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MicroRNA-381 inhibits metastasis and epithelial-mesenchymal transition of glioblastoma cells through targeting LEF1

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Abstract. – OBJECTIVE: Glioblastoma is a common intracranial malignancy that is extremely harmful to human life and health. Various microRNAs (miRNAs) have been reported to be involved in the progression of glioblastoma, except miR-381. Therefore, the purpose of this study is to investigate the role of miR-381 in glioblastoma.

MATERIALS AND METHODS: The expression of miR-381 and LEF1 (lymphoid enhancer-binding factor-1) was quantified using quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot analysis. Transwell and Dual-Luciferase reporter assays were used to investigate the regulatory mechanism of miR-381/LEF1 in glioblastoma.

RESULTS: Downregulation of miR-381 was observed in A172 cells. In addition, the overexpression of miR-381 restrained migration and invasion of glioblastoma cells. Furthermore, overexpression of miR-381 inhibited epithelial-mesenchymal transition (EMT) in A172 cells. Further, miR-381 was confirmed to directly target LEF1 and negatively regulates its expression in glioblastoma cells. Downregulation of LEF1 also inhibited cell migration, invasion, and EMT in glioblastoma cells. More importantly, the upregulation of LEF1 abolished the inhibitory effect of miR-381 in glioblastoma cells.

CONCLUSIONS: MiR-381 inhibits cell metastasis and EMT in glioblastoma by suppressing LEF1 expression.

Key Words:
Cell metastasis, Epithelial-mesenchymal transition, MiR-381, Glioma, LEF1.

Introduction

Glioma is the most common malignant tumor of the central nervous system (CNS). According to pathology, gliomas can be divided into astrocytoma, myelin cell tumor, polymorphic glioblastoma, ependymoma, oligodendrocyte tumor[1]. Glioblastoma is a highly malignant astrocytic tumor. Because glioblastoma is usually invasive, glioblastoma is difficult to completely cure. Making things even more difficult, glioblastoma is difficult to be completely removed. Furthermore, the tumor is not very sensitive to radiotherapy and chemotherapy[2]. In addition, glioblastoma is still easy to relapse after treatment. Recently, targeted therapy and biological therapy have become hot treatments for glioblastoma patients. Although glioblastoma is still difficult to completely cure, the life quality and the survival period of glioblastoma patients can be greatly extended[3]. Therefore, finding new effective therapeutic targets is necessary to improve the survival of patients with glioblastoma.

As a novel target, microRNA (miRNA) has attracted more and more attention. In addition, genomics, biogenesis, mechanisms, and functions of miRNAs have been extensively investigated in human cancers, including glioblastoma[4]. For example, miR-374a was downregulated in glioma and inhibited aggressive cell behaviors[5]. By contrast, miR-744 was upregulated in glioblastoma and promoted glioblastoma cell migration by suppressing MAP2K4 expression[6]. Although the role of many miRNAs in gliomas has been reported, the molecular mechanism of miR-381 in glioblastoma remains unclear. It has been reported that miR-381 regulated cell proliferation, migration, invasion and apoptosis in prostate cancer[7]. In addition, miR-381 expression was found to be decreased in lung adenocarcinoma[8]. Furthermore, miR-381 inhibited malignant behaviors of epithelial ovarian cancer by suppressing YY1 expression[9]. However, it has also been reported that miR-381 was upregulated in osteosarcoma, and low miR-381 expression predicted a good prognosis[10]. These results suggest that miR-381

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is tissue specific. In previous studies, the role of miR-381 in glioblastoma has not been reported. Therefore, the regulatory mechanism of miR-381 was investigated in glioblastoma.

As a member of the high mobility group protein family, LEF1 (lymphoid enhancer-binding factor-1) has been found to express abnormally in human cancers. For example, LEF1 expression was increased in hepatocellular carcinoma and promoted hepatocellular carcinoma cell proliferation and epithelial-mesenchymal transition (EMT) *via* targeting NFIX[11]. In addition, LEF1 was found to promote proliferation and invasion of lung cancer cells[12]. Similarly, LEF1 silencing suppressed tumorigenesis and progression of prostate cancer[13]. Liang et al[14] found that LEF1 can regulate EMT by mediating miR-181a in prostate cancer. EMT is known to be a key process in regulating cancer metastasis[15]. EMT is a biological process in which epithelial cells are transformed into mesenchymal phenotype cells by specific procedures. The main features of EMT are decreased expression of cell adhesion molecules (such as E-cadherin) and increased expression of neuro-cadherin (N-cadherin) expression. Epithelial cells can lose cell polarity and acquire higher migration and invasion abilities through EMT. Therefore, we investigated the effect of LEF1 on cell metastasis and EMT in glioblastoma. Meanwhile, the interaction between miR-381 and LEF1 was also explored in this study. The findings will provide new avenues for the treatment of glioblastoma patients.

