Suppression of microRNA-130b inhibits glioma cell proliferation and invasion, and induces apoptosis by PTEN/AKT signaling

JIAN-JUN GU[1,2*], KAI-CHUN FAN[3*], JIAN-HE ZHANG[2], HONG-JIE CHEN[2] and SHOU-SEN WANG[2]

[1]Stroke Center, Zhengzhou University People's Hospital, Henan Provincial People's Hospital, Zhengzhou, Henan 450000;
[2]Department of Neurosurgery, Fuzhou General Hospital, Fuzhou, Fujian 350025; [3]Department of Gastroenterology,
Chinese PLA General Hospital, Beijing 100853, P.R. China

Abstract. Glioblastoma is the most common malignant brain tumor in adults and is characterized by extensive proliferation and the diffused invasion of tumor cells. Due to the intricate signaling pathways involved in glioma progression, more effective targeted therapies and prognostic biomarkers in clinical practice are required. The suppression of proto-oncogene function or recovery of tumor suppressor gene function remains one of the primary approaches in gene therapy. The close association between the abnormal expression or mutation of microRNA (miRNA) and the tumorigenesis, progression and staging in glioma have been demonstrated previously. However, the expression pattern and specific role of microRNA-130b (miR-130b) in the tumor occurrence and progression of glioma are unclear. In the present study, quantitative polymerase chain reaction was performed to determine the expression level of miR-130b in 30 brain glioma patients and 3 glioma cell lines. An miR-130b inhibitor was transfected into U87 cells to downregulate the expression of miR-130b, and assessments of cell proliferation, cell cycle, apoptosis, cell invasion and migration *in vitro* and nude mouse tumorigenicity *in vivo* were conducted. Western blotting and luciferase reporter gene technology were used to verify the downstream target gene of miR-130b, namely phosphatase and tensin homolog (PTEN). The results demonstrated that miR-130b expression was increased in glioma tissues and cell lines in comparison with non-glioma tissues or cells. The downregulated expression of miR-130b inhibited the proliferation and invasion of glioma cells, induced apoptosis of the cells *in vitro* and inhibited their tumorigenicity *in vivo*. Western blotting and luciferase reporter assays demonstrated that the PTEN gene is a direct target of miR-130b. Western blotting revealed that the miR-130b inhibitor upregulated the expression of PTEN, inhibited AKT pathway activation, upregulated the tumor suppressor gene p27, and suppressed cyclin D1, matrix metalloproteinase 2 and 9 expression. These results suggest that the miR-130b inhibitor suppressed glioma cell proliferation and invasion via the PTEN/AKT pathway. Therefore, miR-130b is suggested to be an effective therapeutic target for glioma.

Introduction

Glioma is the most common primary brain tumor, accounting for 80% of malignant brain tumors, and has a high rate of recurrence, mortality and morbidity (1). The excessive proliferation, resistance to apoptosis and high invasion rate of glioma cells lead to the characteristic lack of boundaries with the surrounding brain tissues, regardless of the differentiation of glioma cells (2). It is thus challenging to completely eradicate the tumor by surgery. With current standard management by surgical resection and precise radiotherapy and chemotherapy, the median survival time of patients with glioblastoma multiforme is only 12-15 months, and the 5-year survival rate remains <3% (3). Therefore, elucidation of the mechanism underlying the tumorigenesis, tumor development and clonal evolution of glioma is essential for the identification of an effective target for gene therapy.

MicroRNAs (miRNAs) are a class of small endogenous non-coding RNAs that are ~22 nucleotides in length. These regulate gene expression post-transcriptionally, primarily through complete or incomplete integration with the mRNA 3' untranslated region (3'UTR) of the target gene. A single miRNA molecule may regulate hundreds of target genes by forming a complex regulatory network with the target genes. miRNAs serve broad roles in embryonic development, cell proliferation, differentiation, apoptosis, cell cycle progression, angiogenesis and a variety of other biological processes (4). Studies have shown that the aberrant expression of specific miRNAs is closely associated with uncontrolled proliferation, apoptosis blockage, invasion and migration, as well as resistance to chemotherapy (5-7). MicroRNA-130b (miR-130b) is an important member of the microRNA-130 family. A previous study demonstrated that in breast tissues, miR-130b

Correspondence to: Dr Shou-Sen Wang, Department of Neurosurgery, Fuzhou General Hospital, 156 Xi'erhuanbei Road, Fuzhou, Fujian 350025, P.R. China
E-mail: wshsen@xmu.edu.cn

*Contributed equally

downregulates p21[Waf1/Cip1], a cell cycle inhibitor, which in turn inhibits mammary epithelial cell apoptosis and promotes tumor growth (8). In addition, Dong *et al* (9) reported that a mutant p53 gene induced epithelial-mesenchymal transition by regulating the miR-130b-zinc finger E-box binding homeobox 1 axis in endometrial carcinoma, resulting in the initiation of tumor development. They also observed that in comparison with normal tissues, miR-130b expression was significantly lower in endometrial carcinoma tissues, and the long-term follow-up of patients with endometrial carcinoma revealed a longer survival time for patients with high levels of miR-130b expression (9). These studies suggest that miR-130b is capable of functioning as either a tumor suppressor or a tumor promoter, depending on the type of tumor involved. A study conducted by Malzkorn *et al* (10) concluded that the expression of miR-130b significantly increased in four patients with glioma as they progressed from World Health Organization (WHO) stage II to IV. However, the functions and mechanisms of miR-130b in glioma require elucidation.

In the present study, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to determine the expression pattern of miR-130b in 30 glioma patients and 3 glioma cell lines. U87 cells were transfected with an miR-130b inhibitor to downregulate the expression of miR-130b. The biological characteristics of the proliferation, cell cycle, cell invasion and migration of the transfected U87 cells were then assessed. Western blotting and luciferase reporter gene technology were used to verify the expression of phosphatase and tensin homolog (PTEN), a downstream target gene of miR-130b, and its underlying mechanism.

