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LABORATORY INVESTIGATION

MiR-198 enhances temozolomide sensitivity in glioblastoma by targeting MGMT

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Abstract Glioblastoma is one of the most frequent and aggressive brain tumors. Accumulating evidence indicates that microRNAs are involved in glioma proliferation, invasion and drug resistance. Previous studies showed that miR-198 is downregulated in glioblastoma. However, the function of miR-198 in glioblastoma is still unclear. In this study, we report that miR-198 levels were greatly downregulated in glioblastoma specimens and decreased expression of miR-198 was associated with poor prognosis in patients with glioblastoma. And overexpression of miR-198 increased chemosensitivity to temozolomide in vitro and in vivo. O^6-methylguanine-DNA methyltransferase (MGMT) was identified as a direct target of miR-198, and miR-198 overexpression prevented the protein translation of MGMT. Furthermore, overexpression of MGMT restored miR-198-induced chemosensitivity to temozolomide. Moreover, the protein levels of MGMT were upregulated in clinical glioblastoma specimens and inversely correlated with miR-198 levels. In conclusion, our studies revealed that MiR-198 induces chemosensitivity in glioblastoma by targeting MGMT and that miR-198 may be used as a new diagnostic marker and therapeutic target for glioblastoma in the future.

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Introduction

Glioma is the most common type of primary brain tumor with poor prognosis. Even treated with surgery, chemotherapy, and/or radiotherapy, the median survival for patients with glioblastoma multiforme (GBM) is only 12–14 months [1]. As a promising chemotherapeutic agent for treating glioblastoma, temozolomide (TMZ) easily crosses the blood–brain barrier and efficiently inhibits GBM cells proliferation and induces apoptosis [2]. Unfortunately, glioblastoma often exhibits resistance against this alkylating agent. The most popular mechanism of TMZ resistance is the expression of O^6-methylguanine-DNA methyltransferase (MGMT) which protects the cellular genome from the mutagenic effects of alkylating agents [3–6]. The expression of MGMT is regulated by MGMT promoter methylation, histone acetylation, transcription factors, microRNAs expression and so on. Therefore, further study should focus on the regulation of MGMT expression.

As a small non-coding RNA molecule (19–25 nucleotides in length), MicroRNAs (miRNAs) regulate gene expression through causing mRNA degradation or translational repression by targeting specific sequences of mRNAs [7–10]. Although several potential miRNAs that might be interacted with the 3′-untranslated region (3′-UTR) of the MGMT gene have been revealed by silico analysis [11], miRNA-dependent regulation of MGMT expression need further investigation in experimental study. Recently, different studies have been conducted in order to associate modulation of expression of specific miRNAs with the response to TMZ in experimental cell models in vitro and in patients

with GBM [12–15]. However, only some of these investigations can be directly related to the therapeutic axis between TMZ and MGMT expression. Among these miRNAs, the level of miR-198 in glioblastoma tissues is much lower than that in normal brain tissues (NBTs) [16]. Therefore, the role of dysregulated miR-198 in glioblastoma biology need to be further illustrated.

In the present study, we seek to answer the following questions: (i) whether miR-198 binds to the seed map site in 3′-UTR of MGMT; (ii) whether miR-198 enhances chemosensitivity to TMZ; and (iii) whether miR-198 increases chemosensitivity to TMZ via reducing the expression of MGMT. The answers to these questions would provide new insights into better understanding of the role of miR-198 in TMZ resistance and provide a novel therapeutic strategy for glioblastoma.

Materials and methods

Patients and samples

The TCGA database was downloaded from the TCGA database (http://tcga-data.nci.nih.gov). The CGGA database was downloaded from the Chinese Glioma Genome Atlas (CGGA, http://www.cgcg.org.cn/). A total of 30 patients (18 males and 12 females, 25 tumor samples and 5 non-tumorous brain specimens) with a median age of 49.6 years (range 21–67 years) who had been treated surgically at the Department of Neurosurgery, The First Affiliated Hospital of Nanjing Medical University (Nanjing, China), between January 2013 and October 2014 were selected for the study. Informed consent was obtained from each subject, and the study was approved by the ethics committee of the hospital.

The glioma tissue samples were histologically diagnosed by the Department of Pathology at The First Affiliated Hospital of Nanjing Medical University according to the WHO classification [17]. As controls, five human non-tumor brain tissue samples were obtained primarily from the cortex of patients with decompressive surgery after physical injury to the brain. Samples were immediately snap-frozen in liquid nitrogen until use. All methods were performed in accordance with the approved guidelines.

