MicroRNA-218 Inhibits Glioma Invasion, Migration, Proliferation, and Cancer Stem-like Cell Self-Renewal by Targeting the Polycomb Group Gene Bmi1

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Abstract

Malignant gliomas are the most common central nervous system tumors and the molecular mechanism driving their development and recurrence is still largely unknown, limiting the treatment of this disease. Here, we show that restoring the expression of miR-218, a microRNA commonly downregulated in glioma, dramatically reduces the migration, invasion, and proliferation of glioma cells. Quantitative reverse transcription PCR and Western blotting analysis revealed that expression of the stem cell-promoting oncogene Bmi1 was decreased after overexpression of miR-218 in glioma cells. Mechanistic investigations defined Bmi1 as a functional downstream target of miR-218 through which miR-218 ablated cell migration and proliferation. We documented that miR-218 also blocked the self-renewal of glioma stem-like cells, consistent with the suggested role of Bmi1 in stem cell growth. Finally, we showed that miR-218 regulated a broad range of genes involved in glioma cell development, including Wnt pathways that suppress glioma cell stem-like qualities. Taken together, our findings reveal miR-218 as a tumor suppressor that prevents migration, invasion, proliferation, and stem-like qualities in glioma cells. Cancer Res; 73(19); 1–10. ©2013 AACR.

Introduction

Malignant gliomas are the most common primary tumors of the central nervous system. They are characterized by high invasion, migration, and proliferation abilities. Surgery, radiation, and chemotherapy are effective therapies for gliomas. However, despite these treatments, the median survival of the patients with the most malignant glioma is approximately 1 to 2 years (1). Many factors influence the effectiveness of glioma therapies, including rapid and invasive tumor growth. Therefore, suppressing proliferation and inhibiting glioma cell migration would be a novel therapeutic strategy.

Materials and Methods

Cell lines and cancer stem-like cell culture

U87MG and U251MG were purchased from the Chinese Academy of Sciences Cell Bank in 2012. LN229 was a generous
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cancer.

All cell lines were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS (GIBCO). The serum-free medium (SFM) was composed of DMEM/F12, 20 ng/mL basic fibroblast growth factor (bFGF; Sigma), 20 μL/mL B27 supplement (Life Technologies), and 20 ng/mL EGF (Sigma).

Cancer stem-like cells (GSC) were isolated from U251 MG glioma cell lines by using SFM. These cells can form neurosphere-like cell aggregates in less than 7 days (17).

Wound healing assays

U251MG cells were seeded in 6-well plates and cultured until they reached confluence. A wound was then created by manually scraping the cell monolayer with a 200 μL pipette tip. The cultures were washed twice with SFM to remove floating cells. The cells were then incubated in DMEM supplemented with 1% FBS. Cell migration into the wound was observed at three preselected time points (0, 12, and 24 hours) in eight randomly selected microscopic fields for each condition and time point. Images were acquired with a Nikon DS-5M Camera System mounted on a phase-contrast Leitz microscope and were processed using Adobe Photoshop 7.0. The distance traveled by the cells was determined by measuring the wound width at different time points and then subtracting it from the wound width at time 0. The values obtained were expressed as a migration percentage, setting the gap width at 0 hour as 0%.

In vitro migration and invasion assays

Cells (5 × 10⁵) were plated on the top side of polycarbonate Transwell filters (without Matrigel for Transwell assay) or plated on the top side of polycarbonate/Transwell filter coated with Matrigel (for Transwell matrix penetration assay) in the top chamber of the QCM 24-Well Cell Invasion Assay (Cell Biolabs, Inc.). For Transwell migration assays, cells were suspended in medium without serum, and medium without serum was used in the bottom chamber. For the invasion assay, cells were suspended in medium without serum, and medium supplemented with serum was used as a chemoattractant in the bottom chamber. The cells were incubated at 37°C for 8 hours (Transwell assay) or 48 hours (invasion assay). The nonmigratory or noninvasive cells in the top chambers were removed with cotton swabs. The migrated and invaded cells on the lower membrane surface were then permeabilized by treatment with ice-cold methanol for 10 minutes, air-dried, then stained with 4', 6-diamidino-2-phenylindole (DAPI) and counted under a microscope. Three independent experiments were conducted and the data were presented as the means ± SEM.

