Original Article

MicroRNA-584 functions as a tumor suppressor and targets PTTG1IP in glioma

Xiang-Peng Wang1, Xing-Li Deng1, Li-Yan Li2

1Department of Neurosurgery, First Affiliated Hospital of Kunming Medical University, Yunnan 650032, China; 2Institute of Neuroscience, Kunming Medical University, Yunnan 650050, China

Received October 17, 2014; Accepted December 1, 2014; Epub December 1, 2014; Published December 15, 2014

Abstract: MicroRNAs (miRNAs) are small noncoding RNA molecules that regulate gene expression at the post-transcriptional level. Compelling evidence shows that there are causative links between miRNAs deregulation and cancer development and progression. In this study, we demonstrated that miR-584 was downregulated in human glioma and could suppress growth of the human glioma cell line U87-MG and U251-MG. Bioinformatics analysis indicated that PTTG1IP was a putative target of miR-584. In a Luciferase reporter system, we confirmed that PTTG1IP was a direct target gene of miR-584. These findings indicate that miR-584 suppresses glioma cell growth by negatively regulating the expression of PTTG1IP, suggesting that miR-584 has a tumor suppressive role in human glioma pathogenesis.

Keywords: Glioma, microRNA, miR-584, PTTG1IP

Introduction

Gliomas are the most common malignant primary brain tumors in adults and exhibit a spectrum of aberrantly aggressive phenotype. A combination of surgery, radiotherapy and chemotherapy is widely used to treat gliomas, particularly malignant gliomas. However, the prognosis of the disease remains poor, with a median survival in the range of 15-17 months [1-4]. Therefore, it is crucial to investigate the mechanism involved in the development and progression of glioma and to find new therapeutic targets.

MicroRNAs (miRNAs) are a class of small (~22 nucleotides) non-coding RNAs that function as negative regulators of gene expression at the post-transcriptional level by binding to complementary sequences in, mainly, the 3’-untranslated regions (3’-UTRs) of specific mRNAs [5-7]. Abnormal expression and the loss of the dynamic balance between oncogenes and tumor suppressor genes typically lead to tumor formation and the development of cancer [8-12]. miRNAs have been shown as important regulators in diverse biological processes of cancer, such as cell proliferation [13], angiogenesis [12], cell differentiation [14], cell apoptosis [15], adhesion, and metastasis [16]. These data emphasize the importance of miRNAs in cancer development and provide new insights into understanding the molecular mechanism of tumorigenesis. In gliomas, various miRNAs such as miR-10b [17], miR-23a [18], miR-92b [19] and miR-17 [20], have been associated with the initiation and progression of glioblastoma and with their invasive nature. In contrast, miR-214 [15], miR-16-1 [21], miR-204 [22] and miR-145 [23] have been implicated as tumor suppressor miRNAs in these tumors. However, the exact role of miR-584 in gliomas has not been revealed yet.

In this study, we focused on the downregulated miR-584 in glial tumors and glioma cell lines, U87-MG and U251-MG. Further investigation revealed that in glioma cell lines miRNA-584 functioned as a tumor suppressor and overexpression of miRNA-584 reduced cell proliferation, decreased cell invasive capacity and increased apoptosis. The PTTG1IP gene predicted by bioinformatics analysis as a target gene of the miR-584, was validated by fluores-
cent reporter assay. These findings suggested that miR-584 could act as a biomarker in glioma and its restoration might be a possible therapeutic approach.

Materials and methods

Clinical specimen and RNA isolation

Tissue specimens and clinical information were obtained as part of an approved study by the Institutional Review Board at the Tianjin Medical University, China. Thirteen human glioma tissues were collected with patient consent at the time of operation, grading of tumors was carried out with WHO criteria (World Health Organization, 2007). The clinical information of patients was shown in Table 1. The matched normal tissue was taken from the distal end of the operative excisions, far from the tumor. Immediately after surgery, samples were snap-frozen and stored in liquid nitrogen. Large and small RNAs were isolated from tissue samples with the mirVana miRNA Isolation Kit (Ambion, USA), according to the manufacturer’s instructions.