Cell culture and reagents

The human GBM cell lines A172, U87, U251, U118, LN229, U138 and T98 were purchased from Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Normal human astrocytes (NHAs) were obtained from Lonza (Walkersville, MD, USA) and cultured in the provided astrocyte growth media supplemented with rhEGF, insulin, ascorbic acid, GA-1000, L-glutamine and 5% FBS. Primary GBM1 and primary GBM2 that derived from GBM surgical specimens were maintained in primary serum-free cultures grown on laminin [18]. Before each experiment, the GBM cells were cultured in DMEM, supplemented with sodium pyruvate, 10% FBS, L-glutamine, nonessential amino acids, and a vitamin solution (Invitrogen life technologies).

Plasmids construction, transfection and stable cell establishment

The entire coding sequence of MGMT was obtained from HUVEC mRNA by RT-PCR. MGMT cDNA were purified by Genechem (Shanghai, China) and were cloned into pcDNA3.1 vector to generate pcDNA3.1-MGMT recombinant plasmid. Transient transfection was performed using Lipofectamine™ 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Lentivirus carrying hsa-miR-NC or hsa-miR-198 was packaged using the lentiviral packaging kit (Open Biosystems) according to the manufacturer's manual. Human embryonic kidney 293T cells were used to packaged lentivirus. Stable GBM cell lines were established as previously described [19].

Quantitative real-time PCR

Total RNA was isolated using TRIzol® Reagent (Invitrogen). Quantitative real-time PCR was performed according to the manufacturer's instructions. The primers for has-MGMT: forward primer, 5′-ACCGTTTGCGACTTGGTACTT-3′; reverse primer, 5′-GGAGCTTTATTTCGTGCAGACC-3′. Has-β-actin mRNA was also amplified in the same PCR reactions as an internal control using the primers: forward primer, 5′-CATGTACGTTGCTATCCAGGC-3′; reverse primer, 5′-CTCCTTAATGTCACGCACGAT-3′. MiRNAs were reverse transcribed using miRNA specific stem-loop RT primers and RT-PCR kit (Takara) according to the manufacturer's protocol. Real-Time PCR was performed using the Applied Biosystems 7500 Sequence Detection System (Applied Biosystems). β-actin or U6 levels were used as an internal control, and fold changes were calculated by relative quantification ($2^{-\Delta\Delta CT}$).

Western blotting

Western blotting was performed as described previously [20], and images were captured by Bio-Rad ChemiDoc XRS+ (Bio-Rad, CA, USA). The antibodies used in this study were: MGMT (Abcam, USA); caspase-3 (Cell Signaling Technology, USA); γ-H2AX and H2AX (Cell Signaling Technology, USA). β-actin (Santa Cruz, USA) was used as a control.

Luciferase reporter assay

The wild-type (WT) and mutated (mut) putative miR-198 target on MGMT 3′-UTR were cloned into pGL3-contral luciferase reporter plasmid (Invitrogen). Firefly and Renilla luciferase signals were determined using a Dual-Luciferase Assay Kit (Promega, Madison, WI, USA).

CCK-8 proliferation assay

The proliferative ability of GBM cells was assessed using Cell Counting Kit-8 (CCK-8, Dojindo, Tokyo, Japan). 5×10^3 GBM cells stably expressing miR-198 or miR-NC were seeded into 96-well cell culture plates followed by being treated with TMZ for indicated concentration. After indicated times, the medium in each well was replaced with 100 µl fresh medium with 10% CCK-8 and then the cells were incubated at 37 °C for an additional 2 h. The absorbance was measured at 450 nm.

Colony formation assay

Cells stably expressing MGMT, miR-198 or miR-NC were independently plated onto 60-mm dishs (1000 in a 60-mm dish) followed by being treated with TMZ (200 µM) for 48 h, and then the culture media were refreshed without TMZ. After 10–12 days, visible colonies were fixed with 100% methanol and stained with 0.1% crystal violet in 20% methanol for 15 min. Colony-forming efficiency was calculated as the number of colonies/plated cells × 100%.

Annexin V-FITC/PI staining

The number of apoptotic cells was counted using an Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences, CA, USA) according to the manufacturer's protocol. Apoptotic cells were analyzed on a Gallios Flow cytometer (Beckman, CA, USA). The results are presented as the percentage of apoptotic cells relative to the total number of cells.

Orthotopic xenograft studies

All experiments involving mice were provided by The Model Animal Research Center of Nanjing University (Nanjing, China). For orthotopic xenograft studies, P-GBM2 cells (2.5×10^5) stably expressing miR-NC or miR-198 were injected intracranially into the striatum of NOD/SCID mice using a stereotactic device (coordinates: 2 mm anterior, 2 mm lateral, 3 mm depth from the dura). At 1 week, the tumor-bearing mice were given TMZ or placebo by oral gavage (66 mg/kg daily for 5 days/week for three cycles) and killed upon signs of tumor formation (rough coat, hunching, and weight loss). Tumors were measured by luminescence imaging (IVIS Spectrum, PerkinElmer, USA) each week.

Immunohistochemistry (IHC)

Immunohistochemistry to detect MGMT in nude mouse xenograft tumor tissues was performed as described previously [21].

