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Original Article

MiR-506-3p inhibits proliferation and invasion by targeting EZH2 in glioblastomas

Yanyao Deng[1], Le Xiao[1], Chao Liu[1], Yuan Li[1], Ziqing Guo[1], Bing Xie[1], Ziqi Jin[1], Zhicheng Lv[2], Hongwei Zhu[3], Aimin Wang[1]

[1]Department of Neurology, The First Hospital of Changsha, Changsha, Hunan, China; [2]Department of Neurosurgery, The First Hospital of Chenzhou, Chenzhou, Hunan, China; [3]Department of Gastroenterology, The Third Xiangya Hospital, Central South University, Changsha, Hunan, China

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Abstract: Background: Increasing evidence has indicated that microRNAs (miRNAs) play an important role in glioblastoma cancer. Deregulation of miR-506-3p has been reported in several cancers. However, the expression and function of miR-506-3p in glioblastomas remain unclear. This study aimed to investigate the roles and underlying mechanisms of miR-506-3p in proliferation and invasion of glioblastoma cancer. Methods: Quantitative real-time PCR (qRT-PCR) and Western blot techniques were used to assess expression of miR-506-3p and enhancer of zeste homolog 2 (EZH2) in glioblastoma cell lines and tissues. Luciferase reporter assays were conducted to investigate the association between miR-506-3p and EZH2. MTT and Transwell invasion assays were performed to evaluate the effects of miR-506-3p on proliferation and invasion, respectively, in glioblastoma cells. Results: Data showed that levels of miR-506-3p were downregulated in glioblastoma tissues and cell lines. Overexpression of miR-506-3p repressed cell growth and suppressed cell invasion in glioblastoma cells, whereas knockdown of miR-506-3p promoted cell growth and increased cell invasion in glioblastoma cells. Moreover, EZH2 was a direct target of miR-506-3p in glioblastoma cells. Enforced expression of EZH2 and suppression of EZH2 alleviated effects of miR-506-3p mimics and inhibitors on proliferation and invasion *in vitro*, respectively. Conclusion: Present results indicate that miR-506-3p plays a tumor suppressor gene role in human glioblastomas by regulating EZH2 genes.

Keywords: MiR-506-3p, glioblastoma, proliferation, invasion, EZH2

Introduction

Glioblastomas, derived from glial cells, have become the most common and most malignant tumors in China [1]. Despite multimodal therapies, such as surgery, radiation and medical therapies for treatment of glioblastoma, the average life expectancy for glioblastoma patients remains limited [2, 3]. Therefore, it is essential to find vital carcinogenic biomarkers and effective therapeutic strategies for glioblastomas.

MicroRNAs (miRNAs) are endogenous noncoding, 19-25 nucleotides RNAs, that negatively regulate a variety of genes expression by binding to the 3'-untranslated region (UTR) of their target mRNAs involved in several cancers [4-6]. Accumulating reports have suggested that miRNAs play important roles in many diverse biological processes, including cell proliferation, migration, invasion, differentiation, and apoptosis [7]. MiRNAs have been reported to serve a tumor-suppressor or oncogenic function by targeting tumor-suppressor or oncogenes genes, respectively [8-10]. It is noteworthy that miRNAs have been implicated in tumor initiation and progression of glioblastomas [11, 12].

MicroRNA-506-3p (miR-506-3p) has been suggested to be downregulated and serve as a suppressive miRNA in colon cancer [13, 14], esophageal cancer [15] and gastric cancer [16]. However, the function and underlying molecular mechanisms of miR-506-3p in glioblastomas remain unclear. Therefore, the aim of the study was to investigate the biological function of miR-506-3p and identify mechanisms of action in glioblastoma cells.

Table 1. Sequences for primers and siRNAs used in the study

Name	Sequence
Primers for qRT-PCR	
EZH2-F	5'-GACCTCTGTCTTACTTGTGGAGC-3'
EZH2-R	5'-CGTCAGATGGTGCCAGCAATAG-3'
GAPDH-F	5'-TTGGTATCGTGGAAGGACTCA-3'
GAPDH-R	5'-TGTCATCATATTTGGCAGGTT-3'
EZH2 siRNA	5'-AAGACTCTGAATGCAGTTGCTd(TT)-3'
	5'-AGCAACUGCAUUCAGAGUCUUd(TT)-3'

F: forward primer, R: reverse primer.

