

# MicroRNA-518b functions as a tumor suppressor in glioblastoma by targeting PDGFRB

XIAOLONG XU<sup>1,2</sup>, FENGLIN ZHANG<sup>2</sup>, XIANZHEN CHEN<sup>1</sup> and QI YING<sup>2</sup>

<sup>1</sup>Department of Neurosurgery, The Tenth People's Hospital Affiliated to Tongji University School of Medicine, Shanghai 200072; <sup>2</sup>Department of Neurosurgery, The 411th Hospital of PLA, Shanghai 200081, P.R. China

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**Abstract.** Glioblastoma (GBM) is the most common and aggressive type of primary human brain tumor in China. Dysregulated microRNA (miRNA/miR) expression has been hypothesized to serve a role in the tumorigenesis and progression of human GBM. To explore the potential mechanisms affecting GBM tumorigenesis, the function of miR-518b in regulating GBM cell proliferation and angiogenesis was examined *in vitro* by CCK-8 and tube formation assay and *in vivo* by xenograft assay. The present study demonstrated that the expression of miR-518b was downregulated in GBM tissues and in GBM cell lines (U87 and U251). In addition, the expression levels of miR-518b were highly associated with tumor size, World Health Organization grade and prognosis. Furthermore, overexpression of miR-518b suppressed GBM cell proliferation and angiogenesis, and induced GBM cell apoptosis *in vitro* and *in vivo*. Overexpression of miR-518b also inhibited the expression of platelet-derived growth factor receptor  $\beta$  (PDGFRB), and the present study confirmed that the 3' untranslated region (3'UTR) of PDGFRB was a direct target of miR-518b. In conclusion, to the best of our knowledge, the present study is the first to present evidence suggesting that miR-518b may serve as a potential marker and target in GBM treatment.

## Introduction

Glioma, which is the most aggressive type of primary human brain tumor, accounts for >44% of all brain cancers in China

and 50% of all malignant brain cancers in the US (1,2). In particular, glioblastoma (GBM), which is also referred to as World Health Organization (WHO) grade IV glioma, is the most common form of glioma. In addition, GBM has the worst survival rate, with an average survival of 12-15 months despite surgical, radio- and chemotherapeutic treatment (3). Although novel therapeutic strategies, including surgical resection, chemotherapy and radiotherapy, have made great progress, the survival rate and prognosis of GBM remain poor (4). Therefore, it is essential to identify potential novel treatments for patients with GBM. It has previously been reported that microRNAs (miRNAs/miR) may be used as biomarkers to estimate GBM prognosis, and may be used as targets in novel therapeutic strategies for the treatment of human GBM (5).

miRNAs are small noncoding RNAs, 16-22 nucleotides in length, which serve an important role in post-transcriptional modification of gene expression by targeting complementary mRNA sequences (6,7). miRNAs are involved in various oncogenic processes, including apoptosis, proliferation, angiogenesis and survival. In recent years, an increasing amount of studies have focused on the roles of miRNAs in human GBM. It has been reported that aberrant expression of miRNAs is closely associated with GBM tumorigenesis, and ectopic expression of specific miRNAs may modulate GBM biological function (7-9). Although these miRNAs have been reported to serve a role in GBM, the potential mechanisms underlying GBM tumorigenesis require further investigation. miR-518b has been reported to be a possible tumor suppressor, which may be used to predict the prognosis of GBM (10). However, information regarding the function and underlying molecular mechanism of miR-518b in GBM angiogenesis is limited.

In order to assess the potential mechanisms of miR-518b in GBM, the present study confirmed that miR-518b expression is downregulated in GBM, and miR-518b overexpression significantly inhibited GBM cell growth and angiogenesis *in vitro* and *in vivo*. In addition, the present study indicated that miR-518b exerts its effect on GBM angiogenesis by inhibiting the expression of platelet-derived growth factor receptor  $\beta$  (PDGFRB). The present study, to the best of our knowledge, is the first to reveal a molecular association between miR-518b and PDGFRB, thus suggesting a novel therapeutic target for GBM.

