Original Article
MiR-182 regulates proliferation and apoptosis by targeting FBW7 in glioma cells

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Abstract: Background: Glioblastoma (GBM) is the most malignant brain tumor and exhibits aggressive phenotype. Studies have shown that microRNA (miR) acts as regulator in GBM, while the effect of miR-182 on GBM is still unclear. The aim of this study was to explore the role of miR-182 in GBM. Methods: miR-182-KO cells were generated using Crispr-cas 9 system. MTS and clonogenic assays were used to determine cell proliferation. Flow cytometry was performed for cell cycle and apoptosis analysis. Western blot and qRT-PCR assays were used to assess the alterations of signaling pathway. Nude mouse xenograft model was used to evaluate the role of miR-182 in vivo. Results: We found that CRISPR-mediated miR-182 knockout enhanced cell cycle arrest and inhibited proliferation. We further identified the F-box and WD repeat domain containing 7 (FBW7) as a target gene of miR-182 in GBM. Furthermore, inhibition of miR-182 induced VEGF-A downregulation. Inhibition of miR-182 promotes apoptosis via FBW-7-mediated Mcl-1 degradation in GBM. In addition, deficiency of miR-182 reduced tumor growth. Finally, our data showed that miR-182 level was inversely correlated with FBW7 in GBM patients. Conclusions: In conclusion, our study suggests that miR-182 is a potential therapeutic target for GBM treatment.

Keywords: miR-182, FBW7, Mcl-1, VEGF-A, GBM

Introduction
Gliomas are the most common primary brain tumors in adult and the survival rate for patients with glioma are still very low [1]. Glioblastoma (GBM) is the most malignant of the glial tumors [2]. Therefore, investigating the underlying molecular mechanism associated with GBM progression has become extremely important for developing novel treatment strategies for GBM [2]. Number of studies have reported the alteration of microRNAs in GBM cells, suggesting that microRNAs can serve as tumor suppressors [3-5].

MiRNAs bind to the 3’-untranslated region (3’-UTR) of target genes to decrease protein expression level by either translation repression or mRNA cleavage [6]. Recent studies demonstrated that miRNAs involved in diverse biological processes, including cell proliferation, apoptosis, tissues homeostasis, organ development and human diseases [6-8]. In cancers, miRNAs may act as tumor suppressors via inhibiting the expression level of cellular oncogenes or tumor promoters by targeting tumor suppressor genes [9]. MiR-182 is one member of the miR-96-182-183 cluster [10, 11]. This miRNA cluster is located on the chromosome 7q32.2 and is highly expressed in the retina during embryo development [10]. The functional importance of miR-182 in the regulation of retina formation was further confirmed in animal study [12]. The potential oncogenic activity of miR182 was firstly shown in human melanoma [13]. MiR-182 was found to be up-regulated in melanoma and repressed tumor suppressors Fork head box O3 (FoxO3) and microphthalmia-associated transcription factor (MITF) to protect cancer cell from apoptosis and to promote cell invasion [13]. Subsequent studies confirmed that miR-182 was overexpressed in many cancers and revealed a number of downstream targets affected by miR-182.
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Overexpression of miR-182 was shown in breast cancer cells and this miRNA affects cell survival, migration and the DNA damage response by inhibiting FoxO1, MIM (Missing in metastasis), MTS1 (Metastasis suppressor 1), and BRCA1 (Breast cancer 1) [15-17].

F-box and WD domain containing 7 (FBW7) is subunit of the SKP1-cullin-F-box (SCF) ubiquitin protein ligases which mediate the degradation of a number of oncoproteins including cyclin E, Notch, c-Myc, Mcl-1 etc [18]. Because most of the substrates of FBW7 are oncoproteins, FBW7 has been suggested to serve as a tumor suppressor [19]. Indeed, down-regulation, mutation or deletion of this gene was frequently found in different cancers [19]. Inactivation of FBW7 by mutation or deletion occurs only in a small portion of cancer patients and many cancers like ovarian, lung and colon cancer rarely exhibit FBW7 mutations [19]. Recent studies suggested that down-regulation of FBW7 due to dysregulation of upstream signaling molecules and transcription factors or up-regulation of miRNAs seems to be a major cause of the reduction of FBW7 protein in tumor tissues [20].