Materials and methods

Clinical samples and cell culture

Fresh glioblastoma tissues and adjacent normal tissues were collected in the Department of Neurosurgery, the First Hospital of Chenzhou. Histological features of all specimens were diagnosis by pathologists, according to WHO criteria. None of these patients received chemotherapy or radiotherapy before surgery. Normal human astrocytes (NHA) were purchased from ScienCell Research Laboratories (Corte Del Cedro Carlsbad, Canada) and cultured, according to manufacturer instructions. Human A172, LN229, U87, and U251 glioblastoma cell lines were obtained from ATCC. All cells were maintained cultured in DMEM (Hyclone, Logan, Utah, USA), supplemented with 10% fetal bovine serum (Hyclone) and incubated in a humidified atmosphere containing 5% CO_2 at 37°C without antibiotics.

Quantitative miRNA and mRNA measurements

Total RNA was extracted using TRIzol Reagent (Ambion), according to manufacturer protocol. cDNA used to measure EZH2 were synthesized using PrimeScriptTM RT reagent kit (TaKaRa), according to manufacturer protocol. cDNA used to measure miR-506-3p were synthesized using miRcute miRNA cDNA first strand synthesis kit (TIANGEN, Beijing), according to manufacturer protocol. Expression of EZH2 was measured using SYBR® Premix Ex TaqTM II (TaKaRa) and GAPDH served as an internal reference. Expression of miR-506-3p was measured using miRcute miRNA qPCR detection kit (TIANGEN, Beijing) and U6 served as an internal reference. All experiments were performed in triplicate. Results are represented as fold induction using the $2^{-\Delta\Delta Ct}$ method. Primers of miR-506-3p and U6 were purchased from GenePharma (China). Primers used to detect expression of EZH2 are listed in **Table 1**.

miR-506-3p mimics, antisense, and transfection

Human miR-506-3p mimics, inhibitors, or negative controls were obtained from Genepharm Company (Shanghai). For transfection, cells were cultured in a 6-well plate and transiently transfected at 70-80% confluence using the LipofectamineTM 2000 reagent (Invitrogen, CA, USA), according to manufacturer instructions.

MTT assay

Cell proliferative impact was measured by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were plated on 96-well plates (2-5×10^3 cells/well). At designated time points, cells were incubated with 2 mg/mL of MTT solution for 2 hours. The resulting formazan product was dissolved in DMSO for O.D. measurement at 570 nm. The experiment was performed in triplicate.

Transwell invasion assay

Cell invasion was determined using 24-well Matrigel invasion chambers (Becton Dickinson), according to manufacturer instructions. Cells (2×10^4) were seeded per well in the upper well of the invasion chamber in DMEM without serum. The lower chamber well contained DMEM supplemented with 10% FBS to stimulate cell invasion. After incubation for 48 hours, non-invading cells were removed from the top well with a cotton swab, while the bottom cells were stained with 0.05% crystal violet and photographed in 5 independent fields for each well.

Western blotting analysis

Total protein was extracted from cells. Cell lysates were prepared in lysis buffer and then separated by 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel Electrophoresis) and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% non-fat milk and then incubated overnight with the primary antibody to EZH2 (Cell

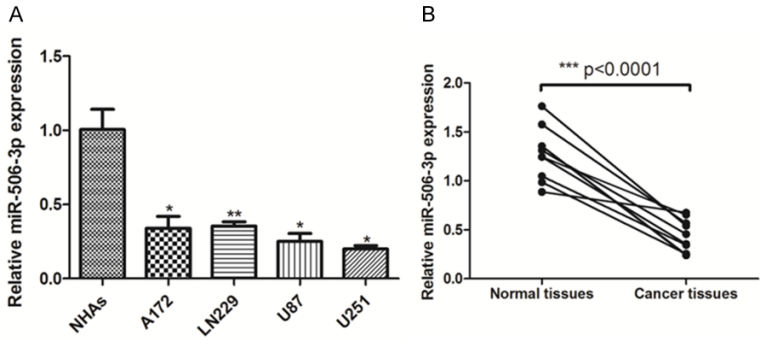

Figure 1. Expression of miR-506-3p was downregulated in glioblastoma cell lines and tissues. A. Relative expression of miR-506-3p in glioblastoma cell lines (A172, LN229, U87, and U251) compared with the normal human astrocytes cell line (NHAs). B. Comparison of the average expression levels of miR-506-3p between glioblastoma tissues and non-tumor tissues.

