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MicroRNA-362 inhibits cell growth and metastasis in glioblastoma by targeting MAPK1

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Abstract. – OBJECTIVE: Glioblastoma (GBM) is a deadly brain cancer that seriously threatens the lives of patients. Moreover, various microRNAs (miRNAs) have been found to be involved in the progression of GBM. The purpose of this study is to preliminarily elucidate the regulatory mechanism of miR-362 in GBM.

PATIENTS AND METHODS: The abnormal expression of miR-362 and MAPK1 was detected by RT-qPCR or Western blot analysis in GBM tissues and cells. CCK-8 and transwell assays were performed to measure cell proliferation, migration and invasion. The relationship between miR-362 and MAPK1 was confirmed by luciferase reporter assay.

RESULTS: MiR-362 expression was reduced in GBM tissues and cells. The decreased expression of miR-362 predicted poor prognosis in GBM patients. Functionally, overexpression of miR-362 inhibited the proliferation and metastasis of GBM cells. In addition, miR-362 directly targets MAPK1. MAPK1 was negatively correlated with miR-362 expression in GBM. Moreover, MAPK1 was upregulated and served as a tumor promoter in GBM. More importantly, the upregulation of MAPK1 weakened the inhibitory effect of miR-362 on cell proliferation and metastasis in GBM.

CONCLUSIONS: MiR-362 restrains cell proliferation and metastasis in GBM by targeting MAPK1, indicating that miR-362 functions as a tumor suppressor in GBM.

Key Words:
MiR-362, Glioblastoma, Proliferation, Metastasis, MAPK1.

Introduction

Glioblastoma (GBM) is the most malignant glioma in astrocytoma, and it is also IV degree glioma[1]. In addition, GBM is growing rapidly and is easy to spread and metastasize. 70% to 80% of GBM patients have a disease course of approximately 3 to 6 months[2]. The peak age of GBM is usually 50-60 years old. Due to its high malignancy, the overall survival rate of GBM patients is still poor. Under the current standardized treatment, the average survival time of GBM patients is only 14.6 months. In addition, the two-year survival rate is only 27%[3]. Although some existing targeted therapies, biological treatments and other methods help to prolong the survival of GBM patients, the survival rate of patients is still not optimistic[4]. Therefore, the development of new effective treatments is still very important for GBM patients.

As an emerging potential target, microRNAs (miRNAs) can regulate the development of different cancers by participating in various physiological activities[5]. Moreover, microRNAs have been reported[6] to be major regulators of gliomas. In addition, many miRNAs have been found to mediate the progression of GBM. For example, miR-615 inhibited cell proliferation, migration and invasion in human GBM by targeting EGFR[7]. In contrast, miR-374b promoted the development of GBM by regulating SEMA3B[8]. Recently, the abnormal expression and role of miR-362 has caught our attention. Previous studies[9,10] have shown that the role of miR-362 depends on the type of cancers. For instance, the upregulation of miR-362 was found in colorectal cancer and lung cancer. Functionally, miR-362 promoted tumor growth and metastasis in hepatocellular carcinoma by targeting CYLD[11]. However, miR-362 was downregulated in cervical cancer and suppressed

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cell proliferation, migration and invasion by directly targeting SIX1[12]. In addition, miR-362 has been found to be downregulated in astrocytoma tissues[13]. But the specific role of miR-362 is still vague in GBM.

As a member of the MAP kinase family, mitogen-activated protein kinase 1 (MAPK1) has been found to be involved in tumors development. Previous studies[14,15] have shown that MAPK1 was upregulated in thyroid cancer and colorectal cancer. Functionally, MAPK1 silencing suppressed the proliferation, migration and invasion of colon adenocarcinoma cells[16]. In addition, some miRNAs have been reported to regulate cancer progression by mediating MAPK1 expression. MiR-22 suppressed the epithelial-mesenchymal transition (EMT) in bladder cancer by inhibiting the MAPK1/Slug/vimentin feedback loop[17]. Moreover, miR-378 was found to inhibit gastric cancer cell proliferation, cell cycle and induced apoptosis by targeting MAPK1[18]. Besides, miR-129-1 has been reported to induce cell cycle arrest in GBM by targeting MAPK1[19]. However, the interaction between, miR-362 and MAPK1 has not been elucidated in GBM. In the current study, the abnormal expression and roles of miR-362 and MAPK1 were investigated in GBM. Furthermore, their relationship was also verified in this study. This study may provide potential treatment for GBM patients.