Statistical analysis

Quantitative data are presented as means ± SEM. The statistical significance levels were set at $^*P<0.05$ and $^{**}P<0.01$. One-way ANOVA and Student's *t* tests were used to perform comparisons. Biostatistical analysis was performed using Office SPSS software (SPSS version 17.0) and GraphPad software (GraphPad Prism 5). All statistical tests were two-sided.

Results

Downregulation of miR-198 expression in human glioblastoma

The CGGA database of glioma showed that miR-198 levels were significantly lower in high-grade tumors (WHO grades III and IV) than those in low-grade tumors (WHO grade II) (Fig. 1a). Next we explored the levels of miR-198 in five NBT samples and 25 GBM samples using QRT-PCR assay. And miR-198 levels were higher in GBM than NBT samples (Fig. 1b). Then, we analyzed the levels of miR-198 in normal human astrocytes (NHAs) and nine GBM cell lines (A172, LN229, T98, U138, U118, U87, U251, primary GBM1, and primary GBM2). The levels of miR-198 were significantly downregulated in these GBM cell lines compared with NHAs (Fig. 1c). Further analysis of miR-198 expression using the CGGA database and TCGA database revealed that decreased expression of miR-198 was associated with poor prognosis in GBM patients (Fig. 1d).

MiR-198 directly targets MGMT

To better understand the underlying function and mechanism of miR-198 in GBM progression, several potential target genes of miR-198 were detected using the bioinformatic algorithm TargetScan. Among the candidates, we detected a predicted binding site for miR-198 in the 3′-UTR of MGMT (Fig. 2a). To verify whether MGMT is a novel direct target of miR-198 in glioma, a luciferase assay was performed in pGL3-contral luciferase reporter plasmids containing the putative wild-type (WT) and mutant (Mut)

