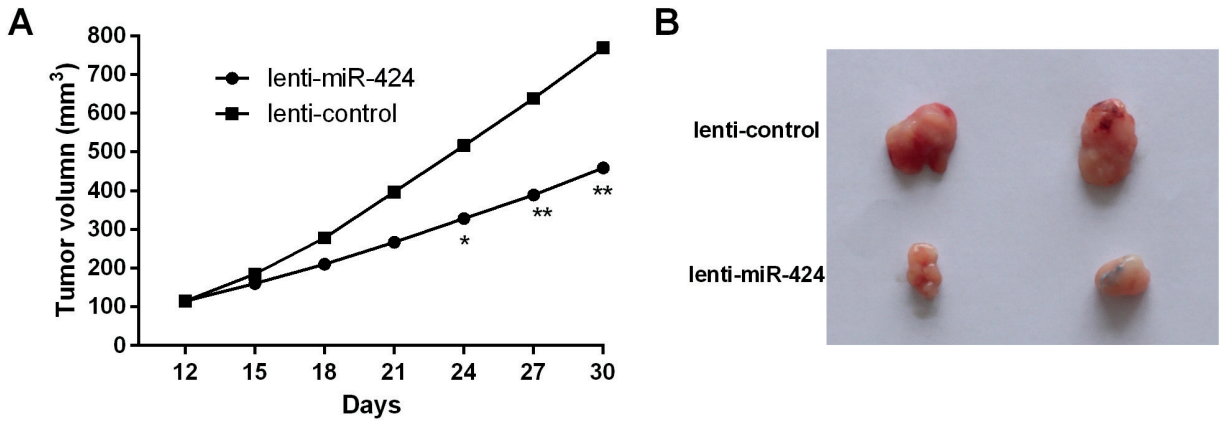

Figure 5. ***A,*** The tumor growth curves of mice which were subcutaneously injected with lenti-miR-424 or lenti-control. ***B,*** The tumor volumes were detected in lenti-miR-424 group and lenti-control group every 3 days from day 12 to 30. $^{**}p<0.01$, $^{*}p<0.05$.

mas is still largely unknown. Some studies have demonstrated that miRNA may be a novel therapeutic approach to diagnose and predict gliomas by regulating specific target genes. Wang et al[23] found that miR-7 inhibited gliomas cell growth and glucose metabolism *via* directly regulating IGF-1R. Huang et al[24] found that serum miR-376 family was diagnostic and prognostic biomarkers for gliomas patients. Shi et al[25] confirmed that miR-181b exerted anti-tumor functions in gliomas *via* regulation of IGF-1R. However, the potential mechanisms associated with the effects of miR-424 on glioma progression are still unclear. This study focused on the roles of the miR-424 in glioma progression.

MiR-424 has been reported to be expressed aberrantly. It plays important roles in multiple malignancies. Yu et al[26] demonstrated that down-regulated miR-424 in hepatocellular carcinoma repressed cell migration and invasion through c-Myb. Li et al[27] reported that miR-424 may exert anti-tumor functions in endometrial carcinoma cells *via* regulating E2F7. Wang et al[28] found that endogenous miR-424 predicted clinical outcome of non-small cell lung cancer and its inhibition acted as tumor suppressor. Moreover, miR-424 has been found to play anti-tumor functions in glioma[29]. Our work provided further evidence that miR-424 suppresses the glioma progression. In brief, we found that miR-424 was downregulated in glioma, which was related to poor OS and worse clinicopathological parameters of glioma patients. Additionally, miR-424 overexpression could suppress glioma cell EMT and metastasis, as well as the tumor growth rate and tumor size of glioma mice.

KIF23 has been demonstrated to be as a therapeutic marker and to be involved in the progression of various cancers, including glioma. For instance, Sun et al[30] found that KIF23 was a prognostic biomarker for glioma and Takahashi et al[31] confirmed that downregulation of KIF23 suppressed glioma proliferation. Their studies, together with our results in this research, suggested a potential critical negative role of KIF23 in the control of glioma progression. In our study, KIF23 was found to be upregulated in glioma tissues and cells, and KIF23 overexpression demonstrated a poor prognosis of glioma patients. Furthermore, KIF23 was implicated in the suppressive functions mediated by miR-424 in glioma cell metastasis.

Conclusions

We demonstrated that miR-424 was downregulated in glioma, which was correlated with poor OS and worse clinicopathological parameters of glioma patients, suggesting that miR-424 acted as a novel cancer suppressor in glioma. KIF23 was showed to be a potential target for miR-424, and miR-424 was demonstrated to function as a negative regulator of KIF23 in glioma. Collectively, miR-424, considered as a tumor-suppressor, inhibited cell metastasis and EMT by targeting KIF23 in glioma, which may provide a novel insight into tumorigenesis and the basis for the development of miRNA-targeting therapies against glioma.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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