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*Correspondence to:* Professor Xianzhen Chen, Department of Neurosurgery, The Tenth People's Hospital Affiliated to Tongji University School of Medicine, 301 Yan-Chang Middle Road, Shanghai 200072, P.R. China  
E-mail: shxianzhenchen@qq.com

Professor Qi Ying, Department of Neurosurgery, The 411th Hospital of PLA, 15 Dongjiangwan Road, Shanghai 200081, P.R. China  
E-mail: 3542808546@qq.com

**Key words:** microRNA-518b, platelet-derived growth factor receptor  $\beta$ , glioblastoma, angiogenesis

## Materials and methods

**Patient samples and cell lines.** Glioma specimens and matched adjacent normal tissues (n=68) were obtained from patients who underwent curative resection at the Department of Neurosurgery, The Tenth People's Hospital Affiliated to Tongji University (Shanghai, China). The specimens were snap-frozen in liquid nitrogen and were then stored at -80°C for RNA extraction and further experiments. None of the patients received interventions or treatments prior to surgery, and all samples were obtained with informed consent. The histopathological diagnoses of these clinical samples were confirmed by two established neuropathologists. The present study was approved by the Ethics Committee of The Tenth People's Hospital Affiliated to Tongji University. Normal human astrocytes (NHAs) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Human GBM cell lines, U87 and U251, were obtained from the American Type Culture Collection (Manassas, VA, USA). All cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator containing 5% CO<sub>2</sub> at 37°C.

**miRNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** RT-qPCR analysis of miRNAs was conducted as previously described (11). Total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), and 1 ng RNA was reverse transcribed into cDNA using reverse transcriptase (Applied Biosystems; Thermo Fisher Scientific, Inc.). The RT protocol was as follows: 30 min at 16°C, 30 min at 42°C, 5 min at 85°C. miRNA expression was detected by qPCR using the SYBR PCR Master Mix (Qiagen China Co., Ltd., Shanghai, China) on the ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). PCR was performed for 25 cycles of: 10 sec at 98°C, 10 sec at 55°C, 20 sec at 72°C. U6 served as a normalization control gene. The 2<sup>-ΔΔC<sub>q</sub></sup> method (12) was used to calculate the relative expression of each gene.

**RNA oligonucleotide and cell transfection.** miR-518b (5'-CAAAGCGCTCCCCTTTAGAGGT-3') and control miRNA (miR-ctrl) mimics (5'-CATGCCGGGTAAACCGTGA GA-3') were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). GBM cells were transfected with the miRNA mimics (50 nM) using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, once the cell density reached 70% confluence. All subsequent assays were performed 48 h post-transfection.

**Cell proliferation assay.** The Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) colorimetric assay was conducted to determine cell proliferative capacity. The cells were seeded onto 96-well plates (5×10<sup>3</sup> cells/well). Subsequently, 10 μl CCK-8 was added to each well at the indicated time-points (1, 2, 3, 4, 5 and 6 days),

Table I. Correlation between miR-518b expression and clinical characteristics.

Characteristic	No.	Relative miR-518b expression		P-value
		Low	High	
Age (years)				
≤50	31	13	18	0.3302
>50	37	21	16	
Sex				
Male	44	20	24	0.4469
Female	24	14	10	
Diameter (mm)				
≤3	35	12	23	0.0146 <sup>a</sup>
>3	33	22	11	
Differentiation				
Well and moderate	35	15	20	0.3319
Poor	33	19	14	
WHO grade				
I-II stage	32	10	22	0.0071 <sup>a</sup>
III-IV stage	36	24	12	
KPS				
≤90	27	13	14	0.8043
>90	41	21	20	

<sup>a</sup>P<0.05. WHO, World Health Organization; KPS, Karnofsky performance status; miR, microRNA.