Materials and methods

Clinical samples and cell lines. Human glioma tumor tissues were obtained from the surgical resection of glioma patients (52-73 years old; 21 males and 9 females) in the Fuzhou General Hospital (Fuzhou, China) between January 2015 and December 2016. A total of 30 glioma samples were thoroughly reviewed by an experienced neuropathologist according to the 2007 WHO classification (11), resulting in 10 glioma samples being classified as grades I and II, 10 as grade III and 10 as grade IV. In addition, 5 non-neoplastic brain specimens were obtained from patients with traumatic brain injury during decompression surgery. All tissue samples were frozen in liquid nitrogen immediately following resection and stored at -80˚C. Prior patient consent was obtained for the use of these clinical materials for research purposes. In addition, ethical approval for the study was obtained from the Ethics Committee of Fuzhou General Hospital. The normal human astrocyte (NHA) cell line was purchased from ScienCell Research Laboratories, Inc. (Carlsbad, CA, USA). The glioma LN229, U87 and U251 cell lines used in this study were obtained in 2013 from the Institute of Biochemistry and Cell Biology (Shanghai Institutes for Biological Sciences, Chinese Academy of Science, Shanghai, China).

Cell culture and transfection. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin in a 5% CO_2 atmosphere at 37˚C. U87 cells ($1x10^5$) were seeded into 6-well plates and transfected with negative control (NC), miR-130b inhibitor and PTEN-targeted small interfering RNA (siPTEN; sequence, 5'-GACUUGAAGGCGUAUACAGtt-3') purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The medium was removed following a 24 h transfection, and the cells were placed in the complete medium and maintained at 37˚C in 5% CO_2.

RNA extraction and RT-qPCR. Total RNAs were extracted from cultured cells and fresh glioma tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) or total miRNAs using mirVana (Ambion; Thermo Fisher Scientific, Inc.) kits according to the manufacturer's protocols. Reverse transcription was then performed by using a Reverse Transcription kit (Promega Corporation, Madison, WI, USA) in accordance with the instructions of the manufacturer. Gene-specific primers were used to synthesize miR-130b cDNA from total RNA, in accordance with the recommended protocol for the miRNA-specific TaqMan miRNA Assay kit (Applied Biosystems; Thermo Fisher Scientific, Inc.); U6 small nuclear RNA (snRNA) was used as an internal control. The expression level of miR-130b and PTEN was examined by qPCR with a SYBR-Green PCR Master Mix kit in conjunction with an ABI-Prism 7300 system (both Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences were as follows: PTEN forward, 5'-TTTGAAGACCATAACCCA CCAC-3' and reverse, 5'-ATTACACCAGTTCGTCCC TTTC-3'; GAPDH forward, 5'-TCGGAGTCAACGGAT TTGG-3' and reverse, 5'-CATGGGTGGAATCATATT GGA-3'; U6 RT-primer, 5'-CGCTTCACGAATTTGCGT GTCAT-3', forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The conditions for PCR were as follows: stage 1, 95˚C for 10 min; stage 2, 40 cycles at 95˚C for 15 sec, 57˚C for 1 min; stage 3 (dissociation stage), 95˚C for 15 sec, 60˚C for 15 sec, 95˚C for 15 sec. The relative expression levels of mature miR-130b and PTEN mRNA were calculated by the $2^{-\Delta\Delta Cq}$ method (12) and normalized to U6 snRNA and GAPDH mRNA levels, respectively.

Western blotting. Total cell lysates from different experiments were obtained by lysing the cells in radioimmunoprecipitation assay buffer (Sigma, St. Louis, MO, USA). Protein concentrations were determined using a BCA protein assay (Pierce; Thermo Fisher Scientific, Inc.). Total protein from each sample (40 μg) was resolved by 10% SDS-PAGE and transferred to PVDF membranes (both from EMD Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies PTEN (1:1,000 dilution; Abcam, Cambridge, MA, USA), phosphorylated (p)-AKT (1:500 dilution; sc-7985-R), p27 (1:500 dilution; sc-528) and cyclin D1 (1:500 dilution; sc-70899) (all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-matrix metalloproteinase (MMP)-2 (1:500 dilution) and anti-MMP-9 (1:500 dilution) (both from Abcam) overnight at 4˚C, followed by incubation with a horseradish peroxidase-conjugated donkey

anti-rabbit secondary antibody (sc-2317; 1:5,000 dilution; Santa Cruz Biotechnology, Inc.) or HRP-conjugated donkey anti-mouse antibody (sc-2318;1:5,000 dilution; Santa Cruz Biotechnology, Inc.) at room temperature for 30 min. Membranes were stripped and then reprobed with a primary antibody against GAPDH (1:1;000 dilution; Santa Cruz Biotechnology, Inc.). The signal intensity was determined with ImageJ gel analysis software version 1.48u (National Institutes of Health, Bethesda, MD, USA). GAPDH was used as an endogenous protein for normalization. Specific bands were visualized using enhanced chemiluminescence detection (Thermo Fisher Scientific, Inc.).

Luciferase reporter assay. The 3'UTR sequence of PTEN predicted to interact with miR-130b or a mutated sequence with the predicted target sites was synthesized and inserted into the *Xba*I and *Fse*I sites of a pGL3 control vector (Promega Corporation) by integrating the search results of TargetScan and miRDB programs (13). These constructs were designated pGL3-PTEN-3'-UTR and pGL3-PTEN-3'-UTR-mut, respectively. For the reporter assay, U87 cells were plated onto 24-well plates and transfected with pGL3, pGL3-PTEN-3'-UTR or pGL3-PTEN-3'-UTR-mut vector and miR-130b inhibitor or NC vectors using the FuGENE HD transfection reagent (Promega Corporation). A *Renilla* luciferase vector pRL-SV50 (Promega Corporation) was co-transfected in order to normalize the differences in transfection efficiency. Subsequent to 48 h transfection, the cells were harvested and assayed using a Dual-Luciferase Reporter Assay system (Promega Corporation), according to the manufacturer's protocol. Transfection was repeated in triplicate in three independent experiments.

