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MiR-376a-3p alleviates the development of glioma through negatively regulating KLF15

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Abstract. – OBJECTIVE: The purpose of this study was to uncover the role of microRNA-376a-3p (miR-376a-3p) in mediating migratory and invasive capacities of glioma, as well as the underlying mechanism.

PATIENTS AND METHODS: MiR-376a-3p levels in 39 collected glioma tissues were detected. After collecting clinical data of included glioma patients, the relationship between miR-376a-3p level and clinical features of glioma was analyzed. Next, regulatory effects of miR-376a-3p on proliferative and metastatic capacities of U251 and T98-G cells were assessed. Downstream genes of miR-376a-3p were searched by bioinformatics approach. At last, the involvement of KLF15 in the development of glioma regulated by miR-376a-3p was explored.

RESULTS: It was found that miR-376a-3p was lowly expressed in glioma tissues. Low level of miR-376a-3p was linked to high metastasis rate and poor prognosis in glioma. Besides, overexpression of miR-376a-3p suppressed proliferative and metastatic capacities of glioma cells. KLF15, the downstream gene binding miR-376a-3p, was highly expressed in glioma, and displayed a negative correlation to miR-376a-3p. Notably, KLF15 was able to abolish the regulatory effects of miR-376a-3p on phenotypes of glioma cells.

CONCLUSIONS: MiR-376a-3p is related to lymphatic metastasis and distant metastasis of glioma, and alleviates metastasis of glioma by negatively regulating KLF15.

Key Words:
MiR-376a-3p, KLF15, Glioma, Metastasis.

Introduction

Glioma is a highly invasive primary tumor of the central nervous system, accounting for 45-55% of intracranial tumors, with incidence of about 6-12/100,000[1-3]. Based on WHO classification, glioma is subtyped into grade I, II, III, and IV[2,4], among which glioblastoma multiforme (WHO grade IV) is the most severe and lethal one. At least 40,000 new cases of glioblastoma multiforme occur worldwide each year, with a median survival of less than one year[5,6]. Glioma is hardly to be resected because of rapid growth and strong invasiveness. Moreover, its high heterogeneity and drug resistance limit the therapeutic efficacy[7]. The development of glioma involves activation of oncogenes and deficiency of tumor suppressors[7,8]. Tumor genetics helps to reveal the genetic information during tumorigenesis. Clarifying molecular mechanisms of glioma can greatly improve clinical outcomes[9,10].

MicroRNAs (miRNAs), have been identified to be vital regulators in tumor development[11-14]. Abnormally expressed miRNAs in tumor species can be utilized as potential tumor biomarkers, which contributes to enhance detective rate and therapeutic efficacy[15,16]. Previous studies[17,18] have shown that miR-376a-3p is a tumor-associated miRNA. In addition, cytoplasmic or nuclear accumulation of miR-376a-3p leads to enhancement of tumor invasiveness, thus influencing the prognosis in glioma[17,18].

Bioinformatics information suggests that KLF15 is the downstream gene binding to miR-376a-3p. Krüppel-Like transcription factors (KLF) belong to the transcription factor family containing C2H2 zinc finger structure. They are abundantly expressed in eukaryotes, with 18 KLF members and 9 specific proteins[19,20]. KLF15 is a key regulator of the respiratory, blood, and immune systems. Meanwhile, KLF15 is reported to affect tumor growth by mediating downstream gene expressions[21]. In this paper, the involvement of miR-376a-3p/KLF15 feedback loop in the development of glioma was explored.

Patients and Methods

Glioma Species

Glioma tissues (n=39) and paracancerous ones (n=39) were surgically resected. Included glioma

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patients were not preoperatively treated. Tumor grade of glioma was determined by the WHO classification. This study was approved by Ethics Committee of Sanbo Brain Hospital Capital Medical University and conducted after informed consent was obtained from each subject.

Cell Culture

Human glioma cell lines (U251, U87, T98-G, A172) and glial cell line (HEB) were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C.

Transfection

Transfection plasmids were purchased from GenePharma (Shanghai, China). Cells were cultured to 50-60% confluence and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). They were collected 48 hours later for the following use.

Cell Proliferation Assay

Cells were inoculated in a 96-well plate with 2×10^3 cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Laboratories, Kumamoto, Japan) for plotting the viability curves.