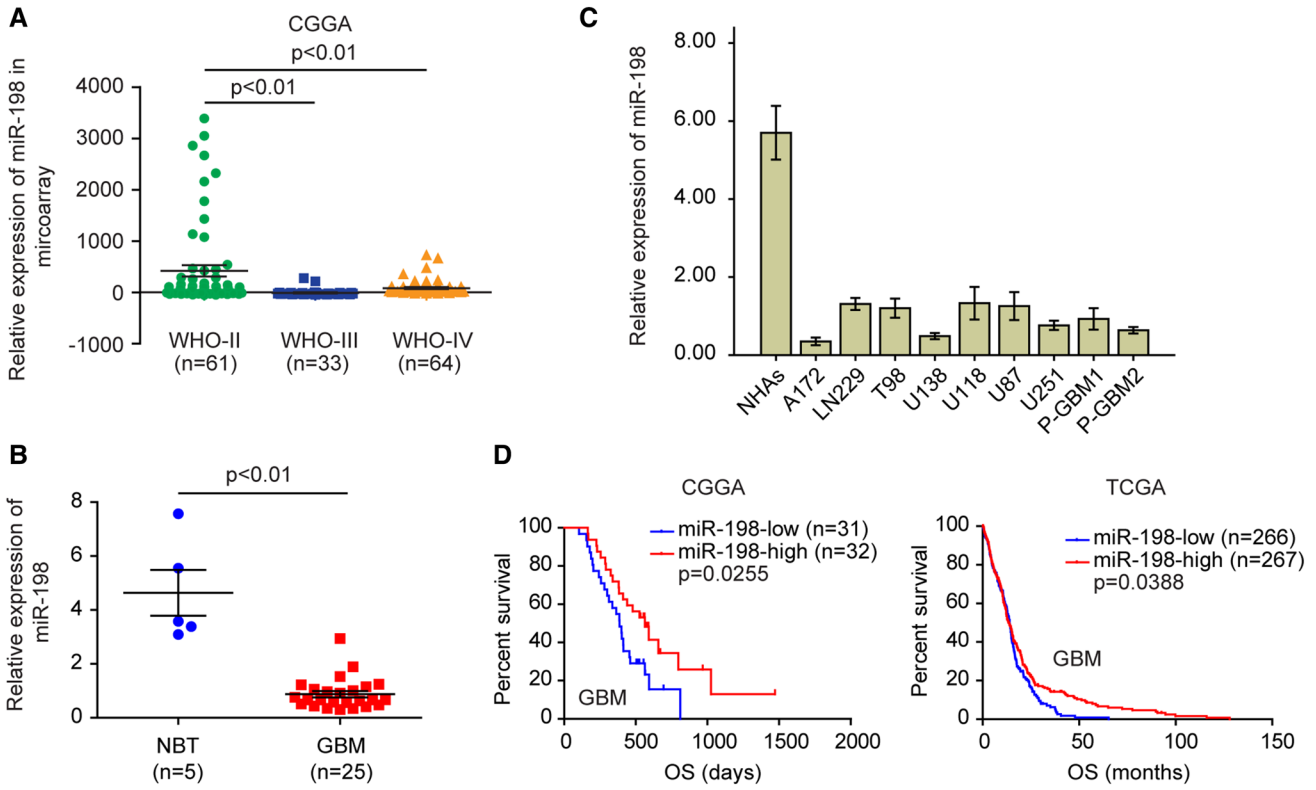


Fig. 1 MiR-198 is downregulated in glioblastoma. **a** Expression levels of miR-198 were analyzed in CGGA database. **b** Expression levels of miR-198 in five NBTs and 25 GBM tissues were analyzed by stem-loop qRT-PCR and normalized to the levels of U6. **c** Expression levels of miR-198 in NHAs, A172, LN229, T98, U138, U118, U87, U251, P-GBM1 and P-GBM2 cells. **d** Kaplan–Meier curves showing the overall survival of patients with high or low expression of miR-198 in GBM patients using CGGA database and TCGA database. *NBT* nontumor brain tissue, *GBM* glioblastoma multiforme, *NHAs* normal human astrocytes, *P-GBM1/2* primary GBM1/2. Student's *t* tests and one-way ANOVA were performed. Data are presented as mean ± SEM

MGMT 3′-UTR binding site. U138 and primary GBM2 (P-GBM2) cells were treated with reporter vector (MGMT-WT or MGMT-Mut) and miR-NC (negative control) or miR-198. Cells cotransfected with MiR-198 and MGMT-WT reporter showed a noteworthy reduction of luciferase activities (Fig. 2b). However, there was no significant difference in the luciferase activities of MGMT 3′-UTR Mut reporter between miR-198 and miR-NC groups (Fig. 2b). To further verify whether miR-198 regulates MGMT expression through mRNA degradation or translational repression, we established U138 and P-GBM2 cells stably expressing miR-198 or miR-NC. And the protein levels of MGMT were downregulated in cells stably expressing miR-198 (Fig. 2c). However, miR-198 overexpression did not affect the mRNA levels of MGMT (Fig. 2d). These results indicated that miR-198 directly targeted MGMT by binding to the 3′-UTR of MGMT and inhibited the translation of the MGMT mRNA into the MGMT protein in GBM cells. Next, we measured the protein levels of MGMT in clinical GBM and NBT specimens. Immunoblotting results revealed that the protein levels of MGMT were observably higher in GBM tissues than that in NBTs (Fig. 2e, f). Moreover, a high correlation between miR-198 and MGMT was observed in these GBM specimens. Spearman's correlation analysis demonstrated that miR-198 levels in GBM tissues were inversely correlated with MGMT protein levels (Fig. 2g, $r = -0.514$). MGMT is the most relevant mechanism of TMZ resistance. These findings suggest a possible relationship between miR-198 expression and TMZ resistance.

MiR-198 overexpression increases chemosensitivity to temozolomide

To explore the potential role of miR-198 in TMZ therapy, we treated A172 cells (TMZ sensitive cells lacking MGMT expression), U138 and P-GBM2 cells (highly expressing the MGMT, Fig. 3a, b) stably expressing miR-198 or miR-NC with different concentrations of TMZ. As shown in Fig. 3c, CCK8 assay detected a significant decrease in cell viability following TMZ exposure in inverse correlation to TMZ concentration. Overexpressing miR-198 only increased chemosensitivity to TMZ in U138 and P-GBM2 cells, but not in A172 cells. Next, we measured the viability