Materials and Methods

Cell Culture

Normal human astrocytes (NHAs) and A172 glioblastoma cell line were obtained from the China Center for Type Culture Collection (Wuhan, China). Growth conditions of these cells included 5% CO_2 at 37°C and Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA).

Cell Transfection

MiR-381 mimics or inhibitor was purchased from GenePharma (Shanghai, China). LEF1 siRNA and overexpression vectors were synthesized by RiboBio (Guangzhou, China). Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for cell transfection. Transfection efficiency was assessed by quantitative Real Time-Polymerase Chain Reaction (RT-qPCR) and Western blot analysis.

qRT-PCR

Total RNA extraction was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was reversely transcribed using a First-Strand cDNA Synthesis kit (Beyotime, Shanghai, China). qRT-PCR assay was performed using SYBR Green qPCR Mix (Beyotime, Shanghai, China) according to the manufacturer's instruction. The relative expression of miR-381 and LEF1 was normalized to U6 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was calculated by the $2^{-\Delta\Delta ct}$ method. The following primers were used: miR-381, (F) 5'-AGT CTA TAC AAG GGC AAG CTC TC-3', (R) 5'-TAT GGT TGT TCT GCT CTC TGT CTC-3'; LEF1 (F) 5'-CGC GGA TCC ATG CCC CAA CTC TCC GGA G-3', (R) 5'-CCG GAA TTC TCA GAT GTA GGC AGC TGT CAT TC-3'; U6, (F) 5'-CTC GCT TCG GCA GCA CA-3', (R) 5'-AAC GCT TCA CGA ATT TGC GT-3'; GAPDH (F) 5'-GAA GGT GAA GGT CGG AGT C-3', (R) 5'-GAG ATG GTG ATG GGA TTT C-3'.

Western Blot Analysis

Protein samples were extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Next, 25 μg protein was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and blocked with 5% non-fat milk. The protein sample was then transferred to polyvinylidene difluoride (PVDF; Beyotime, Shanghai, China) membrane. Next, the membrane was incubated with Vimentin, N-cadherin, E-cadherin, LEF1, and GAPDH primary antibodies (Abcam, Cambridge, MA, USA) overnight at 4°C. After washing, the protein sample was incubated with horseradish peroxidase-conjugated secondary antibodies (Abcam, Cambridge, MA, USA) for 1 h. The protein bands were assessed by enhanced chemiluminescence (ECL) kit (Beyotime, Shanghai, China). Protein was quantified by Image Lab Software (Bio-Rad, Hercules, CA, USA).

Transwell Assay

First, the upper chamber (Corning Incorporated, Corning, NY, USA) was added with 60 μL of diluted Matrigel (BD Biosciences, San Jose, CA, USA) for cell invasion. Meanwhile, the up-

per chamber without Matrigel was used for cell migration. After 30 mins, A172 cell suspension (4×10^3 cells/well) was added to the transwell upper chamber. Next, 500 μL of DMEM medium (10% FBS, Gibco, Rockville, MD, USA) was added to the lower chamber. After 24 h of routine incubation, the moving cells were stained with 0.1% crystal violet (Beyotime, Shanghai, China). Observation and photographing were performed using a light microscope (Olympus Corporation, Tokyo, Japan).

Luciferase Reporter Assay

The Wt-LEF1-3'UTR or Mut-LEF1-3'UTR was inserted into the Luciferase vector pcDNA3.1 (Promega, Madison, WI, USA). A172 cells were transfected with the above vectors and miR-381 mimics. Next, the transfected cells were incubated for 48 h at room temperature. Finally, Luciferase activity was assessed using a Dual-Luciferase assay system (Promega, Madison, WI, USA).

Statistical Analysis

Data were analyzed using GraphPad Prism 6 (La Jolla, CA, USA). The *t*-test is used to analyze the measurement data. The differences between the two groups were analyzed using Student's *t*-test. Comparisons between multiple groups were performed using One-way ANOVA test followed by post-hoc test (Least Significant Difference). Statistical significance was defined as $p<0.05$.