Proliferation assays. Cells ($5x10^3$/well) were plated in 96-well plates and grown for 24, 48, 72 and 96 h following transfection. Cell proliferation was documented every 24 h for 4 days using a Cell Counting kit-8 (CCK-8) assay (Genview, Carlsbad, CA, USA). The absorbance at a wavelength of 570 nm was detected using a microplate reader.

Colony formation assay. The stably-transfected glioma cells (300 cells/well) were seeded into 6-well plates and cultured in cell culture medium for 2 weeks to allow colony formation. The culture medium was refreshed every third day. The colonies were then fixed in 100% methanol for 30 min and stained with crystal violet solution for 30 min. Subsequently, the number of macroscopically observable colonies was recorded.

Cell cycle and apoptosis assays. The effects of miR-310b on the cell cycle and apoptosis of U87 glioma cells were examined by flow cytometry. In brief, the cells were harvested by trypsinization 48 h following transfection. They were then washed three times with ice-cold phosphate-buffered saline (PBS) and fixed with 70% ethanol overnight at 4˚C. The cell cycle and apoptotic rate were measured by flow cytometry (BD Biosciences, San Jose, CA, USA) and the data were analyzed using BD CellQuest™ PRO software (BD Biosciences). Annexin V-APC/7-AAD Apoptosis Detection kit (KaiJi, Nanjing, China) was used.

Transwell and scratch-wound assays. Cell invasion was determined using Transwell and scratch-wound assays. For the Transwell assay, U87 cells were transfected with miR-130b inhibitor or NC. Subsequent to incubation for 48 h, $3x10^4$ cells were transferred to the top of a Matrigel-coated invasion chamber (BD Biosciences) in serum-free DMEM. DMEM containing 10% FBS was added to the lower chamber. After 24 h, the non-invading cells were removed, and the invading cells were fixed using 95% ethanol for 30 min, stained with 0.1% crystal violet for 30 min and images were captured under a magnification of x100. For the scratch-wound assay, miR-130b inhibitor or NC was transfected into the cells in 6-well plates. The cell layers were then scratched using a 20-μl sterile pipette tip to form wound gaps. The wound location in the 6-well plates was marked and the cells were then allowed to migrate into the cell-free wound in serum-free DMEM for 24 h. The tests were repeated in three independent experiments. The wound widths were measured and the relative wound widths were calculated. Data are shown as means ± standard deviation (SD) of 3 independent experiments.

Nude mouse tumorigenicity. U87 cells stably-transfected with miR-130b inhibitor or NC were resuspended in PBS and $1.5x10^6$ cells were injected subcutaneously into the right flanks of male BALB/c athymic (nude) mice (6-8 weeks old; weighing 20 ± 2 g obtained from Shanghai SLAC Experimental Animal Co., Ltd., Shanghai, China). Mice were maintained in a pathogen-free environment. The experiment was conducted using 10 mice (n=5/group). Tumor volumes were measured at different time-points using the formula: Tumor volume = length (mm) x width^2 (mm^2)/2.

Statistical analysis. All data are shown as mean ± standard deviation, and the experiments were repeated three times. Statistical analyses were performed using a two-tailed Student's t-test with SPSS version 12.0 software (SPSS, Inc., Chicago, IL, USA) and multiple-group comparisons were analyzed using one-way ANOVA for the subsequent individual group comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Upregulation of miR-130b in glioma tissues and cells. The expression levels of miR-130b in 30 glioma cases, 5 normal brain tissues and 3 glioma cell lines were detected using RT-qPCR. The results demonstrated that miR-130b expression in glioma tissues was significantly higher than that in normal brain tissues (P<0.05; Fig. 1A), and its expression in high level glioma tissues (III and IV) was significantly higher (P<0.05) than in low grade glioma (I/II). Furthermore, miR-130b expression in the 3 glioma cell lines was significantly upregulated compared with that in the NHAs (P<0.05; Fig. 1B).

PTEN is a potential downstream target of miR-130b. Using the TargetScan bioinformatic database, the target site for miR-130b interaction was detected in the 3'UTR of the PTEN gene. Fig. 2A shows that the target sequence was located at nucleotides 412-418 of the 3'UTR region of the PTEN gene. Then, the 3'-UTR of a PTEN fragment containing the
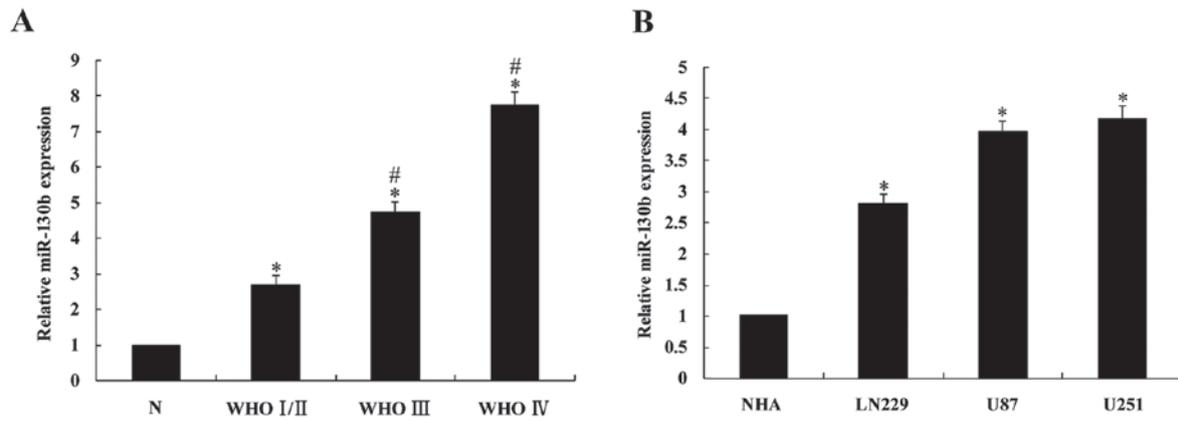
Figure 1. Expression levels of miR-130b in glioma tissues and cell lines. (A) miR-130b was detected in 30 glioma tissues and 5 non-neoplastic brain specimens by reverse transcription-quantitative polymerase chain reaction. miR-130b expression was significantly higher in the glioma tissues compared with the non-neoplastic brain specimens, and the expression in the high level glioma groups is higher than in the low level group. *P<0.05 vs. non-neoplastic brain specimens; #P<0.05 vs. WHO I/II group. (B) miR-130b expression was detected in LN229, U87 and U251 glioma cell lines and primary NHAs. qPCR showed that miR-130b was upregulated in all of the glioma cell lines compared with the NHA cells. *P<0.05 vs. NHA cells. NHA, normal human astrocytes.