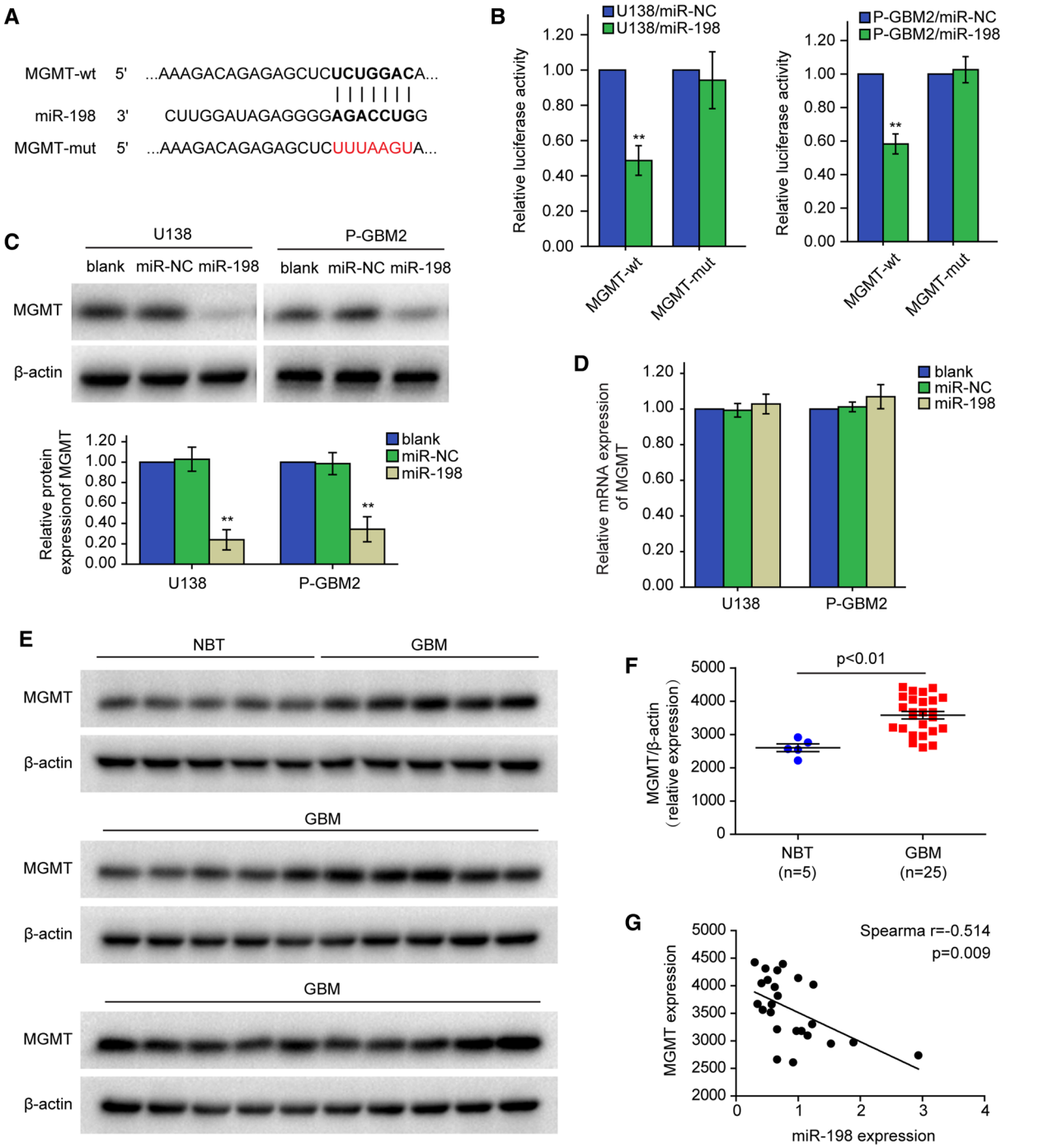
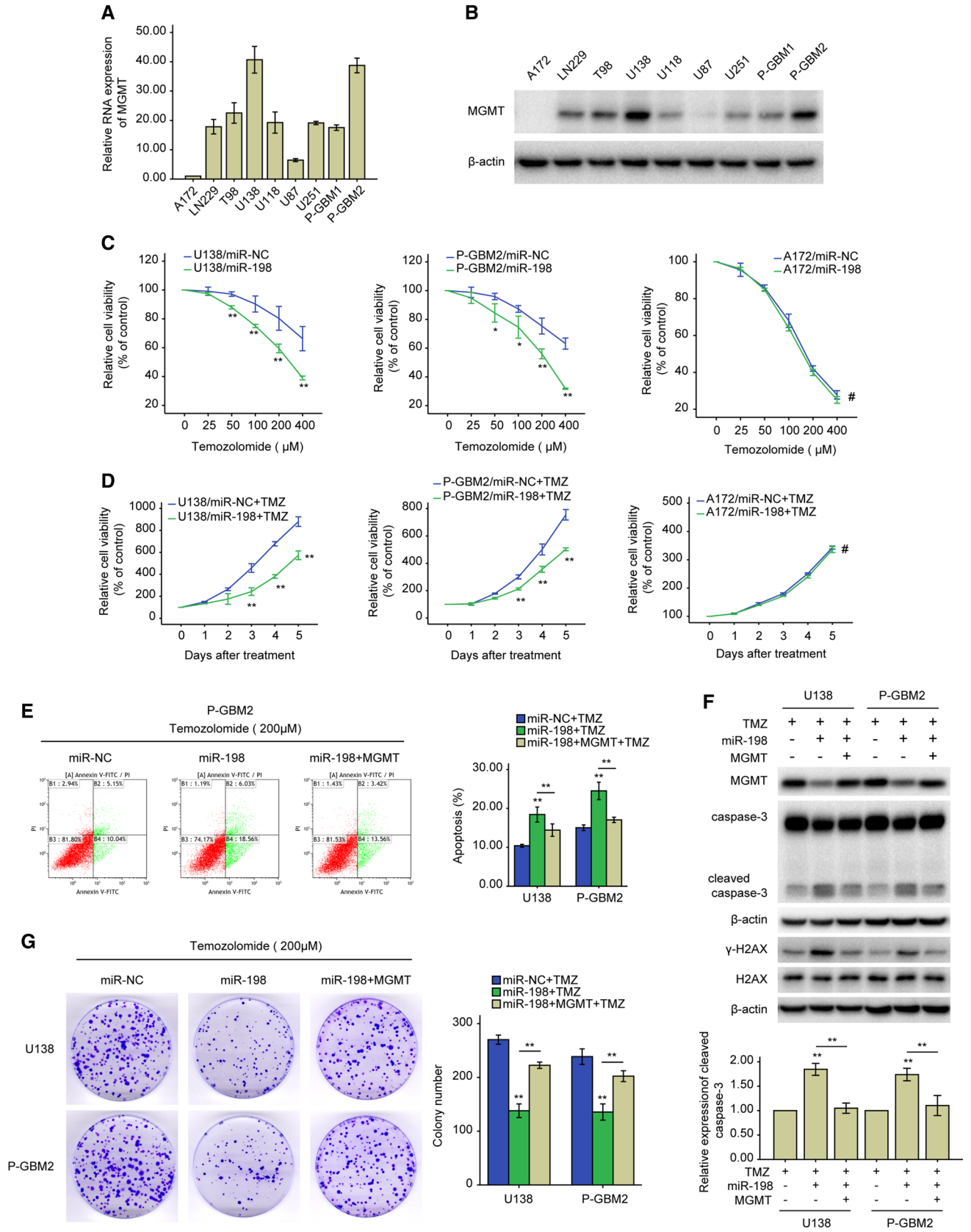