Results

The Dysregulation of MiR-381 in Glioblastoma Cells

First, the expression of miR-381 was examined in glioblastoma cell line A172 and normal human astrocytes NHAs. Downregulation of miR-381 was detected in A172 cells compared to NHAs cells ($p<0.01$; Figure 1A). Next, gain-loss experiment was performed in A172 cells containing miR-381 mimics or inhibitor. We found that the expression of miR-381 was enhanced by its mimics and reduced by its inhibitor in A172 cells ($p<0.01$; Figure 1B). The effect of miR-381 on cell metastasis was assessed by transwell assay. It showed that miR-381 overexpression inhibited cell migration, while miR-381 silencing promoted cell migration in A172 cells ($p<0.01$; Figure 1C). Next, it was found that the upregulation of miR-381 suppressed cell invasion, whereas the downregulation of miR-381 promoted invasion of A172 cells ($p<0.01$; Figure 1D). These results demonstrate that the overexpression of miR-381 inhibits cell migration and invasion in glioblastoma cells.

MiR-381 Overexpression Inhibits EMT in Glioblastoma Cells

Next, the expression of N-cadherin, Vimentin, and E-cadherin (EMT markers) in A172 cells was examined to further elucidate the effect of miR-381 on glioblastoma cell metastasis. Consistent with the above results, overexpression of miR-381 promoted E-cadherin expression and impeded the expression of N-cadherin and Vimentin ($p<0.01$; Figure 2A). By contrast, the knockdown of miR-381 blocked E-cadherin expression and increased N-cadherin and Vimentin expression levels ($p<0.01$; Figure 2B). Combining these results, we consider that the overexpression of miR-381 inhibits metastasis and EMT of glioblastoma cells.

MiR-381 Directly Targets LEF1.

In addition, LEF1 was selected as a candidate downstream target for miR-381. TargetScan database (http://www.targetscan.org/vert_71/) shows that miR-381 has a binding site with the 3'-UTR of LEF1 (Figure 3A). This prediction was confirmed by a Luciferase reporter assay. MiR-381 mimics were found to reduce the Luciferase activity of wt-LEF1 ($p<0.01$; Figure 3B). However, miR-381 mimics had no effect on mut-LEF1 Luciferase activity. Next, LEF1 expression was examined in A172 cells containing miR-381 mimics or inhibitor. qRT-PCR assay showed that miR-381 mimics reduced LEF1 expression, when miR-381 inhibitor increased LEF1 expression ($p<0.01$, Figure 3C, 3D). Similarly, Western blot analysis also showed the same results (Figure 3E). These results indicate that miR-381 directly targets LEF1 and negatively regulates its expression in glioblastoma cells.

The Dysregulation of LEF1 in Glioblastoma Cells

To demonstrate whether LEF1 is involved in glioblastoma, LEF1 siRNA was transfected into A172 cells. First, the upregulation of LEF1 was observed in A172 cells compared to NHAs cells ($p<0.01$; Figure 4A). Next, we found that LEF1 expression was inhibited by its siRNA in A172 cells ($p<0.01$; Figure 4B). Functionally, the downregulation of LEF1 inhibited migration and

