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Original Article

miR-423-5p inhibits the proliferation and metastasis of glioblastoma cells by targeting phospholipase C beta 1

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Abstract: Glioma is a common brain tumor which is highly invasive, responds poorly to therapy, and has a poor prognosis. There is growing evidence that an abnormal expression of many genes is related to glioma and leads to glioma cell growth and metastasis. Phospholipase C beta 1 (PLCB1) plays critical roles in intracellular transduction and regulating signal activation, which are important to tumorigenesis. Therefore, it could bind to miRNA as a target gene. The purpose of our study was to confirm that PLCB1 plays a critical role in suppressing glioma progression. We found that the expression of miR-423-5p was reduced, but the expression of PLCB1 was increased, in glioma tissues and cells. To explore whether miR-423-5p affects PLCB1, a bioinformatics approach suggested that miR-423-5p can directly target PLCB1. Moreover, we observed, using luciferase reporter assays, that miR-423-5p could target PLCB1 3'-UTR. Functionally, the overexpression of miR-423-5p could attenuate the proliferation, invasion, and migration and promote the apoptosis of glioma cells. Furthermore, we found that miR-423-5p could enhance p-ERK expression in glioma cells. Taken together, we deduced that miR-423-5p inhibited proliferation and metastasis by targeting PLCB1, and it also promotes apoptosis in glioma cells. These results suggest that miR-423-5p directly targets PLCB1 3'-UTR and could inhibit cell invasion and migration through the ERK-dependent pathway in glioma, and the miR-423-5p/PLCB1 axis may be a potential target for new potential therapeutic strategies to treat glioma.

Keywords: Glioma, miR-423-5p, PLCB1, metastasis

Introduction

Glioma is the most common subtype of primary brain tumor [1, 2], and it is characterized by diffuse infiltration and is highly metastatic, which explains its fast recurrence and high mortality. Therefore, it is necessary to require available therapy strategies and find novel treatment approaches. Previous findings indicate that the abnormal expression of many genes is related to glioma and leads to glioma cell growth and metastasis [3-5].

PLCB1 plays a vital role in the cell cycle and in cell proliferation, is identified as a member of GPCR, and encodes a protein that catalyzes the formation of phosphatidylinositol 4,5-diphosphate into inositol 1,4,5-triphosphate diester and diacylglycerol [6-8]. It is involved in several kinds of disease, and could be a latent biomarker for disease therapy. Previous studies have shown that PLCB1 overexpression can lead to the proliferation of liver tumor cells and is closely related to the poor prognosis of liver cancer [7]. It also has been detected in colon cancer, and the abnormal expression of PLCB1 is closely related to colon cancer [8].

miRNAs could be tumor suppressors and take part in various cell functions [9, 10]. PLCB1 is reported to be silenced by microRNAs (e.g., miR-1324 and miR-124) [8, 11] and plays a vital role in cell development, proliferation, and apoptosis [12, 13]. Previous research indicates that miR-423-5p has been identified as a novel biomarker in tumors. miR-423-5p has been demonstrated to increase autophagy in hepatocellular carcinoma cells [14]. miR-423-5p levels could inhibit osteosarcoma proliferation and invasion by directly targeting STMN1 [15]. However, the molecular mechanism that regulates miR-423-5p expression is still unclear for glioma.

In this study, we found that PLCB1 was highly expressed in glioma. PLCB1 directly targeted by

miR-423-5p and PLCB1 promotes the proliferation and migration of glioma cells. The overexpression of miR-423-5p suppresses the growth and invasion of glioma. These data suggest that miR-423-5p may be a target for glioma diagnosis and treatment.

Materials and methods

Clinical sample collection

A total of 30 glioma tissue samples and their adjacent tissues were obtained by surgical resection at the Affiliated Hospital of Qingdao University (Qingdao, Shandong, China) from March 2015 to September 2016. None of the patients received any anti-tumor treatment such as chemotherapy or radiotherapy before their surgeries. The samples were immediately frozen in liquid nitrogen following surgical resection and stored at -80°C for subsequent study.

Cell line culture

The human glioma U87 cell line was obtained from the American Type Culture Collection (ATCC). The cells were grown in MEM medium (GIBCO, USA) with 10% FBS (GIBCO, USA) and 1% penicillin/streptavidin (GIBCO, USA) and maintained at 37°C in a humidified atmosphere at 5% CO_2.

siRNA transfection

For the siRNA transfection, 2×10^5 cells per well were plated in a 6-well plate. After adhering for 24 hours, a miR-423-5p duplex mimic, miR-con, PLCB1 siRNA, and control siRNA (RiboBio, Guangzhou, China) were added to the transfection medium for 6 hours at 37°C in a CO_2 incubator. After transfection, the cells were supplemented with a normal culture medium and cultured at 37°C/5% CO_2 for up to 48 hours before harvest.