Plasmids and transfection

The 390 bp sequence of the Bmi1 3’-UTR containing the predicted miR-218 binding sites and its mutant of the miR-218 binding sites were synthesized by Shanghai Generay Biotech Co., Ltd. The DNA fragments were digested with Sac I and Xba I. The resulting fragments were subcloned into the Sac I and Xba I sites of the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega). The srGAP2 3’-UTR containing the predicted miR-218 binding sites was amplified by PCR with the srGAP2-pmirF and srGAP2-pmirR. The PCR fragment was subcloned into the Sac I and Xba I sites of the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega). The fragment containing the Bmi1 CDS was digested with BamH I and Xba I from Bmi1 CDS Clone BC011652 (Protein-tech Group, Inc.) and then subcloned into the BamH I and Xba I sites of the pCDNA3.1(+)Vector (Invitrogen). The cell transfections were carried out with FuGENE HD reagent (Roche) according to the manufacturer’s instructions. For selection of glioma stable cell lines, U251MG was cultured in 400 μg/mL neomycin (G418) for 14 days after transfection. The expression of FZD4 and LEF1 was confirmed by quantitative reverse transcription (qRT)-PCR and Western blotting.

Quantitative RT-PCR

Total RNA from glioma cells was isolated using TRizol reagent (Invitrogen). The RNA was subsequently treated with RNase-free DNase I (Roche). Synthesis of cDNA was done by using the BcaBest RNA PCR kit from TaKaRa according to the manufacturer's instructions. Quantitative RT-PCR was carried out using the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) with Realtime PCR Master Mix (SYBR Green). The PCR primers are listed in Supplementary Table S1. GAPDH was selected as the endogenous control in the assay.

Western blotting

The total cell lysates were prepared in high KCl lysis buffer (10 mmol/L Tris–HCl, pH 8.0, 140 mmol/L NaCl, 300 mmol/L KCl, 1 mmol/L EDTA, 0.5% Triton X-100, and 0.5% sodium deoxycholate) with complete protease inhibitor cocktail (Roche). The protein concentration was determined using a BCA Protein Assay Kit (Pierce). The Western blot assay has been previously described (18). The following primary antibodies were used: Bmi1 (Cell Signaling Technology, 1:2,000), Tri-Methyl-Histone H3 (Lys27/H3K27me3, Cell Signaling Technology, 1:2,000), α-tubulin (Santa Cruz Biotechnology, 1:2,000), PDGFRA (Epitomics, 1:2,000), LEF1 (Abgent, 1:200), SRC (Epitomics, 1:2,000), FZD4 (Santa Cruz Biotechnology, 1:200).

Immunofluorescence and hematoxylin and eosin staining

Cells on poly-L-lysine-coated glass coverslips were fixed with 4% paraformaldehyde for 15 minutes at room temperature and then permeabilized by treatment with ice-cold methanol for 10 minutes. The tumor spheres were fixed in 4% paraformaldehyde, embedded in optimal cutting temperature compound for freezing, and then cryosectioned (10-μm sections). After being blocked by 15% normal donkey serum for 30 minutes, the cells were incubated at room temperature for one hour with primary antibody diluted in antibody buffer (18). The following antibodies were used: Bmi1 (1:200), H3K27me3 (1:200), CD133 (Abgent, 1:50), SOX2 (Epitomics, 1:200), and nestin (R&D, 1:100). After incubation with the primary antibodies, the cells were rinsed and incubated for one hour at room temperature with Alexa Fluor-labeled secondary antibodies (Molecular Probes 1:500). The cells were washed with PBS and the cover slips were mounted with glycerine/PBS containing 0.1 mg/mL.
First, we used a wound-healing assay to examine the effect sequence of miR-218. The infected cells produced high levels of cell lines were infected with a lentivirus encoding the mature cultured glioma cells. To test this hypothesis, several glioma expression of miR-218 would have an opposite effect on GLO-srGAP2