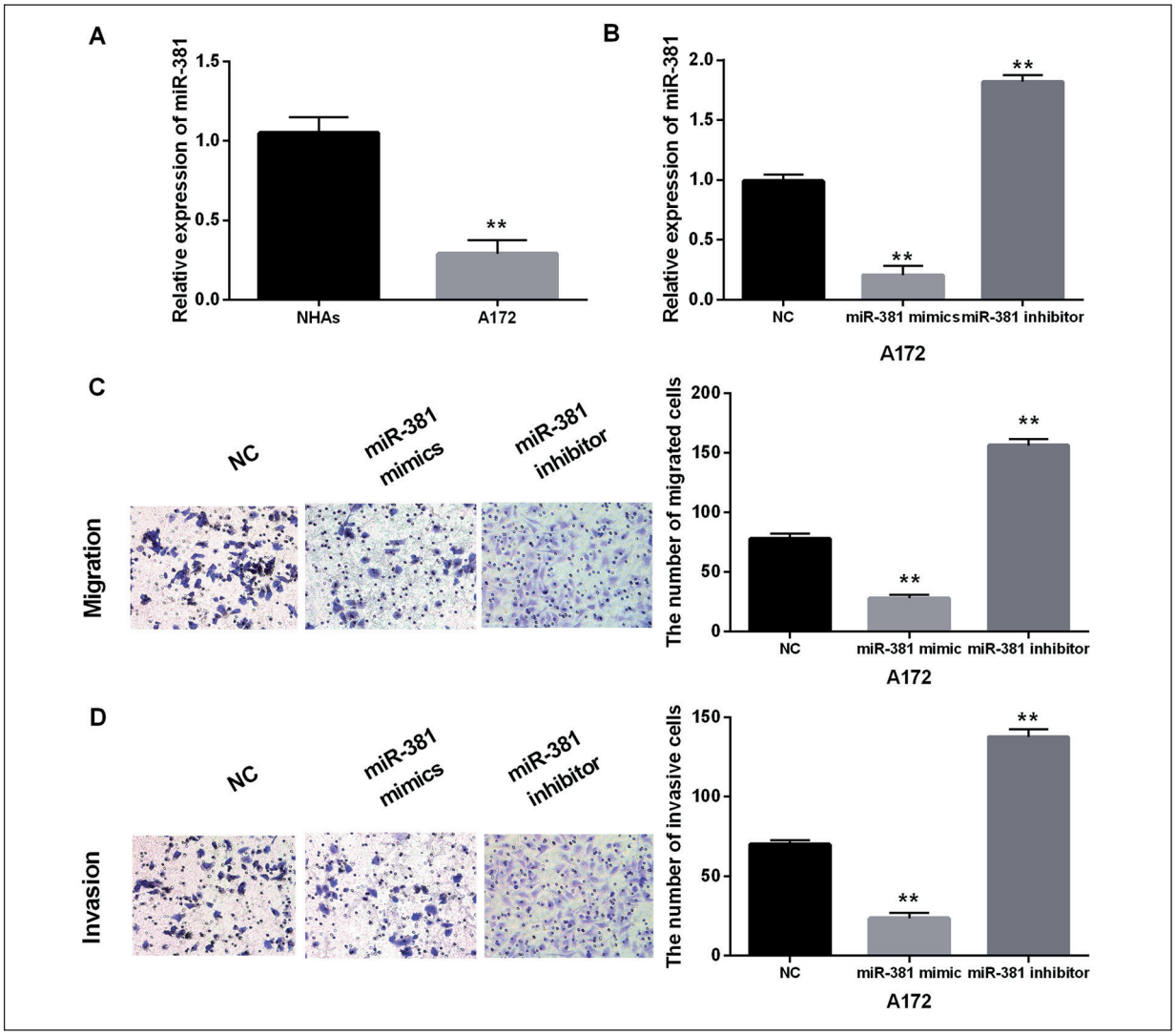

Figure 1. The dysregulation of miR-381 in glioblastoma cells **A,** The miR-381 expression in A172 and NHAs cells. **B,** MiR-381 expression was examined in A172 cells with its mimics or inhibitor. **C, D,** Cell migration and invasion was measured in A172 cells with miR-381 mimics or inhibitor (magnification, 200×). **$p<0.01$.

invasion of A172 cells ($p<0.01$; Figure 4C, 4D). As for EMT, LEF1 siRNA increased E-cadherin expression and decreased N-cadherin and Vimentin expression ($p<0.01$; Figure 4E). Based on these results, the knockdown of LEF1 inhibits metastasis and EMT in A172 cells.

The Suppressive Effect of MiR-381 is Abolished by Upregulation of LEF1.

To explore the interaction between LEF1 and miR-381, the LEF1 vector and miR-381 mimics were transfected into A172 cells. Upregulation of LEF1 was found to restore the reduced expression of LEF1 induced by miR-381 mimics ($p<0.01$; Figure 5A, 5B). Consistently, LEF1 overexpression also abolished the inhibitory effects of miR-381 on cell migration and invasion in A172 cells ($p<0.01$; Figure 5C, 5D). Taken together, we consider that miR-381 inhibits glioblastoma cell metastasis by suppressing LEF1 expression.

Discussion

Recently, many miRNAs have been implicated in the progression of glioblastoma, such as miR-34a-5p and miR-520e[16,17]. In this study, the downregulation of miR-381 was identified in glioblastoma cells. Transwell assay showed that miR-381 inhibited migration and invasion of glio-