Signaling Technology) or Actin (Santa Cruz Biotechnology, Santa Cruz, CA), followed by horseradish peroxidase-labeled secondary antibody incubation. Chemiluminescence signal was developed by ECL Plus Western Blotting Detection Reagents (GE Healthcare Life Sciences, Piscataway, NJ).

Luciferase activity assay

The EZH2 3'-UTR luciferase reporter construct was made by amplifying the EZH2 mRNA 3'-UTR sequence. Cells were co-transfected with pMIR/EZH2 vector or pMIR/EZH2/mut vector containing Firefly luciferase, along with 0.05 μg of the pRL-TK vector (Promega) containing Renilla luciferase and miR-506-3p mimic or scramble oligonucleotide. Luciferase activities were detected using the Dual-Luciferase Reporter Assay System (Promega).

Statistical analysis

Statistical analysis was performed using SPSS 21.0 software (SPSS, USA). Data are expressed as the mean ± SD and differences between groups were analyzed using Student's t test. Data indicates statistical significance when $P < 0.05$.

Results

Expression of miR-506-3p was downregulated in glioblastoma tissues and cell Lines

Expression of miR-506-3p was detected by qRT-PCR in 4 GBM cell lines (A172, LN229, U87, and U251) and NHAs. All 4 tested GBM cell lines showed significantly lower miR-506-3p levels than those in the NHAs (**Figure 1A**). As shown in **Figure 1B**, miR-506-3p was downregulated in glioblastoma tissues, compared with the normal tissues.

MiR-506-3p inhibits the proliferation and invasion in glioblastoma cell lines

The present study then transfected miR-506-3p mimics and inhibitors to observe the possible effects of miR-506-3p on proliferation and invasion of human U251 cells *in vitro*. The efficiency of miR-506-3p mimics and inhibitors was confirmed by qRT-PCR. Compared with the control miRNA, miR-506-3p expression was significantly increased by the mimics while decreased by the inhibitors in U251 cell lines (**Figure 2A**).

MTT assay was performed to measure the effects of miR-506-3p on the proliferation of U251 cells. As shown in **Figure 2B**, miR-506-3p mimic transfection remarkably reduced the proliferation of U251 cells, compared with the control group. In contrast, the proliferation capacity of U251 cell lines was significantly enhanced by miR-506-3p repression induced by inhibitors. This study also confirmed the alteration of U251 cell invasion using Transwell assay. Results showed that the invasion of U251 cell lines was significantly reduced by miR-506-3p overexpression while enhanced by miR-506-3p silencing (**Figure 2C**). Present results indicate that low levels of endogenous miR-506-3p may play an important role in the development of glioblastomas by promoting proliferation and invasion.

EZH2 was a potential target of miR-506-3p in glioblastoma cells

EZH2 has been considered a tumor oncogene in glioblastomas, according to previous studies [17, 18]. To further illuminate the underlying mechanisms of miR-506-3p regulating glioblastoma cells, this study predicted the possible targets of miR-506-3p using target prediction programs (TargetScan, PicTar, and miRanda). Online analysis suggested that EZH2 was