**Luciferase assay and limiting dilution assay**

The Hsa-miR-218 vector (GenePharma Co.) and pmirGLO, pmirGLO-Bmi1 3′-UTR, pmirGLO-Bmi1 3′-UTR-mut, or pmirGLO-srGAP2 3′-UTR were cotransfected into HEK293FT. Cell lysates were prepared at 24 hours posttransfection. The luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). The values obtained from the Hsa-miR-218 vector and pmirGLO were set as 100%. A limiting dilution assay was conducted as described previously (17, 19).

**Animal studies**

U87MG cells stably expressing miR-218 or empty vector controls were implanted in the flanks of athymic mice (3.0 × 10^6 cells per mouse, 6 mice per cell line). Tumor volumes were determined by measuring the length (a) and the width (b). The tumor volume (V) was calculated according to the formula V = (a × b)^2/2 (20). All mouse experiments were carried out in accordance with institutional guidelines and regulations of the government.

**Microarray and bioinformatic analyses**

Total RNA was extracted using TRIzol reagent (Invitrogen). Extracted RNA was labeled and hybridized onto the Agilent One-Color Microarray-Based Gene Expression Analysis platform by Oebiotech Co. Statistical analyses and data normalization were conducted using the Genespring GX software (Agilent Technologies). Genes with 2-fold change in expression were considered differentially regulated by miR-218. Predicted miR-218 nonredundant targets were detected using the miR-ecord Predicted Targets program (http://mirecords.biolead.org/prediction_query.php). Genes were mapped onto KEGG pathways using DAVID version 6.7(http://david.abcc.ncifcrf.gov/). The microarray data reported in this article have been deposited in the Gene Expression Omnibus (accession number GSE46059).

**Statistical analysis**

Data were analyzed using the two-tailed Student t test. P < 0.05 was considered statistically significant.

**Results**

**Overexpression of miR-218 inhibits glioma cell invasion, migration, and proliferation**

Several investigations have shown that miR-218 was strongly downregulated in samples from patients and cell lines of glioma (12–14). Therefore, we hypothesized that the overexpression of miR-218 would have an opposite effect on cultured glioma cells. To test this hypothesis, several glioma cell lines were infected with a lentivirus encoding the mature sequence of miR-218. The infected cells produced high levels of miR-218 in U87MG, U251MG, and LN229 glioma cells (Fig. 1A). First, we used a wound-healing assay to examine the effect of miR-218 on cell migration. As shown in Fig. 1B, compared with the miR-control cells, the miR-218–overexpressing cells showed considerably slower migration. Quantification of wound closure showed that miR-218-U251MG cells closed 19.2% and 34.2% of the wound after 12 and 24 hours, respectively. Conversely, miR-control-U251MG closed 25.1% and 43.2% of the wound after 12 and 24 hours, respectively (Fig. 1C). Furthermore, Transwell migration assays revealed that miR-218 overexpression significantly restrained both glioma cell migration and invasion (Fig. 1C and E). The migration ability of miR-218-U251MG cells was only approximately one-eighth of miR-control-U251MG cells. Likewise, the invasive potential was reduced to one-fifth of miR-218-U251MG cells compared with miR-control cells (Fig. 1D and E). We used WST-1 cell viability assays to examine the effect of miR-218 on cell proliferation. As shown in Fig. 1F, the rate of cell proliferation was decreased in the miR-218 group compared with the other control groups at 48 and 72 hours after transfection (P < 0.05 for each), indicating that miR-218 could significantly inhibit the proliferation of glioma cells.