RNA extraction and quantitative RT-PCR analysis

RNA was isolated from the tissues or cells using a mirVana miRNA Isolation Kit (Ambion, Carlsbad, CA, USA), following the manufacturer's instructions. The first strand of cDNA was synthesized using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Dalian, China). The extraction concentration was measured with a NanoDrop spectrophotometer, with standby preservation at -80°C. The cDNA was amplified using Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) with appropriate primers using an ABI 7500-fast thermocycler (Applied Biosystems, Foster City, CA). U6 and GAPDH served as internal controls. Relative expression was determined using the $2^{-\Delta\Delta Ct}$ method. PLCB1: forward primer: 5'-GATGAGCCCAGATGGCCG-3', reverse primer: 5'-AGTTGAGTCATCATCCCACTTGA-3', miR-423-5p: forward primer: 5'-ATGGTTCGTGGGTGAGGGGCAGAGAGCGAGAGCAGGGTCCGAGGTATTCG-3', reverse primer: 5'-GTGCAGGGTCCGAGGT-3' [16].

Immunohistochemical assays

We performed an immunohistochemical assay for each sample. According to the standard protocol, the major steps included fixing, paraffinizing, dewaxing, dehydrating, antigen retrieval, blocking, incubation with primary antibodies (PLCB1, Cell Signaling Technology), washing, blocking, incubation with secondary antibodies (Santa Cruz), antigen-antibody reactions with diaminobenzidine (Maixin, Fuzhou, China), and observations.

Western blot analysis

U87 cells with specific treatment were harvested and the protein supernatants were isolated using a cell lysis buffer (Cell Signaling Technology; #9803) with added phenylmethylsulfonyl fluoride (PMSF). The extracted proteins were qualified using the BCA method. The total protein content (30 mg) from the cell lysates was resolved by 10% SDS-PAGE and transferred to a 0.45 mm nitrocellulose membrane (Millipore) for 1 hour. The membranes were washed with TBS-T containing 5% (w/v) BSA. The membranes were incubated overnight with specific PLCB1 (Cell Signaling Technology), GAPDH (Cell Signaling Technology), p-ERK (Cell Signaling Technology), ERK (Cell Signaling Technology) and were exposed to secondary antibodies coupled to horseradish peroxidase for 2 hours at room temperature. The membranes were then washed three times with TBST at room temperature. Chemiluminescent signals were generated by the Super Signal West Pico Trial Kit (Thermo Fisher Scientific Inc.) and measured using the Vilber Lourmat imaging system (Vilber Lourmat Corporation, Torcy, France).

Cell viability assay

Cell proliferation was determined using the Cell Titer-Blue Cell viability Assay (Promega). The cells were seeded in a 96-well plate with the density of the optimized cell number (2,000 cells/well). After 24 hours of seeding, the cells were treated with siRNA or diluted chemicals at the indicated working concentration. The cells were incubated for another 48 hours and then measured using a fluorescence microplate reader (Sunrise Remote, Tecan Austria GmbH, Grödig, Austria).

Apoptosis assays

Apoptotic cells were identified using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-based cell detection kit, POD (Roche Applied Science). The cells were fixed on slides with 4% paraformaldehyde for 1 hour, washed with PBS, and then incubated for 10 min with 3% H_2O_2 in methanol at room temperature. After washing with PBS again, the cells were incubated for 2 min on ice with 0.1% Triton X-100 in 0.1% sodium citrate. The slides were then incubated with the TUNEL reaction mixture for 60 min at 37°C and then rinsed with FBS. The stained slides were examined under light microscopy (Motic B1-223A, Motic Deutschland GmbH, Wetzlar, Germany).

Migration and invasion assay

Migration was performed using a wound healing assay. A linear wound was made by scraping the cells using a 200 µl micropipette tip and the debris was washed twice with PBS. At different time points, the cells were photographed with an inverted microscope (Olympus, Japan), and the migration distances were measured. The cell invasion ability was examined using Matrigel-coated Transwell chamber inserts with 8mm pores (Corning, USA). Stably transduced cells were trypsinized, adjusted to 10 10^6/ml in serum-free MEM, and a 200 µl cell suspension (2×10^5 cells) was added to the upper chamber above the Matrigel. The lower chamber was filled with 500 µl 10% FBS-MEM. After 24 h, the upper chamber was removed, and the Matrigel and unmigrated cells were gently scraped with a wet cotton swab. The cells were fixed with 70% ethanol and stained with hematoxylin. Then, the cells were counted under a lighted microscope, randomly selecting three areas for counting.