Patients and Methods

Clinical Tissues

Seventy-two GBM tissues and adjacent tissue were collected from Yantaishan Hospital. All patients involved in this study were histologically diagnosed in line with the 2016 WHO classification of the central nervous system tumors. Moreover, signed written informed consents were obtained from the patients and/or guardians before the experiment. None of the patients received radiation therapy or chemotherapy before surgical resection. The permission of this research was acquired from the Institutional Ethics Committee of Yantaishan Hospital. The study was conducted in accordance with the principles of the Helsinki Declaration.

Cell Culture

Normal human astrocytes NHA (NHAs, BNCC341796) and GBM cell line A172 (ATCC® CRL-1620™) were purchased from ATCC (Manassas, VA, USA). These cells were grown under conditions of 5% CO_2, 37°C and Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Life Technology) supplemented with 10% fetal bovine serum (Gibco, Life Technology, Carlsbad, CA, USA) and 1% penicillin-streptomycin (Gibco, Life Technology, Carlsbad, CA, USA).

Cell Transfection

MiR-362 mimics, miR-362 inhibitor, MAPK1 siRNA or MAPK1 overexpression plasmid was purchased from GenePharma (Shanghai, China). Sequences that have no homology to any known human sequences are used as controls. These sequences were transfected into A172 cells using Lipofectamine 2000 (Invitrogen, CA, USA).

Dual-Luciferase Reporter Assay

The 3'-UTR of wild type or mutant MAPK1 was inserted into pmirGLO Luciferase reporter vector (Promega, Madison, WI, USA). The pmirGLO vector was then co-transfected with miR-362 mimics into A172 cells by Lipofectamine 2000 and incubated for 48 h. Finally, the relative luciferase activity was observed by dual-luciferase reporter assay system (Promega, Madison, WI, USA).

RNA Isolation and RT-qPCR

Total RNA isolation was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA was synthesized by High Capacity RNA-to-cDNA Master Mix (Life Technologies, Carlsbad, CA, USA). PCR amplification was performed by SYBR ExScript RT-PCR kit (TaKaRa, Dalian, China) on an Applied Biosystems AB7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with specific primers. U6 or GAPDH was used as internal normalized control for miR-362 or MAPK1. Relative miRNA or mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method, normalized against U6 or GAPDH and then compared with the control group. The forward primer for miR-362 was 5'-GTCACGAAATCCTTGGAACCTAG-3', and the reverse primer was 5'-TATGGTTGTTCTCGTCTCCTTCTC-3'. The internal control for miR-362 was GAPDH (forward, 5'-CGGAGTCAACGGATTTGGTCGTAT-3'; reverse, 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'). The primers for MAPK1 were 5'-TGGATTCCCTGGTTCTCTCTAAAG-3' (forward) and 5'-GGGTCTGTTTTCCGAGGATGA-3' (reverse).

The internal control was U6 (forward, 5'-CTC-GCTTCGGCAGCACA-3'; reverse, 5'-AAC-GCTTCACGAATTTGCGT-3').

Western Blot Analysis

RIPA lysis buffer (Beyotime, Haimen, China) was used to obtain protein samples. The protein concentration was detected using a BCA protein kit (Beyotime, Haimen, China). Next, A total of 50 μg of protein was separated by 10% SDS-PAGE and electro-transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad). The membrane was then blocked with 5% non-fat milk and incubated with MAPK1 and GAPDH primary antibodies (Abcam, Cambridge, CA, USA) overnight at 4°C. Thereafter, the membrane was washed and incubated with secondary antibodies (Abcam, Cambridge, CA, USA) for 1 h at 37°C. The protein bands were visualized using an ECL kit (Beyotime, Haimen, China).

CCK-8 Assay

The prepared A172 cells were incubated in a 96-well plate for 24 h (37°C, 5% CO_2). Next, A172 (4×10^3/well) cells were incubated for 24, 48, 72 and 96 h. Then, these cells were incubated for 4 h with 10 ml of CCK-8 (Dojindo, Kumamoto, Japan) solution. The absorbance at 450 nm of each sample was determined using a microplate reader (Molecular Devices, San Jose, CA, USA).

