

Original Article

Downregulation of miR-155 inhibits proliferation and enhances chemosensitivity to Temozolomide in glioma cells

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Abstract: Background and aims: MicroRNA-155 (miR-155) is highly expressed in many malignant tumors, including glioma. Overexpression of miR-155 has a poor prognosis in patients with gliomas. Recent study has revealed that miR-155-silencing therapies could be a valuable approach to be associated with anticancer drugs and chemotherapy treatments. This study aimed to investigate its functional role of miR-155 on cell proliferation and chemosensitivity to Temozolomide (TMZ) as well as its possible molecular mechanisms. Methods: Effects of miR-155 overexpression (*miR-155*) or miR-155 silencing (anti-*miR-155*) on proliferation, apoptosis and chemosensitivity to TMZ in U87 or U251 human glioma cell line was detected. Bim cDNA and Bim siRNA transfection was used to assess the relation between miR-155 and Bim. Different protein and mRNA expression was detected by western blotting assay and quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assays. Results: *miR-155* transfection induces cell survival, tumorigenicity and chemoresistance to TMZ, whereas anti-*miR-155* transfection renders cells to apoptosis and enhances chemosensitivity. Further, we identified *Bim* as a target of *miR-155*. Sustained *miR-155* resulted in repression of Bim protein and mRNA levels, and knockdown of *miR-155* increases *Bim*. Introduction of *Bim* cDNA abrogates *miR-155*-induced cell survival and chemoresistance. However, introduction of *Bim* siRNA abrogates anti-*miR-155*-induced cell apoptosis and chemosensitivity. Conclusions: Our study reveals a molecular link between *miR-155* and *Bim* and presents evidence that *miR-155* is a critical therapeutic target in glioma.

Keywords: Glioma, drug resistance, apoptosis, microRNA-155, bim

Introduction

Gliomas are the most frequent primary malignant brain tumors in adults [1, 2], but are still incurable despite current therapy including neurosurgery, alkylating agent based-chemotherapy with temozolomide (TMZ) and ionizing radiation. Yet the survival median of glioma patients remains only around 15 months and the five-year survival is less than 10% [3]. Thus, both new therapeutic targets and improvement of treatments are needed.

MicroRNAs are small noncoding regulatory RNA molecules, with profound impact on a wide array of biological processes [4, 5]. MicroRNAs have been recently implicated in the regulation of tumorigenesis, differentiation, proliferation, and survival through the inhibition of major cellular pathways [6, 7]. miR-155 dysregulation is closely related to cancer [8]. miR-155 transgenic mice develop B-cell malignancy [9] and ele-

vated miR-155 expression was reported in several types of human B-cell lymphomas [10]. A correlation between increased miR-155 and development of tumors such as leukemia, glioblastoma, and breast, lung or gastric cancers has been established recently [11, 12]. Therefore, targeting miR-155 has been proposed as a promising approach to treat both hematopoietic and solid cancers [13-15].

Previous study has found that miR-155 has been shown to be upregulated in human glioma tissues versus normal brain tissues [16]. Furthermore, targeting miR-155 induced apoptosis and increased the chemosensitivity in GBM cells [17-19]. However, the role and mechanism of miR-155 in regulating apoptosis and chemosensitivity are still to be further elucidated.

BIM is a pro-apoptotic BH3-only Bcl2 family member that induces apoptosis via the mitochondria. Among Bcl-2 family members, BIM is

remarkable in that it can play a major role in mediating apoptosis on its own [20]. Translation of Bim mRNA is negatively regulated by a series of microRNAs (e.g., miR-9, -181a, -17~92, -25, -32, -221/222 and -301a) and RNA-binding proteins (e.g., Hsc70 and Hsp27). The importance of microRNAs in Bim regulation is demonstrated in embryonic stem cells deficient in the Argonaute (Ago) 1-4 proteins that are the core effectors of the microRNA pathway [21]. These embryonic stem cells are defective in microRNA silencing, show upregulated expression of the three isoforms of Bim (BimEL, BimL and BimS) and undergo apoptosis [21]. The upregulation of Bim was sufficient to induce apoptosis that could be prevented by simultaneous expression of activated Akt [21]. Reintroduction of any single Ago into Ago-deficient cells was able to rescue the endogenous miRNA silencing defect and apoptosis [21]. We therefore suggested that targeting miR-155 also promotes BIM-mediated apoptosis.