Software support and statistical analysis

All images were formatted for optimal presentation using Adobe Illustrator CS4 (Adobe Systems). To determine the statistical significance, the *P*-value from t-statistic was calculated.

Results

PLCB1 was significantly highly expressed in glioma

To examine the level of PLCB1 in tissues and cell lines, we quantified PLCB1 expression in the glioma tissues and the cell lines. qPCR was performed so that the mRNA expression level of PLCB1 had a significantly high expression in the glioma tissues compared with the corresponding adjacent tissues (**Figure 1A**). Similarly, the high protein expression of PLCB1 was verified by immunohistochemistry and Western-blot (**Figure 1B** and **1C**). These results suggest that PLCB1 may play a vital role in glioma.

PLCB1 and cell viability evaluation

We detected PLCB1 expression in the glioma U87 cells and the astroglia (AS) cells using qPCR and Western-blot (**Figure 2A** and **2B**). The PLCB1 expression level was clearly higher in the U87 cells compared with the AS cells. To assess the biological role of PLCB1, PLCB1-specific small interfering RNAs (PLCB1 siRNAs) or the corresponding control siRNA (Con siRNA) were introduced into the glioma cells and the efficiency of PLCB1 siRNA was also tested by qPCR and Western-blot (**Figure 2C** and **2D**). As a result, the knockdown of PLCB1 exhibited significantly decreased cell growth, invasion and migration in theU87 cells (**Figure 2E-G**). This indicates that PLCB1 plays a vital role in anti-apoptotic activity and in cell proliferation in glioma cells.

miR-423-5p directly targets PLCB1 3'-UTR in glioma cells

Using TargetScan (http://www.targetscan.org), we found that PLCB1 could be a potentially direct target gene of miR-423-5p (**Figure 3A**). We cloned the 3'-UTR wide-type or the 3'-UTR mutant-type of PLCB1 into a pMIR-REPORT vector. As expected, the luciferase activity of 3'-UTR wild-type in cells transfected with miR-423-5p was much lower than in the cells transfected with the miR-control, while the 3'-UTR

