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RESEARCH

miR-19a and miR-19b Overexpression in Gliomas

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Abstract Astrocytic gliomas are the most common type of human primary brain tumors with poor prognosis. MicroRNAs(miRs) are frequently deregulated in gliomas and play an oncogenic or tumor suppressor role. In our previous study we found that miR-19a and miR-19b were upregulated in malignant glioma cell lines by microRNA array. For further validation of this finding, the expression of miR-19a and miR-19b was detected by qRT-PCR and in situ hybridization(ISH) in 8 malignant glioma cell lines, 43 freshly resected glioma samples and 75 archival paraffin embedded glioma specimens with different grades of malignancy in the present study. The results demonstrate that miR-19a and miR-19b are overexpressed in glioma cell lines and astrocytic glioma tissues, and their expression level is positively correlated with tumor grades. Additionally, the tumor suppressor gene PTEN is identified as the target of miR-19a and miR-19b by Luciferase assay. It is speculated that miR-19a and miR-19b may have an oncogenic role in gliomagenesis at least partially via the negative regulation of PTEN and the molecular mechanism of gliomagenesis in which miR 19a and miR-19b involved should be investigated further.

Keywords Glioma · miR-19 expression · Real-time PCR · In situ hybridization · PTEN

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Introduction

Astrocytic gliomas are the most common type of primary brain tumor. Astrocytic gliomas can be classified into four different grades according to the World Health Organization (WHO) classification system [1], glioblastoma multiforme (GBM, grade IV) is the most malignant and highly aggressive form of astrocytic gliomas. Current treatment of astrocytic gliomas includes surgical resection, radiation therapy and chemotherapy, but patient's response is poor and average survival of patients with aggressive form of gliomas is less than 2 years [2]. So it is imperative to gain an in-depth understanding of the molecular pathology that results in gliomagenesis and tumor progression,and the findings may lead to novel therapeutic interventions for malignant astrocytic gliomas.

MicroRNAs are a class of short noncoding RNAs which act as posttranscriptional regulators of gene expression. By binding to the 3′-untranslated region of target mRNAs, miRs could trigger translational downregulation and/or increase degradation of mRNA from target genes.

MicroRNAs are thought to play important roles in many physiologic and pathological processes, including cancer. MiR-19a and miR-19b belong to mircoRNA-17-92 cluster, which locate on chromosome 13q31.3, and are reported to be tumor associated miRs involving in tumorigenesis. Several studies have shown enhanced expression of miR-19a and miR-19b in different types of tumors, such as multiple myeloma, in which miR-19a and miR-19b are induced by Myc and directly suppress SOSC-1 [3]. MiR-19a and miR-19b are also overexpressed in anaplasic thyroid cancer cells, and inhibition of miR-19a suppresses anaplastic thyroid cancer cell proliferation [4].

We previously studied miR-19a and miR-19b expression in glioma cell lines U251, H4 and U87 by miRNA microarray. As compared to normal brain tissue, the expression of miR-19a and miR-19b was highly upregulated in glioma cell lines(data not shown). For further evaluating the dysregulation of miR-

19a and miR-19b in gliomas, we detected miR-19a and miR-19b in 8 glioma cell lines, 43 freshly resected glioma tisuse samples and 75 archival paraffin embedded glioma specimens with different grade of malignancy in the present study.

PTEN is one of the most frequently deregulated tumor suppressor genes in human cancers. Functionally, PTEN is a phospholipid phosphatase which acts as a negative regulator of cell survival via inhibition of the AKT/mammalian target of rapamycin (mTOR) pathway, even modest modulation of PTEN expression level by miRNAs may have functional consequences. miRNA target prediction algorithms (TargetScan, Pictar, HuMiTar [5]) predict that PTEN is the potential target of miR-19. In this study, we will identify PTEN as the target gene of miR-19 in glioma cell lines by luciferase asssay.