In the present study, we investigate the role of miR-155 on cell apoptosis, proliferation and chemosensitivity to Temozolomide (TMZ) as well as its possible molecular mechanisms.

Materials and methods

Cell culture

Human malignant glioma cell lines LN229, U87 and U251 were obtained from ATCC (the American Type Culture Collection, Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 2 mM L-glutamine (Invitrogen), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Invitrogen). All cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C.

Transfection of miRNA mimics, inhibitor, siRNA and cDNA

U87 and U251 cells were seeded in 6-well or 10-cm dishes. Transient transfections of miR-155 mimic [miR-155] (U87 cells) or inhibitor [anti-miR-155] (U251 cells) and negative control oligonucleotides (mimic-ctrl, or inhibitor-ctrl, NC) (Genepharma, Shanghai, China) at a final concentration of 50 nM were accomplished with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), following the manufacturer's

protocol. For stable miR-155 (anti-miR-155) transfection, we infected cells for 24 h and added 0.1% puromycin into medium 48 h after transfection. Cells were analyzed by fluorescence microscopy 48 h after transfection.

Similarly, cells were transiently transfected with plasmid pcDNA3.1 BIM cDNA (U251 cells) or BIM siRNA (U87 cells) and negative control scramble (pcDNA3.1, control siRNA, Genepharma) at a final concentration of 25 nM for 24 h. Protein assays and RT-PCR analyses were conducted 48 h after transfection. In addition, the stable miR-155 transfected U87 cells or stable anti-miR-155 transfected U251 cells and its controls were transiently transfected with BIM cDNA (U87 cells) or BIM siRNA (U251 cells) and negative control scramble (Genepharma) at a final concentration of 25 nM for 48 h. Protein assays and RT-PCR analyses were conducted 48 h after transfection.

Temozolomide (TMZ) treatment

U87 and U251 cells were treated with 1-100 µM TMZ for 24 h, or 100 µM TMZ for 2-24 h. Cells were transiently transfected with BIM cDNA (U251 cells) or BIM siRNA (U87 cells) and negative control scramble at a final concentration of 25 nM for 24 h, then treated with 1-100 µM TMZ for 24 h, or 100 µM TMZ for 2-24 h. The stable miR-155 transfected U87 cells were transiently transfected with BIM cDNA for 24 h, then treated with 100 µM TMZ for 24 h. The stable anti-miR-155 transfected U87 cells were transiently transfected with BIM siRNA for 24 h, then treated with 100 µM TMZ for 24 h. Cell apoptosis, Soft-agar colony formation, viability assays were performed at 24 h after TMZ treatment.

Reverse transcriptase-PCR (RT-PCR) and real-time PCR analysis

Total RNA was extracted from cells with Trizol Reagent (Invitrogen) in accordance with the protocol specified by the manufacturer, and its quality was assessed with a BioPhotometer (Eppendorf). First-strand cDNA was synthesized from 5 µg of total RNA using Superscript II reverse transcriptase (Invitrogen). 100 ng of total RNA was reverse transcribed into cDNA using the MirVana MiRNA Detection Kit (Ambion). Fifty nanograms of cDNA were used to perform a TaqMan Gene Expression Assay in a

final volume of 20 μ L. The expression analysis was carried out in a Prism 5700 Sequence Detection System (Applied Biosystems) and 96-well MicroPlates (Applied Biosystems). For all reactions, the TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) was utilized. Primers and probes for TaqMan PCR were obtained from Applied Biosystems predesigned TaqMan Gene Assays. Applied PCR conditions were 50°C for 2 min and 95°C for 10 min followed by 40 cycles at 95°C for 15 s and at 60°C for 40 s. All assays were run in triplicates. Analysis of relative gene expression data was performed employing the $2^{-\Delta\Delta C_t}$ method with the 18S rRNA as endogenous control. Semiquantitative RT-PCR was done to amplify *BIM* and *-actin*. The molecules were amplified using the pairs of primers: *BIM*: forward 5'-CTTCGGCCCGCTCTACCT-3' and reverse 5'-CCTCGCGTTACGTGGCTTAGG-3'; *-actin*: forward 5'-ACTACCATCACCATCTCTC-3' and reverse 5'-CTACACGCCAACGTTTCC-3'.