To analyze the role of miR-218 in glioma carcinogenesis, we further assessed the effects of miR-218 overexpression on tumor growth in vivo. miR-218-U87MG cells and their respective control cells were implanted into the right and left flanks (3.0 × 10^6 cells per flank) of nude mice by subcutaneous injection. At 14 days postinjection, the mean volumes of xenograft tumors generated from miR-218-U87 cells were significantly smaller than those originating from miR-control-U87MG cells (n = 6 animals per group, P = 3.18 × 10^-5; Fig. 1G and H). H&E staining showed decreased cell density in miR-218-U87MG xenografts (Fig. 1I). Immunohistochemical staining for Ki67 showed decreased expression in miR-218-U87MG tumors compared with miR-control-U87MG tumors (Fig. 1J). Thus, miR-218 overexpression significantly inhibited the proliferation of glioma cells both in vitro and in vivo.

**Bmi1 is a direct target of miR-218**

We used several computational methods to search for the potential targets of miR-218 in humans. The TargetScan Program identified two conserved binding sites for miR-218 in the 3′UTR region of the Bmi1 gene, a known core regulator governing the stemness of glioma cells (Fig. 2A; refs. 9, 21). To validate if Bmi1 is a direct functional target of miR-218, we used qRT-PCR and Western blotting to compare Bmi1 expression in the three glioma cell lines transfected with miR-218 or miR-control. As shown in Fig. 2B, there was significantly decreased Bmi1 RNA and protein expression after overexpression of miR-218 (Fig. 2B and C).

To obtain further direct evidence that miR-218 alters Bmi1 expression by posttranscriptional effects on the 3′UTR of the gene, we constructed a luciferase reporter plasmid containing the 3′UTR of Bmi1. As shown in Fig. 2D, the luciferase activity in theLuc–Bmi1-UTR-transfected cells was significantly decreased compared with the luciferase activity in the miR-218 target site mutant Bmi1 3′UTR and negative control cells. Finally, immunofluorescence was used to compare the expression levels and pattern of Bmi1 between miR-control-U87MG and miR-218-U87MG cells. The results showed that
overexpression of miR-218 only reduced the Bmi1 expression level and did not change the Bmi1 expression pattern (Fig. 2E). It has also been reported that, in Bmi1−/− mice, the levels of H3K27me3 are reduced (22). Consistent with the findings, Fig. 2C showed that glioma cells overexpressing miR-218 had reduced H3K27me3 levels compared with controls, and we also confirmed that Bmi1 levels were reduced.

**miR-218 inhibits self-renewal capacity of glioma stem-like cells**

It has been reported that the presence of GSC in the U251MG cell line (17) and Bmi1 could regulate glioma cell stemness (9, 23). To examine whether the effects of miR-218 on glioma self-renewal were consistent with Bmi1 downregulation, U251MG cells were cultured in SFM and tumor sphere formation was observed. We found a significant decrease in the volume of U251MG neurospheres stably expressing miR-218 compared with controls (Fig. 3A and B). The self-renewal capacity of cancer stem-like cells in the U251MG cells was detected with a limiting dilution assay. The number of cells required to generate at least one tumor sphere per well was calculated as 231.74 ± 6.9 in miR-218-U251MG cells and 142.88 ± 2.64 in miR-control-U251MG cells (Fig. 3C). We examined stem cell markers, such as CD133, SOX2, nestin, and Bmi1 in these neurospheres by immunofluorescence staining, and found that miR-218-U251MG–derived neurospheres showed decreased GSC markers expression compared with miR-control-U251MG neurospheres (Fig. 3D), suggesting that the overexpression of miR-218 reduced the glioma stem-like cell stemness. Immunohistochemical staining for Bmi1 and nestin showed decreased expression of these genes in miR-218-U87MG xenograft tumors compared with miR-control-U87MG xenograft tumors (Supplementary Fig. S1), suggesting miR-218 regulates glioma stemness in vivo. Taken together, these data
indicate that miR-218 reduced self-renewal capacity of glioma stem-like cell.