Material and Methods

Cell Culture

U251, SNB19, LN308, U87 and LN229 human glioblastoma cell lines were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science. Human glioblastoma cell lines TJ905 and TJ899 were established and characterized in Laboratory of Neuro-oncology, Tianjin Neurological Institute [6]. Human glioblastoma cell line A172 was kindly provided by Prof. Jinhuan Wang (Tianjin First Central Hospital, China). The miR-19a/b antisense oligonucleotides(miR-19a/b As) as well as scramble miRNA were obtained from Gene Pharma Co, Ltd (Shanghai, China).

All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10 % fetal bovine serum(Invitrogen, USA), 2 mM glutamine (Sigma, USA), 100 units of penicillin/ml (Sigma, USA), and 100 μg of streptomycin/ml (Sigma, USA), incubated at 37 °C with 5 % CO2, and subcultured every other day.

Tissue Microarray and In-Situ Hybridization

Tissue microarray was prepared by ChaoYing Biotechnology (Shanxi, China) from archival paraffin embedded glioma specimens. In each tissue microarray slide, there were 75 cases of astrocytic gliomas including: 10 cases of WHO I, 13 cases of WHO II, 34 cases of WHO III, 18 cases of WHO IV, and 5 cases of normal brain tissue.

For in-situ hybridization staining, Using antisense locked nucleic acid (LNA)-modified oligonucleotides detection probe, 5′-DIG labeled(Exqon, Denmark), The sequences of detction probes were as follows: hsa-miR-19a, 5′-TCAGTTTTGCA TAGATTTGCACA-3′.(product No: 18091-01) hsa-miR-19b, 5′-TCAGTTTTGCATGGATTTGCACA-3′, (product No: 38092-01). In-situ hybridization was performed with In-situ hybridization kit (Boster Sci corp, Wuhan, China) according to the manufactures' protocol. The result was visualized by FluoView Confocal Laser Scanning Microscopes-FV1000 (Olympus, Japan) and analyzed by IPP5.1 (Olympus, Japan).

Freshly Resected Tissue Samples and qRT-PCR

Tissue specimens and clinical information were obtained according to the regulations and internal biosafety and bioethics guidelines of Tianjin Medical University and Tianjin Municipal Science and Technology Commission. Forty-three freshly resected astrocytic glioma samples were collected with patient's consent at the time of operation and classified according to 2007 WHO categories. There were 12 cases of WHO grade I–II, 15 cases of WHO grade III, 16 cases of WHO grade IV (Glioblastoma). Each tumor tissue sample was taken from three separate viable areas of tumors. Five normal brain tissue specimens were obtained from internal decompression of patients with cerebral hemorrage and temporal lobe resection for intractable epilepsy. Each tissue sample was snap frozen following resection and stored in the liquid nitrogen. All the tumor samples and normal brain tissues were diagnosed by two independent neuropathologists.

Total RNA was extracted from glioma tissues, normal brain tissues and glioma cell lines by Trizol reagent(Invitrogen, USA), reverse transcription (RT) reaction and real-time PCR was conducted according to the instruction of mirVana™ qRT-PCR miRNA detection kit (Gene Pharma Co. Ltd, Shanghai). Amplification reaction was performed using qRT-PCR thermocycler (BioRad, USA), the protocol was performed as 95 °C for 3 min, then 40 cycles consisting of 95 °C for 12 s and 62 °C for 30 s. Both RT and PCR primer were purchased from Ambion, USA. Relative expression were shown as fold change ($2^{-\Delta\Delta Ct}$) and normalized to the expression of U6. All qRT-PCRs were performed in triplicate, and the data were presented as means ± standard errors of the means (SEM). Since highly similar sequence of miR-19a and miR-19b, they could be amplified by the same primers.

miR-19a (19b)primer	Upper: TCATCACGCTGTGCAAATCT
	Lower: TATGGTTGTTCTGCTCTCTGTCTC
U6snRNA primer	Upper: ATTGGAACGATACAGAGAAGATT
	Lower: GGAACGCTTCACGAATTTG

Plasmid Constructs and Luciferase Reporter Assay

The human 3′-UTR of the PTEN gene which contains the putative binding site for miR-19a/b was amplified by PCR, using the primers carrying XbaI sequence as follows:

Forward: 5′CC TCTAGA aga ttt atg atg cac tta 3′
Reverse: 5′ CC TCTAGA ttt aat ata aac att ata 3′

The product was digested using XbaI enzyme and cloned into the XbaI treated PGL3 vector (Promega, USA), then the 3′-UTR of the PTEN just located at the site immediately downstream of the luciferase gene in the reporter gene vector. The ligated product was transduced into E. coli JM109 and PCRs were used to screen for the clones harboring the forwardly oriented insert, The desired construct was subsequently sequenced to generate the vector PGL3-PTEN-3′-UTR(PGL3-PTEN).

For the luciferase reporter assay, the human glioblastoma cell lines U251, SNB19 and LN229 . Were culture in 96-well plates. They were transfected with 0.2 μg each of the PGL3 or PGL3-PTEN plasmids and 5 pmol of the miR-19a/b antisense oligonucleotide(AS-miR-19a/b) using lipofectamine 2000. At 48 h after transfection, luciferase activity was measured using the Luciferase Assay System (Promega, USA).

Western Blot Analysis

At 48 h after transfection with As-miR-19a/b, total proteins from the parental and transfected LN229 cells were extracted and the protein concentration was determined by Lowry method. A total of 40 μg protein lysates from each sample was subjected to SDS-PAGE on 10%SDS-polyacrylamide gel. Separated proteins were transferred to a PVDF membrane (Millipore, USA). The membrane was incubated with primary antibodies against PTEN(1:1000 dilution, Santa Cruz, USA) followed by incubation with HRP-conjugated secondary antibodies(1:1000 dilution, Zymed, USA). The specific protein was detected using a SuperSignal protein detection kit (Pierce, USA). After washing with stripping buffer, the membrane was reprobed with antibody against GAPDH (1:1000 dilution, Santa Cruz, USA). The band density of specific proteins was quantified after normalization with the density of GAPDH.

Statistical Analysis

Data were expressed as means ± SEM. Statistics was determined by ANOVA and Spearman correlation test using SPSS11.0. Statistical significance was determined as $P<0.05$.

Results

Detection of miR-19a and miR-19b by qRT-PCR Assay

The Expression of miR-19a and miR-19b in Glioma Cell Lines

We employed qRT-PCR to evaluate the expression level of miR-19a and miR-19b in 8 glioma cell lines. The comparative Ct (ΔΔCt) method was used to determine the change of miR-19a/b expression in glioma cell lines relative to that in normal brain tissue. The results demonstrated increased expression of miR-19a and miR-19b in glioma cell lines relative to normal brain tissues($p<0.05$), i.e. 1.71±0.03 fold in A172 cell, 9.70±0.15 fold in LN229 cell, 11.32±0.19 fold in LN308 cell, 3.33±0.23 fold in SNB19 cell, 6.69±0.23 fold in U251 cell, 9.48±0.2 fold in U87, 3.47±0.38 fold in TJ899 and 2.19±0.23 fold in TJ905 (Fig. 1).

The Expression of mir-19a and mir-19b in Glioma Tissues

The expression of miR-19a and miR-19b was detected in 43 glioma samples by qRT-PCR. As compared with normal brain tissue samples, miR-19a and miR-19b were up-regulated in glioma samples ($p<0.05$), and positively correlated with the tumor grades. The expression of mir-19a and miR-19b in different grades of astrocytic gliomas was: 2.48±1.54 fold in WHO I~II grade glioma, and 4.89±3.27 fold in WHO III grade glioma and 10.06±7.44 fold in WHO IV grade glioma relative to normal brain tissues (Fig. 2).

Detection of mir-19a and mir-19b by In-Situ Hybridization

The Expression of mir-19a and mir-19b in Glioma Cell Lines

We also detected the expression of miR-19a and miR-19b in seven glioma cell lines by in-situ hybridization. The fluorescent signals of miR-19a and miR-19b in glioma cell lines were stronger than normal brain tissues that indicated miR-19a and miR-19b upregulated significantly in U251, LN229, SNB19, LN308, U87, TJ899 and TJ905 cells (Fig. 3).