and the plates were incubated at 37°C for 2 h. The data were measured at 560 nm using a Benchmark Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Cell cycle and apoptosis analysis.** The role of miR-518b in cell cycle progression and apoptosis was detected by flow cytometry. According to the manufacturer's protocol, cells were cultured for 48 h, harvested and rinsed with phosphate-buffered saline (PBS), before being fixed in 70% ethanol at 4°C overnight. Subsequently, cells were rinsed with PBS again, and were treated with PBS containing 100 mg/l bovine pancreatic RNase A (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37°C for 2 h, followed by incubation with propidium iodide (Sigma-Aldrich; Merck KGaA) for 30 min at room temperature. Cell apoptosis was detected using the Annexin V fluorescein isothiocyanate Apoptosis kit (Calbiochem; EMD Millipore, Billerica, MA, USA) according to the manufacturer's protocol. Cell cycle progression and apoptosis were analyzed on a BD FACSCalibur flow cytometer with ModFit 3.0 software (both from BD Biosciences, Franklin Lakes, NJ, USA).

**Tube formation assay and xenograft assay.** U87 and U251 cells transfected with miR-518b or miR-ctrl mimics were grown in Dulbecco's modified Eagle's medium for 6 h at 37°C in a 24-well plate coated with Matrigel (Sigma-Aldrich; Merck

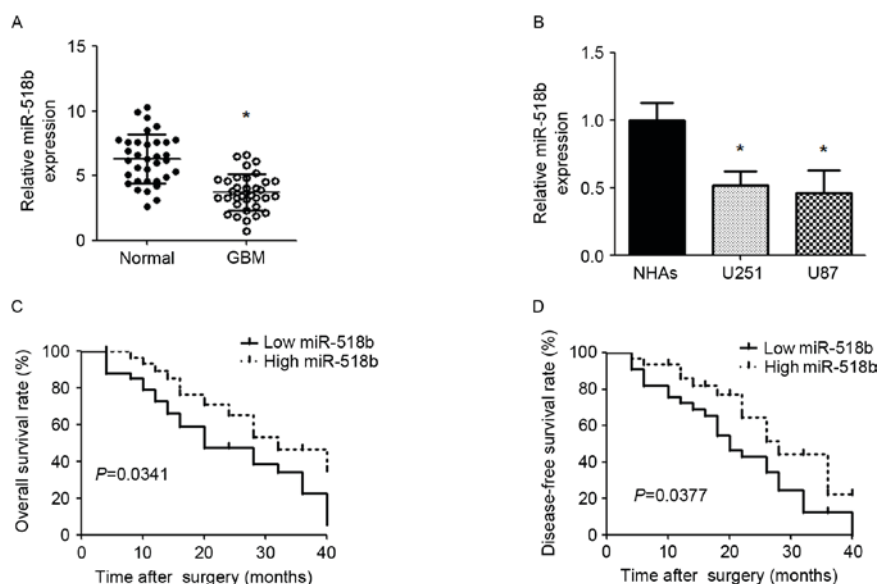


Figure 1. miR-518b expression in GBM and its association with survival. (A) Mature miR-518b expression was determined by qPCR in human GBM tissues and adjacent noncancerous tissues. Data are presented as the mean  $\pm$  standard deviation. \* $P < 0.05$  vs. normal group (n=34). (B) Expression of miR-518b was detected in cell lines by qPCR. Data are expressed as the mean  $\pm$  standard deviation. \* $P < 0.05$  vs. NHAs. All experiments were repeated 5 times. Kaplan-Meier analyses of correlations between miR-518b expression and (C) overall survival rate or (D) disease-free survival rate. GBM, glioblastoma; miR-518b, microRNA-518b; qPCR, quantitative polymerase chain reaction; NHA, normal human astrocytes.

KGaA). For the tube formation assay, human umbilical vein endothelial cells (HUVECs;  $5 \times 10^3$  cells/well) were cultured in Ham's F12K basal medium (Gibco; Thermo Fisher Scientific, Inc.) with 200 ml transfected U87 or U251 cell-conditioned medium. Tube formation was analyzed to determine the capability of transfected cells to develop a vascular phenotype under a light microscope. The branch points of the formed tubes, which represent the degree of *in vitro* angiogenesis, were quantified by eye in 5 high-power fields using a light microscope. For the xenograft assay, the indicated cells ( $2 \times 10^6$ ) were subcutaneously injected into the flanks of female athymic nude mice (5 per group; 8-weeks old; weight, 18-22 g; obtained from Tongji University Animal Centre). All mice were allowed *ad libitum* access to an autoclaved chow diet and water in filter-topped cages in an SPF animal room (12-h light: Dark cycle; temperature, 25°C; humidity, 40-60%). A total of 30 days after injection, the nude mice were sacrificed by cervical dislocation, and the xenograft tumors were removed and weighed.