miR-218 regulates glioma cell development by downregulating Bmi1 expression

We used short-hairpin RNA (shRNA) targeting Bmi1 mRNA (shBmi1) to specifically suppress the expression of Bmi1 in U251MG (Fig. 4A and B) and U87MG cells (Fig. 4C and D). The knockdown effect of shRNA was tested by qRT-PCR and Western blot analysis (Fig. 4E and F). Knockdown of Bmi1 expression can dramatically inhibit migration and proliferation of glioma cells (Fig. 4). This result is consistent with the effect of miR-218 overexpression (Fig. 1B–E), which further supports that the suppression of Bmi1 might be the key mechanism of miR-218 regulation of glioma development.
miR-218 regulates a broad range of genes involved in development of glioma cells

To investigate the differences between miR-218-U251MG and miR-control-U251MG cells in gene expression, we conducted expression profiling on the Agilent One-Color Microarray-Based Gene Expression Analysis platform (Agilent-028004 SurePrint G3 Human GE 8 x 60 K Microarray). There were a total of 1,546 genes with at least a 2.0-fold change in the miR-218 versus control. Of these genes, 456 genes were upregulated and 1090 genes were downregulated. Because miR-218 direct functional targets should be downregulated by miR-218 overexpression, we analyzed the genes that were downregulated on the microarray. First, we used miRecord Predicted Targets program (http://mirecords.biolead.org/prediction_query.php) to predict the hsa-miR-218 nonredundant targets in the whole human genome. The miR-218 targets were predicted by at least three target prediction programs and 2,319 genes were identified. We found that 106 predicted genes had at least a 2.0-fold change in the microarray (Fig. 5A and Supplementary Table S2). Pathway analysis of the differentially expressed genes in the miR-218 versus control revealed significant enrichment for genes involved in several important cellular processes such as cancer development, cell junctions, insulin, and chemokine signaling pathways (Fig. 5B). We firstly confirmed the fidelity of microarray results by analyzing the mRNA expression of 10 representative genes with qRT-PCR (Fig. 5C). Subsequently, we investigated the protein expression levels of 4 of the 10 genes in Fig. 5C by Western blotting analysis (Fig. 5D). The expression levels were consistent with the microarray data for weakly expressed genes, but some differences were seen for highly expressed genes.

To assess the contribution of the identified target genes on glioma development, we used shRNA targeting LEF1 and FZD4, which belong to the Wnt pathway, to specifically suppress the expression of LEF1 and FZD4 in U251MG (Supplementary Fig. S2). We found a significant decrease in the volume of U251MG neurospheres in which efficient knockdown of LEF1 and FZD4 was compared with controls (Fig. 5E–H). These results further support that miR-218 may regulate glioma cells development by regulating many genes besides Bmi1.

Discussion

Cancer development is a highly orchestrated process that requires complex transcription and posttranscriptional regulation of gene expression (24). miRNAs target multiple genes and play important roles in many cancer processes (25). Researchers have shown that miR-218 is drastically downregulated in human glioma compared with normal brain tissues (12–14). It has also been shown that miR-218 regulates glioma cell invasion by downregulating IkB kinase-β and LEF1 (26). Our results showed that miR-218 bound to the complementary sites within the 3'UTR of Bmi1 and dramatically decreased Bmi1 mRNA and protein expression. In addition, a significant inverse correlation between the levels of miR-218 and mRNA expression of Bmi1 was
observed in glioma (Fig. 2). We have shown that Bmi1 is a direct functional target of miR-218 in glioma, and Bmi1 is involved in regulating the invasion and migration of glioma cells.