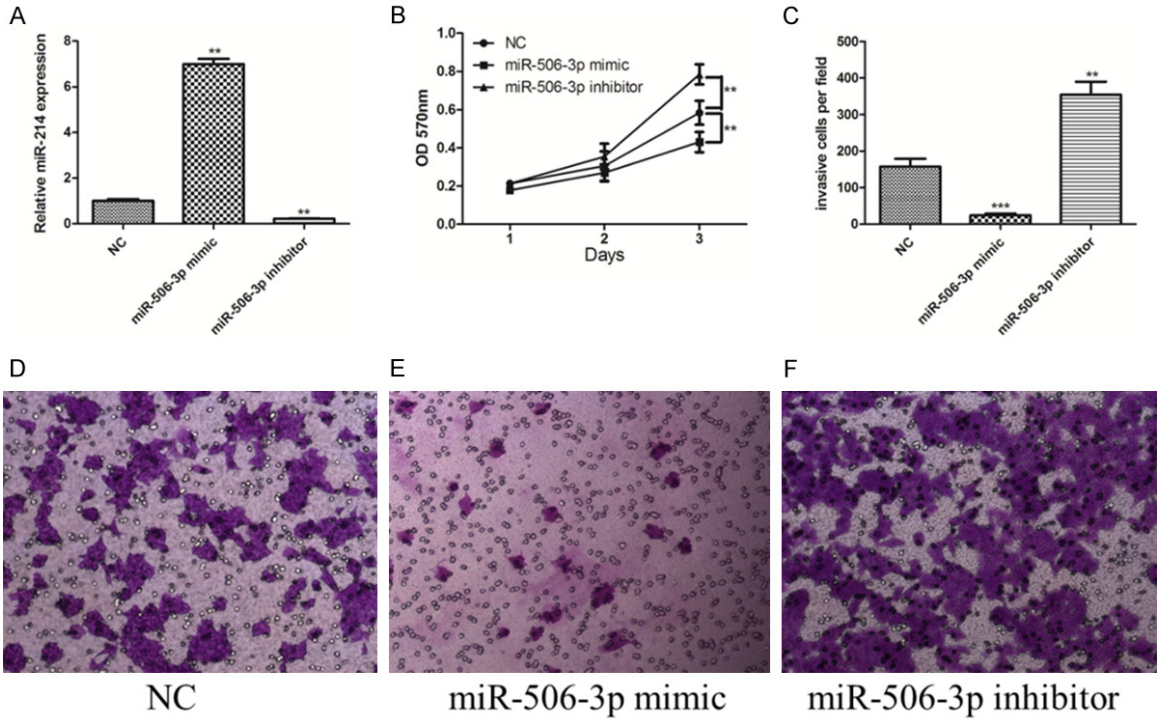

Figure 2. miR-506-3p inhibits proliferation and invasion. A. Relative expression of miR-506-3p in U251 cells transfected with miR-506-3p mimics or inhibitors. B. Cell proliferation was measured by MTT assay. U251 cells were transfected with miR-506-3p mimics or inhibitors. C-F. Cell invasion ability was assessed by Transwell assay. U251 cells were transfected with miR-506-3p mimics or inhibitors.

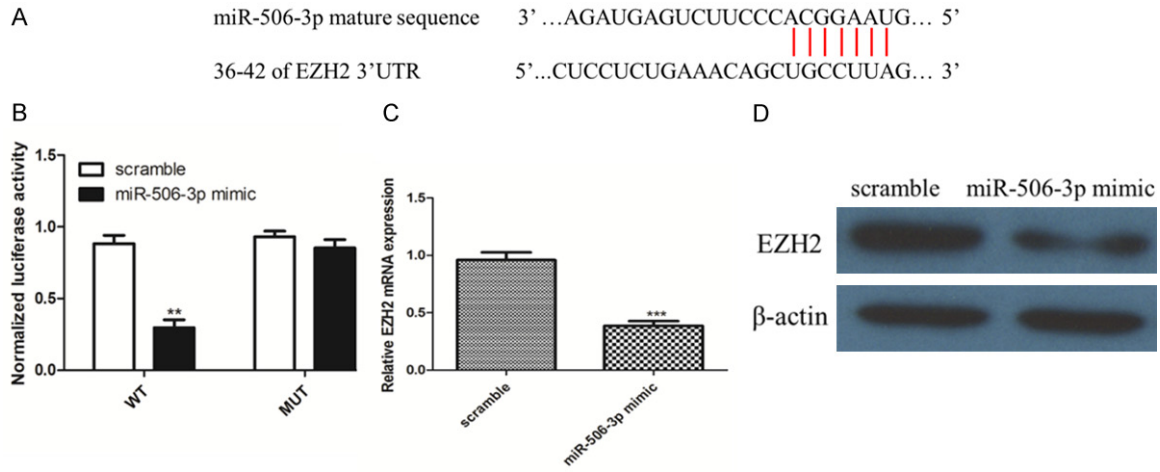

Figure 3. EZH2 is a potential target of miR-506-3p in glioblastoma cells. A. Schematic representation of the putative binding sites in EZH2 mRNA 3'UTR for miR-506-3p. B. miR-506-3p mimic repressed luciferase activities controlled by wild-type EZH2-3'-UTR but did not affect luciferase activity controlled by mutant EZH2-3'-UTR. C. Relative mRNA expression levels of EZH2 were detected by real-time PCR. D. Western blot analysis was performed to evaluate expression levels of EZH2 in the U251 cells, which was transfected with miR-506-3p mimic or scramble, respectively. β-actin was used as a loading control.