Transwell Assay

For detection of cell migration, A172 cells (4×10^3 cells/well) were suspended in 500 μl of medium without FBS and placed in the upper chambers of the Transwell (Costar, Cambridge, MA, USA)., The lower chambers were filed with medium containing 10% FBS as a chemoattractant. For detection of cell invasion, the upper chambers were coated with Matrigel (BD Biosciences, San Jose, CA, USA). After 24 h at 37°C, the moved cells were stained with 0.1% crystal violet. The cells were observed and counted under a microscope (Olympus, Tokyo, Japan).

Statistical Analysis

Data are expressed as mean ± SD. Statistical analyses were analyzed by SPSS 13.0 or Graphpad Prism 6 (La Jolla, CA, USA). Differences between groups were analyzed using Student's t-test and one-way analysis of variance (ANOVA) with Tukey's post-hoc test. The difference was considered significant at $p < 0.05$.

Results

MiR-362 Expression Was Reduced in GBM Tissues and Cells

First, the abnormal expression of miR-362 was detected in GBM tissues and cells. RT-qPCR showed that miR-362 expression was decreased in GBM tissues compared to normal tissues ($p<0.01$, Figure 1A). Consistently, the downregulation of miR-362 was also found in A172 cells compared to NHA cells

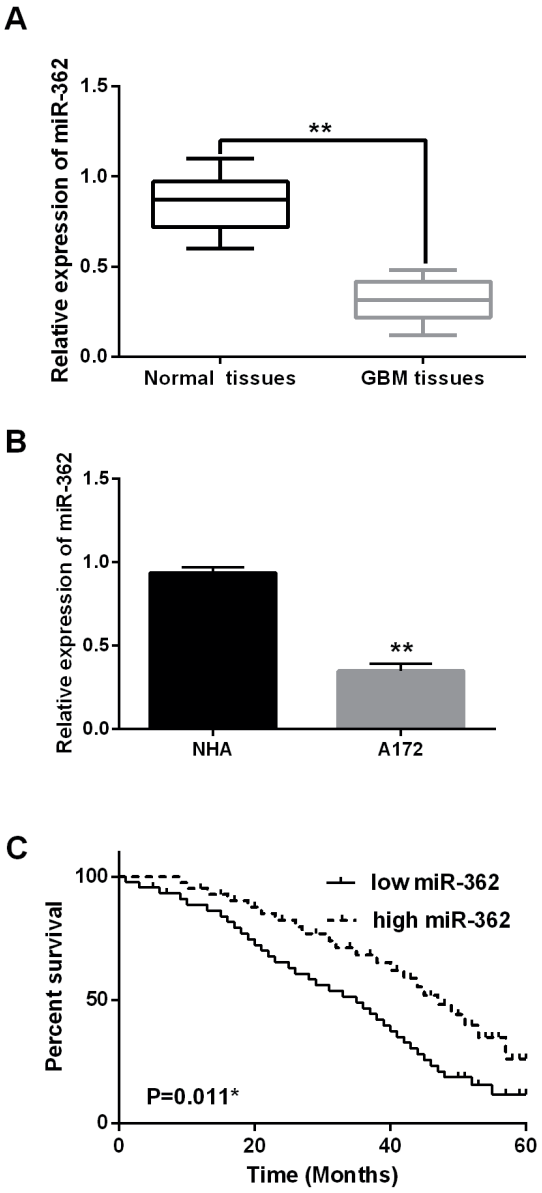

Figure 1. MiR-362 expression was reduced in GBM. **A,** The mRNA miR-362 expression in GBM tissues. **B,** Te miR-362 expression in A172 and NHA cells. **C,** Low miR-362 expression was related to shorter overall survival in GBM patients *$p < 0.05$, **$p < 0.01$.