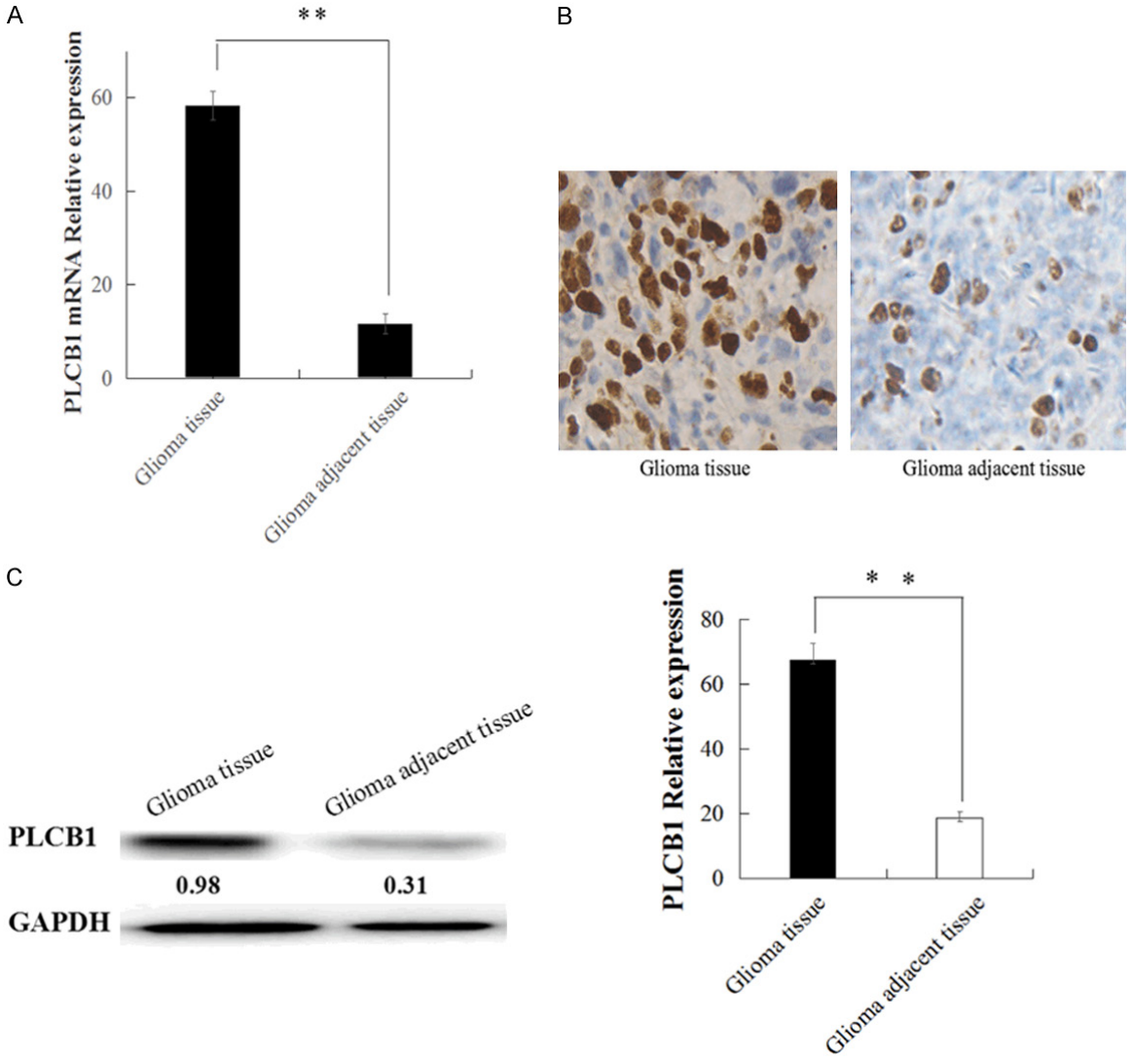


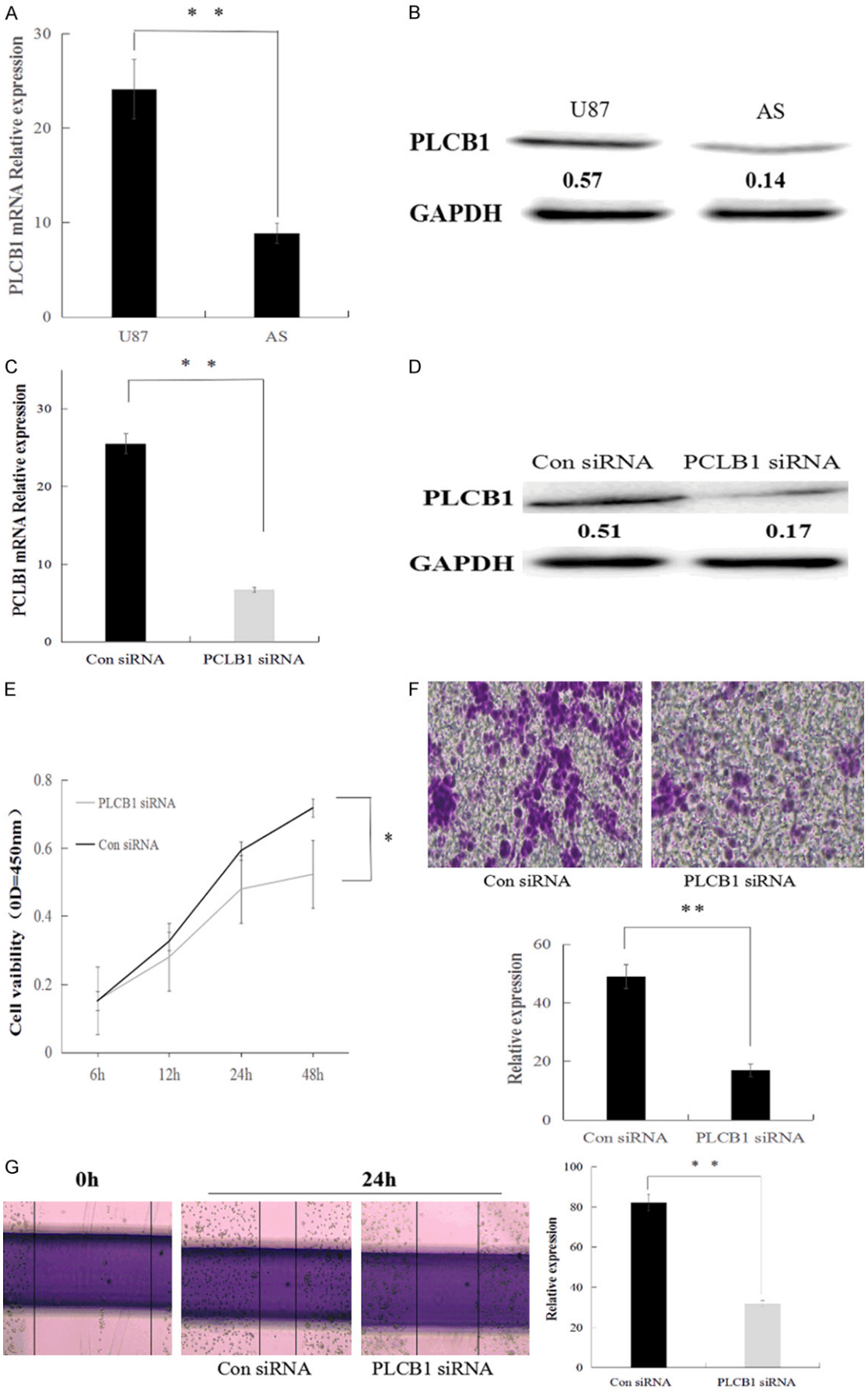
Figure 1. PLCB1 was highly expressed in glioma tissue. A. qRT-PCR was used to examine the relative mRNA expression level of PLCB1 in glioma tissues compared with the adjacent tissues. B, C. Relative protein expression of PLCB1 in glioma tissues compared with normal tissues by immunohistochemical and Western-blot assays. **P < 0.01.