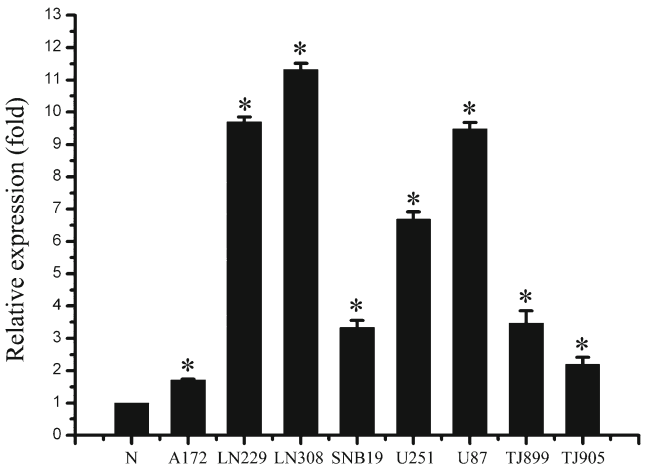

Fig. 1 miR-19a/b expression in glioma cell lines detected with qRT-PCR. $^{*}p<0.05$, compared with normal brain tissue. The expression level of miR-19a and miR-19b in 8 glioma cell lines is up-regulated relative to normal brain tissues

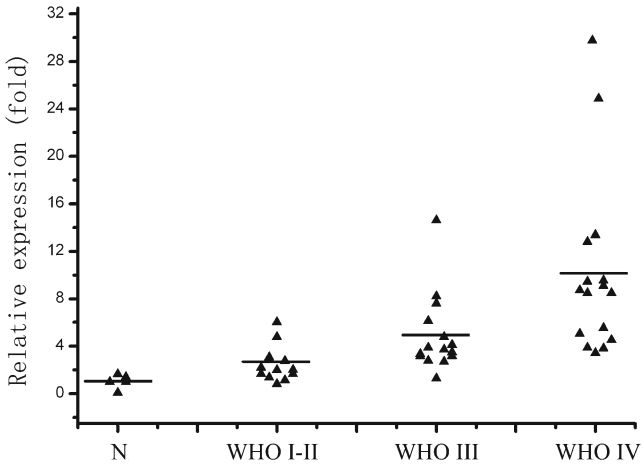

Fig. 2 miR-19a/b expression in glioma samples detected with qRT-PCR. miR-19a and miR-19b is up-regulated in glioma samples compared with normal brain samples

The Expression of miR-19a and miR-19b in Glioma Tissues

We examined the expression of miR-19a and miR-19b in 75 astrocytic glioma specimens by tissue array. The results of in-situ hybridization demonstrated that the expression of miR-19a and miR-19b was significantly increased relative to normal brain tissues (Fig. 4). The positive expression rate was 69.6 % in grade I–II tumors and 100 % in grade III~IV tumors (Table 1).

By evaluation of the staining intensity, we analysed the correlation between miR-19a/b expression level and glioma grades with Spearman Rank Correlation test. The staining intensity was scored as (−) negative staining, (+) weak staining, (++) positive staining and (+++) strong staining. As a result, the miR-19a and miR-19b expression level was positively correlated with glioma grade, r=0.542, P=0.000 (Table 1) and this result was concordant with that of qRT-PCR detection.