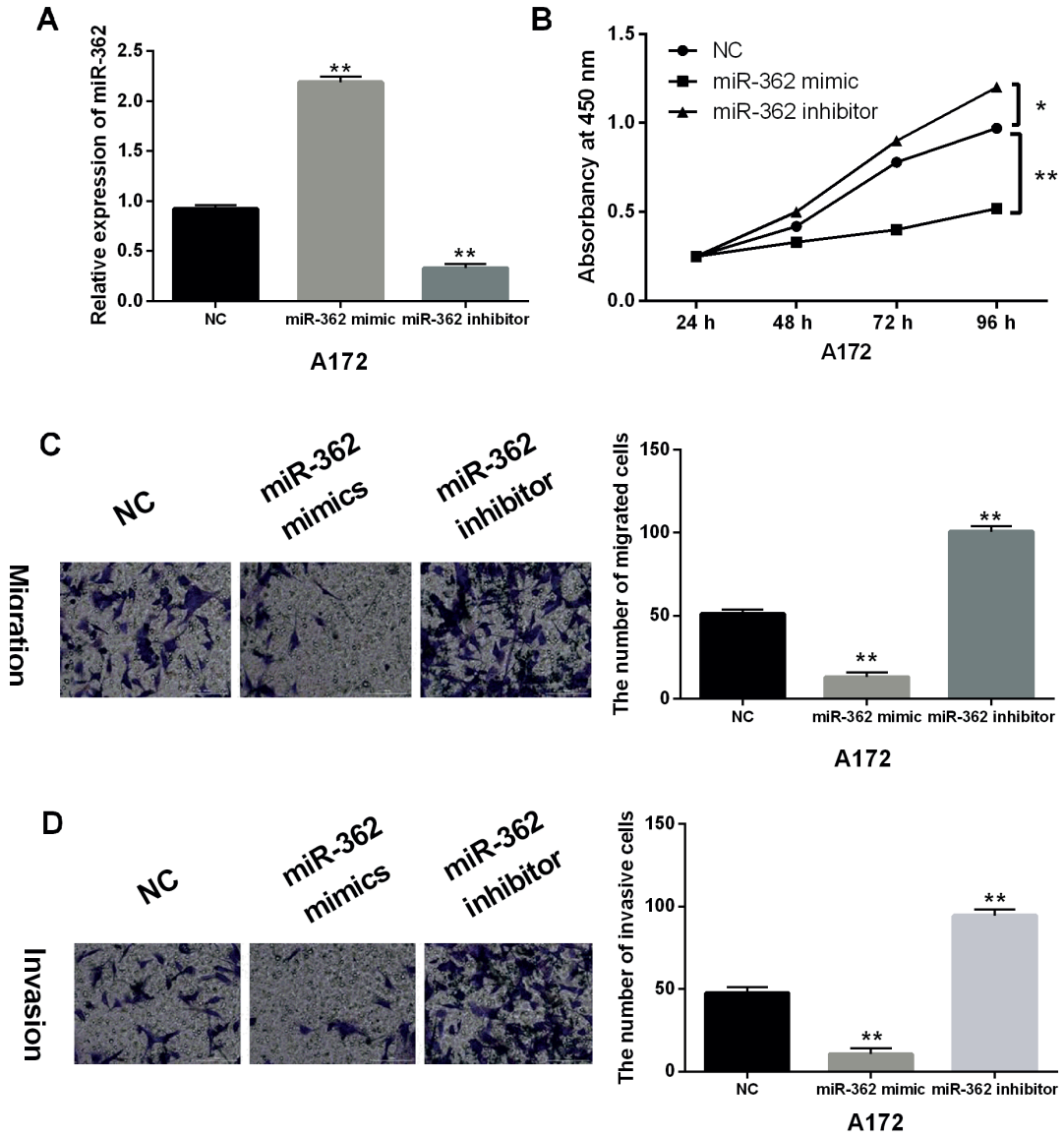

Figure 2. MiR-362 overexpression restrained GBM cell growth and metastasis. **A,** MiR-362 expression was examined in A172 cells with its mimics or inhibitor. **B,** Cell proliferation was measured in A172 cells with miR-362 mimics or inhibitor. **C-D,** Cell migration and invasion were measured in A172 cells with miR-362 mimics or inhibitor (magnification, 200x). ** p <0.01.

(p<0.01, Figure 1B). In addition, the correlation between miR-362 and the prognosis of GBM patients was analyzed. We found that the low expression of miR-362 was associated with shorter overall survival of GBM patients (p<0.05, Figure 1C). Based on these results, we suspect that miR-362 may regulate malignant behavior in the progression of GBM.

Overexpression of MiR-362 Restrained GBM Cell Proliferation and Metastasis

Next, miR-362 mimics or inhibitor was transfected into A172 cells to explore the function of miR-362 in GBM. RT-qPCR showed that miR-362 mimics increased its expression level, while miR-362 inhibitor decreased its expression in A172 cells (p<0.01, Figure 2A). Functionally, cell proliferation was inhibited by overexpression of miR-362, and was promoted by downregulation of miR-362 in A172 cells (p<0.01, Figure 2B). Similarly, the overexpression of miR-362 suppressed cell migration, while the downregulation of miR-362 promoted cell migration in A172 cells (p<0.01, Figure 2C). In addition, miR-362 mimics were found to inhibit cell invasion, whereas

miR-362 inhibitor promoted A172 cell invasion (p<0.01, Figure 2D). These results indicate that the overexpression of miR-362 inhibits cell proliferation and metastasis in GBM.