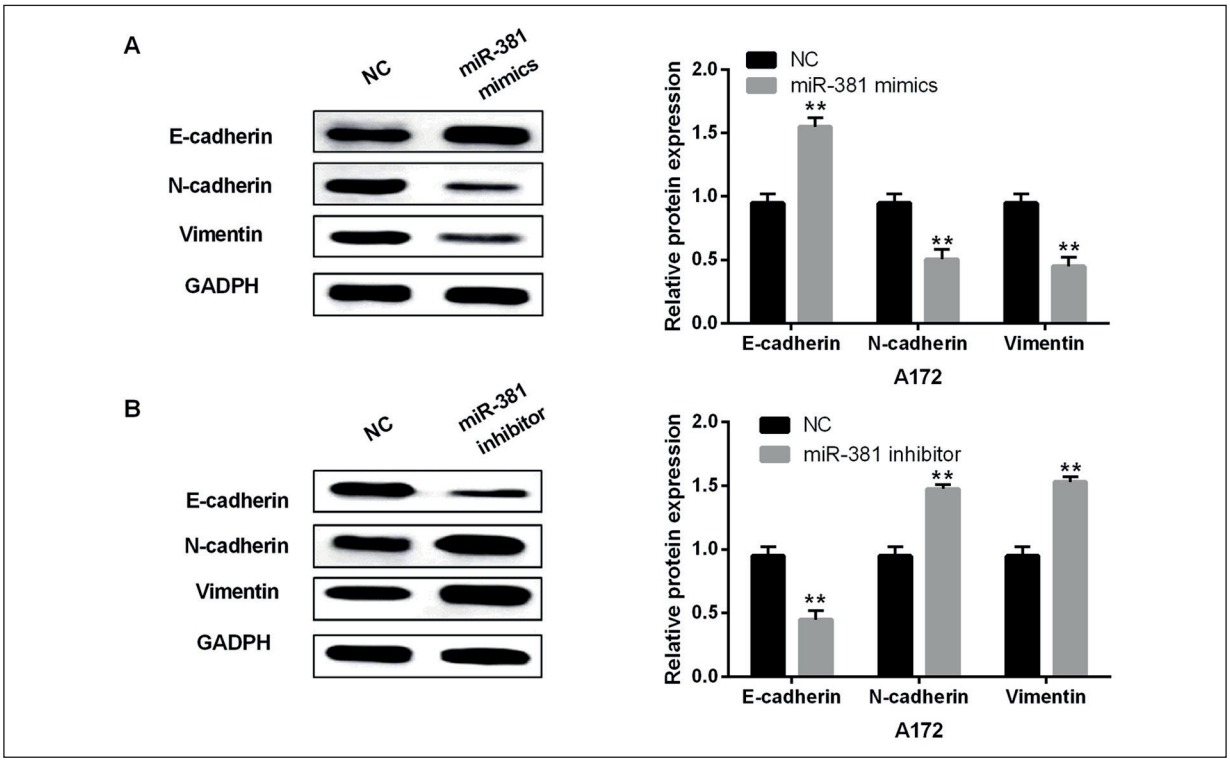

Figure 2. Overexpression of miR-381 inhibited EMT in glioblastoma cells. **A, B,** MiR-381 regulated expressions of E-cadherin, N-cadherin and Vimentin in A172 cells. **p<0.01.