Cell culture and transfection

Two human glioma cell line U87-MG and U251-MG were maintained in Dulbecco’s modified Eagle’s medium (Gibco, USA), and supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Cells were incubated at 37°C in a humidified chamber supplemented with 5% CO₂. The transfection was performed using the Lipofectamine 2000 Reagent (Invitrogen, USA) following the manufacturer’s instructions.

All the RNA oligonucleotides were purchased from GenePharme (Shanghai, China).

Cell viability and proliferative capacity assay

To determine cell viability and proliferative capacity, cells were examined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and colony formation assays as described previously. U87-MG and U251-MG cells were seeded in 96-well plates at a density of 5000 cells per well, and then transfected with miR-584 mimics or miR-control on the next day. The MTT assay was used to determine relative cell viability at 0, 12, 24 and 48 h. Ten microliters of MTT (at a final concentration of 0.5 mg/ml) solution was added to 100 µl of culture medium, and incubated for 4 h at 37°C; the absorbance at 570 nm (A570) was then measured using an uQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, USA).

For the colony formation assay, the number of viable cell colonies was determined after 15 days after inoculation of 150 cells/well in triplicate in 12-well plates. The cells were stained with crystal violet. The rate of colony formation was calculated with the equation: colony formation rate = (number of colonies/number of seeded cells) × 100%

Cell apoptosis assay

The apoptotic ratios of cells were determined with the Annexin V-7-ADD apoptosis detection kit (Roche, Switzerland). Briefly, 48 hours after transfection, the cells were collected and washed twice with cold PBS buffer, resuspended in 200 µl of binding buffer, incubated with 20 µl of Annexin-V-R-PE for 20 minutes in an dark ice bath, and then 7-AAD 10 μl before analyzing by flow cytometry. Cells treated with DMSO were used as the negative control.

Western blot analysis

Total cellular extracts were extracted using RIPA buffer. Proteins were separated by 10% SDS denatured polyacrylamide gel, and then transferred onto a nitrocellulose membrane. Membranes were incubated with an antibody against PTTG1IP or an antibody against glycer-aldehyde-3-phosphate dehydrogenase (GAP-
MicroRNA-584 and glioma

Table 2. The primers used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>U6 RT</td>
<td>5’-GTCGTATCCAGTGCAGGGTGCGAATTTGCTGAGATACGACAATATGGAA-3’</td>
</tr>
<tr>
<td>U6 forward</td>
<td>5’-TCGGTGCTGGCTGGCTGGCGAC-3’</td>
</tr>
<tr>
<td>miR-584 mimics</td>
<td>5’-UUAUUGUGUUGCCUGGUGACUGAG-3’</td>
</tr>
<tr>
<td>miR-584 control</td>
<td>5’-AAACGGUUAUAAUAGCCUCGCCG-3’</td>
</tr>
<tr>
<td>GAPDH forward</td>
<td>5’-CTGTAGACATTAGGAGAGCCTG-3’</td>
</tr>
<tr>
<td>GAPDH reverse</td>
<td>5’-CTAGAAGCATTGCGGTGGAC-3’</td>
</tr>
</tbody>
</table>

Figure 1. Depressed expression of miR-584 in glial tumors, glioma cell lines. A. Endogenous level of miR-584 in glioma samples and normal brain tissues by real-time qRTPCR by qRTPCR. B. The relative abundance of miR-584 in glioma cell lines, U87-MG and U251-MG, and the normal glial cell line. The expression of miR-584 is normalized to U6 small nuclear RNA (*P < 0.05).

DH) overnight at 4°C. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody. Protein expression was assessed by enhanced chemiluminescence and exposure to chemiluminescent film. Lab works image acquisition and analysis software was used to quantify band intensities. Antibodies were purchased from Abcam (Cambridge, UK).