PTEN is a Target Gene of miR-19a/b

To explore the target gene of miR-19a/b, miRNA targets prediction databases were searched, including Targetscan, Pictar and HuMiTar [5], 3′UTR of PTEN was found to contain the highly conserved putative binding sites of miR-19a/b. To verify PTEN is one of the target genes of miR-19a/b, we constructed the PGL3-PTEN plasmid containing 3′UTR of PTEN with miR-19a/b putative binding site and conducted a reporter gene assay. As shown in Fig. 5, Reporter assay revealed that reduction of miR-19a/b led to a remarkable increase of luciferase activity in PGL3-PTEN combined with AS-miR-19a/b transfected cells(6.52±2.12 fold

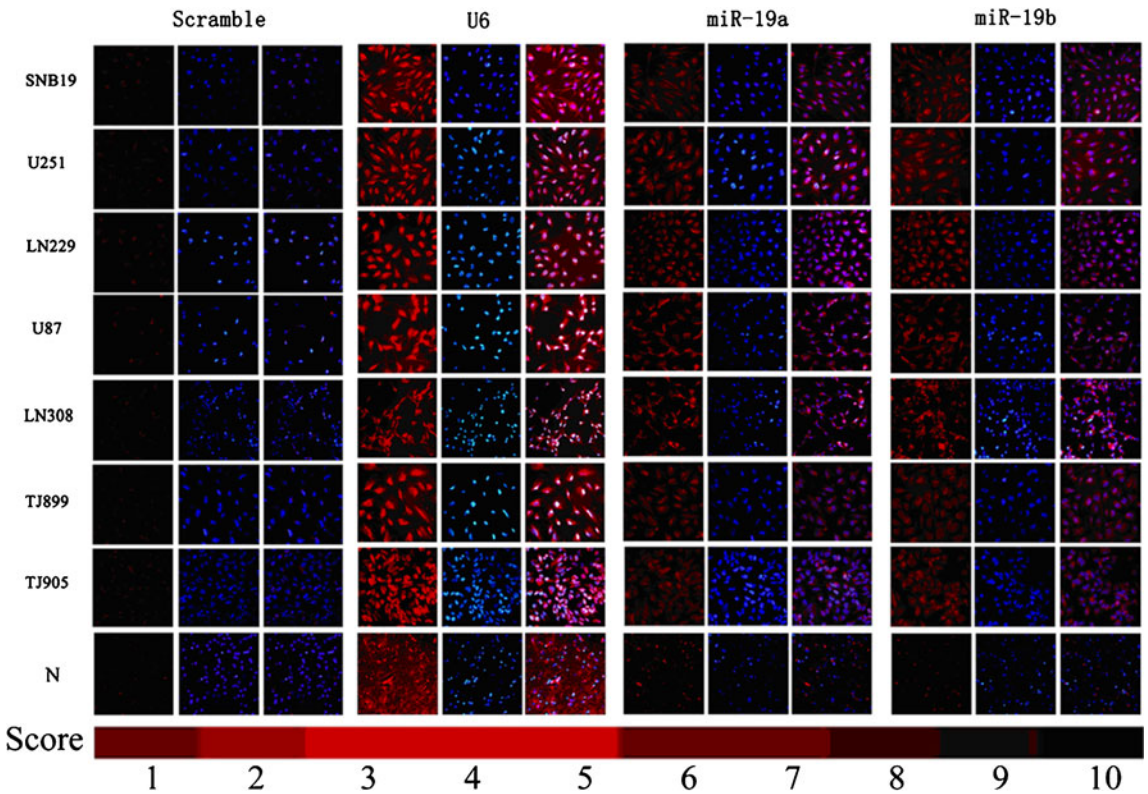

Fig. 3 miR-19a/b expression in human glioma cell lines examined by in-situ hybridization (×200). The fluorescent signals of miR-19a and miR-19b in glioma cell lines: SNB19, U251, LN229, U87, LN308, TJ899, TJ905 are stronger than normal brain tissues

