Abstract. A prospective method of treatment for cancer is to inhibit oncogene signaling pathways with microRNA (miRNA or miR). In the present study, whether the expression of STAT2, AdipoR1/AMPK/SIRT1 pathway of glioma is regulated by miR-3908 was explored. To confirm whether the predicted miR-3908 is matched with STAT2 and AdipoR1, 3'UTR luciferase activity of STAT2 and AdipoR1 was assessed. In the presence of the mimics or inhibitors of miR-3908, cell function of glioma cells, such as proliferation, growth, migration, invasion and apoptosis were analyzed. The expression of AdipoR1 and its downstream AMPK/SIRT1 pathway proteins or STAT2 were examined. Luciferase reporter analysis showed that miR-3908 directly target STAT2 and AdipoR1. miR-3908 suppressed expression of STAT2 and AdipoR1 and downregulated AdipoR1 pathway genes, including AMPK, p-AMPK and SIRT1. miR-3908 inhibited tumorigenicity, migration, growth and invasion in glioma cell lines U251 and U87 as well as increased apoptosis of these cells. The pathways related to tumorigenicity and tumor progression, STAT2 and AdipoR1/AMPK/SIRT1 could be restrained by miR-3908. In conclusion, restoration of miR-3908 expression induced suppression of cancer progression and glioblastoma tumorigenicity. The present study discovered novel tumorigenesis associated with miR-3908, which may represent a new target in treatment for glioblastoma.

Introduction

The primary malignant tumors in CNS (central nervous system) contain 80% of glioma (1). Based on the diagnostic criteria of WHO (World Health Organization) in 2007, four grades are classified: AGI (pilocytic astrocytomas) or grade I, AGII (diffuse astrocytomas) or grade II, AGIII (anaplastic astrocytomas) or grade III and GBM (glioblastomas) or AGIV (grade IV) (2).

Because invasive malignant tumor cells surround normal brain tissue and GBM is diffusely infiltrating, it could not be cured by resection (2-5). Moreover, the prognosis of GBM after treatment with conventional therapeutic methods is not satisfactory (5,6), the 5-year survival rate is lower than 5%, especially in elderly patients (7). It is a challenge to identify the potential targets for treatment on GBM. The mechanistic insight associated with establishment and progression of tumor was provided according to novel overexpressed or mutated molecules, which might be the new research aims to explore anticancer drugs. In addition, there are specific mutations in cancer cells providing both mechanistic insight associated with tumor establishment and progression, as well as potential targets on the development of new anticancer drugs (8).

The miRNAs (MicroRNAs) are a group of tiny molecules and non-coding RNA, 22-25 nucleotides in length that function on regulation of gene expression at post-transcriptional level. MicroRNAs (miRNAs) control gene expression by pairing with incompletely matching aim sites of the 3' untranslated regions (UTRs) of mRNA, and cause translational repression and/or mRNA destabilization, thereby downregulating the expression of the targeted gene (9). Growing literature indicates the vital function of miRNAs on expression regulation at post-transcriptional level. Moreover, the regulated expression
of genes involve in numerous biological processes, especially for different pathogenic disorders, including variety of tumors, especially in GBM (10).

STAT2 belongs to the protein family of STAT (11,12), which play various roles of growth factors and cytokines. The transcription activator could be phosphorylated by kinases related to receptor, and then translocated to nucleus of cells based on heterodimers or homodimers (12).

Based on epidemiologic studies, it is well known that the development of multiple cancers or advanced progression of disease was inversely related to the expression levels of adiponectin (13). Two adiponectin single nucleotide polymorphisms have been shown to increase prostate, colon and breast cancer risk (14-16). Adiponectin deficiency through the use of knockout mice has shown accelerated hepatic tumor formation (17) and increased colon formation (18), yet, it delayed tumor growth in a mammary MMTV-PyV mT model due to decreased vascularization and increased apoptosis in early stages of disease (19,20). Tumor promoting effects are likely secondary to initiation, but no clear studies have implicated adiponectin as an initiator of cancer development.

