MicroRNA-16 inhibits glioma cell growth and invasion through suppression of BCL2 and the nuclear factor-κB1/MMP9 signaling pathway

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Glioma is one of the most common types of primary brain tumors in adults, and represents one of the most aggressive and lethal human cancer types.1 Over the past decades, an increasing amount of evidence has shown that microRNA (miRNA), a class of non-coding RNA, plays a pivotal role in glioma. They can act as both tumor suppressors and oncogenes by negatively regulating their mRNA targets through degradation or translational repression.2–5 Through using the microRNA.org database (http://www.microrna.org/microrna/home.do) and the Database on Predicted and Published MicroRNAs (miRWalk: http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/), we previously identified miRNA-16 (miR-16) is most strongly correlated with malignancy in nearly all analyzed human tumors. Other authors have shown the downregulation of miR-16 in a wide range of cancers, including breast, prostate, and lung cancers, as well as in chronic lymphocytic leukemia.6–9 These findings suggest that miR-16 is a possible tumor suppressor that acts in a variety of cancers.

The prognosis of human glioma is poor, and the highly invasive nature of the disease represents a major impediment to current therapeutic methods. At the molecular level, tumor cell invasion is mediated by a set of factors that initiate or promote cell motility, matrix destruction, angiogenesis, and other biological events.10–13 Nuclear factor-κB (NF-κB) mediates cell proliferation, cell migration, and angiogenesis.11,10,11 Aberrant activation of NF-κB has been observed in many types of human cancer, including glioma. Nuclear localization of p50, an indicator of NF-κB activation, has also been demonstrated in clinical specimens of glioblastoma multiform.13–15 Nuclear factor-κB signaling orchestrates several key biological processes during the development and progression of cancer by inducing the transcription of a variety of target genes regulating cell apoptosis, cell cycle, and invasion.14–16 Among NF-κB-regulated genes, MMPs are closely associated with tumor invasion.16–19 In particular, MMP9 and MMP2 levels increase with tumor progression in gliomas, and are thus known as key enzymes for invasion.20,21 By analyzing the homology between miR-16 and the NF-κB1 mRNA sequences on the microRNA.org database (http://www.microrna.org/microrna/getMrna.do?gene=4790&utr=3116&organism=9606&), we found that 15 nucleotides are complementary. Therefore, miR-16 may inhibit the expression of NF-κB1 and MMP proteins, and may reduce glioma invasiveness. BCL2 is an oncogene, an anti-apoptotic protein residing in the mitochondria, and is also involved in glioma development.23,24

The aim of the present study was to explore miR-16 expression in human brain gliomas and in three malignant glioma
cell lines (SHG44, U87, and U373), as well as the correlation between miR-16 and NF-xB1 expression, cell apoptosis, and invasiveness. This study also aimed to lay the foundation for future in-depth study of the mechanisms of action of miR-16 in human glioma.

Materials and Methods

Human tissue samples. For the study, 29 glioma samples were obtained from 29 Chinese patients between March 2009 and September 2011 from the Department of Neurosurgery, Brain and Nerve Research Laboratory of The First Affiliated Hospital of Soochow University (Suzhou, China). Patients included 18 men and 11 women. There were seven cases of grade I glioma (pilocytic astrocytoma), eight cases of grade II (diffuse astrocytoma), seven cases of grade III (anaplastic astrocytoma), and seven cases of grade IV (primary brain glioblastoma), according to the 2007 WHO classification system. The mean ages of the patients at the time of surgery were 48.1 years for men and 47.9 years for women. Six samples of non-neoplastic brain tissue samples were obtained from adult patients with craniocerebral injuries, for whom a partial resection of brain tissue were required as decompression treatments. There were seven cases of grade I glioma (pilocytic astrocytoma), eight cases of grade II (diffuse astrocytoma), seven cases of grade III (anaplastic astrocytoma), and seven cases of grade IV (primary brain glioblastoma), according to the 2007 WHO classification system. The mean ages of the patients at the time of surgery were 48.1 years for men and 47.9 years for women. Six samples of non-neoplastic brain tissue samples were obtained from adult patients with craniocerebral injuries, for whom a partial resection of brain tissue were required as decompression treatments. All samples were collected 24, 48, and 72 h after transfection. Then the proportion of cells in early-stage apoptosis was detected using the annexin V–PE Apoptosis Detection Kit (eBioscience, San Diego, CA, USA) and flow cytometry (FACSCanto; BD Biosciences, Franklin Lake, NJ, USA). Cell viability was assessed in PBS, and once in 1× binding buffer. Cells were resuspended in 1× binding buffer at 1–5 × 10^6/mL. Fluorochrome-conjugated annexin V (5 µL) was added to 100 µL cell suspension. Cells were incubated for 10–15 min at room temperature. Cells were washed in 1× binding buffer, and resuspended in 200 µL of 1× binding buffer. The 7-AAD Viability Staining Solution (5 µL) was added, and the cells were analyzed by flow cytometry. All analyses were carried out in triplicate.