mutate-type showed little change in luciferase activity (**Figure 2B**). These results reflected that PLCB1 directly targets miR-423-5p.

miR-423-5p inhibits cell proliferation and promotes cell apoptosis

To observe the effects of miR-423-5p in glioma, we determined the expression level of miR-423-5p in glioma tissues and cell lines using qPCR. The expression of miR-423-5p in the tumor samples was lower than in the adjacent tissue samples (**Figure 4A**). Consistent with the cancer tissue results, the miR-423-5p level was lower in the U87 cells compared with the As cells (**Figure 4B**). These data show that miR-423-5p may play a regulator role in glioma.

Moreover, U87 cells were transfected with miR-423-5p mimics and miR-con. The transfection efficiency was determined by doing a qPCR analysis in the U87 cells. As shown in **Figure 4C**, the expression level of PLCB1 was distinctly suppressed in the U87 cells transfected with the miR-423-5p mimics compared to the control group. CCK-8 assays were used to evaluate cell proliferation, and the results demonstrated that an overexpression of miR-423-5p could inhibit cell growth significantly compared with the control group (**Figure 4D**). These data indi-


A
* *
PLCB1 mRNA Relative expression
30
20
10
0
U87
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U87
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Figure 2. PLCB1 was highly expressed in glioma cells and is essential for cell proliferation, invasion and migration. A, B. Relative mRNA and protein expression of PLCB1 in glioma cells compared with AS cells. C, D. Transfection of PLCB1 siRNA reduced the PLCB1 mRNA and protein levels. E-G. Cell proliferation, invasion, and migration were inhibited by siRNA-PLCB1 in glioma cells. **P < 0.01, *P < 0.05.

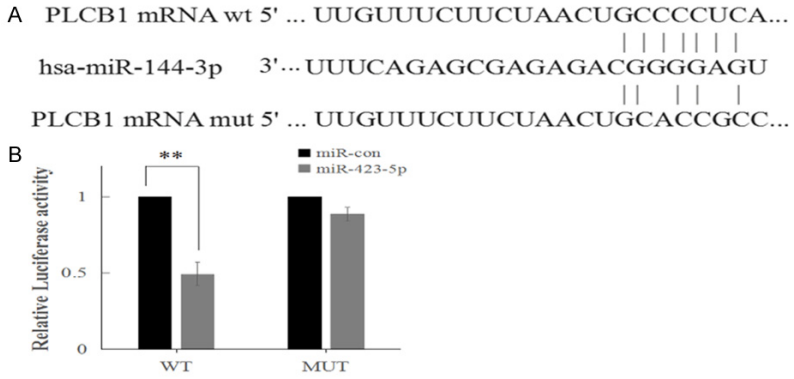

Figure 3. miR-423-5p directly targeted PLCB1 3'-UTR. A. The binding sites of miR-423-5p in PLCB1 mRNA 3'-UTR were predicted by Targetscan. The PLCB1 wild type and mutant type are shown in diagram. B. U87 cells were transfected with pMIR-3'-UTR or pMIR-3'-UTRMut together with miR-con or miR-423-5p. After 48 h, relative luciferase activity was examined. **P < 0.01.

cated that the overexpression of miR-423-5p in U87 cells markedly reduced their tumor growing ability.