The adiponectin receptors have been detected in gastric, colon, prostate, breast, pancreatic and many other cancers (21-25). Adiponectin receptor detection in gastric cancers was associated with longer overall survival (26). Two single nucleotide polymorphisms of adiponectin receptor 1 associates with prostate and breast cancer risk (15,16). Six genetic associations in the AdipoR1 and AdipoR2 genes have been detected in diabetic patients (27). Deletion of the AdipoR1, but not AdipoR2, resulted in promotion of epithelial cell proliferation and increased number of aberrant crypt foci in a murine model (18). Future studies addressing the development.

miRNA transfection. The manufacturer's protocol was followed, and Lipofectamine 3000 was performed for transient transfection of miRNA inhibitors or precursors. The mimics of hsa-miR-3908 (HMI1345) (Sigma-Aldrich), a negative control (miR-NC; AM17110) and inhibitors of hsa-miR-3908 (HLTUD1345) (Sigma-Aldrich) were used for the experiments.

Materials and methods

Cells. U251 and U87 of Homo sapiens glioma cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). These cells were cultured with Dulbecco's modified Eagle's medium (DMEM) contained fetal bovine serum (FBS) and PSN in a humidified environment of 5% CO₂.

Agents. TaqMan miRNA assay, Human miRNA precursors, inhibitors of miRNA, Lipofectamine 2000 and mirVana™miRNA isolation kit were all obtained from Invitrogen (Carlsbad, CA, USA). The mitochondrial MTS and apoptosis assay was performed with CellTiter 96® AQueous One Solution Cell Proliferation assay and Caspase-GloH® 3/7 assay (Promega, Madison, WI, USA), respectively. The constructs of luciferase reporter gene contained 3’UTR of STAT2 and AdipoR1 was purchased from GeneCopoeia (Rockville, MD, USA).

QRT-PCR. following the manufacturer's instructions, total RNA of cells was extracted with TRIzol reagent (Invitrogen), and then reverse transcription was performed. The mirVana™ miRNA isolation kit (Life Technologies) was used to determine intrinsic expression of miR-3908 from cells with TaqMan miRNA assay (normalized with RNU6B values) based on its manufacturer's instructions.

Western blotting. Cells were lysed, and then total proteins were extracted with RIPA lysis buffer. Total proteins were analyzed with electrophoresis method using SDS-PAGE (sodium dodecyl sulfate-polyacrylamide) gel, and then transferred to a PVDF (polyvinylidene fluoride) membrane with 0.45 µm pore size (Roche, Branchburg, NJ, USA). The membranes were blocked with 5% skim milk at room temperature and then washed three times with TBST (28). The membranes were probed with primary antibodies: STAT2 (1:2,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA); AdipoR1, AMPK, p-AMPK and SIRT1 (1:3,000 dilution; Cell Signaling Technology, Danvers, MA, USA); or β-actin (1:3000 dilution; Cell Signaling Technology), at 4°C, overnight. Then, they were incubated at room temperature with appropriate secondary antibodies (1:5,000 dilutions; Santa Cruz Biotechnology).

Analysis of proliferation and clonogenicity in glioma cells. The ability of cell proliferation was detected with MTS assay. For clonogenicity assay, cells colonies were stained with crystal violet and fixed with formalin. The different amounts of developed colonies were evaluated.

Cell migration assay. The ability of mitosis in glioma cells were measured with wound scratch assay. Cells were scratched, and then the movement of cells was observed and measured at 48 h. The migration cells were counted and the total number was quantified.

Cell invasion assay. For invasion assay, Matrigel invasion chambers with 4 µm pores were used. Cells were seeded in the upper chambers (coated in Matrigel) at 2x10⁵ concentration in serum-free medium. The chemo-attractant was added to the
culture media, and then incubated for 48 h at 37˚C. In the top
Transwell, non-invaded cells were removed with a cotton swab.
After fixing with formalin, those translocated successfully
were stained with crystal violet, and then numbered under a
microscope.

**Apoptosis assays.** According to protocol (29), the staining
with Annexin V was implemented to determine cell apoptosis.
Cells (3x10^5) were cultured for 48 h, subsequently washed
with PBS, and then incubated with Annexin V solution. Cells
were washed again and fixed with 1% paraformaldehyde.
7-AAD was used for dual staining of cells. The total number
of 7-AAD+/Annexin-V double-positive cells was tested with a
flow cytometer (29).

**Statistical analysis.** Data are displayed as mean ± SD. The
significant differences between data were estimated with
two-tailed Student's t-test or one-way ANOVA analysis. For
comparison of quantitative data, t-tests or ANOVA (analysis
of variance) were conducted between groups. Medians were
compared between groups through Kruskal-Wallis ANOVA.
SPSS software (version 18.0; SPSS, Inc., Chicago, IL, USA)
was used to perform the analyses. P<0.05 was considered
statistically significant.