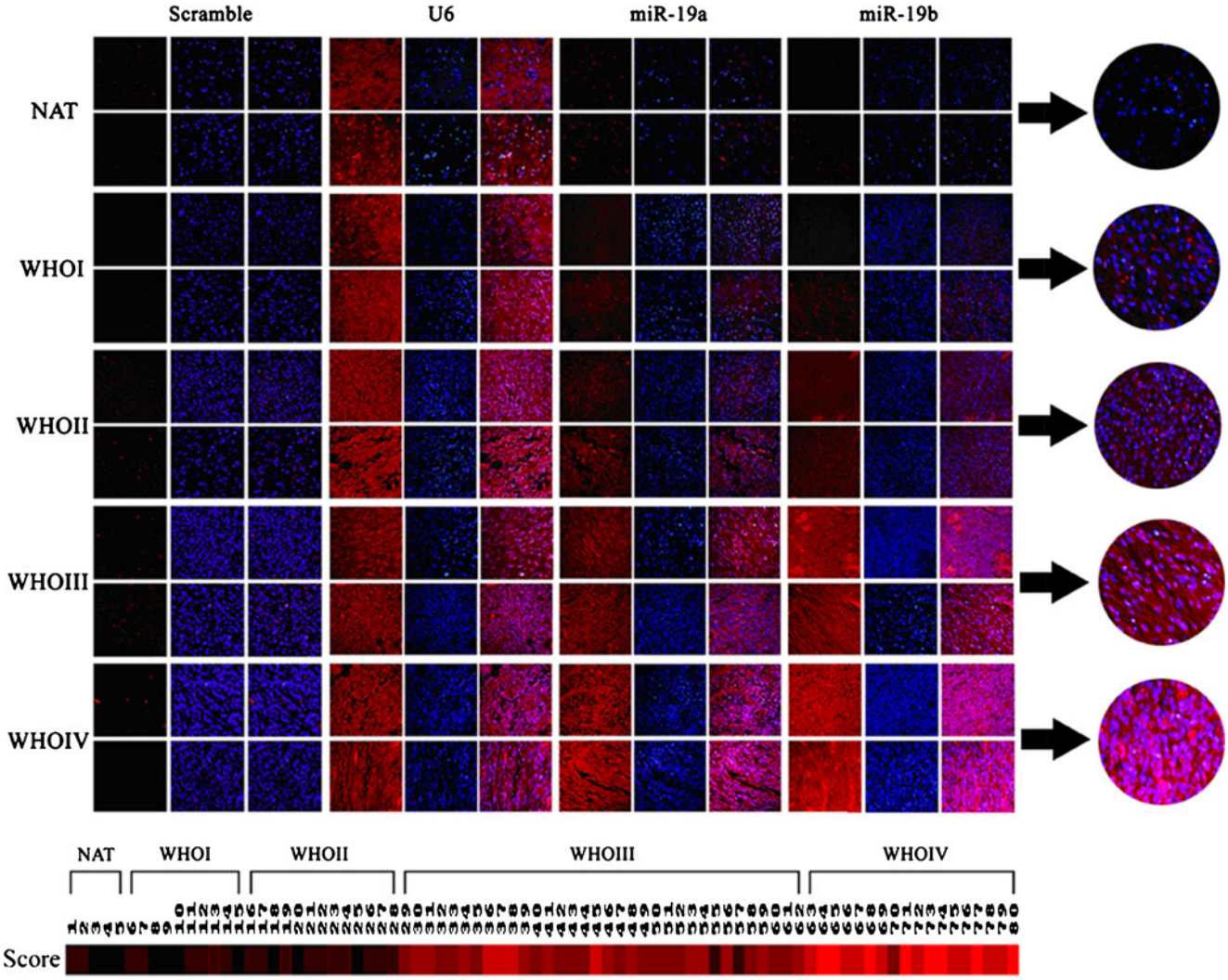

Fig. 4 miR-19a/b expression in human glioma samples examined by in-situ hybridization (×200). miR-19a and miR-19b exhibit increased expression compared with normal brain tissues detected by in-situ hybridization

for SNB19 cells, 9.85±2.72 fold for LN229 cells and 5.54±1.92 fold for U251 cells), whereas no change of luciferase activity was found in PGL3-PTEN with scr ODN transfected cells(1.08±0.27 fold for SNB19 cells, 0.91±0.29 fold for LN229 cells and 0.92±0.24 fold for U251 cells). Moreover, Western blot analysis showed that PTEN expression was up-regulated in glioma cells treated with AS-miR-19a/b (Fig. 6) compared to the cells treated with scr ODN or control cells. These evidences indicated that miR-19a/b directly modulated PTEN expression by binding to 3′UTR of PTEN.