miR-130b interaction site was ligated into a vector at a region downstream of the luciferase reporter gene. The luciferase reporter assay revealed that following transfection with the miR-130b inhibitor, a significant increase in wild-type luciferase activity was observed (P<0.05). By contrast, the luciferase activity of the mutant showed no significant change compared with that of the NC (P>0.05; Fig. 2B). The present study thus shows that miR-130b directly binds the 3'UTR of the PTEN gene, thereby inhibiting its expression.

Inhibition of the proliferation and induction of apoptosis of U87 cells by miR-130b. To confirm that U87 glioma cell proliferation is inhibited by miR-130b, U87 cells were transfected with miR-130b inhibitor, and the CCK-8 colorimetric assay and colony formation proliferation experiments were conducted to examine the inhibitory effects of the miR-130b inhibitor on U87 cell proliferation. At ~24 h after transfection, the cells were harvested and seeded at a density of $1x10^5$ cells/well in 6-well plates. The cells were collected at 24, 48, 72 and 96 h after plating, and cell proliferation was examined. The results demonstrated that following transfection of the miR-130b inhibitor, cell proliferation at 48, 72 and 96 h was significantly inhibited, with a lower proliferation rate compared with that of the NC (P<0.05; Fig. 3A). Similarly, the results of the colony formation assay suggested that the downregulation of miR-130b significantly inhibited the colony formation of U87 cells, compared with that of the NC (P<0.05; Fig. 3B and C). The cells transiently transfected with the miR-130b inhibitor were subjected to cell cycle analysis by flow cytometry. Compared with the NC, U87 cells transfected with the miR-130b inhibitor underwent G0/G1 cell cycle arrest (Fig. 3D and E). Furthermore, transient transfection with the miR-130b inhibitor significantly induced apoptosis of the U87 cells (Fig. 3F and G). These *in vitro* experiments suggest that the reduced expression of miR-130b suppressed glioblastoma cell proliferation via cell cycle arrest and the promotion of cell apoptosis.

Inhibition of nude mouse tumorigenicity by miR-130b. To substantiate the effect of the miR-130b inhibitor in glioma
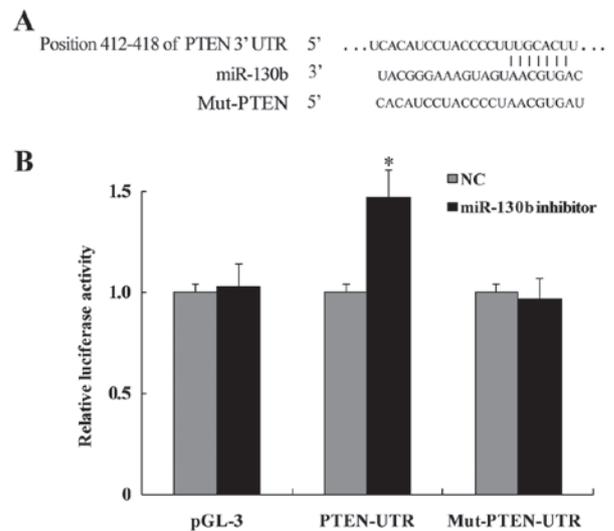
Figure 2. Identification of PTEN as the target of miR-130b. (A) Identification of target sites in the 3'UTR of PTEN was performed in TargetScan. (B) A luciferase assay was performed to determine the target association in U87 cells transfected with the control pGL-3 vector, a vector containing the PTEN sequence that was predicted to interact with miR-130b (PTEN-UTR) or a vector containing a mutated PTEN sequence with the predicted target sites (Mut-PTEN-UTR). The reported data are representative of at least three independent experiments. *P<0.05 vs. NC. PTEN, phosphatase and tensin homolog; UTR, untranslated region.

carcinogenesis, U87 cells transfected with miR-130b inhibitor or miR-130b NC were implanted into the right flanks of BALB/c athymic mice by subcutaneous injection. At 28 days post-injection, the mean volumes of tumors generated from the miR-130b cells were significantly smaller than those originating from NC cells (Fig. 4A-C). Western blotting for the glioma xenograft tissues revealed that expression of PTEN was higher in the miR-130b inhibitor group compared with the NC group (Fig. 4D).