Western blot analysis

The primary antibody used was anti-BIM (Abcam, Qingdao, China). Protein samples were separated with 12% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were incubated with primary antibodies overnight at 4°C. Membranes were washed and incubated for 2 h with HRP-conjugated anti-rabbit secondary antibodies (ProSci, Poway, CA, USA), followed by detection and visualization using ECL Western blotting detection reagents (Pierce antibodies; Thermo Fisher Scientific).

MTT assay

For the MTT assay, 50 μ L of 0.25 mg/ml MTT-tetrazolium salts (Sigma-Aldrich Corp., Hangzhou, China) in PBS was added to each well. After 3 hours of incubation, the formazan crystals were dissolved by adding 2-propanol. The absorption of the formazan solution was measured using an Infinite M1000PRO Tecan spectrophotometer at a wavelength of 570 nm.

Soft-agar assays

The stable miR-155 transfected U87 cells or stable anti-miR-155 transfected U251 cells and its controls (10^5 per well) were mixed with 0.4% agarose in growth medium, plated on top of a solidified layer of 0.5% agarose in growth medium, in a 24-well plate, and fed every 3 d with

growth medium. After 2-3 wk, the colonies were dyed with Cristal Violet (0.01% solution), washed with PBS, and imaged by using a custom, automated plate imager with a digital camera.

Flow cytometry assay

The quantification of cell death was determined by flow cytometry using the Annexin V-FITC apoptosis detection kit according to the manufacturer's instructions (BD Pharmingen, BD Bioscience, USA). Briefly, 1×10^6 of the HT-29 cells were seeded into each Petri dish (30 mm) and after a 24 h incubation, various concentrations of the test compound were added and incubated for 24 h, 48 h, and 72 h, respectively. The cells were then washed with PBS, suspended in annexin V binding buffer and then added to an annexin V-FITC solution and propidium iodide (PI) for 10 minutes at room temperature. The samples were then analyzed using FACScalibur (BD Bioscience, USA) using CellQuest Pro analysis software (Becton Dickinson, USA).

Statistical analysis

The data are reported as the mean $\pm$ standard deviation. Comparisons were analyzed using Student's *t* test or ANOVA. *P* < 0.05 was considered to indicate statistical significance.

Results

Effect of miR-155 on proliferation, apoptosis and tumorigenicity of gliomas cells in vitro

We first evaluated *miR-155* expression in NL229, U87 and U251 cell lines by qRT-PCR. U251 cell line had significantly elevated levels of *miR-155*, whereas the U87 cell line had lower expression of *miR-155* (Figure 1A). So we used U251 and U87 cells for further study below.

To investigate the biological role of miR-155 expression in the development and progression of gliomas, U87 cells were transfected with *miR-155* and U251 cells were transfected with anti-*miR-155* oligonucleotides, respectively. The cells infected with scrambled oligonucleotides (NC) were used as controls. After 48 h of incubation, miR-155 was significantly increased in miR-155-transfected U87 cells as compared with that of negative control (NC) transfected cells, and miR-155 was significantly decreased in miR-155-transfected U251 cells as com-