**Immunohistochemistry.** Tissues were fixed in 4% paraformaldehyde for 24 h at room temperature and embedded in paraffin. Tissue sections (5  $\mu$ m) were then dewaxed, rehydrated in graded alcohol, and incubated with 0.3% hydrogen peroxide to block endogenous peroxidase activity. Subsequently, the sections were treated with rabbit monoclonal anti-PDGFRB (cat no. 3169, 1:500; Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-cluster of differentiation (CD) 34 (cat no. 3569, 1:500; Cell Signaling Technology, Inc.) antibodies at 4°C overnight, followed by incubation with a secondary anti-mouse immunoglobulin G antibody (cat no. sc-3749, 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 15 min at room temperature, followed by diaminobenzidine staining. Each step was conducted according to the manufacturers' protocols. Microvessel density (MVD) was

assessed using light microscopy by counting vessel density in 5 high-power fields.

**Western blot analysis.** Total protein was isolated from the cultured cells and lysed with radioimmunoprecipitation assay lysis buffer. Protein concentration was determined using the bicinchoninic acid method, after which equal protein samples (50  $\mu$ g) were separated by 10-15% SDS-PAGE and were transferred onto polyvinylidene fluoride membranes. After blocking the membranes with 5% skim milk at room temperature for 1 h, the membranes were incubated with primary antibodies against PDGFRB (cat no. 3169, 1:2,000; Cell Signaling Technology, Inc.) and GAPDH (cat no. sc-25778, 1:2,000; Santa Cruz Biotechnology, Inc.) at 4°C overnight. The membranes were subsequently incubated with secondary goat anti-rabbit antibody (cat no. 1662408; 1:3,000; Bio-Rad Laboratories Inc., Hercules, CA, USA) at 37°C for 1 h. Protein bands were analyzed using an enhanced chemiluminescence system (Bio-Rad Laboratories, Inc.).

**Plasmid construction.** Sequences containing wild-type and mutant 3' untranslated regions (3'UTRs) of PDGFRB (2,671 bp; NCBI Reference Sequence, XM\_005268464.2) were amplified by PCR to construct the reporter vectors. The PCR amplicons were then cloned downstream of the luciferase open reading frame in the pMIR-REPORT vector (Ambion; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The correct clones were confirmed by sequencing.

**Dual-luciferase reporter assay.** For the luciferase assay, U87 and U251 cells were grown to 70-80% confluence in 24-well plates and were co-transfected with miR-518b/miR-ctrl and wild type/mutant recombinant luciferase reporter plasmids using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