Fig. 2 MiR-198 directly targets related MGMT. **a** The sequence of miR-198 binding sites within MGMT. The reporter constructs of the MGMT 3′-UTR sequences and the mutated 3′-UTR sequences are shown in the schematic diagram. **b** Luciferase reporter assay was performed to detect the relative luciferase activities of WT and Mut MGMT reporters. **c** The protein levels of MGMT in GBM cells overexpressing miR-198 or miR-NC. **d** The mRNA levels of MGMT in GBM cell transfected with miR-198 or miR-NC. **e, f** The protein levels of MGMT in NBT and GBM specimens were determined by immunoblotting; the fold changes were normalized to β-actin. **g** Spearman's correlation analysis was used to determine the corrections between the MGMT expression and miR-198 levels in human GBM specimens. Student's *t* tests and One-way ANOVA were performed. Data are presented as mean ± SEM (**P < 0.01, #P > 0.05)

of GBM cells treated with TMZ (200 μM) using CCK8 assay at different time points. The results showed that miR-198 overexpression significantly inhibited cell survival of both U138 and P-GBM2 cells in the presence of TMZ (Fig. 3d). However, overexpression of miR-198 did not affect cell survival of A172 cells, indicating that miR-198


A
Relative RNA expression of MGMT
50.00
40.00
30.00
20.00
10.00
0.00
A172
LN229
T98
U138
U118
U87
U251
P-GBM1
P-GBM2
B
MGMT
β-actin
C
U138/miR-NC
U138/miR-198
P-GBM2/miR-NC
P-GBM2/miR-198
A172/miR-NC
A172/miR-198
Relative cell viability (% of control)
Temozolomide (μM)
D
U138/miR-NC+TMZ
U138/miR-198+TMZ
P-GBM2/miR-NC+TMZ
P-GBM2/miR-198+TMZ
A172/miR-NC+TMZ
A172/miR-198+TMZ
Days after treatment
E
P-GBM2
Temozolomide (200μM)
miR-NC
miR-198
miR-198+MGMT
Annexin V-FITC
PI
miR-NC+TMZ
miR-198+TMZ
miR-198+MGMT+TMZ
Apoptosis (%)
U138
P-GBM2
F
U138
P-GBM2
TMZ
miR-198
MGMT
caspase-3
cleaved caspase-3
β-actin
γ-H2AX
H2AX
Relative expressionof cleaved caspase-3
G
Temozolomide (200μM)
miR-NC
miR-198
miR-198+MGMT
U138
P-GBM2
Colony number