Inhibition of the invasion and migration of U87 cells by the miR-130b inhibitor. Following the transfection of U87 cells with the miR-130b inhibitor for 24 h, cell invasion ability was
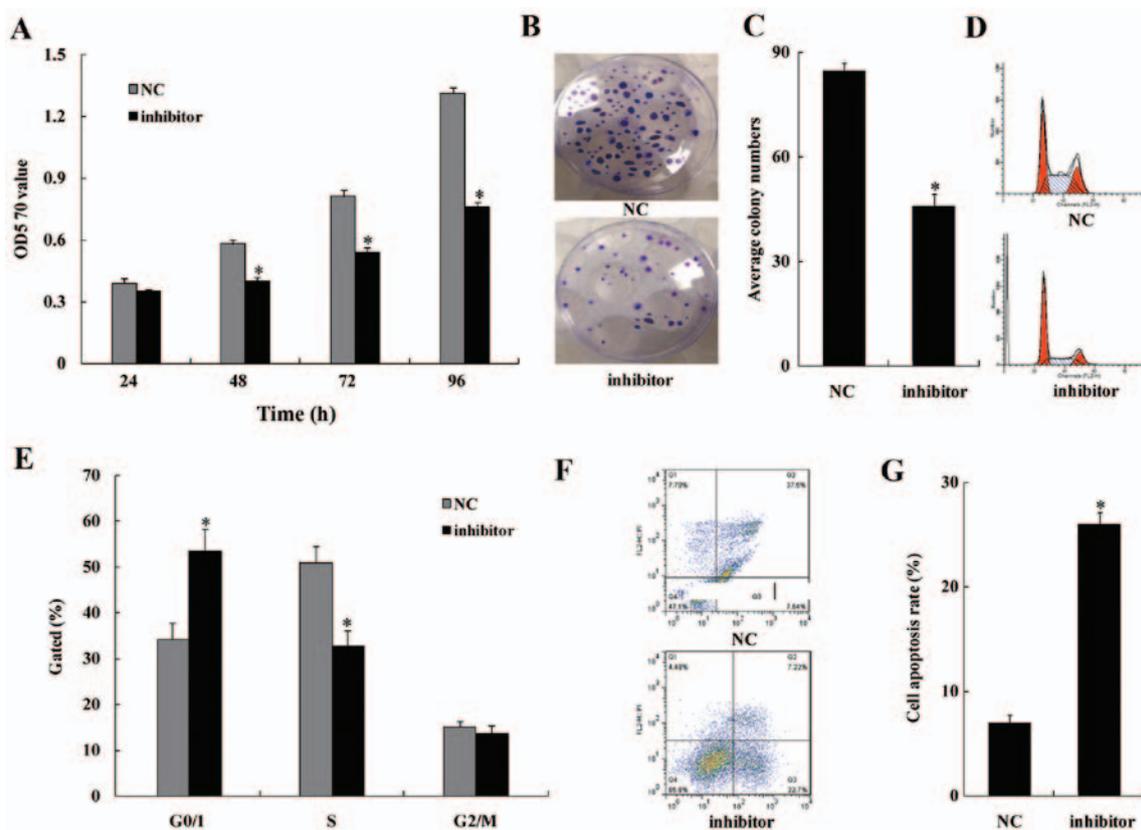
Figure 3. Downregulation of miR-130b inhibits U87 cells proliferation *in vitro* and suppresses tumorgenesis *in vivo*. (A) miR-130b inhibitor inhibited the proliferation of U87 cells at 48, 72 and 96 h. (B and C) Downregulation of miR-130b reduced the number of surviving colonies from the U87 cells compared with the NC cells. (D and E) Downregulation of miR-130b exhibited an increased G0/G1 phase and an decreased S phase. (F) Flow cytometric analysis of apoptosis using Annexin V/PI double staining. The lower left quadrant shows living cells, the upper right quadrant shows late apoptotic cells, and the lower right quadrant shows early apoptotic cells. (G) Downregulation of miR-130b induced the apoptosis of U87 cells. Data are the mean ± standard deviation of 3 independent experiments. *P<0.05 vs. NC group. NC, negative control.
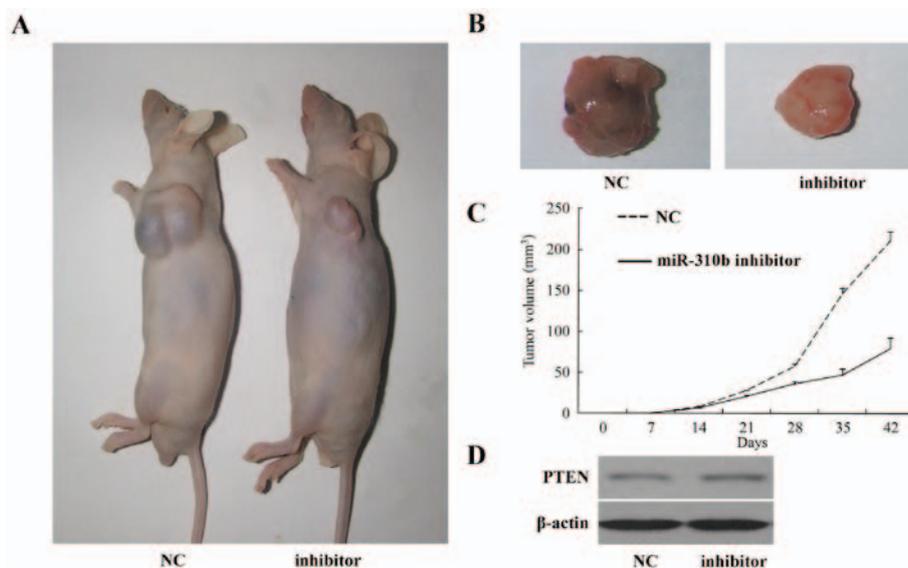
Figure 4. Evaluation of U87 cell growth *in vivo*. U87 cells stably transfected with miR-130b inhibitor or NC (1.5x10⁶ cells) were subcutaneously injected into nude mice and the tumor volume was calculated every week after injection. Representative images of (A) the mice and (B) the tumors, and (C) the tumor volume over time are shown. (D) Western blotting of PTEN protein expression in the xenograft tumor tissues. NC, negative control; PTEN, phosphatase and tensin homolog.