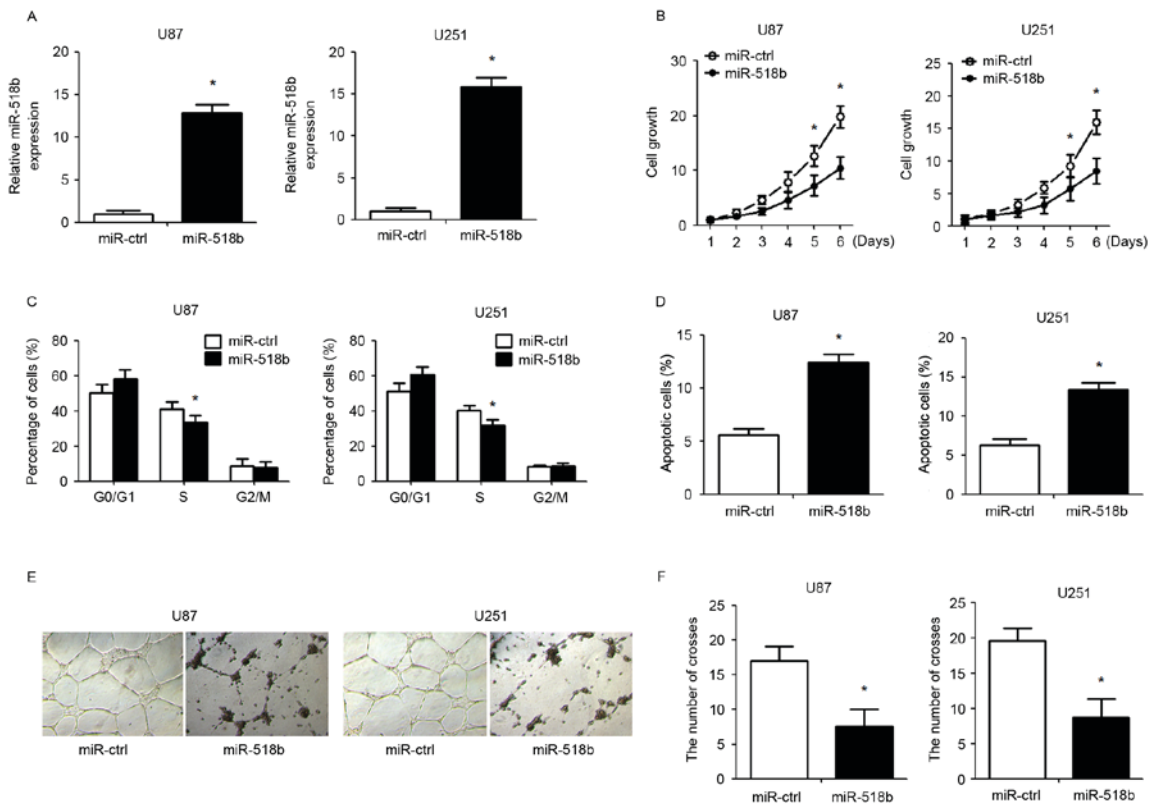


Figure 2. Effects of miR-518b on cell proliferation, apoptosis and angiogenesis. U87 and U251 cells were transfected with miR-518b or miR-ctrl mimics for 48 h. (A) miR-518b overexpression in U87 and U251 cells was validated by quantitative polymerase chain reaction. (B) Cell Counting Kit-8 assays were performed to evaluate cell proliferation at the indicated time-points. (C) Proportion of cells at each stage of the cell cycle. (D) The rate of apoptosis was detected by flow cytometry. (E and F) Capillary tube formation ability was determined by calculating the branch points of the newly formed tubes. Data are presented as the mean  $\pm$  standard deviation (n=5). \*P<0.05 vs. miR-ctrl group. miR, microRNA; ctrl, control.

After 24 h, luciferase activity was measured using the Dual-Luciferase Reporter Assay system (Beyotime Institute of Biotechnology, Beijing, China) according to the manufacturer's protocol. Values were normalized against *Renilla* luciferase gene activity.

**Statistical analysis.** SPSS 15.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze the data and results are presented as the mean  $\pm$  standard deviation. Student's t-test was used to analyze the statistical differences between two groups. Following one-way analysis of variance, the Tukey test was used to compare the differences between three groups. Fisher's exact test was performed to identify the differences between categorical variables. Kaplan-Meier method and log-rank test were used to conduct a univariate survival analysis. P<0.05 was considered to indicate a statistically significant difference.

## Results

**miR-518b expression and clinicopathological features of GBM.** The expression levels of miR-518b were lower in GBM tissues compared with in adjacent nontumor tissues (Fig. 1A). The expression levels of miR-518b were also significantly reduced in U87 and U251 cell lines compared with in NHAs (Fig. 1B). miR-518b expression levels were classified low or high based on the median expression level of the cohort. As presented in Table I, a clinical characteristics analysis revealed that low miR-518b expression was associated with larger tumor

size (P=0.0146) and a higher WHO grade (P=0.0071); however, miR-518b expression was not significantly associated with other characteristics, including age, gender, differentiation and Karnofsky performance status. Kaplan-Meier analysis revealed that low miR-518b expression was significantly associated with worse overall survival and disease-free survival in patients with GBM (P=0.0341 and P=0.0377, respectively; Fig. 1C and D).