Wound Healing Assay

5.0×10^4 cells suspended in culture medium containing 1% FBS were inoculated per well of 6-well plates and an artificial wound was created. 24 hours later, the percentage of wound closure was calculated.

Transwell Migration and Invasion Assay

3×10^5 cells were inoculated in the upper transwell chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate, with 500 μL of medium containing 10% FBS in the bottom. After 48-h incubation, bottom cells were reacted with 15-min methanol, 20-min crystal violet, and captured using a microscope. Thereafter, migratory cells were counted in 10 random fields per sample (magnification 200×). Finally, invasion assay was similarly conducted in the transwell chamber pre-coated with diluted Matrigel.

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

RNAs extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNAs underwent qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were the internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. MiR-376a-3p: forward: 5'-TGCACCTAAAAGGAG-3', reverse: 5'-GTGCAGGGTCCGAGGT-3', U6: forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCG-3', KLF15: forward: 5'-CAAAGCAGCCATCAAG-3', reverse: 5'-TCAGAGGAGAAACCTC-3', and GAPDH: forward: 5'-GCACCGTCAAGGCTGAGAAC-3', reverse: 5'-TGGTGAAGACGCCAGTGGA-3'.

Western Blot

Cells were lysed for isolating cellular protein and electrophoresed. Protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h, and the membranes were reacted with primary and secondary antibodies for indicated time. Band exposure and analyses were finally conducted.

Dual-Luciferase Reporter Assay

Cells inoculated in a 24-well plate were co-transfected with miR-376a-3p mimic/NC mimic and KLF15-WT/KLF15-MUT using Lipofectamine 2000. Cells were lysed for determining relative Luciferase activity 48 h later.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. The differences between groups were analyzed by the *t*-test. Chi-square test was used for analyzing the relationship between miR-376a-3p level and clinical data of glioma. Later, Pearson correlation test was applied for evaluating the relationship between miR-376a-3p and KLF15 levels in glioma species. Kaplan-Meier curves were depicted for survival analysis. $p<0.05$ represented that the difference was statistically significant.

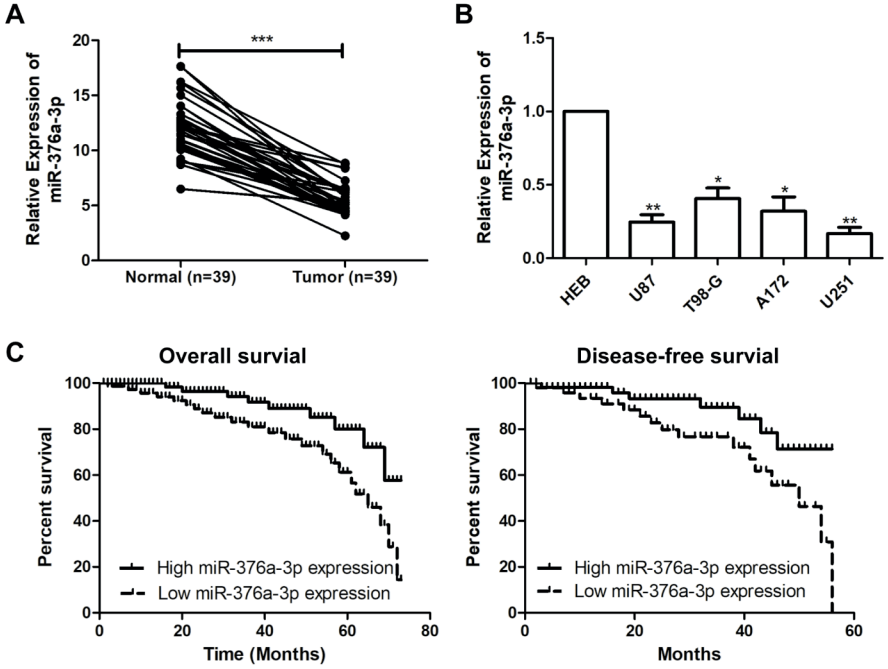

Figure 1. MiR-376a-3p is lowly expressed in glioma. **A,** MiR-376a-3p level in normal tissues (n=39) and glioma tissues (n=39). **B,** MiR-376a-3p level in glioma cell lines. **C,** Kaplan-Meier curves showed overall survival (left) and disease-free survival (right) in glioma patients expressing high or low level of miR-376a-3p. Data are expressed as mean ± SD. *p<0.05, **p<0.01, ***p<0.001.