MiR-362 Directly Targets MAPK1

In addition, TargetScan database (http://www.targetscan.org/) shows that miR-362 has a site that binds to the 3'-UTR of MAPK1 (Figure 3A). Next, a Luciferase reporter assay was performed to verify this prediction. It was found that miR-362 mimics reduced the luciferase activity of Wt-MAPK1. However, Mut-MAPK1 Luciferase activity was not affected by miR-362 mimics in A172 cells (p<0.01, Figure 3B). Meanwhile, a negative correlation between miR-362 and MAPK1 was detected in GBM tissues (p<0.01, R^2=0.5248; Figure 3C). Consistently, MAPK1 expression was reduced by miR-362 mimics and enhanced by miR-362 inhibitor in A172 cells (p<0.01, Figure 3D, 3E). Briefly, miR-362 directly targets MAPK1. And MAPK1 is negatively correlated with miR-362 expression in GBM.

MAPK1 was Upregulated in GBM Tissues and Cells

To investigate whether MAPK1 is involved in the progression of GBM, the abnormal expres-

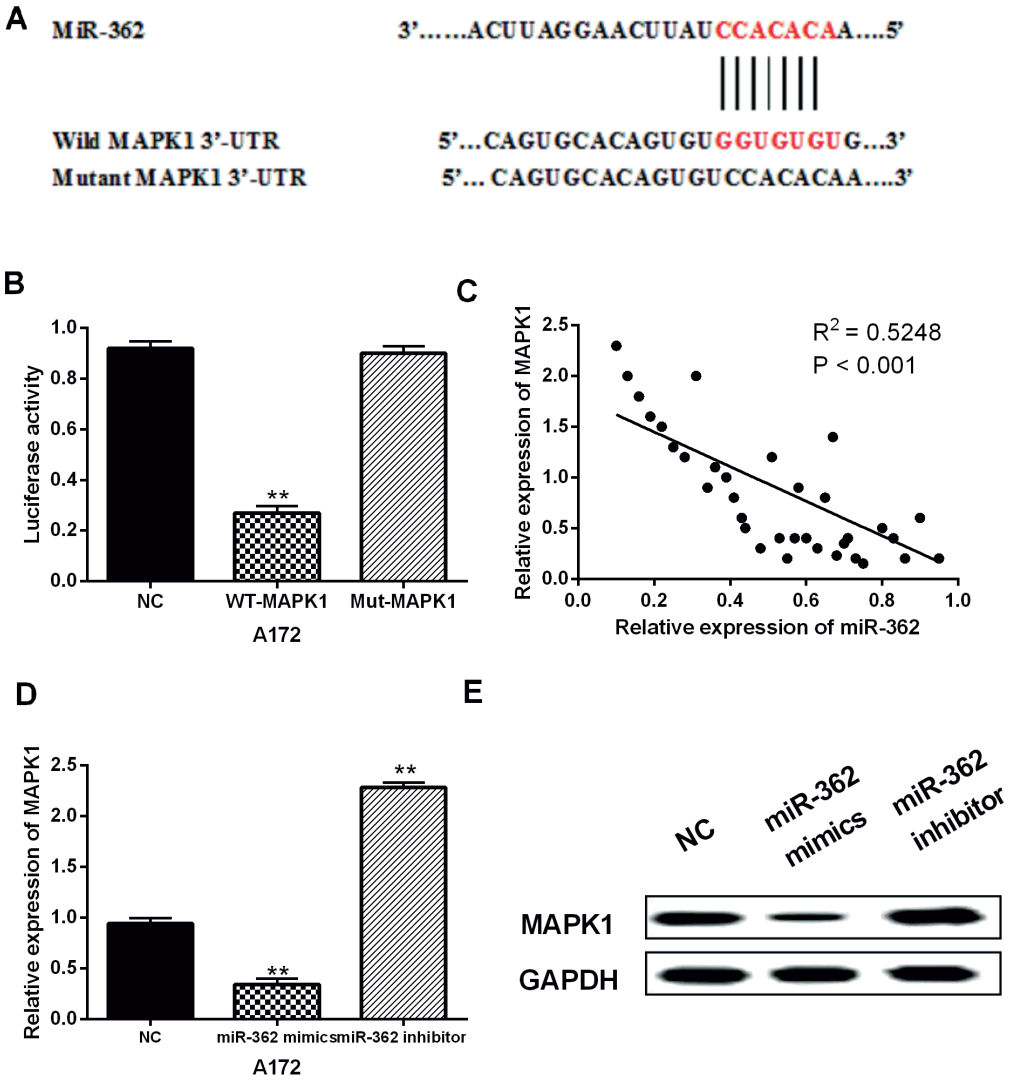

Figure 3. MiR-362 directly targets MAPK1. **A,** The binding sites between MAPK1 with miR-362. **B,** Luciferase reporter assay (**C**) MiR-362 was negatively correlated with MAPK1 in GBM tissues. **D-E,** MAPK1 expression regulated by miR-362 mimics or inhibitor ** p <0.01.

sion and role of MAPK1 was detected in GBM. RT-qPCR showed that MAPK1 was upregulated in GBM tissues and cell lines ($p<0.01$, Figure 4A, 4B). In addition, RT-qPCR showed that MAPK1 siRNA significantly reduce MAPK1 expression in A172 cells ($p<0.01$, Figure 4C). CCK-8 assay showed that downregulation of MAPK1 restrained the proliferation of A172 cells ($p<0.01$, Figure 4D). Similarly, cell migration and invasion were also suppressed by knockdown of MAPK1 in A172 cells ($p<0.01$, Figure 4E, 4F). These results reveal that MAPK1 is upregulated and serves as a tumor promoter in GBM.

MiR-362 Regulated GBM Progression by Targeting MAPK1