**miR-518b affects GBM cell proliferation, apoptosis and angiogenesis in vitro and in vivo.** To investigate the role of miR-518b in GBM cells, miR-518b overexpression was induced in human GBM cells (U87 and U251) by transfection with miR-518b mimics. The transfection efficiency was confirmed qPCR (Fig. 2A). CCK-8 assay demonstrated that overexpression of miR-518b suppressed the growth of U87 and U251 cells in a time-dependent manner (Fig. 2B). Compared with the control group, flow cytometry revealed that transfection with miR-518b mimics markedly reduced the percentage of cells in S phase and enhanced the percentage of apoptotic cells (Fig. 2C and D). To determine whether miR-518b may regulate angiogenesis in GBM, human umbilical vein endothelial cells were resuspended in GBM cell-conditioned medium and were cultured in Matrigel to allow for tube formation. The results indicated that overexpression of miR-518b significantly reduced the formation of tube-like structures (Fig. 2E and F). To clarify the effects of miR-518b on GBM cell proliferation and angiogenesis *in vivo*, nude mice were inoculated with GBM cells post-transfection. The results demonstrated that



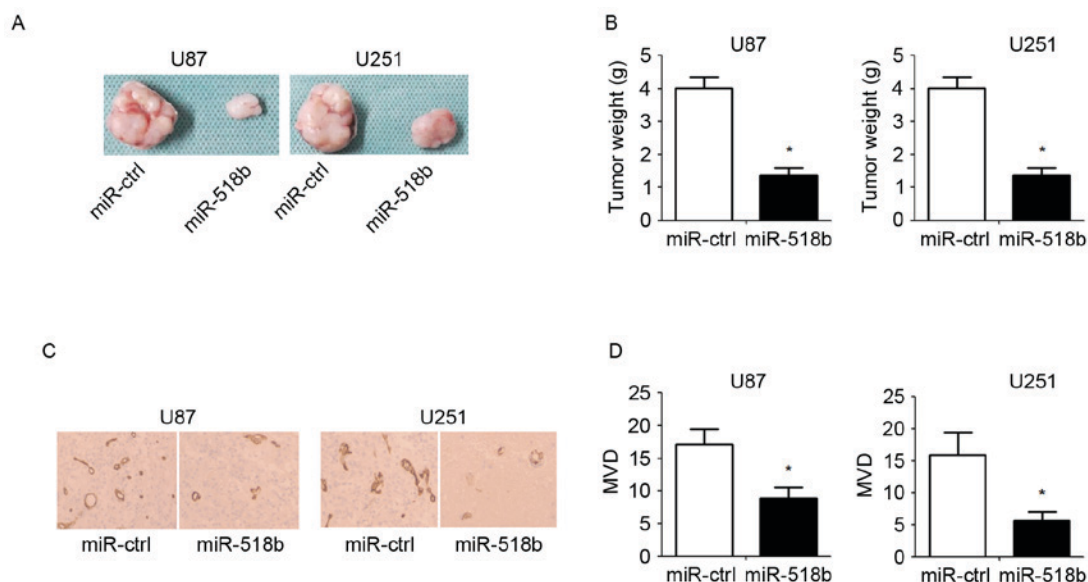


Figure 3. miR-518b inhibits the growth and angiogenesis of glioblastoma cell lines *in vivo*. Transfected cells were subcutaneously injected into the flanks of athymic mice for 30 days. (A) Representative images of xenografts from nude mice are presented. (B) Tumor weight of the excised tumors. (C) Immunohistochemical staining of CD34 in xenograft tumors (magnification, 200x). (D) MVD analysis was performed according to CD34 staining. Data are presented as the mean  $\pm$  standard deviation (n=5). \*P<0.05 vs. miR-ctrl group. miR, microRNA; ctrl, control; CD, cluster of differentiation; MVD, microvessel density.