Results

MiR-376a-3p Was Lowly Expressed In Glioma

MiR-376a-3p levels in glioma and parancancerous tissues were detected, and it was lowly expressed in the former (Figure 1A). Similarly, miR-376a-3p was lowly expressed in glioma cell lines (Figure 1B). Clinical data of included glioma patients were collected. The data showed that miR-376a-3p level was negatively linked to rates of lymphatic metastasis and distant metastasis, while it was unrelated to age, gender, and tumor staging in glioma patients (Table I). Besides, the survival analysis uncovered worse overall survival and disease-free survival in glioma patients expressing a low level of miR-376a-3p (Figure 1C).

MiR-376a-3p Suppressed Proliferative and Metastatic Capacities In Glioma

MiR-376a-3p overexpression and knockdown models were constructed in U251 and T98-G cells, respectively (Figure 2A). The results showed that viability was markedly decreased in U251 cells overexpressing miR-376a-3p, which was enhanced in T98-G cells with miR-376a-3p knockdown (Figure 2B). In addition, overexpression of miR-376a-3p markedly reduced migratory and invasive capacities, as well as wound closure ability in U251 cells. Conversely, knockdown of miR-376a-3p yielded the opposite results (Figure 2C, 2D).

MiR-376a-3p Directly Bound KLF15

Through online prediction, binding sites in the promoter regions of miR-376a-3p and KLF15 were identified (Figure 3A). Luciferase activity was decreased in glioma cells co-transfected with KLF15-WT and miR-376a-3p mimic, suggesting the binding relationship between KLF15 and miR-376a-3p (Figure 3B). Moreover, protein level of KLF15 was downregulated in U251 cells overexpressing miR-376a-3p and upregulated in T98-G cells with miR-376a-3p knockdown (Figure 3C). In addition, miR-376a-3p level was found to be negatively correlated to that of KLF15 in glioma tissues (Figure 3E). Compared with normal tissues, KLF15 was upregulated in glioma tissues (Figure 3D).