a potential direct target of miR-506-3p with a binding site in the 3'-UTR (**Figure 3A**). To further confirm whether this prediction was right, luciferase reporter assay in U251 cells was performed. It was found that overexpression of miR-506-3p repressed the activity of pMIR-

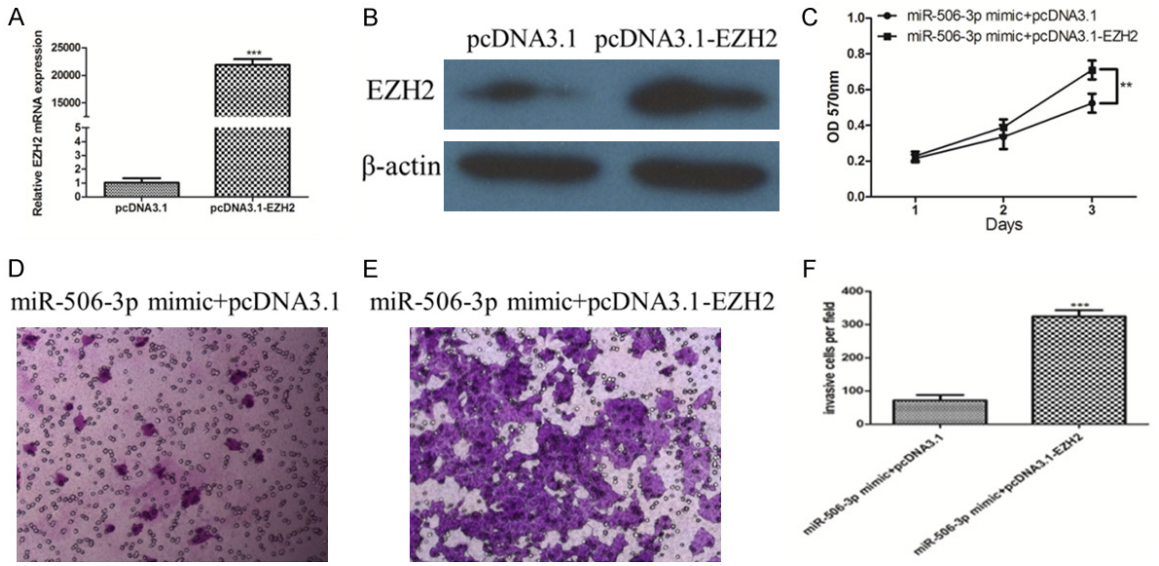

Figure 4. Enforced expression of EZH2 alleviates effects of miR-506-3p on proliferation and invasion in U251 cells. A. The mRNA expression level of EZH2 was successfully increased after transfection with pcDNA3.1-EZH2. B. The protein expression level of EZH2 was successfully decreased after transfection with pcDNA3.1-EZH2. C. The effects of pcDNA3.1-EZH2 plus miR-506-3p mimic or pcDNA3.1 plus miR-506-3p mimic on cell proliferation by MTT assay. D-F. The effects of pcDNA3.1-EZH2 plus miR-506-3p mimic or pcDNA3.1 plus miR-506-3p mimic on cell invasion by Transwell assay.

WTEZH2-3'UTR plasmid in U251 cells, without changes in luciferase activity of pMIR-MUT-EZH2-3'UTR plasmid (**Figure 3B**). Moreover, miR-506-3p inhibited mRNA expression of EZH2 in U251 cells (**Figure 3C**). Ectopic expression of miR-506-3p repressed protein levels of EZH2 in the U251 cells (**Figure 3D**). Present results indicate that miR-506-3p mediated regulation of EZH2 expression depended on its binding to a specific seed region in the EZH2 3'UTR.