Bmi1 is a polycomb group epigenetic gene silencer that is highly expressed in various types of human cancers (27). In addition, it is involved in the development and progression of cancers (28, 29). It was recently reported that Bmi1 expression correlated with the poor prognosis and glioma progression in patients (30). This result is consistent with our findings that Bmi1 was a functional target of miR-218. It was reported that Bmi1 also plays a role in neural stem cell self-renewal (21, 31). There is substantial evidence that the signaling pathways regulate cancer stem cell-like properties are similar to those governing neural stem cell stemness. It is now recognized that the development of stem cell-like properties in glioma contributes to disease progression (32). We observed a significant decrease in the self-renewing capacity, expression of stem cell markers, and the volume of U251MG neurospheres stably expressing miR-218 (Fig. 3). These results suggest that miR-218 regulates glioma stem-like cells in part by blocking Bmi1-associated pathways.

Slit2 is one of host genes of miR-218 (16), which is downregulated in glioma cells. The promoter region of Slit2 is usually hypermethylated in glioma cell lines and neuroblastomas (33, 34). Moreover, low Slit2 expression in glioma is associated with glioma cell migration (35). These results are consistent with miR-218 regulation of migration and invasion of glioma cells. Recently, He and colleagues reported that Slit2 promoter hypermethylation may contribute to the silencing of miR-218 in human colon cancer (36), and glioma may have a similar mechanism.

Bmi1 is a part of the PRC1 complex, which is able to recognize the trimethylated H3K27(H3K27me3), and this interaction could bring neighboring nucleosomes into the proximity of the PRC2 complex (37). Ezh2 is a part of the PRC2 and directly interacts with DNA methyltransferases (DNMTs) for the maintenance of...
DNA methylation (38). Bmi1 could affect DNA methylation through H3K27 methylation. The overexpression of miR-218 in glioma cells downregulated Bmi1 expression and reduced H3K27 methylation, which further reduces miR-218 expression (Fig. 2C and E), and a reduced expression of miR-218 caused high expression of Bmi1 in glioma cells. The high expression of Bmi1 may cause enhanced methylation of the slit2 promoter. These factors may form a positive feedback loop to accelerate glioma development from low to high grade.

To identify more mRNA targets of miR-218 in glioma cells, we screened through the list of differentially regulated downregulated genes after miR-218 overexpression to identify potential oncogenes (Fig. 5A). It has been reported that LEF1 was a miR-218 target gene in glioblastoma multiforme cells that regulates glioblastoma cell invasion (26), and we further proved LEF1 also regulated glioma stem cell stemness (Fig. 5E and F). Vav3 is also involved in affecting the invasive behavior of glioblastoma (39) and tumor cells stimulated with PDGF (40). In addition, Frizzled 4 regulates glioma cell invasion (41) and stemness (Fig. 5G and H), whereas reduced SRC expression impairs growth of malignant glioma (42) and MMP24 (MT5-MMP) overexpressed in brain tumors (43). Reduced expression levels of these genes were consistent with our microarray data (Fig. 5C).

The Wnt pathway significantly has been found to correlate with the invasion and proliferation of tumor cells (44). Wnt proteins bind receptors of the Frizzled (Fz) family and regulate formation of the β-catenin–LEF1 complex in the nucleus, which activates many downstream target genes regulating cell invasion, migration, and proliferation. FZD4 and LEF1 are involved in the Wnt pathway, and we provided evidence that these genes also regulate glioma stem cell stemness (Fig. 5E–H). These results showed that miR-218 could regulate the Wnt pathway to influence glioma development. Pathway analysis of the differentially expressed genes in the miR-218 versus control revealed that miR-218 could regulate several important cellular pathways such as cancer development, cell junctions, and signaling pathways (Fig. 5B). Taken together, our results suggest that miR-218 regulates a complex regulatory framework to coordinately downregulate many oncogenes regulating glioma cell invasion, migration, proliferation, and self-renewal.
of glioma cancer stem-like cells. Our findings confirmed the potential of miR-218 overexpression for glioma therapy. The pleiotropic functions of miR-218 in glioma development require further research.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: Y.-Y. Tu, X.-C. Gao, W.-L. Jin, Y.-S. Zhang
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X.-C. Gao, G. Li, D.X. Cui, W.-L. Jin
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