The present study aimed to explore the role of miR-182 in the cell proliferation and invasion of GBM. We identified FBW7 as the target of miR-182 in GBM. In addition, we revealed that hypoxia-induced vascular endothelial growth factor-A (VEGF-A) production was significantly reduced in miR-182 KO cells. Collectively, our findings indicate that miR-182 is an oncogenic miRNA in GBM and promotes tumorigenesis by increasing proliferation, invasion and angiogenesis.

Materials and methods

Cell culture

U87, U251, HEK-293T cells were got from ATCC. The cells were cultured in DMEM Medium (Gibco) with 10% FBS (Gibco), 0.5% penicillin streptomycin, and 1% glutamine at 37°C containing 5% CO₂. The miR-182 inhibitor, mimics, negative controls were got from Gene Pharma.

DNA constructs

The lentiviral packaging plasmid pCMV-ΔR8.2 (Addgene: 12263) and pCAG-VSVG were obtained from Addgene (Addgene: 35616). The lentiviral vector pLentiCRISPR, which expresses Cas 9 and gRNA, was obtained from Addgene as well. The gRNA that targets miR-182 genomic sequence was subcloned into the lentiCRISPR vector according to the instruction.

Generation of miR-182 GKO clones

HEK-293T cells were transfected with 1 μg pLentiCRISPR containing miR-182 gRNA, 1 μg pCMV-ΔR8.2, and 0.5 μg pCAG-VSVG to package virus. U87 cells were then infected with 100 μl lentivirus for three days. After limiting dilution, single clone was selected out with 2 μg/ml puromycin for three weeks. To determine the mutation, genomic DNA was purified and sequenced.

Transfection

Transfection of cells was conducted using the Geneln reagent (Amsbio) according to the instructions. FBW7 siRNA (Santa Cruz) was delivered by lipofectamine 2000 according to instructions from manufacturer.

Clonogenic and proliferation assays

Indicated cells were seeded on 6-well plates and the colonies grown on the plates after two week were stained with Giemsa solution, and quantified by using the Image ProPlus software. For MTS assay. The indicated cells were seeded in 96 well plates (1×10⁴ cells per well). MTS assay was performed according to the instructions of MTS assay kit (Promega). Luminescence was recorded by 1420 Multi-label Counter (Perkin Elmer). Each assay was repeated three times.

Flow cytometry

The stable cell lines were collected by cell dissociation buffer and fixed using 70% ethanol in -20°C overnight. Then the cells were stained by propidium iodide (25 μg) in the presence of RNase (100 mg/ml) for 30 mins at 37°C. The cells in various phases of cell cycle were detected by flow cytometry.

Bioinformatics prediction

We used different public databases including miRWalk, TargetScan and Pictar to identify the crucial tumor suppressor genes that are target-
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by miR-182 and confirmed the expression level of these genes in the stable cells including U87 and U251.

Real time PCR (RT-PCR)

Total RNA was extracted using the TRIzol RNA Kit (Invitrogen, CA) according to the protocol of manufacturer. Briefly, cDNA was generated using reverse transcriptase (Invitrogen). PCR was performed using SsoFast™ Probes Supermix (Bio-Rad) with specific primer, and a cycling procedure (38 cycles) on a CFX96-TM RT-PCR System (Bio-Rad). Relative levels of gene mRNA were evaluated by TaqMan Gene Expression RT-PCR assays. The threshold cycle (Ct) was shown as the result. The quantification of transcripts was calculated by Ct (ΔΔCt). Control experiment was performed to make sure that the RNA was uncontaminated. β-actin was applied to act as internal control for mRNA quantification. The primer sequences were as follows: FBW7 F: 5’-GCGCGGAATGGTGAACTT-3’, FBW7 R: 5’-CGTTCTGGTCTCCAGGCCT-3’; GLUT1 F: 5’-CATCATCTTCATCCCGGC-3’, GLUT1 R: 5’-CTCCTCGTTGCGGTTGGA-3’; HK2 F: 5’-CGGCGGTGCTACAATAGG-3’, HK2 R: 5’-CTCGGGATCATTGTGAGGG-3’; LDHA F: 5’-AGCCCGATTCCGT-TACCT-3’, LDHA R: 5’-CACCAGCAACATTCATTC-3’; VEGF F: 5’-CATTACGCAGTCTGGATCTT-3’, β-actin F: 5’-TGACAGCAGTGTTGGGA-3’, β-actin R: 5’-CAAATCGTCGCGCCACAT-3’.