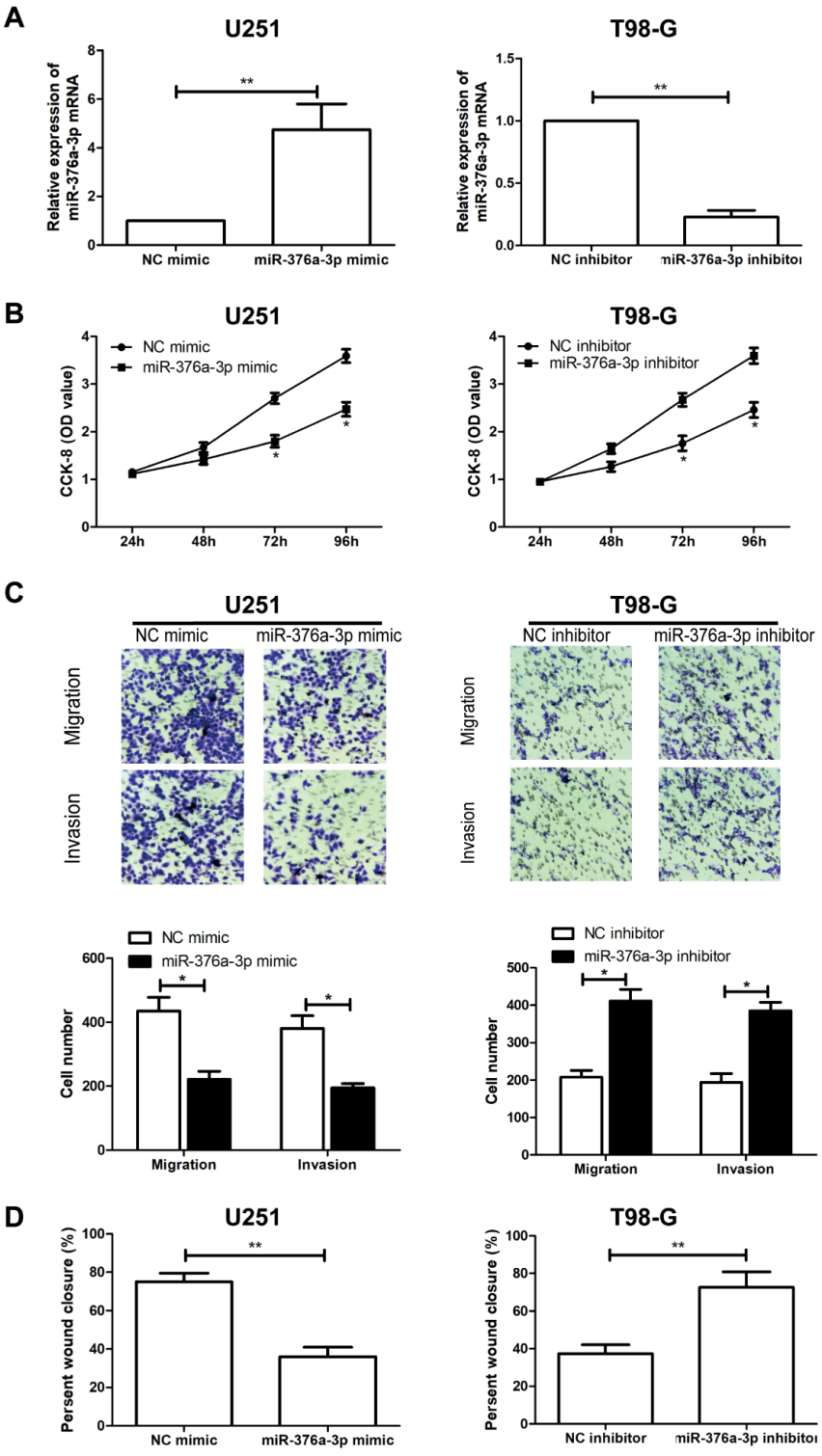

Figure 2. MiR-376a-3p suppresses proliferative and metastatic capacities in glioma. **A,** Transfection efficacy of miR-376a-3p mimic and inhibitor in U251 and T98-G cells, respectively. **B,** Viability in U251 and T98-G cells influenced by miR-376a-3p. **C,** Migration and invasion in U251 and T98-G cells influenced by miR-376a-3p (magnification 200×). **D,** Wound healing percentage in U251 and T98-G cells influenced by miR-376a-3p. Data are expressed as mean ± SD. *p<0.05, **p<0.01.

Table I. Association of miR-376a-3p expression with clinicopathologic characteristics of glioma.

Parameters	No. of cases	MiR-376a-3p expression		*p*-value
		High (%)	Low (%)	
Age (years)				0.656
<60	21 12	9		
≥60	18 9	9		
Gender				0.429
Male	19 9	10		
Female	20 12	8		
T stage				0.757
T1-T2	25 13	12		
T3-T4	13 8	6		
Lymph node metastasis				0.016
No	27 18	9		
Yes	12 3	9		
Distance metastasis				0.041
No	25 17	9		
Yes	13 4	9		

MiR-376a-3p Regulated Metastasis of Glioma by Targeting KLF15

Rescue experiments were conducted to uncover the involvement of KLF15 in the development of glioma, and the transfection efficacy of pcDNA-KLF15 and si-KLF15 in glioma cells was determined (Figure 4A, 4B). Notably, decreased migratory cell number (Figure 4C) and wound closure percentage (Figure 4D) were partially reversed by overexpression of KLF15. The above data demonstrated that KLF15 was able to abolish the regulatory effect of miR-376a-3p on metastatic potential of glioma.

Discussion

Glioma is featured by high mortality, disability, and recurrence[3-5]. Currently, glioma is mainly treated by surgical procedures combined chemotherapy or radiotherapy, but it is highly recurrent[4,7]. Early diagnosis and intervention contribute to improve life quality of glioma patients[6,7]. Preclinical diagnosis of glioma is mainly based on imaging examination, which is confirmed by postoperative pathology[3-5,8]. Nevertheless, imaging examination fails to elucidate the severity degree and lacks specificity, and pathological examination is performed after the surgery. It is urgent to seek biomarkers that are effective and sensitive in glioma screening and treatment[8-10].