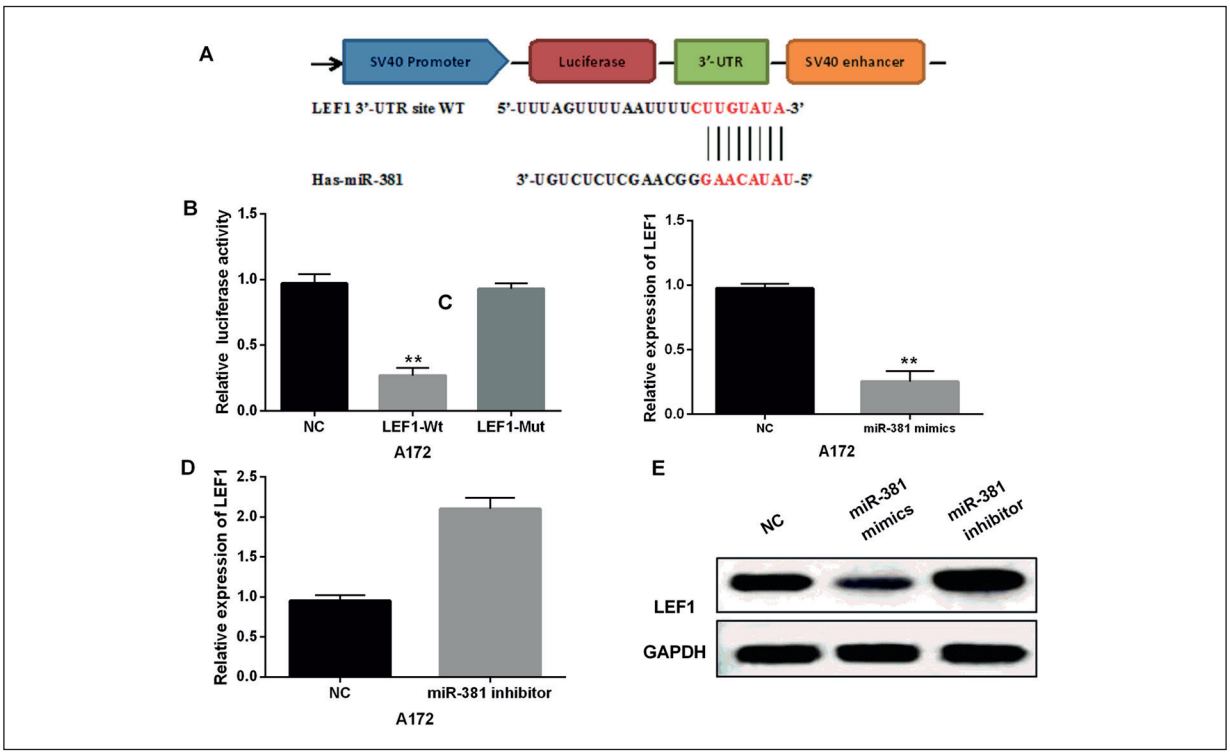

Figure 3. MiR-381 directly targeted LEF1. **A,** The binding sites between miR-381 and LEF1. **B,** Luciferase reporter assay. **C, D, E,** The mRNA and protein expression of LEF1 in A172 cells with miR-381 mimics or inhibitor **p<0.01.