Western blot (WB)

WB was conducted as previous description [18], with antibodies including anti-Mcl-1, anti-FBW7 (Abcam), anti-β-actin, anti-HIF1α, anti-Notch (santa cruz), anti-cyclin E (Cell signaling).

Apoptosis assay

Cells were stained with Hoechst 33258 (Invitrogen), then performed apoptosis analysis by nuclear staining. Annexin V and propidium iodide (PI) staining was conducted by annexin-Alexa 488 (Invitrogen) and PI followed by flow cytometry.

3’-UTR reporter and dual-luciferase assay

Cells were transfected with 3’-UTR reporter of FBW7. Mutations were introduced into the two predicted binding sites of miR-182 using QuikChange Lightning kit to mutate the two targeting sequences on 5’-TTGCCCAACATTTGCCAA-3’ to 5’-TTGCCGGCCAGGGCCAA-3’. After 48 hr, the cells were detached by 0.05% trypsin-EDTA and collected. Then cells were dissolved in Passive Lysis Buffer (Promega) and 20 μl of cell lysate was transferred into luminometer plate. Luciferase activity and Renilla luciferase activity (as an internal control) were measured by a Luminometer. FBW7 3’-UTR reporter activity was normalized by Renilla luciferase activity and the results from three independent assays in different cell lines were compared.

In vivo animal study

In this study, the NOD/SCID mice were 6~8 week-old. All procedures regarding animal experiments were approved by Capital Medical University of Animal care and the Use Committee. The subcutaneous tumor growth assay was performed under specific pathogen-free conditions. Briefly, 1×10⁶ WT and miR-182-KO U87 cells were suspended in 100 μl of serum-free medium and subcutaneously injected into the back of nude mice. The tumor volumes were recorded every 5 days and calculated using the formula 1/2 × Length × Width². Mice were euthanized after 30 days injection. The xenografts were harvested for the detection of miR-182, FBW7, and Mcl-1 expression by Western blot.
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Figure 2. Knockout of miR-182 increased cell cycle arrest, inhibited proliferation and clonogenicity of GBM cells. A. Knockout of miR-182 increased the population of cells at G2/M phase in miR-182-KO cells. B. Proliferation was reduced in miR-182-KO cells. C. miR-182-KO cells exhibited lower clonogenicity than the parental U87 cells. D. Overexpression of miR-182 decreased the population of cells at G2/M phase in U251 cells. E. Overexpression of miR-182 increased the growth of U251 cells. F. Overexpression of miR-182 in U251 cells showed increased clonogenicity than that of parental cells. The results are the mean ± SD of triplicate determinations; statistical significance of results was evaluated by student’s t-test. *P<0.05, **P<0.01.
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Statistical analysis

Statistical analysis was conducted using GraphPad Prism software. P values were calculated by student’s t-test and were considered statistically significant if P<0.05. Data were presented as mean ± SD in the figures unless otherwise indicated.