examined using a Transwell assay. U87 cells transfected with the miR-130b inhibitor exhibited a significant reduction in the

number of cells that traversed the membrane compared with that of the NC (P<0.05; Fig. 5A and B). The wound healing
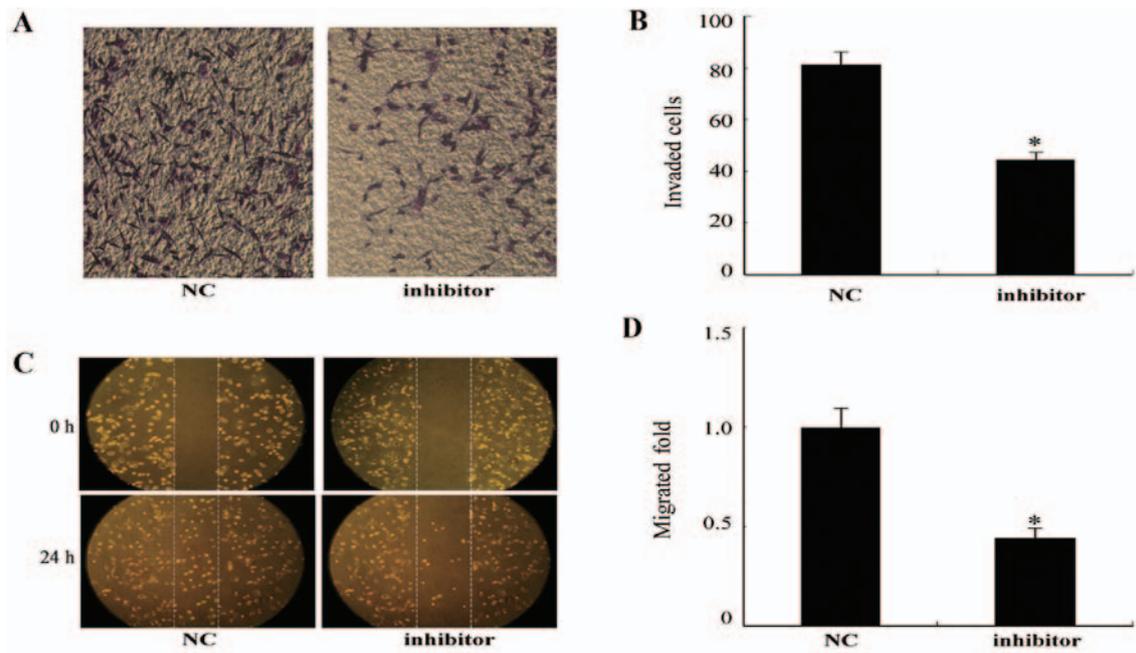
Figure 5. Downregulation of miR-130b inhibits the migration and invasion of U87 cells. (A and B) U87 cells were examined for cell invasion in 24-well plates with Transwell chambers. Invasiveness was attenuated by the downregulation of miR-130b. (C and D) Wound healing was photographed at 0 and 24 h, and wound gaps were analyzed by measuring the distance of migrating cells from different areas for each wound. *P<0.05 vs. NC group. NC, negative control
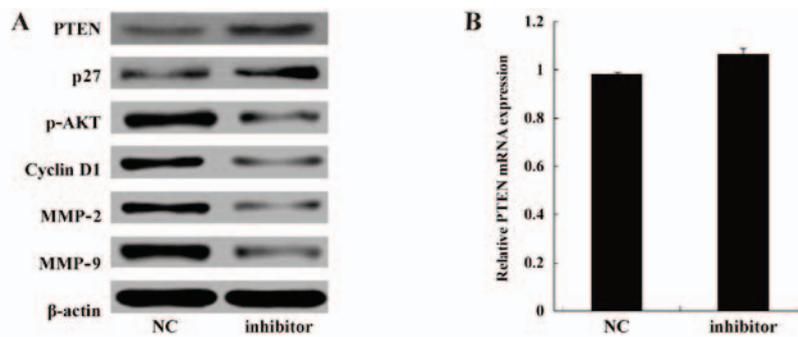
Figure 6. Downregulation of miR-130b upregulates the expression of PTEN and inhibits the AKT signaling pathway. (A) U87 cells were treated with NC and miR-130b inhibitor, and the levels of PTEN, p-AKT, p27, cyc1in D1, MMP-2 and -9 proteins were measured by western blotting. (B) The levels of PTEN mRNA in U87 cells were measured by quantitative polymerase chain reaction. NC, negative control; PTEN, phosphatase and tensin homolog; MMP, matrix metalloproteinase
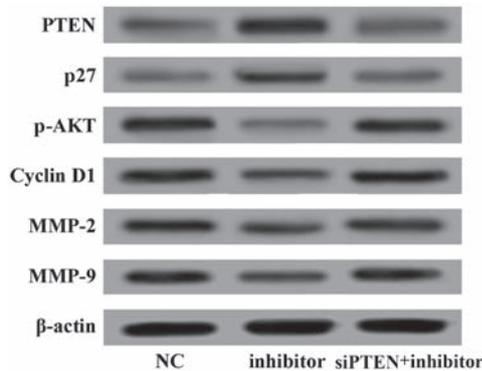
Figure 7. PTEN siRNA decreases the expression of PTEN and activates the AKT signaling pathway in U87 glioma cells. (A) Western blotting revealed that PTEN was increased with miR-130b inhibitor transfection, whereas co-transfection with PTEN siRNAand miR-130b inhibitor decreased PTEN expression and activated the AKT signaling pathway. NC, negative control; PTEN, phosphatase and tensin homolog; siRNA, small interfering RNA; siPTEN, siRNA targeting PTEN; MMP, matrix metalloproteinase.

assay also demonstrated that cell migration was inhibited in the U87 cells transfected with the miR-130b inhibitor compared with the NC (Fig. 5C and D).