MiRNAs are single-chain RNA molecules that were initially discovered in nematode embryo[11-13,22]. With the development of sequencing technologies and high-throughput analysis, miRNAs have been well concerned in tumor diseases[15,16]. Every miRNA can target hundreds of target genes and thus influence their biological functions[14,16]. In this study, miR-376a-3p was lowly expressed in glioma tissues, and the low level of miR-376a-3p was linked to high metastasis rate and poor prognosis in glioma. *In vitro* researches uncovered that overexpression of miR-376a-3p suppressed proliferative and metastatic capacities of glioma cells. It was believed that miR-376a-3p might be a tumor suppressor in glioma.

Subsequently, potential target genes binding to miR-376a-3p were searched. As bioinformatics information discovered, binding sites existed in the promoter regions of miR-376a-3p and KLF15. Furthermore, Dual-Luciferase reporter assay confirmed their binding relationship. Previous studies reported that KLF15 accumulation is closely linked to tumor invasiveness and prognosis. KLF15-based target therapy contributes to inhibit tumor growth, and KLF15 is considered as an ideal tumor biomarker[19-21]. The findings of this study showed that KLF15 was upregulated in glioma species, and displayed a negative correlation to miR-376a-3p. Of note, KLF15 was able to abolish the regulatory effects of miR-376a-3p on metastatic capacities of glioma cells.

A

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score
Position 897-903 of KLF15 3' UTR hsa-miR-376a-3p	5' ...GGUUUAUUUCACAUCCUAUGAAU... 3' UGCACCUAAAAGGA--GAUACUA	7mer-A1	-0.18