**Results**

**Expression of STAT2 and AdipoR1 was suppressed by
miR-3908 in glioma cells.** To study if microRNA modulates STAT2 and AdipoR1, or downstream pathway of
AdipoR1 in glioma cells, the online software DIANA Tools (microT v4.0) was used to predict miRNAs that target AdipoR1. Of these miRNAs, miR-3908 potentially targeted mRNAs of AdipoR1 3'UTRs. (Fig. 1). Further experiments

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<table>
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<th>Predicted consequential pairing of target region (top) and miRNA (bottom)</th>
<th>Site type</th>
<th>Context++ score</th>
<th>Context++ score percentile</th>
<th>Weighted context++ score</th>
<th>Conserved branch length</th>
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Figure 1. miR-3908 potentially targeted mRNAs of AdipoR1 3'UTRs. To study if microRNA modulates STAT2 and AdipoR1, or downstream pathway of AdipoR1 in glioma cells, the online software DIANA Tools (microT v4.0) was used to predict miRNAs that target AdipoR1. Of these miRNAs, miR-3908 potentially targeted mRNAs of AdipoR1 3'UTRs.

Figure 2. STAT2 and AdipoR1 mRNAs are direct targets of miR-3908 in glioma cell lines U251 and U87. To study if microRNA modulates STAT2 and AdipoR1, or downstream pathway of AdipoR1, firstly we used DIANA microT v4.0 online software (http://diana.cslab.ece.ntua.gr/) to predict if one or more miRNAs target STAT2 and AdipoR1, the key factor that regulates homeostasis and fatty acid, cholesterol and lipid biosynthesis. Among the miRNAs, miR-3908 was retrieved because miR-3908 potentially co-targeted the 3'UTRs of STAT2 and AdipoR1 mRNAs. To further prove whether miR-3908 directly binds with the 3'UTRs of STAT2 and AdipoR1, we carried out a 3'UTR luciferase reporter assay in miR-3908 transfected glioma cell line U251 and U87, respectively. (A) The relative 3'UTR luciferase activities of STAT2 and AdipoR1 significantly decreased in miR-3908 transfected glioma cell line U251 compared to control (P<0.01). (B) The relative 3'UTR luciferase activities of STAT2 and AdipoR1 significantly decreased in miR-3908 transfected glioma cell line U87 compared to control (P<0.01).
were carried out with luciferase reporter gene assay to identify whether 3'UTRs of STAT2 and AdipoR1 was bonded straight with miR-3908. The results showed that the relative luciferase activities of STAT2 and AdipoR1 3'UTR were obviously downregulated in glioma cell line U251 and U87 transfected with miR-3908 (Fig. 2A and B), respectively. However, it was identified that miR-3908 could not directly bond with AMPK and SIRT1 3'UTRs (Fig. 2C and D). In glioma cell line U251 and U87, which were only cultured after 48 h, the expression levels of endogenous miR-3908 in these cell lines were detected and are shown in Fig. 2E. The results confirmed that mRNAs of STAT2 and AdipoR1 are direct goals of miR-3908.

To verify if miR-3908 affects the mRNAs and protein expression of STAT2, AdipoR1, AMPK and SIRT1 in glioma cells, we accomplished relative quantification analyses with qRT-PCR or western blot analysis, respectively (Fig. 3). The results indicated that miR-3908 inhibited the mRNA expression of STAT2 or AdipoR1 in U251 or U87 glioma cell lines, but not AMPK and SIRT1 (Fig. 3A). Moreover, the inhibitors of miR-3908 promoted mRNA expression of STAT2 or AdipoR1 in U251 or U87 glioma cell lines, but not AMPK and SIRT1 (Fig. 3C). Altogether, our results demonstrated that miR-3908 directly regulates the protein expression of STAT2 and AdipoR1 by interacting with its 3'UTR.

miR-3908 suppresses cell growth and clonogenicity in glioma cells. For evaluating the possible biotic outcomes induced by miR-3908, we overexpressed or downregulated miR-3908, and then conducted several functional experiments associated with cancer progression and tumorigenicity in U251 and U87 cells. Compared with miR-NC cells, proliferation of U251 (Fig. 4A) and U87 (Fig. 4B) cells were inhibited when they were transfected with miR-3908, respectively. Moreover, the inhibitors of miR-3908 accentuated the process of these glioma cells (Fig. 4).