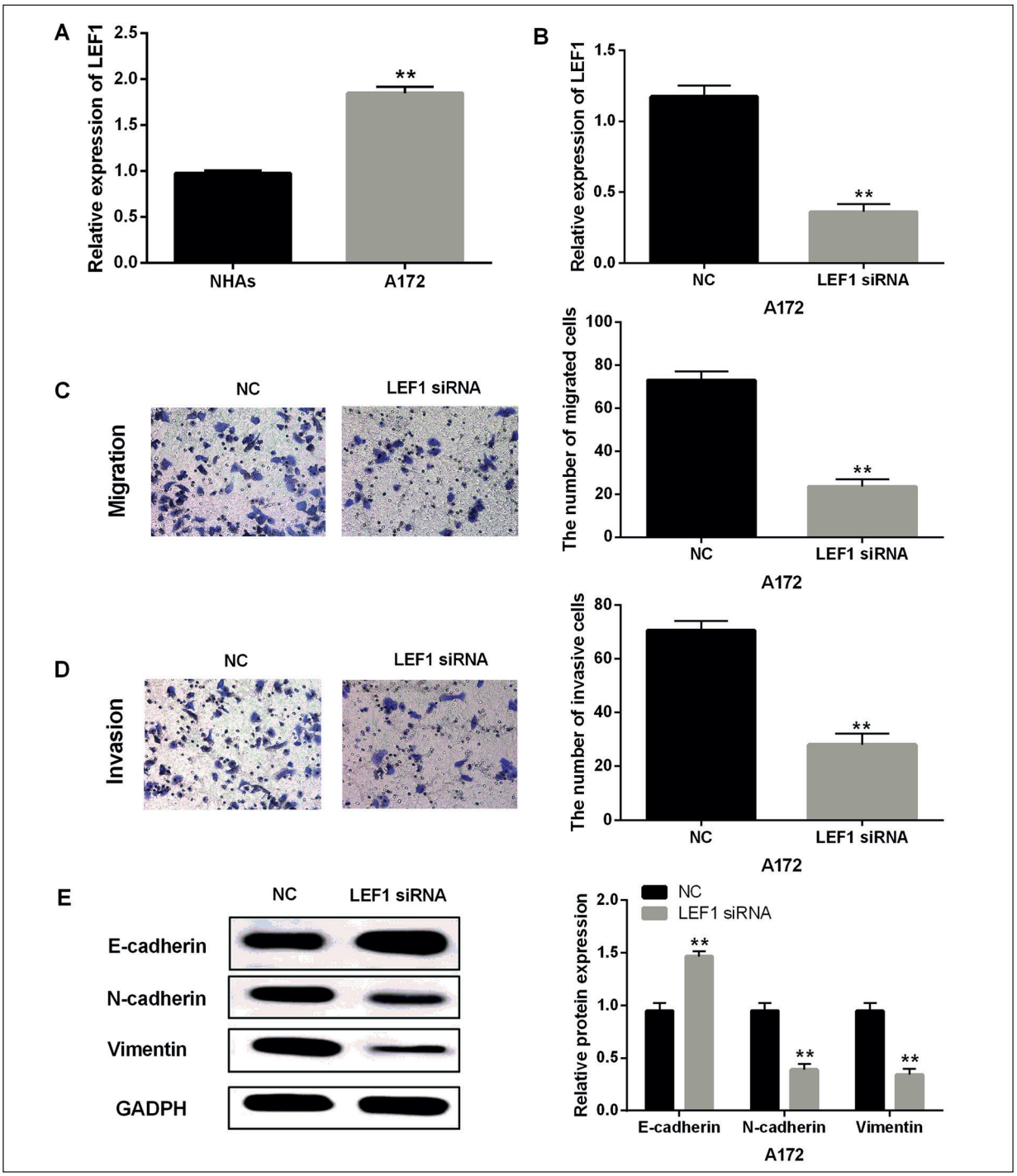

Figure 4. The dysregulation of LEF1 in glioblastoma cells. **A,** LEF1 expression was measured in A172 and NHAs cells. **B,** LEF1 expression was measured in A172 cells with LEF1 siRNA. **C, D,** Cell migration and invasion in A172 cells with LEF1 siRNA (magnification, 200×). **E,** Western blot analysis of E-cadherin, N-cadherin and Vimentin in A172 cells with LEF1 siRNA. ***p*<0.01.

blastoma cells. Furthermore, the overexpression of miR-381 inhibited EMT in glioblastoma cells. In addition, miR-381 was found to directly target LEF1 and negatively regulate its expression in glioblastoma cells. At the same time, the upregulation of LEF1 was identified in glioblastoma cells. Furthermore, LEF1 silencing suppressed cell migration, invasion, and EMT in glioblasto-