Furthermore, a TUNEL assay showed that the apoptosis rate was increased markedly in the U87 cells with miR-423-5p (**Figure 4E**). It was shown that the accumulation of miR-423-5p could increase the number of apoptotic cells. Taken together, miR-423-5p can inhibit cell proliferation and promote cell apoptosis.

miR-423-5p attenuates cell invasion and migration in glioma cells through the ERK-dependent pathway

Cell invasion is a crucial process in cancer metastasis, so a Transwell assay was used to assay the invasion of glioma cells. miR-423-5p at high levels resulted in a significant decrease in cell invasion in transfection with miR-423-5p cells compared with the miR-control group (**Figure 5A**). The result was consistent with those from a migration assay (**Figure 5B**). Moreover, we detected invasion-associated protein by Western-blot. The results reflected that invasion-associated protein expression was attenuated in U87 cells with miR-423-5p transfection. Several studies have suggested that PLCB1 is corrected with the ERK pathway in many kinds of tumor cells, including lung cancer, glioma and breast cancer [17-19], but the mechanism is not clear in glioma. To reveal whether miR-423-5p suppressed the invasion and migration by the ERK-dependent pathway, U87 cells were transfected with miR-423-5p mimics, miR-Con, for 24 h. Western blotting was carried out to analyze the levels of p-ERK and total ERK, and the results reflected that miR-423-5p inhibited p-ERK (**Figure 5C**). These results demonstrate that ERK-dependent signaling mediates the creased levels of invasion and migration in U87 cells. Combined with the above data, these results show that miR-423-5p directly targets PLCB1 3'-UTR and could inhibit cell invasion and migration through the ERK-dependent pathway in glioma cells.

Discussion

Glioma, with its low, 5-year survival rates, is the most common and aggressive brain tumor. The main treatment for glioma is surgical, but there is no clear boundary with brain tissue, so it is difficult to remove the tumor. The effect of clinical therapy is very poor at present. Therefore, there is a pressing need for better therapies that enable the precise targeting of genes in glioma cells.

Previous studies supported the idea that most cancers result from the accumulation of genetic changes (mutations) in cells over time [20, 21]. Previous studies demonstrated that PLCB1 had a higher expression in liver cancer and colon cancer, so it could serve as a biomarker for these cancers [7, 11]. In our glioma samples, a majority of the tumor tissues expressed high levels of PLCB1 mRNA compared to adjacent tissues. Moreover, we found both high PLCB1 mRNA expression and protein expression in the glioma cells. Furthermore, we determined that high PLCB1 expression was associ-