◂**Fig. 3** MiR-198 increases chemosensitivity of GBM cells to TMZ through MGMT. **a** The RNA levels of MGMT in nine GBM cell lines. **b** Expression of MGMT protein was analyzed in nine GBM cell lines. **c** Cell proliferation was evaluated in GBM cells stably expressing miR-NC or miR-198, with or without the TMZ treatments at different concentration. CCK8 assay was performed 48 h after treatment. **d** Cell proliferation in 200 μM TMZ treatments were tested every 24 h in U138, P-GBM2 and A172 cells overexpressing miR-198 and miR-NC. **e** GBM cells were cotransfected with miR-198 or miR-NC, pcDNA3.1-MGMT plasmid and cultured in 200 μM TMZ, then subjected to apoptosis analysis by flow cytometry 48 h later. **f** Western blot analysis of MGMT, caspase-3, cleaved caspase-3 and γ-H2AX expression in GBM cells cotransfected with miR-198 or miR-NC, pcDNA3.1-MGMT plasmid and cultured in 200 μM TMZ. **g** GBM cells cotransfected with miR-198 or miR-NC, pcDNA3.1-MGMT plasmid were exposed to 200 μM TMZ for 48 h and the proliferation was measured by colony formation assays. Student's *t* tests and One-way ANOVA were performed. Data are presented as mean ± SEM (*P < 0.05, **P < 0.01, #P > 0.05)

sensitized GBM cells to TMZ might through MGMT. Then, Flow cytometry was performed to test whether miR-198 overexpression enhance the pro-apoptotic effect of TMZ in GBM cells. Cell apoptosis rates were increased in U138 and P-GBM2 cells treated with TMZ plus miR-198, whereas the ascending cell apoptosis rates induced by TMZ plus miR-198 were partially abolished by forcing expression of MGMT (Fig. 3e). In addition, after TMZ treatment, GBM cells expressing miR-198 had a significant decrease in the expression of MGMT and increase in the levels of phosphorylated histone H2AX (γ-H2AX) and cleaved caspase-3 compared with vector control cells. However, ectopic expression of MGMT significantly reversed the enhancement effect of miR-198 on γ-H2AX and cleaved caspase-3 in the presence of TMZ (Fig. 3f). Furthermore, MGMT overexpression observably restored the reduction of colony forming capacity induced by miR-198 plus TMZ (Fig. 3g). These results suggest that miR-198 enhances GBM cells more sensitive to TMZ through targeting MGMT.

MiR-198 overexpression enhances temozolomide-induced growth inhibition in vivo

Next, we investigated the role of miR-198 overexpression in the reversal of TMZ resistance in vivo. 2.5×10^5 luciferase-labeled P-GBM2 cells stably expressing miR-198 or miR-NC were intracranially injected into NOD/SCID mice. Representative images for luminescence images of tumor-bearing xenografts and paralleled HE-stained orthotopic xenograft tumors are given in Fig. 4a–c. Xenografts carrying miR-198-overexpression P-GBM2 cells displayed a significant regression of tumor growth following TMZ treatment (Fig. 4a, b). Histologic analysis confirmed that all mice bearing tumors derived from miR-198-overexpression P-GBM2 cells had a significant decrease in MGMT expression (Fig. 4c). These findings were further confirmed by the survival curves, in which TMZ-treated miR-198-overexpression xenografts exhibited significantly increased survival as compared with TMZ-treated miR-NC xenografts (Fig. 4d). Together, these data suggest that upregulation of miR-198 sensitizes GBM cells to TMZ in vivo.

Discussion

Dysregulated miRNAs have been frequently indicated in diverse human cancers, including brain tumors such as glioblastoma [22]. Previous studies showed that miRNAs play important role in cancer progression, i.e., proliferation, invasion, metabolism, drug resistance and so on, and may act as candidate oncogene or tumor-suppressor gene in diverse cancers. In our study, we found that miR-198 was downregulated in human GBM tissues compared with NBTs. And decreased expression of miR-198 was associated with poor prognosis in GBM patients. Moreover, we demonstrated that miR-198 overexpression decreased the protein levels of MGMT through directly targeting its 3′-UTRs.

The DNA repair protein MGMT recovers the structural integrity of O^6-meG bases by transferring the methyl group to a cysteine residue (Cys145) within its own active site. Although O^6-meG only accounts for a minor proportion of the TMZ-induced base lesions, it is thought to be the most cytotoxic one since it strictly obstructs DNA replication. Preclinical studies demonstrated that MGMT expression mediates increased cellular resistance towards O^6-alkylating agents such as TMZ both in vitro and in vivo [23]. The level of MGMT is downregulated by different mechanisms, namely methylation of the CpG islands in the promoter regions [24], di-methylation of histone H3K9 [25], interference with protein translation by miR-648 [14], degradation of mRNA by miR-181d, -767-3p, -221, -222, -603 [13, 14, 26, 27]. And the expression of MGMT is upregulated by a variety of mechanisms, i.e., the acetylation of histones H3 and H4 [28], different nuclear transcription factors (AP-1, HIF-1α, CEBP, Sp1 and NF-κB) [29–34] and the stabilization by binding of N-myc downstream regulated gene 1 protein [35].The present study confirmed that miR-198 decreased the protein expression of MGMT through inhibiting the translation of the MGMT mRNA into the MGMT protein in vitro and in vivo. Moreover, analysis of clinical samples revealed that MGMT levels were upregulated in GBM specimens, and the increased protein levels of MGMT were inversely correlated with the decreased miR-198 levels, indicating that MGMT were downregulated by miR-198. Meanwhile, miR-198 overexpression rendered GBM cells more sensitive to TMZ