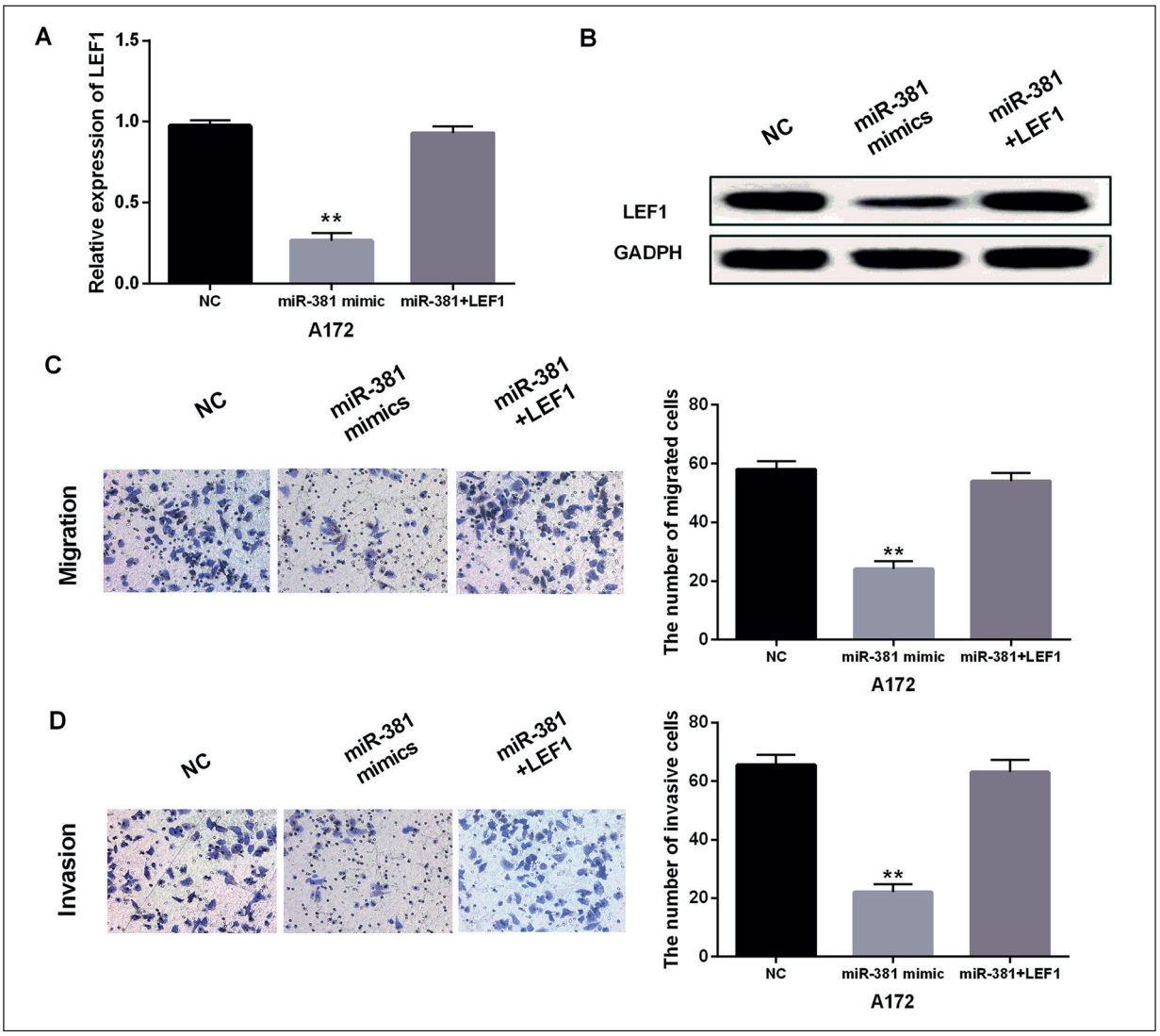

Figure 5. LEF1 abolished the inhibitory effect of miR-381 in glioblastoma cells. **A, B,** LEF1 expression was observed in A172 cells containing miR-381 mimics and LEF1 vector. **C, D,** Cell migration and invasion in A172 cells containing miR-381 mimics and LEF1 vector (magnification, 200×) $^{**}p<0.01$.

ma. Notably, the upregulation of LEF1 abolished the inhibitory effect of miR-381 in glioblastoma. These findings indicate that miR-381 inhibits the metastasis of glioblastoma cells.

Previous studies[18-20] have shown that miR-381 expression was reduced in oral squamous cell carcinoma, non-small cell lung cancer, and colon cancer, which is consistent with our results. In addition, miR-381 has been shown to act as a tumor inhibitor in colorectal cancer by targeting Twist1[21], which is similar to our results. MiR-381 was also found to inhibit cell migration and invasion in papillary thyroid carcinoma by regulating LRP6 expression[22]. In particular, Xue et al[23] found that miR-381 restrained EMT and cell metastasis in breast cancer *via* inhibiting CXCR4 expression. The same effect of miR-381 on cell metastasis and EMT was also found in glioblastoma. Many previous studies have shown that miR-381 acts as a tumor inhibitor in human cancers by regulating the expression of target genes, such as SOX4 and LRH-1[24,25]. To further explain the regulatory mechanism of miR-381 in glioblastoma, LEF1 was confirmed to be a direct target for miR-381 in this study.

In this study, LEF1 was found to be upregulated in glioblastoma and act as an oncogene. Similar to our results, the upregulation of LEF1 has been identified in colon cancer[26]. Functionally, LEF1 has been reported to inhibit tumor progres-

sion and induce myo-differentiation in a subset of rhabdomyosarcoma[27]. Furthermore, LEF1 was also found to promote EMT in prostate cancer[28]. As a target gene, LEF1 expression has been reported to be negatively correlated with some miRNAs, including miR-557 and miR-708[29,30]. A negative correlation between LEF1 and miR-381 expression was also identified in this study. In addition, the interaction between miR-381 and LEF1 was also investigated in glioblastoma. We found that miR-381 restrained glioblastoma cell metastasis *via* targeting LEF1. Consistent with our results, miR-219-5p has been found to inhibit EMT and cell metastasis in colorectal cancer *via* inhibiting LEF1 expression[31]. Especially, Liu et al[32] demonstrated that miR-218 suppressed glioblastoma cell invasion by inhibiting the expression of oncogenic gene LEF1. Moreover, it has been reported[33] that miR-381/LEF1 axis inhibited renal carcinoma cell invasion. The results of previous studies supported our conclusion. In this study, the relationship between miR-381 and LEF1 was first proposed in glioblastoma, which has not been reported in previous studies. Furthermore, inhibition of cell metastasis induced by miR-381 was confirmed in glioblastoma.

Conclusions

The downregulation of miR-381 was observed in glioblastoma cells. Furthermore, overexpression of miR-381 inhibited metastasis and EMT of glioblastoma cells by downregulation of LEF1. The results suggest that miR-381 have a potential to provide a new approach for the treatment of glioblastoma patients.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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