Target prediction and Luciferase reporter assays

Based on bioinformatic prediction (TargetScan, RNA22 and microrna.org), PTTG1IP was selected as candidate target of miR-584. The 3’UTR segments of PTTG1IP containing putative binding sites for miR-584 were obtained by PCR and inserted into pmirGLO vector. The wild-type reporter construct pmirGLO/PTTG1IP-3’UTR and the mutant reporter construct pmirGLO/PTTG1IP-3’UTR mut, in which the site of perfect complementarity to miR-584 was mutated using site-directed mutagenesis PCR, were used for miRNA functional analysis. Wild-type and mutant insertions were confirmed by DNA sequencing. All primer information is available in Table 2. For Luciferase reporter experiments, U87-MG cells were co-transfected with the miR-584 mimics in a 48-well plate followed by the pmirGLO/PTTG1IP-3’UTR reporter vector or the pmirGLO/PTTG1IP-3’UTR mut. Firefly luciferase and Renilla luciferase levels were measured at 48 h after transfection. Each experiment was repeated at least three times.

Real-time quantitative PCR

Small RNA (5 µg) was reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega, USA) with the specific primers. The cDNA was used as template to amplify either
Figure 2. Overexpression of miR-584 suppresses cell growth and promotes apoptosis of the glioma cell lines. A. Measurement of miR-584 expression levels by real-time RT-PCR. Small RNA was extracted from U87-MG and U251-MG cells transfected with miR-584 mimics or miR-control, and U6 snRNA served as an endogenous normalize. The relative miR-584 expression level (mean ± SD) is shown (*P < 0.05). B and C. Cell viability was detected through MTT assay. After U87-MG and U251-MG cells were transfected with the miR-584 mimics or miR-control, the MTT assay was used to determine the relative cell growth activity at 0, 12, 24 h and 48 h post-transfection (*P < 0.05). The relative cell growth activity was normalized to the growth activity of U87-MG and U251-MG cells in the control groups. D and E. Cell proliferation ability was evaluated by a colony formation assay. U87-MG and U251-MG cells transfected with miR-584 mimics or miR-control were seeded in 12-well plates. On the 8th day after seeding, the number of colonies was counted (*P < 0.05). F and G. Apoptosis of U87-MG and U251-MG cells following miR-584 mimics transfection was analyzed by flow cytometry. The cells were stained with annexin V-fluorescein isothiocyanate and counterstained with 7-ADD (*P < 0.05).
MicroRNA-584 and glioma

mature miR-584 or an endogenous control U6 snRNA by PCR. The PCR was performed as follows: 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 30 s. The real-time PCR was performed using SYBR Premix Ex Taq (TaKaRa, Japan) on the iQ5 Real-Time PCR Detection system (Bio-Rad). The relative expression of miR-584 was defined as follows: quantity of miR-424/quantity of U6 within the same sample. Briefly, a cDNA library was generated through reverse transcription using M-MLV reverse transcriptase (Promega) with large RNA (5 µg). The cDNA was used to amplify the genes PTTG1IP and the β-actin gene, which served as an endogenous control. The PCR was performed as follows: 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s. Real-time PCR was performed as described above, and the relative gene expression level was defined as follows: quantity of gene/quantity of β-actin within the same sample.

Statistical analysis

Data are expressed as the means ± standard deviation (SD), and $P \leq 0.05$ is considered to be statistically significant using the Students-Newman-Keuls test.

Results

Depressed expression of miR-584 in glial tumors, glioma cell lines

To determine the expression of miR-584 in human glioma tissues and adjacent normal tissues, we used quantitative real time RT-PCR to detect 13 pairs of glioma samples (Figure 1A). Furthermore, miR-584 expression was significantly low in glioma cell line U87-MG and U251-MG by real-time RT-PCR (Figure 1B). It was shown that miR-584 expression level was generally and significantly downregulated in glioma.