In vitro invasion assay. SHG44, U87, and U373 cells were transfected with miR-16 mimics or negative control oligonucleotide. Human glioma cell lines SHG44, U87, and U373 were collected 24, 48, and 72 h after transfection. Then the proportion of cells in early-stage apoptosis was detected using the annexin V–PE Apoptosis Detection Kit (eBioscience, San Diego, CA, USA) and flow cytometry (FACSCanto; BD Biosciences, Franklin Lake, NJ, USA). Cell viability was assessed in PBS, and once in 1× binding buffer. Cells were resuspended in 1× binding buffer at 1–5 × 10^6/mL. Fluorochrome-conjugated annexin V (5 µL) was added to 100 µL cell suspension. Cells were incubated for 10–15 min at room temperature. Cells were washed in 1× binding buffer, and resuspended in 200 µL of 1× binding buffer. The 7-AAD Viability Staining Solution (5 µL) was added, and the cells were analyzed by flow cytometry. All analyses were carried out in triplicate.

Western blot analysis. The primary antibodies used were anti-NF-xB1 (Abcam, Tokyo, Japan), anti-BCL2 (Abcam), anti-MMP2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-MMP9 (Abcam). Protein samples were separated with 12% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were incubated with primary antibodies overnight at 4°C. Membranes were washed and incubated for 2 h with HRP-conjugated anti-rabbit secondary antibodies (ProSci, Poway, CA, USA), followed by detection and visualization using ECL Western blotting detection reagents (Pierce antibodies; Thermo Fisher Scientific).

 Luciferase reporter assay. The 3′-UTR of the NF-xB1 and Bcl2 gene, which contains one putative miR-16 targeting site, was amplified by chemical synthesis and inserted into the XhoI/SacI and SacI/HindIII sites of the pMIR-REPORT vector (Ambion, Austin, TX, USA). To express miR-16, a genomic fragment encompassing the coding region was cloned by PCR and inserted into the XhoI and KpnI sites of pCMV vector. In total, 40 000 cells were seeded in 24-well plates 24 h prior to
transfection. Cells were cotransfected with 0.1 \( \mu \)g pMIR-NF-xB1, pMIR-BCL2, or pMIR-REPORT, together with 40 nM miR-16 precursor molecule or 40 nM negative control. The pRL-TK vector (Promega, Madison, WI, USA) containing Renilla luciferase was also cotransfected as a reference control. Firefly and Renilla luciferase activities were measured by using the dual-luciferase reporter assay (Promega) 24 h after transfection. Firefly luciferase activity was normalized to Renilla luciferase activity.

Effect of miR-16 in vivo. Computer-designed oligonucleotide sequences expressing the pre-miR-16 were connected to the pcDNA6.2-GW/EmGFP-miR plasmid. The plasmid was transfected in U87 cells using Lipofectamine 2000. In order to select stably transfected U87 cells, the medium was supplemented with 10 \( \mu \)g/mL blasticidin S HCl (Invitrogen), cultured for 2 months and subcultured for more than 10 generations. We established an intracranial and a subcutaneous xenograft nude mouse model using U87 cells (1 \( \times \) 10⁶, respectively) transfected with plasmid connected pre-miR-16 or negative control oligonucleotide, respectively. On day 17 post-implantation, caliper measurements were carried out to assess tumor growth.