Furthermore, the colony formation assay revealed that miR-3908 exhibited significantly decreased ability for colony formation compared with the control group in U251 (Fig. 5A and C) and U87 cell lines (Fig. 5B and C) (P<0.01). But,
miR-3908 downregulation facilitated significantly the ability of colony formation in both cell lines (Fig. 5). These results suggested that miR-3908 also lowered clonogenicity of U251 and U87 cells (Fig. 5).

**miR-3908 suppresses migration and invasion of glioma cells.** It was demonstrated that the migration ability was obviously inhibited by miR-3908 compared to control in both cell lines U251 (Fig. 6A and C) and U87 (Fig. 6B and C) (P<0.01). Moreover, downregulation of miR-3908 promoted significantly the ability of migration in both cell lines (Fig. 6). Furthermore, the present study suggested that miR-3908 also inhibited the invasion ability of U251 (Fig. 7A and C) and U87 cells (Fig. 7B and C), respectively. Besides, the inhibitors of miR-3908 enhanced obviously the invasion of the two cell lines (Fig. 7). The capability of invading surrounding tissues and migrating efficiently is a sign of progressive and metastatic cells.

**Apoptosis of glioma cells.** To verify whether apoptosis of glioma cells could be induced by miR-3908, dual-staining measurement with 7-AAD and Annexin V-FITC was conducted, as well as microplate assays for caspase activity. Our results showed the increased fractions of apoptotic glioma cells induced by miR-3908 (P<0.01) in U251 (Fig. 8A) and U87
miR-3908 suppresses cancer progression and tumorigenicity of glioma through STAT2 and AdipoR1/AMPK/SIRT1 pathway. The expression of STAT2, AdipoR1, AMPK, p-AMPK and SIRT1 proteins was detected by western blot analysis. Upregulation of miR-3908 in gliomacell lines U251 (Fig. 9A) and U87 (Fig. 9B) significantly inhibited the expression of STAT2, AdipoR1, AMPK, p-AMPK and SIRT1. Furthermore, treatment with inhibitors of miR-3908 in glioma cells significantly increased the expression of STAT2, AdipoR1, AMPK, p-AMPK and SIRT1 (Fig. 8). It showed that miR-3908 suppressed tumorigenicity and cancer progression of glioma through restraining STAT2 and AdipoR1/AMPK/SIRT1 pathways.

Discussion

GBM is the primary and deadliest malignancy of the brain and extremely common (30-32) with near highest fatality in two years after diagnosis (33,34). The cellular capacity, which reconstitute the tumor entirely, and tumorigenicity of cell subpopulations are the major reasons of therapeutic failure (31). Growing evidence indicates cell subpopulations of GBM induce its lethality based on the properties of its tumorigenicity and self-renewal (35). It demonstrated that the primary tumor could generate phenocopies (36,37). Key transcription factors could be regulated with epigenetic modifications and then control transformation between therapeutic developments and tumorigenic states of GBM (31).
MicroRNAs suppress translation of messenger RNA or promote degradation of it (38,39) and are vital regulators for oncogenesis. Moreover, their regulations on signaling in tumor cells are sophisticated. Most expression of microRNAs is inhibited in tumors (40-43). However, some amplified expression of microRNAs act as oncogenic in variety of tumors (43-46). The essential signaling activation of STATs and NF-κB pathways induce regulation of Notch pathway genes in glioma, which was identified as the novel targets for treatment on GBM (47). STAT2 plays a vital role on carcinogenesis of skin and colon, and could activate the signaling pathway of oncogenic STAT3 (48).

In the present study, we studied if microRNA modulates STAT2 and AdipoR1, or downstream pathway of AdipoR1 in glioma cells. The online software DIANA Tools (microT v4.0) were used to predict miRNAs that target STAT2 and AdipoR1. Within these miRNAs, miR-3908 potentially co-targeted mRNAs of STAT2 and AdipoR1 3'UTRs. Then, the 3'UTR luciferase reporter assays were carried out. It identified that miR-3908 could directly bind with 3'UTRs of STAT2 and AdipoR1, but not AMPK and SIRT1. Moreover, miR-3908 directly regulated the protein expression of STAT2 and AdipoR1 by interacting with its 3'UTR. The protein expression of AMPK and SIRT1 was regulated indirectly by STAT2 and AdipoR1 in glioma cells. Why the protein expression levels of AMPK and SIRT1 are decreased, but their mRNA
levels are not been affected? It may be due to the change in the translation of these mRNAs induced by mir-3908. mir-3908 may act as translational modulators on mRNAs of AMPK and SIRT1. It is known to influence the evolution and stability of AMPK and SIRT1 mRNAs but they can also affect translation efficiency.