Inhibition of the AKT signaling pathway by an miR-130b inhibitor via PTEN upregulation. The miR-130b inhibitor was transiently transfected into U87 cells to reduce miR-130b expression, and then total cellular RNA and protein were extracted following 48 h of transfection. PTEN expression at the RNA and protein levels was measured. The results demonstrated that compared with cells transfected with NC, when miR-130b expression was downregulated via transfection with the miR-130b inhibitor, PTEN was clearly upregulated at the protein level (Fig. 6A), but no significant difference in the level of mRNA expression was observed (Fig. 6B). The phosphorylation of AKT was significantly inhibited, and the tumor suppressor gene p27 was upregulated by the miR-130b inhibitor. In addition, cyclin D1, MMP-2 and MMP-9 expression was decreased.
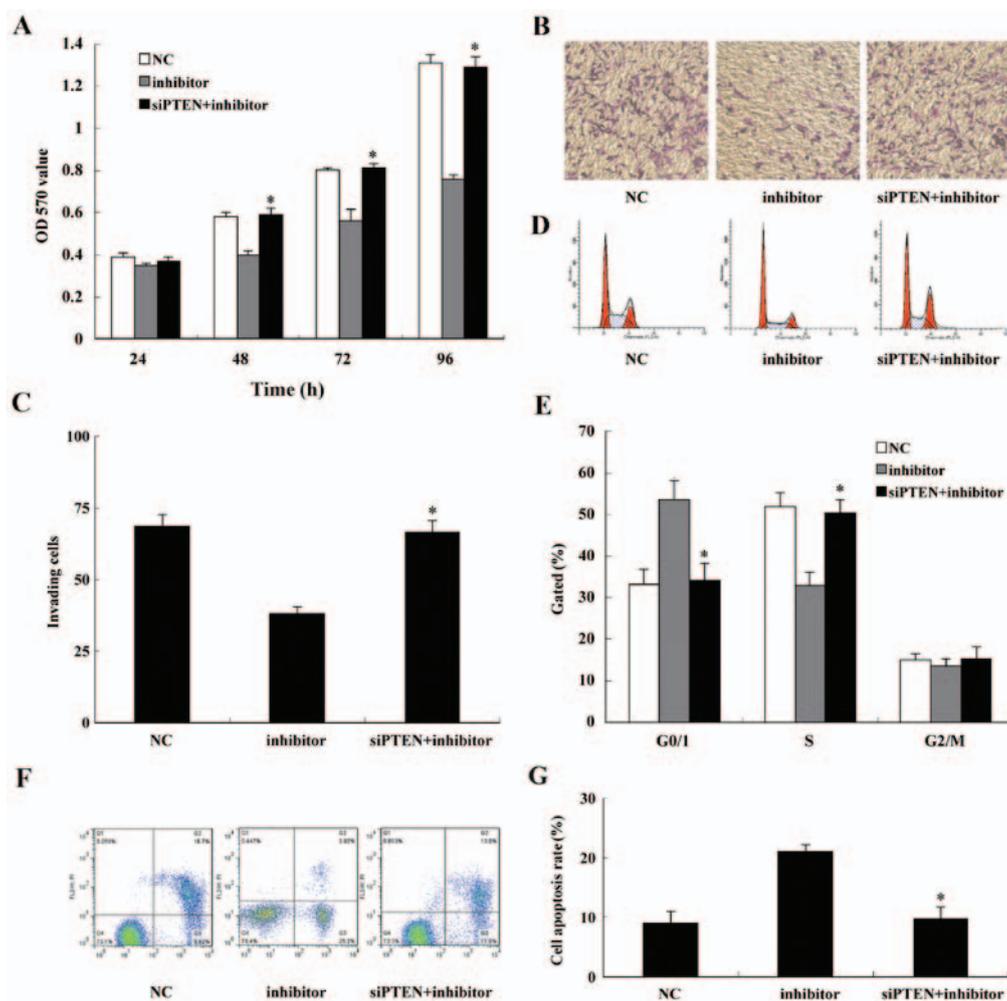
Figure 8. PTEN siRNA reverses the effect of the miR-130b inhibitor in U87 glioma cells. (A) PTEN siRNA attenuated the inhibition of U251 cell proliferation induced by the miR-130b inhibitor at 48, 72 and 96 h. (B and C) Transwell assays revealed that the miR-130b inhibitor attenuated the invasion of U87 cells and PTEN siRNA promoted the invasion. (D and E) Flow cytometry demonstrated that PTEN siRNA co-transfected with the miR-130b inhibitor significantly decreased the percentage of cells in the G0/G1 phase and increased the percentage of cells in the S phase. (F and G) Flow cytometry indicated that the miR-130b inhibitor induced the apoptosis of U87 cells and PTEN siRNA attenuated this effect. Data are mean ± standard deviation of three independent experiments. *P<0.05 vs. the inhibitor group. NC, negative control; PTEN, phosphatase and tensin homolog; siRNA, small interfering RNA; siPTEN, siRNA targeting PTEN.

Knockdown of endogenous PTEN reverses the effect of the miR-130b inhibitor. To confirm that miR-130b and PTEN serve a role in glioma cell proliferation and invasion, U87 cells were co-transfected with the miR-130b inhibitor and siPTEN. At 48 h after transfection, total proteins were extracted and western blotting demonstrated that the protein expression level of PTEN was markedly reduced (Fig. 7). The protein of PTEN was markedly reduced when U87 cells were co-transfected with the miR-130b inhibitor and siPTEN, the phosphorylation of AKT was signifcantly activated, and the tumor suppressor gene p27 was downregulated. In addition, cyclin D1, MMP-2 and MMP-9 expression was increased. CCK-8 and Transwell assay results indicated that following co-transfection with the miR-130b inhibitor and siPTEN U87, cell proliferation and cell invasion were markedly increased compared with that of the cells transfected with miR-130b inhibitor alone (Fig. 8A-C). Cell cycle analysis demonstrated that U87 cells co-transfected with the miR-130b inhibitor and siPTEN presented a lower percentage of cells in the G1 phase and a higher percentage of cells in the S phase (Fig. 8D). The analysis of apoptosis by

flow cytometry revealed that the knockdown of endogenous PTEN attenuated the apoptosis-inducing effect of the miR-130b inhibitor (Fig. 8E and F). These findings confirm that miR-130b inhibits glioblastoma cell proliferation and invasion and induces their apoptosis via the downregulation of PTEN expression.