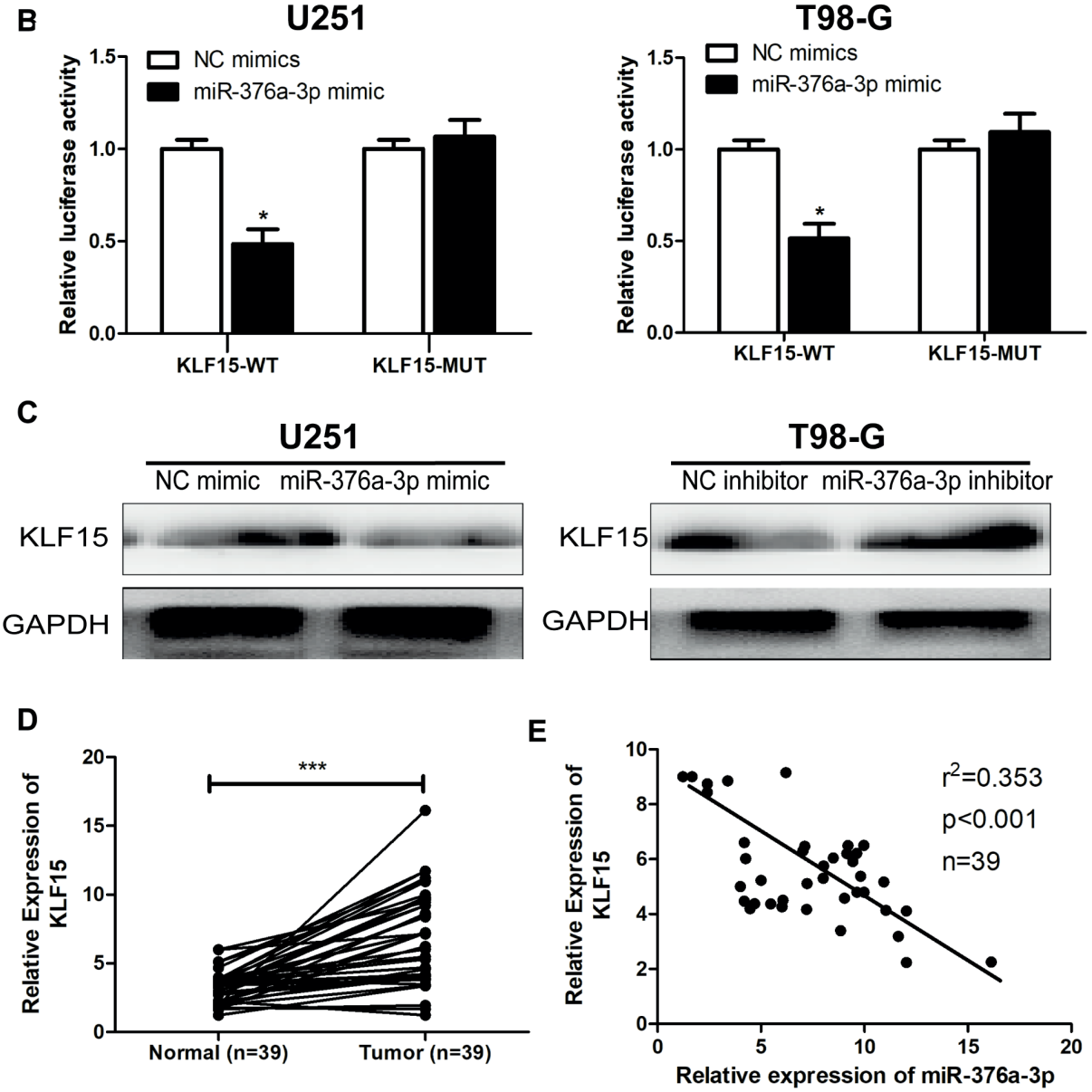

Figure 3. MiR-376a-3p directly binds to KLF15. **A,** Binding sites in the promoter regions of miR-376a-3p and KLF15. **B,** Luciferase activity in U251 and T98-G cells co-transfected with KLF15-WT and miR-376a-3p mimic. **C,** Protein level of KLF15 in U251 and T98-G cells influenced by miR-376a-3p. **D,** KLF15 level in normal tissues (n=39) and glioma tissues (n=39). **E,** A negative correlation between expression levels of miR-376a-3p and KLF15 in glioma species. Data are expressed as mean ± SD. *p<0.05, ***p<0.001.