Finally, miR-362 mimics and MAPK1 vector were transfected into A172 cells to explore their interaction. RT-qPCR showed that the decreased expression of MAPK1 induced by miR-362 mimics was recovered by MAPK1 vector in A172 cells (Figure 5A). Functionally, the inhibitory effect of miR-362 on cell proliferation was weakened by upregulation of MAPK1 in A172 cells (Figure 5B). Similarly, the upregulation of MAPK1 also impaired the inhibitory effect of miR-362 on cell migration and invasion in A172 cells (Figure 5C, 5D). Taken together, the upregulation of MAPK1 weakens the anti-tumor effect of miR-362 in GBM.

Discussion

GBM is a highly malignant tumor that can easily cause human death. Many studies have shown that miRNAs are associated with the tumorigenesis of GBM. In fact, miR-590 inhibited the migration, invasion and EMT of glioblastoma cells by targeting ZEB1 and ZEB2[20]. Here, miR-362 was also found to be a tumor suppressor in the pathogenesis of GBM. Specifically, miR-362 was downregulated in GBM tissues and cells. The downregulation of miR-362 predicted worse prognosis in GBM patients. The abnormal expression of miR-362 in GBM and the relationship between miR-362 and prognosis in GBM patients are first reported in this study. Functionally, the overexpression of miR-362 restrained cell proliferation and metastasis in GBM. In addition, miR-362 directly targets MAPK1. MAPK1 expression was negatively correlated with miR-362 expression in GBM. More importantly, the upregulation of MAPK1 impaired the anti-tumor effect of miR-362 in GBM. These results indicate that miR-362 acts as a tumor suppressor in GBM by targeting MAPK1.

MiR-362 plays an inhibitory effect in other cancers and diseases by regulating the expression of target genes[21,22]. First, the downregulation of miR-362 was found in renal cancer and atherosclerosis, which is consistent with our results. Functionally, the downregulation of miR-362 promoted tumor progression in human breast cancer[23]. In addition, miR-362 functioned as a tumor suppressor in cervical adenocarcinoma by targeting MCM5[24]. In this work, miR-362 also inhibited cell proliferation and metastasis in GBM by suppressing MAPK1 expression. And the negative correlation and interaction between miR-362 and MAPK1 are confirmed for the first time in our study. In addition, we also found that MAPK1 was upregulated and exerted carcinogenesis in GBM, which has not been reported in previous studies.