Immunohistochemistry and immunofluorescence. Formalin-fixed paraffin-embedded U87 tumors were cut with a microtome into 6-µm sections. Antigen retrieval was carried out in 10 mM sodium citrate buffer of pH 6.0 for 16 min at 96–98°C. Slides were incubated with primary antibodies against MMP9, Ki-67 (Boster Bioengineering, Wuhan, China) and NF-xB1. Sections were subsequently incubated with the Cell and Tissue Staining Kit HRP-DAB system (R&D Systems, Minneapolis, MN, USA), according to the manufacturer’s instructions. Immunostainings were run with known positive and negative tumor controls, and were blindly evaluated by a pathologist.

Statistical analysis. Statistical analyses were carried out using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA), and significance determined with two-tailed Student’s \( t \)-test. Data were considered statistically significant at \( P < 0.05 \).

Results

Expression of miR-16 and NF-xB1 negatively correlated in glioma. To assess miR-16 and NF-xB1 expression in non-cancerous brain tissues and in different grades of glioma, we detected miR-16 expression and mRNA levels of NF-xB1 in six non-neoplastic brain tissues and in 29 human glioma tissue samples. Data showed that miR-16 expression in non-cancerous brain tissues was lower than in human brain glioma tissues (\( P < 0.01 \)) and decreased with the increasing degree of malignancy in glioma (low-grade gliomas vs. high-grade gliomas, \( P < 0.05 \); Fig. 1a). In addition, we found that the expression of miR-16 in glioma cell lines SHG44, U87, and U373 was lower than in non-cancerous brain tissues (\( P < 0.01 \); Fig. 1c). However, the expression of NF-xB1 increased with the increasing degree of malignancy in glioma (low-grade gliomas vs. high-grade gliomas, \( P < 0.01 \); Fig. 1b). Therefore, the expression of miR-16 and NF-xB1 was negatively correlated in glioma.

MicroRNA-16 directly targets NF-xB1 in human glioma cells. By analyzing the homology between miR-16 and NF-xB1 mRNA sequences, we observed that the 15 nucleotides from the 5' end of miR-16 were complementary to bases 255–279 of the NF-xB1 cDNA (\( Homo sapiens \), NM_003998 AK122850 M58603; Fig. 2a). A dual-luciferase reporter assay system was used to validate whether miR-16 directly recognizes the 3'-UTR region of NF-xB1 mRNA. As shown in Figure 2(b), the relative luciferase activities of the pre-miR-16 groups transfected with NF-xB1 3'-UTR constructs were significantly decreased, compared to those transfected with pMIR-NF-xB1 constructs, for the 293T cell line, implying that NF-xB1 is a direct target of miR-16. To further demonstrate the correlation between miR-16 and NF-xB1, mRNA and protein expression of NF-xB1 were detected by qRT-PCR and Western blotting. Data showed that miR-16 upregulation dramatically reduced the protein expression of NF-xB1. Meanwhile, we observed that NF-xB1 expression was increased by downregulating miR-16 (Fig. 2c,d). Thus, miR-16 inhibited NF-xB1 expression in human glioma.

Upregulation of miR-16 induces apoptosis in human glioma cells by directly downregulating BCL2 expression. The micro-
RNA.org database (http://www.microrna.org/microrna/get Mrna. do?gene=596&utr=5412&organism=9606) shows that BCL2 is a target of post-transcriptional repression by miR-16 (Fig. 3a).