Results

Generation of miR-182 knockout cells in GBM

To explore the role of miR-182 in GBM, we generated miR-182-KO cells using Crispr-cas9 system. The PAM sequence are within the miR-182 sequence (Figure 1A). The gRNA was sub-cloned into lenti-CRISPR vector that drives the expression of both CAS9 and gRNA. Next, we packaged lentivirus to mutate endogenous miR-182 gene. U87 cells were infected and selected. After three weeks, a number of clones were obtained (Figure 1B). We subsequently isolated RNA from the derived clones and determined the abundance of mature miR-182 using real-time RT-PCR. As shown in Figure 1C, miR-182 levels were reduced in miR-182-KO cells.

Knockout miR-182 induced cell cycle arrest, inhibited proliferation and clonogenicity of GBM U251 cells

We next explored the effect of miR-182 on cell cycle and cell

Figure 3. FBW7 was targeted by miR-182. A. FBW7 protein levels were detected in WT and miR-182-KO U87 cells by Western blot. B. FBW7 mRNA and protein expression levels were detected in WT and miR-182-OE U251 cells by Western blot and RT-PCR. C. The miR-182 targeting sequence in the FBW7 3’-UTR was shown and mutagenesis was performed. WT or mutated FBW7 3’-UTR reporters were transfected into WT and miR-182-KO cells and the reporter activities were compared. D. The FBW7 3’UTR reporter vector was transfected into WT or miR-182-OE U251 cells and the reporter activities were compared. E. The protein levels of Mcl-1, cyclin E and c-myc were detected by Western blot. The results are the mean ± SD of triplicate determinations; statistical significance of results was evaluated by student’s t-test. **P<0.01.
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Flow cytometry analysis demonstrated that significant increase of cells at G2/M phase were found in miR-182-KO cells, indicating that deficiency of miR-182 induced cell cycle arrest (Figure 2A). In consistent with this data, proliferation of miR-182-KO cells was lower than that of WT cells (Figure 2B). Additionally, the clonogenicity of miR-182-KO cells was also decreased (Figure 2C). On the contrary, overexpression of miR-182 in U251 cells increased the number of cells at S and G2/M phase that was accompanied with enhanced cell proliferation and clonogenicity (Figure 2D-F). Our results suggested that miR-182 acted as an oncogenic miRNA and simultaneously enhanced growth and invasiveness of GBM.

FBW7 was targeted by miR-182

Since miR-182 was approved as an oncogenic miRNA in GBM, we used bioinformatics analysis to search the tumor suppressor genes which could be targeted by this miRNA and identified FBW7, a component of the E3 ubiquitin-protein ligase that mediates the degradation of various onco-proteins, as a potential target. We assessed the expression level of FBW7 in different GBM. The level of FBW7 protein was increased in miRNA-182-KO cells (Figure 3A). Overexpression of miR-182 in U251 cells reduced FBW7 protein level but not mRNA level. (Figure 3B). We further verified miR-182 could target FBW7 directly by using FBW7 3′UTR reporter assay. Moreover, the FBW7 3′UTR reporter activity of miR-182-KO cells was upregulated by 50% when compared to the control cells and mutation of the two miR182-binding sites abolished this activity (Figure 3C). We next transfected FBW7 3′UTR reporter into miR-182 overexpressing U251 cells and found that the reporter activity was repressed (Figure 3D). Our results also showed that overexpres-
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miR-182 promotes HIF1α expression in glioma cells

Next, expression level of HIF1α in U251 cells was determined. As expected, treated with miR-182 mimics, HIF1α protein level was increased,
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Even in normoxic conditions, when basal level of HIF1α was low (Figure 4A). However, mRNA level of HIF1α was not affected (Figure 4B), indicating that miR-182 regulated HIF1α at post-transcription level. Expression levels of glycolytic enzymes, including Glut1, HK2 and LDHA, were increased by miR-182 (Figure 4C). It is well-known that HIF-1α induces vascular endothelial growth factor (VEGF) expression to promote tumor angiogenesis. Interestingly, VEGF was also up-regulated by miR-182 (Figure 4C). Moreover, expression levels of target genes of HIF1α were inhibited by miR-182 inhibitor under hypoxic conditions (Figure 4D and 4E). Together, these data indicate that miR-182 promotes HIF1α expression in glioma cells.