Enforced expression of EZH2 alleviates effects of miR-506-3p on proliferation and invasion

The present study investigated whether overexpression of EZH2 affects the role of miR-506-3p in U251 cells. pcDNA3.1-EZH2 was co-transfected with miR-506-3p, then the effects of proliferation and invasion of U251 cells were measured. pcDNA 3.1 empty vector served as control. Overexpression of EZH2 was confirmed by qRT-PCR and Western blot (**Figure 4A**, **4B**). Present data showed U251 cells transfected with pcDNA3.1-EZH2 plus miR-506-3p mimics grew much faster (**Figure 4C**) and performed much stronger invasion (**Figure 4D**) ability than those transfected with pcDNA3.1 empty vector plus miR-506-3p mimics. Results indicate that enforced expression of EZH2 alleviates effects of miR-506-3p on proliferation and invasion.

Silencing EZH2 alleviates effects of miR-506-3p inhibitor on proliferation and invasion

To further confirm the finding that EZH2 mediates the role of miR-506-3p in U251 cells, this study investigated if knockdown of EZH2 could affect the effects of miR-506-3p inhibitors on proliferation and invasion. Knockdown of EZH2 in U251 cells using siRNA targeted EZH2 was confirmed by qRT-PCR and Western blot (**Figure 5A**, **5B**). Data showed that U251 cells, in which expression of EZH2 and miR-506-3p were both repressed, grew much slower (**Figure 5C**) and performed more weaker invasion (**Figure 5D**) ability than cells in which only expression of miR-506-3p was inhibited. Present results indicate that knockdown of EZH2 alleviates effects of miR-506-3p inhibitors on proliferation and invasion, supporting the hypothesis that miR-506-3p regulates proliferation and invasion of glioblastoma cells through regulating EZH2.

Discussion

Increasing evidence has suggested that miRNAs play important roles in the development