Our data showed that miR-16 expression was inversely correlated with BCL2 in the same tissue samples of human glioma (Fig. 3b). A dual-luciferase reporter assay system was used to validate whether miR-16 directly recognizes the 3′-UTR region of BCL2 mRNA. As shown in Figure 3(c), the relative luciferase activities of the pre-miR-16 groups transfected with BCL2 3′-UTR constructs were significantly decreased, compared to those transfected with pMIR-BCL2 constructs, for the 293T cell line, implying that BCL2 is a direct target of miR-16. To examine if miR-16 facilitates apoptosis in human glioma cells, flow cytometry data indicated that increasing miR-16 expression induced early apoptosis, compared with the negative control oligonucleotide and mock, and that the proportion of early apoptotic cells in the miR-16 treatment group was markedly increased (\( P < 0.01, n = 3 \)) (Fig. S1). We further investigated the mechanism of transfection of the miR-16 mimics and inhibitors on inducing apoptosis in human glioma cells. BCL2 was detected by Western blot. Data showed that the upregulation of miR-16 led to obvious downregulation of BCL2 expression (Fig. 3d), and that miR-16 induces apoptosis in human glioma cells in vitro.

Upregulation of miR-16 reduces invasiveness of glioma cells in vitro. To assess the effects of miR-16 on the invasiveness of glioma cells, we used a Transwell invasion system. The numbers of invasive cells with miR-16 mimics were significantly reduced compared with those with the negative control oligonucleotide and mock (Fig. 4a,b).

Upregulation of miR-16 leads to downregulation of MMP9 expression. The Transwell invasion system clearly showed that the upregulation of miR-16 reduced the invasiveness of glioma cells. To further explore the molecular associations between...
miR-16 and invasiveness in human glioma, MMP9 and MMP2 were detected by Western blot analysis. Data showed that MMP9 expression was clearly inhibited by the upregulation of miR-16 and that the opposite result was observed by inhibiting miR-16 expression. However, miR-16 had little effect on MMP2 expression (Fig. 4c).

Upregulation of miR-16 reduces glioma growth and invasiveness in an in vivo mouse model. Finally, to test the effects of miR-16 on glioma growth in vivo, we used a mouse model of human glioma. As shown in our experiments, all tumors from U87 cells transfected with plasmid-connected pre-miR-16 were efficiently suppressed compared with the negative control (Fig. 5c,d). The intracranial tumors were removed and sectioned. Hematoxylin–eosin staining showed that miR-16 reduced glioma growth and invasion in the encephalic nude mouse model (Fig. 5a). Immunofluorescence showed that miR-16 reduced glioma invasion and MMP9 expression in the nude encephalic mouse model (Fig. 5b). Sections were stained for Ki-67, MMP9, and NF-κB1. Immunohistochemistry showed that tumors with U87 cells transfected with miR-16 reduced Ki-67 (Fig. 6a), MMP9 (Fig. 6b), and NF-κB1 stainings (Fig. 6c), compared with negative control oligonucleotide. Thus, the data indicated that miR-16 upregulation led to the inhibition of glioma cell proliferation and invasion in tumor xenografts, and that the upregulation of miR-16 reduced glioma growth in the mouse model in vivo.

Discussion
Previous studies showed that proto-oncogene activation and inhibition of tumor suppressor genes play an important role in the occurrence and development of glioma. In the present

![Fig. 4. miR-16 reduces invasiveness of glioma cells. (a,b) Transwell invasion system showed that the numbers of invasive cells were significantly reduced compared with the cultures transfected with the negative control oligonucleotide. Each bar represents mean values ± SD from three independent experiments. **P < 0.01. (c) Expression of MMP9 was clearly inhibited by upregulation of microRNA-16 (miR-16).](image1)

![Fig. 5. miR-16 reduces glioma growth and invasion in an in vivo mouse model using U87 cells. (a) Hematoxylin–eosin staining showed that microRNA-16 (miR-16) reduced glioma growth and invasion in an encephalic glioma nude mouse model. (b) Immunofluorescence showed that miR-16 reduced glioma invasion and MMP9 expression in a nude encephalic glioma mouse model (yellow arrow). (c,d) MicroRNA-16 reduced glioma growth in an s.c. glioma nude mouse model.](image2)
study, we showed that miR-16 is a tumor suppressor gene that inhibits glioma cell growth through the downregulation of BCL2. Moreover, the key finding of this study is that miR-16 levels are inversely correlated with NF-κB1 protein expression, and that miR-16 directly reduces the expression of NF-κB1 protein, inhibiting glioma cell invasion through the NF-κB/MMP9 signaling pathway.