Discussion

Glioma is the most common malignancy and the most lethal adult brain tumor (3). Due to the abnormal cellular and molecular heterogeneity of tumor cells, the treatment of glioma using surgery combined with radiotherapy and comprehensive chemotherapy with temozolomide has remained unsatisfactory, often resulting in short survival times and poor prognosis. In the past decade, the median survival time of patients with glioblastoma was only 15 months (14,15). Research on the diagnosis and treatment of glioma is now particularly focused at the molecular mechanism underlying glioma tumorigenesis and development, particularly in the

evolution of a variety of genes. Therefore, the discovery of the fundamental disease genes associated with glioma may provide an effective treatment for the targeted prevention and control of the disease at the genetic level. A number of studies have shown that miRNAs serve an important role in the initiation and progression of human cancer, acting as either oncogenes or tumor suppressor genes (16,17). It has been confirmed that a single miRNA molecule may act on multiple or even hundreds of target genes, and several different miRNAs may be able to regulate the same gene, so that miRNAs and their targeting proteins form a complex regulatory network that is important in regulating growth, development and tumorigenesis (18).

The miR-130 family includes miR-130a and b. The human miRNA-130b gene consists of 22 nucleotides (5'-CAGUGCA AUGAUGAAAGGGCAU-3'). miR-130a and miR-130b are co-localized on chromosome 22, located 10 kb apart, and can be expressed in cells in tandem, suggesting that they may have similar biological functions (19). The present study detected abnormal miR-130b expression in a variety of gliomas, which was consistent with the increased expression of miR-130b in melanoma (20), gastric cancer (21), bladder cancer (22), colorectal cancer (23), metastatic renal carcinoma (24) and glioma (10), whereas miR-130b has been found to be downregulated in papillary thyroid carcinomas (25), endo-metrial cancer (9), pituitary adenoma (26) and pancreatic cancer (27). In addition, miR-130b has an association with neurodegenerative diseases such as Alzheimer's and Parki-nson's diseases (28), and inhibits lipogenesis (20). Tu *et al* (29) detected the increased expression of miR-130b in human hepatocellular carcinoma (HCC) and liver cancer cell lines, which was associated with a poor prognosis in liver cancer patients. They also demonstrated that the inhibition of miR-130b expression upregulated its target genes through PPAR-γ, which resulted in inhibition of the invasion and migration of HCC. Furthermore, a study conducted by Zhao *et al* (27) demonstrated that the expression of miR-130b was reduced in pancreatic cancer, and identified an association of the downregulation of miR-130b with poor prognosis in pancreatic cancer patients. The study also found that the over-expression of miR-130b inhibited the proliferation and invasion of pancreatic cancer cells by downregulating the target gene signal transducer and activator of transcription 3. However, the expression pattern of miR-130b and the specific mechanisms underlying its effects in the pathogenesis of glioma have not been fully elucidated. The purpose of the present study was to investigate the mechanism by which miR-130b acts in glioma cell proliferation and invasion, and to determine whether it regulates the target gene, PTEN. In the present study RT-qPCR detected significantly higher miR-130b expression in human glioma tissues than in normal brain tissues, as well as significantly higher expression in the LN229, U87 and U251 cell lines compared with the NHA cell line. The downregulation of miR-130b in U87 glioma cells inhibited cell proliferation, invasion and migration and induced apoptosis. These findings indicate that miR-130b functions as an oncogene in glioma cells.

The PTEN gene is a tumor suppressor gene that is located on chromosome 10q23.3 and has dual specificity (30). PTEN, as a lipid phosphatase, dephosphorylates the second messenger phosphatidylinositol 3,4,5-triphosphate,

blocks the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway, upregulates the tumor suppressor gene p27 and inhibits cyclin D1 accumulation to regulate the cell cycle, which in turn causes cell G0/G1 arrest that prevent cells from entering the S phase. p27 is the key mediator in the repression of the G1 phase by PTEN, as well as a down-stream target for PTEN-dependent cell cycle arrest (31,32). PTEN also reduces AKT phosphorylation and inhibits MMP secretion, thereby inhibiting tumor invasion and migration (33). Human malignant glioma (astrocytoma and glioblastoma) has a high level of PTEN gene deletions and mutations, which leads to abnormal activation of the PI3K/Akt pathway, particularly an increase in Akt phos-phorylation and activity that promotes tumor cell growth, proliferation, invasion and metastasis (34). The present study demonstrated that the transfection of U87 cells with an miR-130b inhibitor promoted the expression of the target gene PTEN and reduced the phosphorylation of AKT. Glioma cells transfected with the miR-130b inhibitor also exhibited increased expression of the tumor suppressor gene p27, suppressed cyclin D1 expression and reduced cell proliferation with G0/G1 arrest, which prevented cells from entering the S phase. Finally, the miR-130b inhibitor also reduced Akt phosphorylation levels to inhibit MMP-2 and -9 expression, thereby suppressing U87 invasion and migration. The siRNA silencing of PTEN reversed the inhi-bition of cell proliferation and invasion and promoted the apoptosis-inducing effect of the miR-130b inhibitor. Thus, the present study demonstrated that miR-130b functions in the tumorigenesis and development of glioma via the PTEN/AKT signaling pathway.

In conclusion, the present study has elucidated the onco-genic functions of miR-130b in glioma, which involved the negatively regulation of the expression of PTEN. These results indicate that miR-130b is important in the proliferation, apop-tosis and invasion of glioma, and suggest that the inhibition of miR-130b expression may be an effective treatment approach for human glioma.

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