Inhibition of miR-182 promotes apoptosis via FBW7-mediated Mcl-1 degradation in GBM

We next examined whether miR-182 inhibitor associated with induction of apoptosis. In apoptotic assays, miR-182 inhibitor treatment induced apoptosis of U87 cells (Figure 5A). miR-182 inhibitor increased the number of Annexin V positive U87 cells (Figure 5B). We detected cleaved caspase 3 and 9 in miR-182 inhibitor treated U87 cells and found that miR-182 inhibitor activates caspase 3 and 9 in U87 cells (Figure 5C). These data indicated that miR-182 inhibitor induced caspase-dependent apoptosis in glioma cells. Next, we detected whether FBW7/Mcl-1 involved in miR-182 inhibition induced apoptosis. As shown in Figure 5D and 5E, knockdown of FBW7 or overexpression of Mcl-1 blocked miR-182 inhibitor induced apoptosis in U87 cells. The above study demonstrated miR-182 inhibition induced FBW7/Mcl-1-dependent apoptosis in glioma cells.

Deficiency of miR-182 reduces tumor growth

To evaluate the potential role of miR-182 in vivo, nude mice were subcutaneously inoculated with WT or miRNA-182-KO U87 cells. The tumor volume of the miR-182-KO group was significantly smaller than that of the control group (Figure 6A and 6B). Consistently, protein levels of FBW7 were also up-regulated in miR-182-KO tumors, while expression of Mcl-1 was decreased (Figure 6C).

Discussion

In this study, we generated miR-182 knockout in GBM cells using Crispr-cas9 system. We showed that knockout miR-182 significantly reduced the cell proliferation and clonogenicity of GBM. These data suggest that miR-182 serves as an oncogenic miRNA and may affect multiple functions in GBM cells. We also identified FBW7 as a target of miR-182 in GBM. FBW7 is a crucial component of the SCF E3 ubiquitin protein ligase that mediates the degradation of a number of oncoproteins. We found that deficiency of miR-182 increased FBW7 expression which leads to reduction of Mcl-1, cyclin E and Notch protein levels.

Another important finding of this study is the less sensitivity of miR-182-KO cells to hypoxia. Hypoxia in cancer cells up-regulates the expression of HIF-1α to adapt this hypoxia stress and subsequently HIF-1α induces the production of angiogenic factors like VEGF-A to trigger angiogenesis to provide oxygen and nutrient and to sustain tumor growth [21]. HIF-1α activity is regulated by prolyl hydroxylase enzymes (PHD) [21, 22]. In the presence of oxygen, PHD enzymes hydroxylate HIF1α and facilitates the binding of HIF-1α to the von Hippel-Lindau (VHL) E3 that ubiquitinates and degrades HIF1α [23, 24]. Under oxygen deprivation, PHD enzymes decrease HIF-α hydroxylation and results in HIF-α accumulation and nuclear translocation to activate gene transcription and angio-

Figure 6. Knockout miR-182 reduces tumor growth in vivo. A. Nude mice were injected s.c. with 4×10⁶ WT or miR-182-KO U87 cells. Three weeks later, tumor volume was calculated. B. Penetrative tumors at the end of the experiment. C. The levels of FBW7 and Mcl-1 in tumors were determined by Western blot. N=5 mice per group. Statistical significance of results was evaluated by student’s t-test. *P<0.05.
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Among the angiogenic factors investigated, VEGF-A was up-regulated by miR-182 in hypoxia condition. We also demonstrated that the dramatic increase of HIF-α in miR-182-overexpressing cancer cells produced a large amount of VEGF-A in vitro. Results of our study support the notion that miR-182 controls the HIF-α/VEGF-A axis to enhance tumor angiogenesis.

In conclusion, results of our study imply a positive feedback loop may exist in GBM cells to amplify miR-182 and HIF-α signaling in the cells. Collectively, we conclude that miR-182 serves as an oncogenic miRNA in glioma and may target multiple genes to promote glioma tumorigenesis.

Disclosure of conflict of interest

None.

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