MicroRNA-16 is one of the most commonly implicated miRNAs in cancer development. Its expression is strongly downregulated in a variety of solid tumors. For example, the loss of the genomic locus at 13q14 encompassing the miR-16 gene has been associated with a number of malignancies, including high-grade prostate carcinoma, breast cancer, non-small-cell lung cancer, and chronic lymphocytic leukemia, suggesting that this deletion is of pathogenetic significance. A further study found that miR-16 induced apoptosis through the downregulation of BCL2 in chronic lymphocytic lymphoma and gastric cancer, indicating that it may be essential for apoptosis by targeting BCL2 and the caspase signaling pathway. However, current knowledge of the molecular mechanisms mediating the functions of miR-16 in cancer, and more specifically in gliomas, is limited at best. Our results also suggest that miR-16 expression in human brain glioma tissues and in human glioma cell lines were lower than in non-cancerous brain tissues, and that miR-16 expression decreased with the increasing degree of malignancy.

BCL2 is an oncogene, an anti-apoptotic protein residing in the mitochondria, and is also involved in glioma development. Hussein et al. proved that the expression of BCL2 protein gradually decreased with the increasing degree of malignancy in glioma. These data imply that miR-16 levels are inversely correlated with BCL2 protein expression in human glioma. Our data further showed that miR-16 directly downregulated BCL2 expression and induced the apoptosis of human glioma cells. Taken together, our results suggest that the upregulation of miR-16 may contribute to human glioma growth because of decreased levels of BCL2.

Most current therapies for glioma treatment are ineffective against invading cells. The molecular mechanisms mediating invasion may provide a foundation for decreasing glioma recurrence, and for prolonging patients’ survival. Tumor cell invasion and metastatic spread are indicative of advanced tumor progression, and depend on changes in cell–cell and cell–matrix adhesion. Thus, the NF-κB signaling pathway plays a crucial role in tumor development through the transcriptional regulation of genes associated with tumor growth, invasion, and metastasis.

Expression of MMPs, especially MMP2 and MMP9, are upregulated and correlated with progression, tumor aggressiveness, and poor prognosis in glioma. Dimerization of the NF-κB transcription factor at the κB sequence in the MMP9 promoter initiates MMP9 transcription, thus upregulating MMP9 protein expression. Therefore, blocking NF-κB transcriptional activity could inhibit glioma cell invasion. Shin et al. found that miR-16 and miR-21 are directly regulated by the transcription factor NF-κB. In our study, we further observed that 15 nucleotides are complementary between miR-16 and the NF-κB1 mRNA sequence. Therefore, we hypothesized a putative connection between miR-16 and glioma progression, invasion, and metastasis. Indeed, our findings suggest that the levels of miR-16 were inversely correlated with mRNA expression of NF-κB1 in the same human glioma patient samples, and that miR-16 had a tumor-suppressive function in glioma through the suppression of NF-κB1 and MMP9 in vitro. In our study, we proved that miR-16 suppressed glioma growth, expression of Ki-67 and invasion by regulating the NF-κB/MMP9 signaling pathway in vitro and in vivo.

In summary, we identified miR-16 as a negative regulator of tumor growth and invasion in vitro and in vivo in glioma. These in vitro and in vivo findings were further validated in patient samples, where miR-16 expression was inversely correlated with the grade of glioma. Mechanistically, the tumor-suppressive role of miR-16 can be attributed to inhibition of the
BCL2 and NF-κB1/MMP9 signaling pathway. In conclusion, our results add a mechanistic insight regarding the role of miR-16 in the growth and aggressiveness of glioma. As glioma presents a therapeutic challenge due to the lack of molecular targets, a therapeutic miRNA approach, where oncogenic pathways are directly targeted, might provide new insights into the development of therapeutic strategies against cancer using miR-16.

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Disclosure Statement
The authors have no conflict of interest.

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