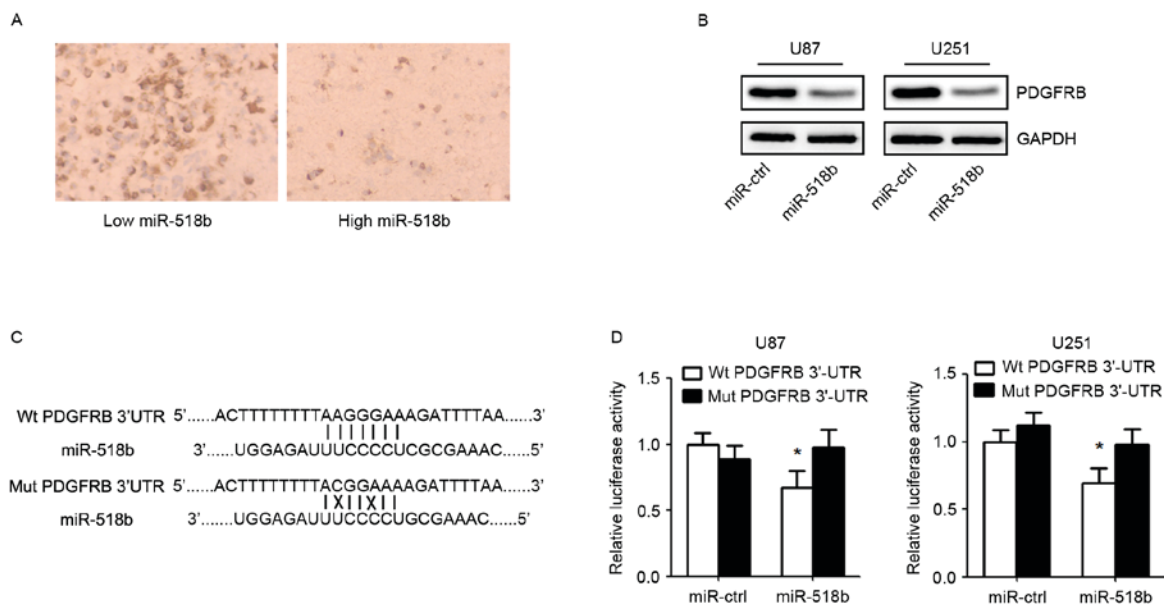


Figure 4. PDGFRB is a direct target gene of miR-518b. (A) Inverse relationship between the expression of miR-518b and PDGFRB was detected in GBM specimens (magnification, 200x). (B) Western blot analysis of PDGFRB expression in U87 and U251 cells 48 h post-transfection with miR-518b and miR-ctrl. (C) PDGFRB mRNA 3'UTR contains binding sites for miR-518b. (D) U87 and U251 cells were co-transfected with the dual-luciferase reporter plasmid carrying the Wt or Mut 3'UTR sequences of PDGFRB and miR-518b or miR-ctrl mimics. A luciferase reporter system analysis was performed. Data are presented as the mean  $\pm$  standard deviation (n=5). \*P<0.05 vs. miR-ctrl group. miR, microRNA; ctrl, control; PDGFRB, platelet-derived growth factor receptor  $\beta$ ; Wt, wild-type; Mut, mutant; 3'UTR, 3' untranslated region.

overexpression of miR-518b significantly reduced tumor growth, as reflected by decreased tumor weight (Fig. 3A and B). In addition, CD34 immunohistochemical staining indicated that miR-518b overexpression significantly decreased MVD compared with in the control group (Fig. 3C and D).

*miR-518b directly inhibits PDGFRB expression via targeting its 3'UTR.* To identify the target gene of miR-518b,

putative protein-coding gene targets of miR-518b were searched for using bioinformatics prediction software. PDGFRB was identified as a potential target by TargetScan ([http://www.targetscan.org/vert\\_71/](http://www.targetscan.org/vert_71/)) and miRBase (<http://www.mirbase.org/>) algorithms. Immunohistochemistry demonstrated that the expression of PDGFRB was markedly reduced in GBM samples with high miR-518b expression compared with in GBM samples with low miR-518b expression (Fig. 4A). Western

blot analysis indicated that the expression of PDGFRB was decreased in cells transfected with miR-518b mimics (Fig. 4B). Furthermore, to investigate whether PDGFRB was a direct target of miR-518b, PDGFRB wild-type or mutant 3'UTRs were cloned into a dual-luciferase reporter assay system (Fig. 4C). The results suggested that miR-518b mimics transfection, in U87 and U251 cell lines, significantly suppressed the luciferase activity of wild-type PDGFRB 3'UTR but not that of mutant PDGFRB3'UTR (Fig. 4D).