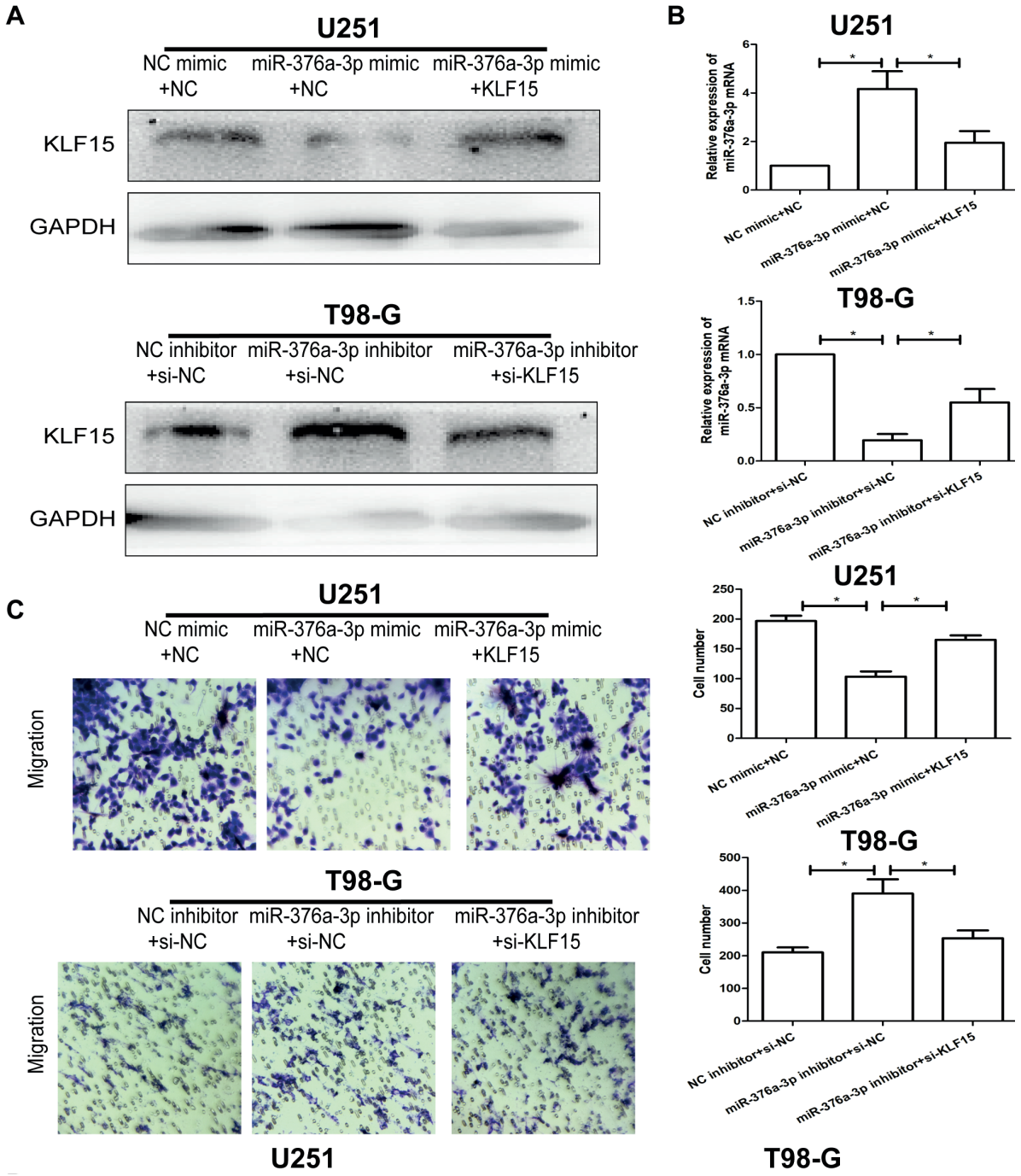

Figure 4. MiR-376a-3p regulates metastasis of glioma by targeting KLF15. **A,** Protein level of KLF15 in U251 and T98-G cells influenced by both miR-376a-3p and KLF15. **B,** MiR-376a-3p level in U251 and T98-G cells influenced by both miR-376a-3p and KLF15. **C,** Migration in U251 and T98-G cells influenced by both miR-376a-3p and KLF15 (magnification 200×).

Figure continued

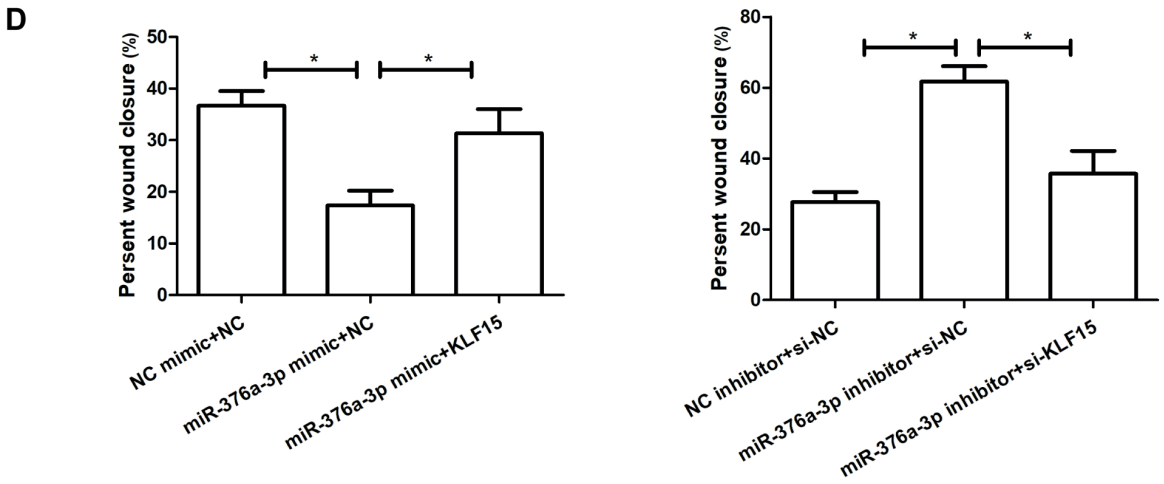

Figure 4 (continued). D, Wound closure percentage in U251 and T98-G cells influenced by both miR-376a-3p and KLF15. Data are expressed as mean ± SD. *$p<0.05$.

Conclusions

To sum up, miR-376a-3p is related to lymphatic metastasis and distant metastasis of glioma, which alleviates metastasis of glioma by negatively regulating KLF15.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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