Overexpression of miR-584 suppresses cell growth and promotes apoptosis of the glioma cell lines

First, we transfected either miR-584 mimics or an miR-control into U87-MG and U251-MG cells, and detected miR-584 levels by real time RT-PCR. Expression of miR-584 was increased

Figure 3. MiR-584 decreased the Invasion and Migration Abilities of Glioma Cells. A and B. Transwell assays showed that cells transfected with miR-584 mimics presented less invasion ability than those with miR-control (*$P < 0.05$). C and D. Wound healing assays showed that glioma cells transfected with miR-584 mimics may close the wound more slowly when compared with those with miR-control (*$P < 0.05$).
MicroRNA-584 and glioma

Figure 4. MiR-584 targets PTTG1IP and negatively regulates its expression. A. The predicted miR-584 binding site on the PTTG1IP mRNA 3'-UTR and the deletion mutation at the miR-584 "seed region" binding site, on the PTTG1IP mRNA 3'-UTR are shown. B. U87-MG cells were transfected with the wild type or mutated version of the luciferase-PTTG1IP 3'-UTR reporter vector as well as the miR-584 mimics or miR-control. The miR-584 mimics reduced the intensity the luciferase-PTTG1IP 3'-UTR reporter vector, while the mutant luciferase-PTTG1IP 3'-UTR failed to alter the luciferase intensity (\(*P < 0.05\)). C and D. Measurement of PTTG1IP mRNA expression levels by qRT-PCR. RNA was extracted from U87-MG and U251-MG cells transfected with the miR-584 mimics or miR-control. The endogenous expression levels of the β-actin mRNA were used for normalization, and the relative PTTG1IP expression levels are shown (\(*P < 0.05\)). E. Measurement of PTTG1IP expression levels by Western blot analysis. Protein was extracted from U87-MG and U251-MG cells transfected with the miR-584 mimics or miR-control. The endogenous expression levels of the GAPDH protein were used for normalization, and the relative PTTG1IP protein expression levels are shown (\(*P < 0.05\)).

12 fold in the U87-MG cells and 13 fold in U251-MG cells transfected with miR-584 mimics as compared with controls (Figure 2A). Cell viability of glioma cells transfected with miR-584 mimics was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay; miR-584 mimics reduced cell viability at 12, 24, 48 or 72 h after transfection (Figure 2B and 2C). In parallel, we analyzed colony formation and cellular proliferation to assess the effect of miR-584 on the proliferative capacity of glioma cells. The colony formation rate of U87-MG and U251-MG cells transfected with miR-584 mimics, meanwhile, were significantly lower than that of the control group (Figure 2D and 2E). The fluorescence activated cell sorting (FACS) analysis showed that the forced expression of miR-584 lead to glioma cell apoptosis. The percentage of total apoptotic cells (early apoptotic + late apoptotic) significantly increased in response to miR-584 overexpression compared with miR-control overexpression in U87-MG and U251-MG cells (Figure 2F and 2G). These results indicated that miR-584 suppressed the ability to proliferate and promoted apoptosis in glioma cells.

MiR-584 decreased the Invasion and migration abilities of glioma cells

Cell migration and invasion are two essential processes of cancer metastasis. Thus, we first examined the invasive ability of glioma cells transfected with miR-584 and found that cells transfected with miR-584 presented less invasion ability than those with control miRNA (Figure 3A and 3B). There was clear difference
MicroRNA-584 and glioma

in the migration ability between glioma cells transfected with miR-584 and those with miR-control, revealing that glioma cells transfected with miR-584 may close the wound more slowly when compared with those with miR-control (Figure 3C-E). These data demonstrated that miR-584 might inhibit migration and invasion in glioma.

**MIR-584 targets PTTG1IP and negatively regulates its expression**

MicroRNAs regulate a variety of cellular activities through regulation of the expression of target genes. To determine the mechanism of miR-584-mediated cell cycle dysregulation in glioma cells, we next identified target genes that could be responsible for the effect of miR-584. Thus, bioinformatic analyses (TargetScan, RNA22 and miRcorna.org) were used to identify potential target genes of miR-584. To further confirm that miR-584 directly targets PTTG1IP, we performed luciferase reporter assays to examine whether miR-584 interacts directly with its target PTTG1IP. We constructed a series of 3’UTR fragments, including the full-length wild-type PTTG1IP 3’UTR and a binding site mutant (Figure 4A). These fragments were then inserted into the pmirGLO luciferase reporter plasmid. In U87-MG cells, we found that the cotransfection of miR-584 and the wildtype PTTG1IP 3’UTR caused a significant decrease in luciferase units compared with the controls. However, the cotransfection of the mutant PTTG1IP 3’UTR and miR-584 mimics failed to alter the luciferase intensity (Figure 4B). Furthermore, overexpression of miR-584 reduced PTTG1IP mRNA and protein expression in U87-MG and U251-MG cells (Figure 4C-E). Taken together, these results suggest that miR-584 binds directly to the 3’UTR of PTTG1IP, thereby repressing gene expression.