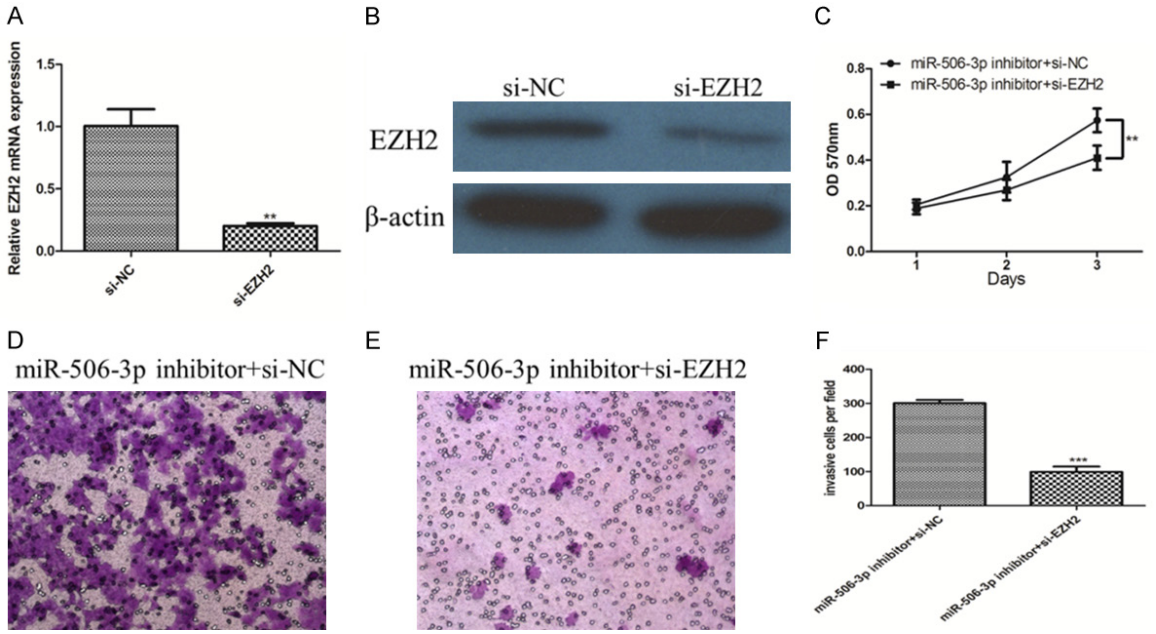

Figure 5. Silencing EZH2 alleviates effects of miR-506-3p inhibitor on proliferation and invasion in U251 cells. A. The mRNA expression level of EZH2 was successfully decreased after transfection with si-EZH2. B. The protein expression level of EZH2 was successfully decreased after transfection with si-EZH2. C. The effects of si-EZH2 plus miR-506-3p inhibitor or si-NC plus miR-506-3p inhibitor on cell proliferation by MTT assay. D-F. The effects of si-EZH2 plus miR-506-3p inhibitor or si-NC plus miR-506-3p inhibitor on cell invasion by Transwell assay.

and progression of tumors. The present study found that expression of miR-506-3p was downregulated in glioblastoma cell lines and tissues. Furthermore, *in vitro* experiments verified that miR-506 inhibits the proliferation and invasion of glioblastoma cells. EZH2 was identified as a direct target of miR-506-3p via the 3'UTR of EZH2. This study also confirmed that the miR-506-3p-EZH2 axis modulates proliferation and invasion in glioblastoma cells.

To date, the roles of miR-506-3p in cancer cells have not been well clarified. miR-506-3p plays contradictory roles in several cancers. In melanoma, overexpression of miR-506-3p was critical for promoting cancer growth, migration, and invasion [19], indicating that miR-506-3p serves as an oncogene in melanomas. In contrast, in ovarian cancer, miR-506-3p, suppressing cell migration and invasion, was demonstrated as a key EMT inhibitor. In addition, miR-506-3p expression was positively relative with early FIGO stage and extended survival [20, 21]. Similar results have been found in cervical cancer [22], liver cancer [23, 24], and breast cancer [25], suggesting that miR-506-3p functions as a tumor suppressor gene in some tumors. The function of miR-506-3p in glioblastomas remains poorly understood. In the present study, miR-506-3p was downregulated in glioblastoma cell lines and tissues. Based on *in vitro* experiments, it was verified that miR-506-3p serves as a tumor suppressor in glioblastomas.

MiRNAs typically perform their functions by repressing expression of target mRNAs. In ovarian cancer, miR-506-3p could inhibit proliferation and promote senescence by directly targeting the CDK4/6-FOXM1 axis [20]. Further studies have shown that miR-506-3p could suppress cervical cancer growth by directly targeting the hedgehog pathway transcription factor Gli3 [22]. Moreover, recent profile studies demonstrated that miR-506-3p could regulate the biological behavior of cancer cells by targeting GATA6 and FLOT1 in oral squamous cell cancer [26] and renal cell cancer [27]. Present findings confirmed that miR-506-3p serves as a tumor suppressor in glioblastomas, but the underlying mechanisms remain still unclear. Therefore, TargetScan, PicTar, and miRanda databases were used to identify target genes of miR-506-3p in glioblastomas. All three databases indicated that EZH2 may be a candidate miR-506-3p target gene. Moreover, previous stud-

ies have reported that EZH2 was a tumor oncogene in several cancers [28, 29], including glioblastomas [30, 31]. The present study conducted a luciferase reporter assay to ensure whether EZH2 is a direct target of miR-506-3p. Results suggested that regulation of EZH2 by miR-506-3p depended on its binding to the 3'UTR of EZH2. To establish whether the effects of miR-506-3p were exerted via direct inhibition of EZH2, this study restored EZH2 expression in miR-506-3p overexpressing cells and measured the proliferation and invasion of these cells. It was found that effects of proliferation and invasion were clearly increased. Moreover, this study also knock-downed EZH2 expression in miR-506-3p inhibiting cells and measured proliferation and invasion of these cells. It was found that effects of proliferation and invasion were clearly decreased. Results indicate that EZH2 is a mediator of miR-506-3p function.

In conclusion, present results indicate that miR-506-3p, downregulated in glioblastoma cell lines and tissues, inhibits proliferation and invasion *in vitro* and that EZH2 is a direct target of miR-506-3p.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Aimin Wang, Department of Neurology, The First Hospital of Changsha, 311 Yinpan Road, Changsha 410005, Hunan, China. Tel: 17188613755012597; Fax: 073188618339; E-mail: 13755012597@163.com

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