Furthermore, the possible biological outcomes induced by miR-3908 in glioma cell lines U251 and U87 were investigated with the mimics and inhibitors of miR-3908. Several functional experiments associated with cancer progression and tumorigenicity was performed. Our results demonstrated that, compared with miR-NC cells, proliferation and colony formation of U251 and U87 cells would be suppressed when they were transfected with miR-3908. Moreover, the inhibitors of miR-3908 accentuated these processes of glioma cells. These results suggested that miR-3908 inhibited the proliferation of U251 and U87 cells, also lowering their clonogenicity.

The migration experiment and wound scratch assay were also conducted. These results demonstrated that miR-3908 obviously repressed the invasion and migration ability compared to control in the cell lines U251 and U87. Moreover, downregulation of miR-3908 promoted significantly the ability of invasion and migration in U251 and U87 cells. The
capability of invading surrounding tissues and migrating efficiently is a sign of progressive and metastatic cells. In vitro, the expression of miR-3908 significantly restrained migration (Fig. 6) and invasion (Fig. 7) in U251 and U87 cells, respectively. In turn, as miR-3908 inhibitors block miR-3908 in glioma cells, the ability of cell migration and invasion was enhanced. These data demonstrated that miR-3908 constrains pathways concerned with cancer progression and tumorigenicity in glioma cells.

To verify whether apoptosis of glioma cells could be induced by miR-3908, Dual-staining measurement with 7-AAD and Annexin V-FITC was conducted, as well as the microplate assays for caspase activity. Our results showed increased fractions of apoptotic glioma cells induced by miR-3908 (P<0.01) in U251 (Fig. 8A) and U87 (Fig. 8B). Obviously, miR-3908 also induced caspase-3/7 activities in U251 (Fig. 8C) and U87 cells (Fig. 8D). Conversely, inhibitors of miR-3908 suppressed these activities (Fig. 7). As the results show, miR-3908 induces glioma cell apoptosis.

AMPK is the vital energy sensor responsible for the homeostasis of cellular energy (49). While cellular energy is exhausted by hypoxia, starvation, stress or other reasons, intracellular AMP increase, and then activates AMPK through α-subunit phosphorylation caused by upstream kinases (50). Activated AMPK stimulate pathways of energy-consuming catabolics (such as glucose transport and fatty acid oxidation) and suppress anaerobic pathways of energy-consuming, and then homeostasis of cellular energy is restored. Sirtuins are deacetylases dependent on NAD+, and a response to shift of stress or energy metabolism. SIRT1 (sirtuin 1) is almost characterized in this family. AMPK and SIRT1 could emphatically manage the activities of each other (51). In liver or skeletal muscle, adiponectin modulates energy metabolism by the AMPK/SIRT1 pathway (52). Osmotin regulates AdipoR1, and then activates SIRT1 and AMPK to block production of Aβ (53).

The expression of STAT2, AdipoR1, AMPK, p-AMPK and SIRT1 proteins was detected by western blot analysis. Upregulation of miR-3908 in glioma cell lines U251 (Fig. 9A) and U87 (Fig. 9B) significantly inhibited the expression of STAT2, AdipoR1, AMPK, p-AMPK and SIRT1. Furthermore, treatment with inhibitors of miR-3908 in glioma cells significantly increased the expression of STAT2, AdipoR1, AMPK, p-AMPK and SIRT1 (Fig. 8). It showed that miR-3908 suppressed tumorigenicity and cancer progression of glioma through restraining STAT2 and AdipoR1/AMPK/SIRT1 pathways.

In conclusion, STAT2 and AdipoR1 are direct targets of miR-3908. miR-3908 could inhibit tumorigenicity and tumor progression of glioma through suppressing STAT2 and AdipoR1/AMPK/SIRT1 pathways. When miR-3908 is restored it will induce suppression of cancer progression and glioblastoma tumorigenicity. miR-3908 is a novel discovery of a research target for treatment in glioblastoma.

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References