**Quantitative analysis of PTTG1IP expression in glioma tissue**

To determine the expression of PTTG1IP in glioma and adjacent normal tissue, real-time RT-PCR for PTTG1IP was performed on thirteen tissue pairs, each consisting of an glioma and adjacent normal tissue. In general, PTTG1IP expression levels were significantly higher in glioma tissues than in the matched normal tissues (Figure 5). In contrast, miR-584 expression levels were predominantly downregulated in glioma tissue (Figure 1A).

**Discussion**

There are a lot of studies focusing on the relationship between cancer and deregulated miRNA expression. Several miRNAs have been identified as oncogenes and tumor suppressors that are involved in glioma development. For instance, miR-21 [24], and miR-27b [25], have been shown to be expressed in glioma and to increase cell growth and invasion. In contrast, decreased expression of miR-205 [26], miR-34c [27], miR-451 [28] and miR-218 [29] may inhibit glioma cell proliferation and invasion. For miR-584, our findings are consistent with studies on other types of malignancy, such as breast [30], kidney [31] and colon [32]. Fils-Aimé N et al reported that TGF-β silences the expression of miR-584, resulting in enhanced PHACTR1 expression, and further leading to actin rearrangement and breast cancer cell migration [30]. Ueno K et al found that Expression of miR-584 in RCC (A-498 and 769-P) cells was downregulated compared with HK-2 cells. It demonstated that miR-584 is a new tumour suppressor miR in ccRCC (clear cell renal cell carcinoma) and inhibits cell motility through downregulation of ROCK-1 [31].

PTTG Binding Factor (PBF or PTTG1IP) is a little characterised proto-oncogene and is identified through its ability to interact with PTTG1, the human securin [33, 34]. PBF is widely expressed...
in normal human tissues, including normal thyroid [33, 35]. Whilst expression is low in normal breast tissue, immunohistochemical analysis demonstrated that PBF was strongly expressed in epithelial cells of all types and grades of breast tumour assessed [36]. MiR-584 inhibits cancer growth by blocking the expression and activity of PTTG1IP. The loss of miR-584 leads to the upregulation of PTTG1IP, consequently resulting in malignant transformation.

The current study identified that miR-584 were significantly down-regulated in glioma samples compared with normal samples. To further realize the role of miR-584 in glioma, we enhanced miR-584 expression in U87-MG and U251-MG cells using the miR-424 mimics, and found that overexpressed miR-584 suppressed the growth of glioma cells and induced cell apoptosis. We used the Dual-Luciferase reporter assay to confirm the target gene of miR-584 were PTTG1IP. In this study, we confirmed that miR-584-PTTG1IP signalling pathway represents a functional mechanism by which miR-584 suppresses glioma. However, miRNAs usually work in the regulation of multiple targets and we could not tell that there is no other signalling pathway working by miR-584 in glioma.

In summary, we demonstrated that miR-584 expression was markedly decreased in human glioma cell lines, and for the first time, we described the roles of miR-584 in cellular proliferation, migration, and invasion abilities in glioma cells. In addition, this study suggested that PTTG1IP is one of putative target genes of miR-584.

Acknowledgements

This work was support by Fund of scientific research in department of education of Yunnan (No. 2014Y174).

Disclosure of conflict of interest

None.

Address correspondence to: Xing-Li Deng, Department of Neurosurgery, First Affiliated Hospital of Kunming Medical University, 295 Xichang Road, Yunnan 650032, China. Tel: +86-871-65324888-2201; Fax: +86-871-65336015; E-mail: dengxin-gli1015@163.com

References

MicroRNA-584 and glioma


MicroRNA-584 and glioma