Discussion

MicroRNAs are a family of highly conserved, small noncoding RNAs that posttranscriptionally repress gene expression via degradation or translational inhibition of their target

Table 1 Detection of miR-19a/b expression by ISH across the WHO classified human gliomas

	Grade	N				Positive rate	total	$\chi 2$	*P*
		−	+	++	+++				
miR-19a/b	I–II	7	16	0	0	69.6 %	23	1.135	0.000
	III	0	2	28	4	100 %	34		
	IV	0	0	1	17	100 %	18		

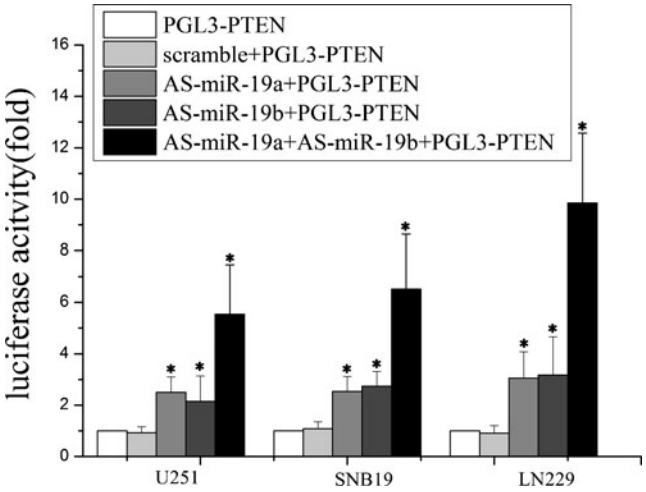

Fig. 5 The luciferase activity increased in U251, SNB19 and LN229 glioblastoma cells co-transfected with PGL3-PTEN and AS-miR-19a/b. $^{*}p<0.05$, compared with control cells transfected with pGL3-PTEN. Luciferase activity was significantly increased in cells transfected with pGL3-PTEN vector and miR-19a/b AS compared with cells transfected with PGL3-PTEN and scr ODN, the result demonstrates that PTEN is the target gene of miR-19a/b

mRNAs. A large number of miRNAs have been identified to be involved in the pathogenesis of a variety of malignancies, including glioma. The expression of a group of miRNAs is significantly altered in gliomas, such as mir-21, miR221/222, mir-181, mir-26a, mir-296, mir-146b, mir-125b are up/down-regulatd in glioma cell lines [7, 8], and they may function as oncogenes or tumor suppressor genes.

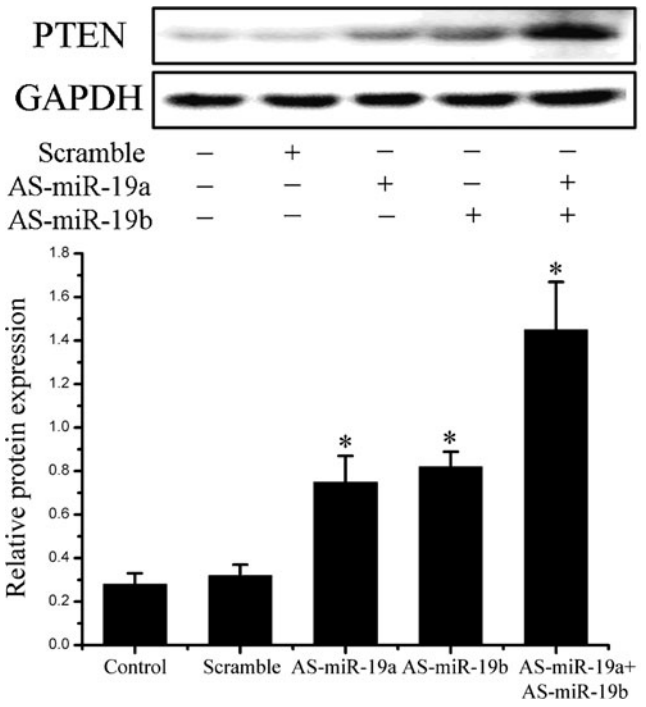

Fig. 6 The PTEN protein expression in LN229 cells detected by Western blot analysis. PTEN protein expression was upregulated in LN229 cells transfected with miR-19a/b AS group compared with the cells treated with scr ODN or control cells. GAPDH served as endogenous normalizer