## Discussion

GBM is a common type of tumor that affects children and adults, which is associated with poor prognosis, high relapse frequency and unsatisfactory therapeutic effects in response to clinical comprehensive treatment (13). Previous studies have indicated that miRNAs serve an important role in the occurrence and progression of GBM (14), and various miRNAs exert their impact on GBM carcinogenesis (15,16). The present study focused on the aberrant expression of miR-518b in GBM and its newly identified relationship with PDGFRB in GBM.

miR-518b has been deemed a tumor suppressor in carcinogenesis, as demonstrated by the observation that downregulation of miR-518b has frequently been detected in esophageal squamous cell carcinoma (17) and chondrosarcoma (18). In a recent study, a miRNA microarray demonstrated that miR-518b, which targets PDGFRB, is downregulated in microvascular proliferation, which may be the mechanism underlying the high expression of PDGFRB in GBM microvascular proliferation (19). The present study demonstrated that the expression of miR-518b was reduced in GBM tissues and cell lines. Low miR-518b expression was also associated with unfavorable clinical characteristics, including tumor size and WHO grade, suggesting that miR-518b may be closely associated with the occurrence and development of GBM. In addition, the patients with GBM and low miR-518b expression exhibited a worse survival rate, which indicated that miR-518b may be a prognostic marker in patients with GBM. Furthermore, overexpression of miR-518b *in vitro* markedly inhibited cell growth by regulating cell cycle progression and apoptosis. Tube formation and xenograft assays provided direct evidence to suggest that miR-518b suppressed the development of GBM by inhibiting angiogenesis. Therefore, *in vitro* and *in vivo* findings indicated that overexpression of miR-518b in GBM inhibited tumor proliferation and angiogenesis. These results were consistent with the findings of previous studies (17,18,20), which indicated that miR-518b suppressed the tumorigenesis of various cancer cells. The results of the present study revealed that miR-518b acted as a tumor suppressor gene in GBM pathogenesis.

PDGFRB, which is a cell-surface tyrosine kinase receptor, is an important target for anticancer therapeutics (19,21). It has been reported that PDGFRB is an angiogenic factor, which is implicated in numerous cellular processes, including angiogenesis, proliferation and migration (22,23). Previous studies have suggested that PDGFRB may induce GBM tumorigenesis, and enhance tumor proliferation by modulating the expression of tumor suppressor miRNAs in U87 human GBM cells (21,24). Furthermore, the expression levels of PDGFRB are significantly increased in GBM microvascular proliferation than in

GBM tumor cells indicating that PDGFRB serves a critical role in GBM microvascular proliferation (19). In the present study, dual-luciferase reporter assay and western blotting demonstrated that PDGFRB is a direct target of miR-518b, and PDGFRB is negatively regulated by miR-518b in GBM tissues. These findings indicated that miR-518b may inhibit GBM proliferation and angiogenesis by suppressing PDGFRB expression. PDGFRB overexpression is a notable feature that may be considered a critical event in GBM tumorigenesis. The present study confirmed that PDGFRB is a target gene of miR-518b in GBM; however, more relevant functions and downstream regulatory mechanisms remain to be explored.

In conclusion, the present study revealed that miR-518b expression is decreased in GBM, and its reduced expression is associated with tumor size and WHO grade. Furthermore, miR-518b exerts critical effects on prognosis, proliferation, apoptosis and angiogenesis in GBM by directly targeting PDGFRB. These findings indicated that miR-518b may function as a tumor suppressor by targeting PDGFRB in the occurrence and development of GBM. Taken together, this newly identified target of miR-518b may also be considered a prognostic marker and therapeutic target for patients with GBM.

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