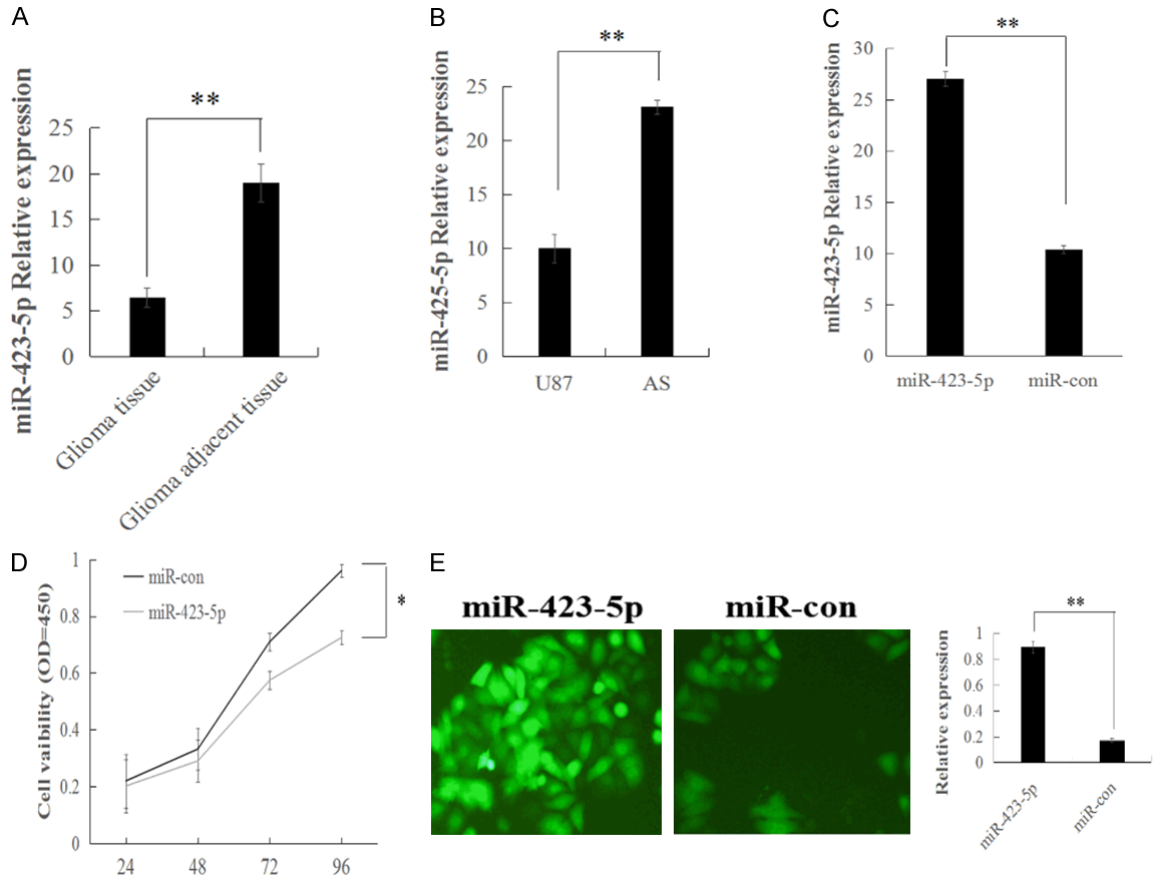

Figure 4. miR-423-5p was downregulated in glioma cell proliferation and apoptosis. A. The relative expression of PLCB1 was measured in glioma tissue compared to adjacent tissues using qRT-PCR. B. The relative mRNA expression of miR-423-5p in glioma cells and AS cells. C. After being transfected with miR-con or miR-423-5p, qRT-PCR was performed to determine the expression of miR-423-5p in glioma cells. D. A CCK-8 assay was used to examine the effect of miR-423-5p on the cell proliferation of glioma cells. E. Cell apoptosis was inhibited by miR-423-5p in glioma cells. **P < 0.01, *P < 0.05.

ated with cell proliferation, invasion, and migration in glioma cells. However, the precise mechanism and the therapeutic targets of glioma have not yet been fully elucidated.

MicroRNAs (miRNA) are a new class of noncoding RNAs, which regulate a variety of biological processes, and their functions are involved in cell proliferation, cell apoptosis, development, and metabolism [22-24]. Nowadays, many studies have reported that miRNAs are associated with glioma. For example, MiR-150-3p targets SP1 and suppresses the growth of glioma cells [25]. MicroRNA-1231 exerts a tumor suppressor role by regulating the EGFR/PI3K/AKT axis in glioma [26]. In the present study, we showed that there were few studies finding that miR-423-5p is related to glioma, and even less data indicating the specific mechanism between them. In our study, we measured a low expression of miR-423-5p in glioma tissue samples compared to adjacent tissues. These results were in line with the cell results. Moreover, we found that miR-423-5p high expression can distinctly inhibit glioma cell growth and invasion. Taken together, this indicates that miR-423-5p expression is gradually decreased in line with the increased malignancy of glioma.

Previous research has shown that miRNAs, as a tumor inhibitor, are incorporated into an RNA-induced silencing complex that binds to 3'-UTRs of objective genes, causing the degradation or translational repression of mRNA [27]. miR-423-5p, widely considered a tumor suppressor, is bound to the 3'-UTRs of the target genes and plays an important role in the regulation of the cell cycle, cell differentiation, and apoptosis in