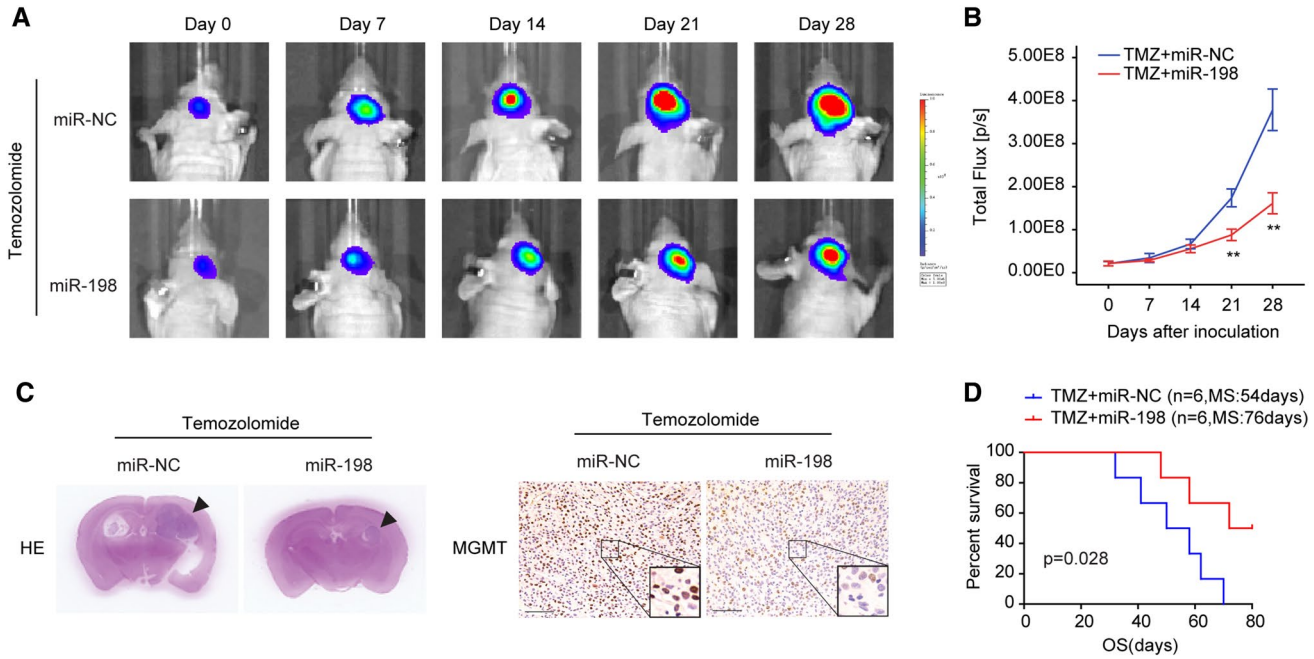


Fig. 4 Overexpression of miR-198 increases chemosensitivity of GBM cells to TMZ in vivo (**a**), representative pseudocolor bioluminescence images of intracranial xenografts bearing miR-198-overexpression P-GBM2 cells or control cells in the presence of TMZ on the days as indicated. **b** Growth curve of subcutaneous xenograft tumors were drawn on bioluminescence images to quantify photon fluxes per second. **c** Representative H&E staining for tumor cytostructure. IHC analysis of MGMT expression in intracranial xenografts. **d** survival curve of miR-198-overexpression P-GBM2 cells or control cells-derived intracranial xenografts treated with TMZ. Student's *t* tests were performed. Data are presented as mean ± SEM, *scale bar* 100 μm

through targeting MGMT in vitro and in vivo. Thus, the miR-198 plus TMZ combination could be an effective therapeutic strategy for glioblastoma.

It is known that each miRNA can modulate the expression of hundreds of gene transcripts [36]. MiR-198 also modulates the expression of c-MET [37], cyclin D2 [38] and PLAU [39]. Our study only focuses on miR-198 as a regulator of MGMT in glioma. Therefore, it will be interesting to explore whether miR-198 impact the expression of other genes and other functions of miR-198 in glioma.

Collectively, we showed that miR-198 rendered GBM cells more sensitive to TMZ by negatively regulating MGMT expression. Our study further illustrated the role of microRNA in TMZ resistance in glioblastoma. Although miRNA-based therapeutics are still in the initial stages of development, our findings present miR-198 involved in TMZ resistance as possible targets for cancer therapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Animal experiments were approved by the Animal Management Rule of the Chinese Ministry of Health (documentation 55, 2001) and were in accordance with the approved guidelines and the experimental protocol of The First Affiliated Hospital of Nanjing Medical University (Nanjing, China).

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