miR-19a and miR-19b are members of miR-17-92 cluster which consists of seven miRs (miR-17-5p, miR-17-3p, miR-18, miR-19a, miR-20, miR-19b, and miR-92-1). Ota et al. found for the first time that the miR-17-92 cluster which resides in intron3 of c13 orf 25 gene at 13q31.3 is frequently overexpressed in B-cell lymphoma cell lines [9], this evidence suggests that miR-17-92 cluster is involved in tumorigenesis.

Subsequently, miR-17-92 miR cluster is reported to be a potent microRNA-encoding oncogene and demonstrates enhanced expression in lung cancer and myc-rearranged lymphoma [10, 11].

The studies about miR-19 are limited. It has been reported that miR-19 is overexpressed in multidrug resistance(MDR) cell lines of breast cancer and is related to sensitivity of MDR cells to cytotoxic agents [12]. MiR-19 is also highly expressed in T-cell acute lymphoblastic leukemia (T-ALL) and cell lines, Transfectd T-ALL cell lines with miR-19 mimics, CYLD expression is repressed while NF-κB expression increased [13]. Expression level of miR-19b and miR-92a is increased in cerebospinal fluid(CSF) of primary central nervous system lymphoma (PCNSL). Overexpresssion of MiR-21, miR-19, and miR-92a has diagnostic value in distinguishing PCNSL from inflammatory CNS diseases and other neurologic disorders [14]. High expression of miR-19 is observed in retinoblastoma [15]. MiR-19 regulates tissue factor expression in breast cancer cells, inhibition of the miR-19 induces endogenous tissue factor expression in MCF-7 cells, and enhanced expression of miR-19 downregulates tissue factor expression in MDA-MB-231 cells [16].

In this study, we explore the expression of miR-19a/b in glioma cell lines and specimens, miR-19a/b expression is significantly up-regulated in glioma, and positively correlates with the tumor grade as detected by qRT-PCR and in-situ hybridization.

We also identify that PTEN is the target gene of miR-19 by luciferase reporter assay. PTEN is up-regulated when miR-19 is knocked down by miR-19 antisense oligonuclieotide in LN229 cell line. PTEN has also been reported to be the target gene of miR-19 in breast cancer, T-cell lymphoma and B-cell lymphoma [12, 17, 18]. Increased miR-19 expression dampens the expression of the tumor suppressor PTEN, thus activating the Akt–mTOR signaling, so miR-19 has been identified as oncogenic miRNA. The miR-19 is essential to mediate the oncogenic activity of the entire miR-17-92 cluster, the oncogenic activity of miR-19 is at least partially mediated by the PI3K–Akt–mTOR pathway. It is well recognized that AKT/mTOR pathway plays an important oncogenic role in gliomagenesis, and it also involved in modualtion of organism

life spans, this finding suggests that miR-19 might be an important regulator on the cross-roads between aging and cancer [19].

In conclusion, we evaluate the expression level of miR-19a and miR-19b in malignant glioma cell lines and astrocytic glioma tissue samples, and identify both miR19a/b are up-regulated in gliomas. To our knowledge, this study for the first time indicates that miR-19a/b is overexpressed in gliomas and may be associated with gliomagenesis. PTEN has been detected as the target gene of miR-19a/b in LN229 glioma cell line, so one of the possible mechanism is that miR-19a/b exert their effect on gliomagenesis by negative regulation on PTEN. Certainly, it should be further studied on the role and mechanism of miR-19a/b impacting on gliomas.

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Conflict of Interest The authors have not conflict of interest.

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