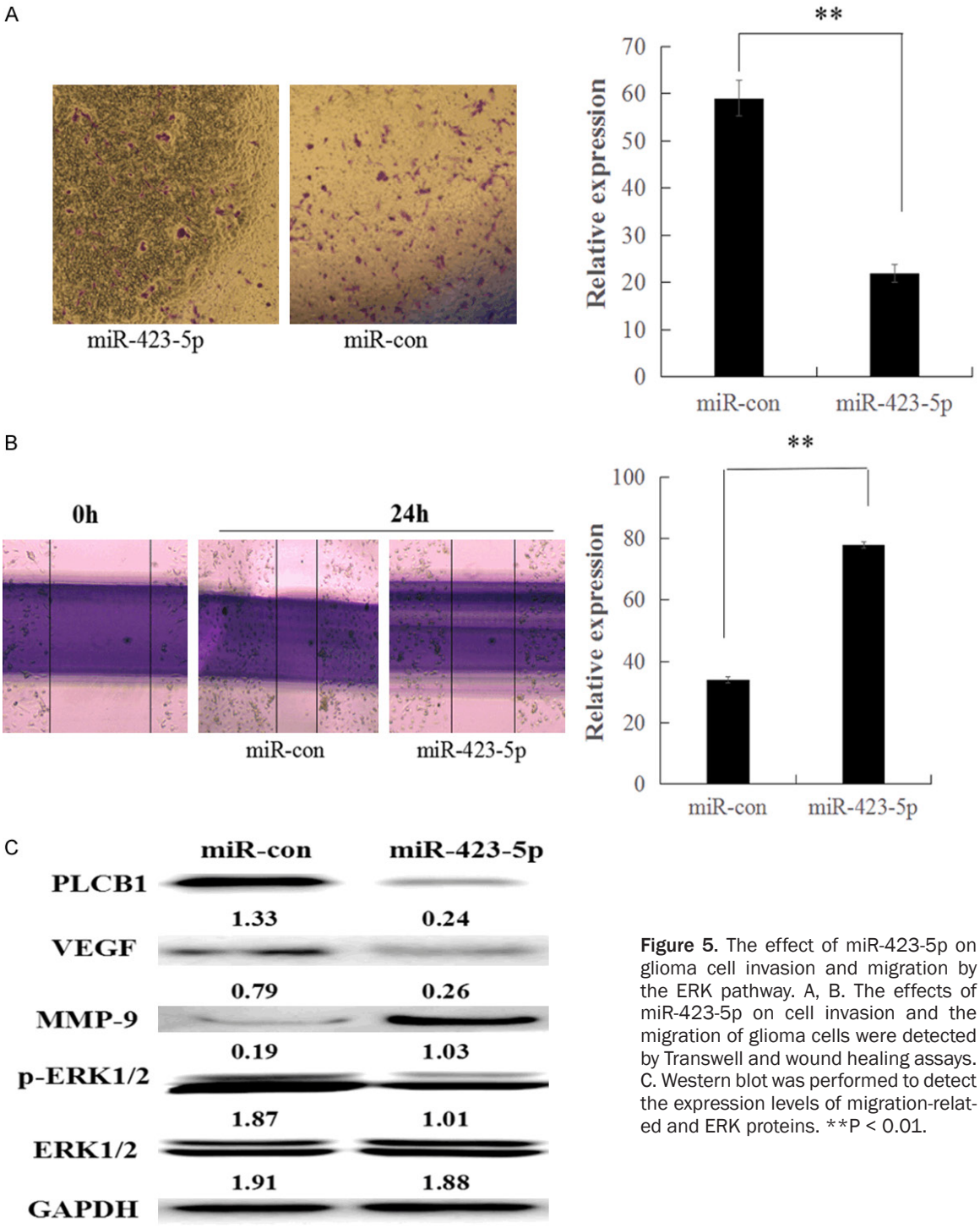

Figure 5. The effect of miR-423-5p on glioma cell invasion and migration by the ERK pathway. A, B. The effects of miR-423-5p on cell invasion and the migration of glioma cells were detected by Transwell and wound healing assays. C. Western blot was performed to detect the expression levels of migration-related and ERK proteins. **P < 0.01.

some cancers, including ovarian cancer, glioblastomas, and colon cancer [28-30]. In our study, we identified PLCB1 as a direct target gene of miR-423-5p. The enforced expression of miR-423-5p was explored to create low expression levels of PLCB1 in glioma cells and promote their malignant progression. It is suggested that miR-423-5p attenuated the viability and invasion of glioma by targeting PLCB1. Our data also showed that the accumulation of miR-423-5p affected cell apoptosis, specifically, it increased cell apoptosis. Furthermore, the upregulation of miR-423-5p was determined to alter ERK signaling pathways. In concordance

with data implicating miR-423-5p induces cell apoptosis in glioma cells by targeting PLCB1, thereby inhibiting colon tumor cell growth, invasion, and migration.

In general, these results reveal that miR-423-5p could serve as a potential therapeutic target involved in the regulatory roles of PLCB1 in glioma cells. Moreover, our results demonstrated that miR-423-5p plays a vital role in the growth and metastasis of glioma, and affects cell apoptosis. Therefore, these findings may indicate that miR-423-5p could be a diagnostic and prognostic marker in the treatment of glioma.

Disclosure of conflict of interest

None.

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