As a target gene, MAPK1 has been reported[25] to be negatively regulated by various miRNAs, such as miR-508. In addition, miR-585 suppressed cell proliferation and migration in gastric cancer by targeting MAPK1[26]. More importantly, downregulation of MAPK1 inhibited cell proliferation, migration and invasion in endometrial cancer[27]. These results are similar to our results. In addition, downregulation of miR-362 has been detected in astrocytoma of human brain tumors[13], which is consistent with our result. Meanwhile, it has been found that MAPK1 is involved in the tumorigenesis of GBM[19]. Nevertheless, previous researches did not specifically investigate the role of miR-362 and its relationship with MAPK1 in GBM. However, these studies prove the accuracy of our research. Therefore, we consider that the miR-362/MAPK1 axis serves as a tumor suppressor in the tumorigenesis of GBM.

Conclusions

In conclusion, downregulation of miR-362 was found in GBM and associated with poor prognosis of GBM patients. In addition, miR-362 inhibited cell proliferation, migration and invasion in GBM by downregulating MAPK1. Our findings will help to understand the molecular mechanism of GBM. However, despite this, there is still a need

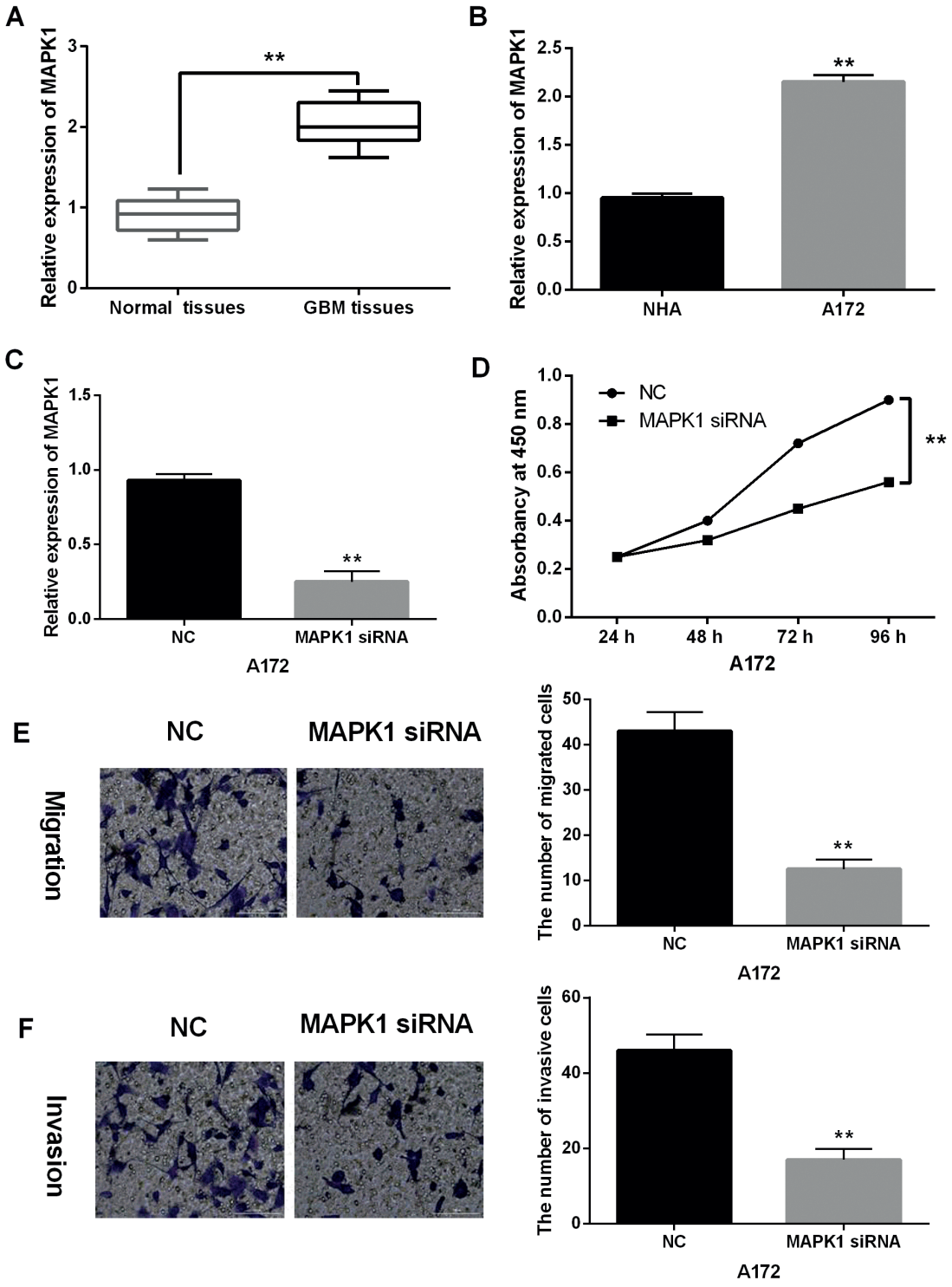

Figure 4. MAPK1 was upregulated in GBM. **A,** MAPK1 expression in GBM tissues (**B**) MAPK1 expression was measured in A172 and NHA cells (**C**) MAPK1 expression was measured in A172 cells with its siRNA. **D,** Cell proliferation in A172 cells with MAPK1 siRNA (**E -F**) Cell migration and invasion in A172 cells with MAPK1 siRNA (magnification, 200x). ** $p < 0.01$.

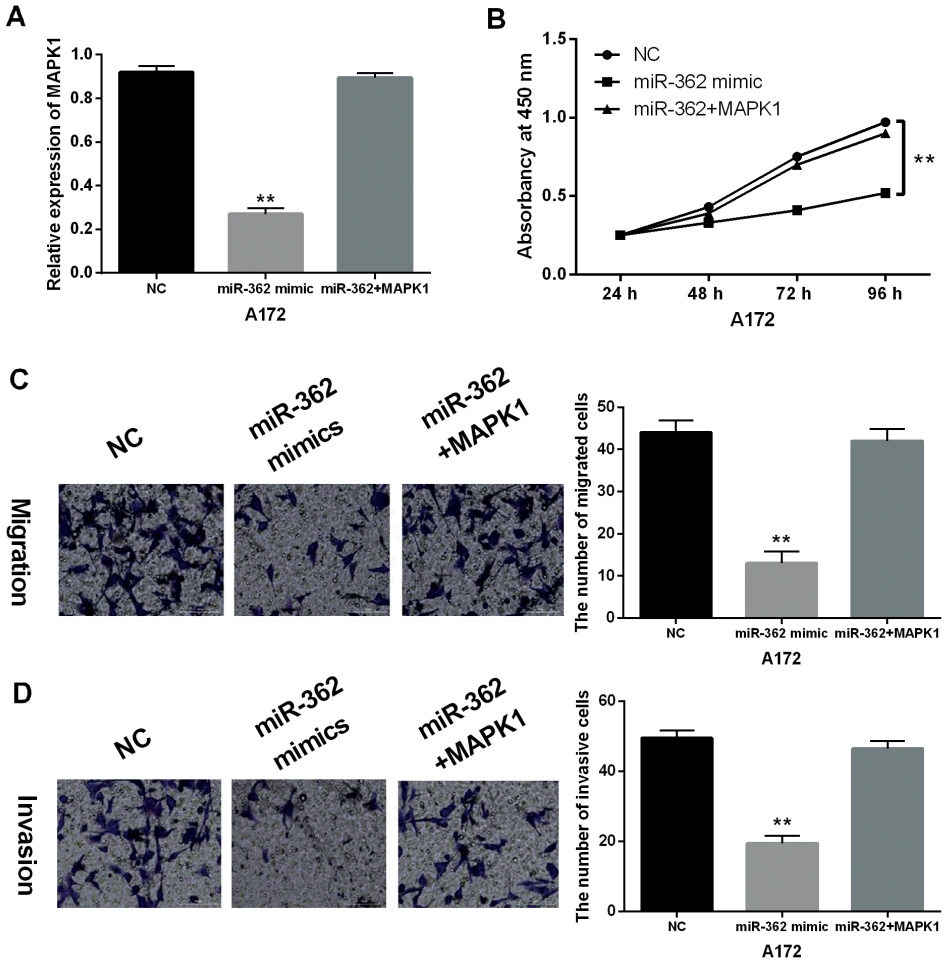

Figure 5. MiR-362 regulated GBM progression by targeting MAPK1. **A,** MAPK1 expression was observed in A172 cells containing miR-362 mimics and MAPK1 vector. **B,** Cell proliferation in A172 cells containing miR-362 mimics and MAPK1 vector (**C-D**) Cell migration and invasion in A172 cells containing miR-362 mimics and MAPK1 vector (magnification, 200x). ** $p < 0.01$.

for more in-depth research on the pathogenesis of GBM.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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