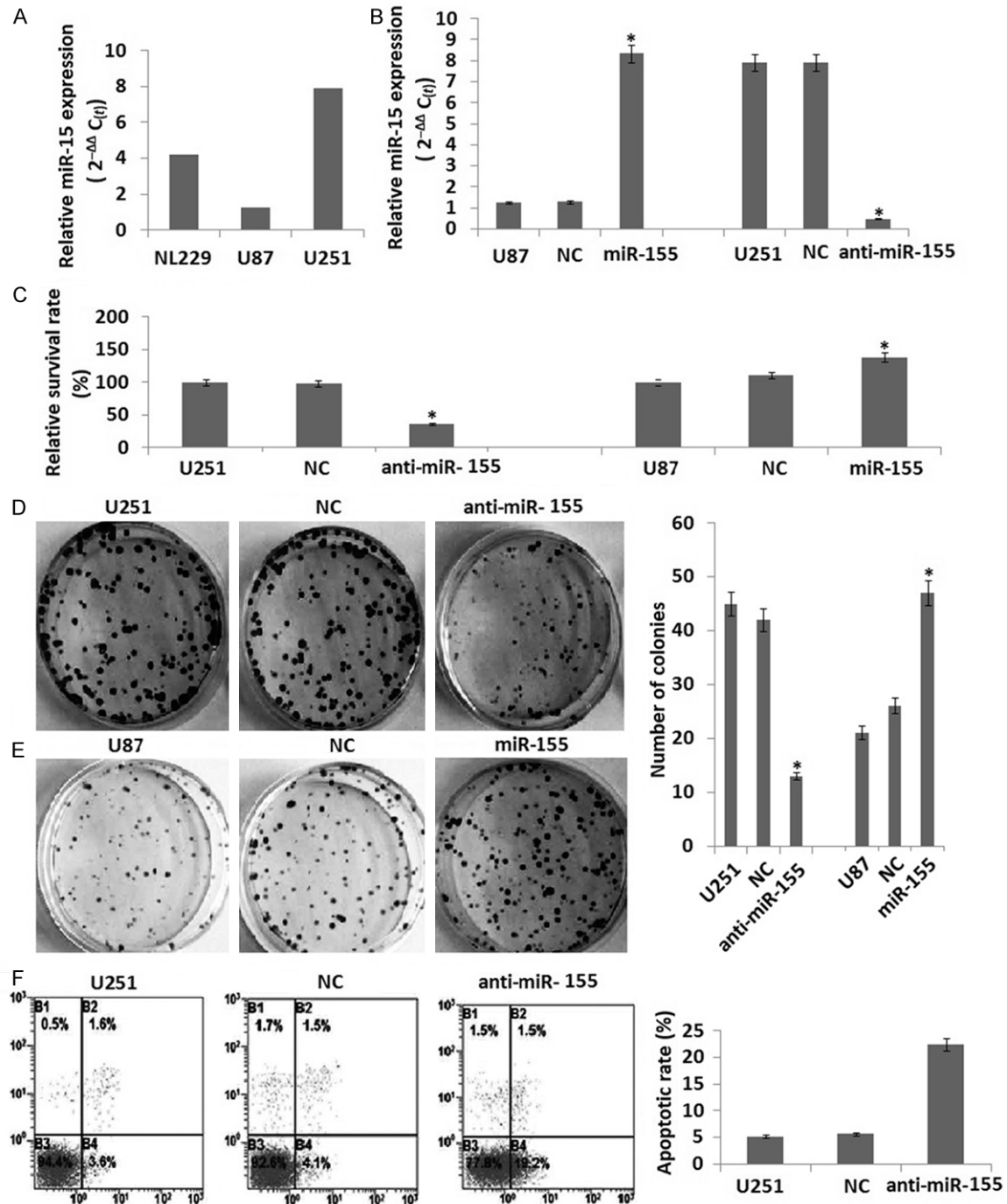


Figure 1. Effect of miR-155 on proliferation, apoptosis and tumorigenicity of gliomas cells. A. Real-time PCR analysis of miR-155 expression in LN229, U87 and U251 cell lines. B. Real-time PCR analysis of miR-155 expression in miR-155 or miR-155-transfected gliomas cell lines. Each bar represents the mean of three independent experiments. C. Effects of miR-155 overexpression or miR-155 downregulation on the growth of U87 or U251 cells. MTT assays revealed that miR-155-transfected cells proliferated more rapidly than the vector control cells, and anti-miR-155-transfected cells proliferated more slowly than the vector control cells. D. Representative micrographs (left) and quantification (right) of crystal violet stained U87 cell colonies. E. Representative micrographs (left) and quantification (right) of crystal violet stained U251 cell colonies. F. Flow cytometric analysis of apoptosis in U87 or U251 cells. This assay could discriminate between intact cells, early apoptotic cells, late apoptotic cells, and necrotic cells. The bar graph shows the average apoptosis rate (early apoptosis and late apoptosis) of anti-miR-155-transfected cells. Each bar represents the mean of three independent experiments. *P < 0.05.

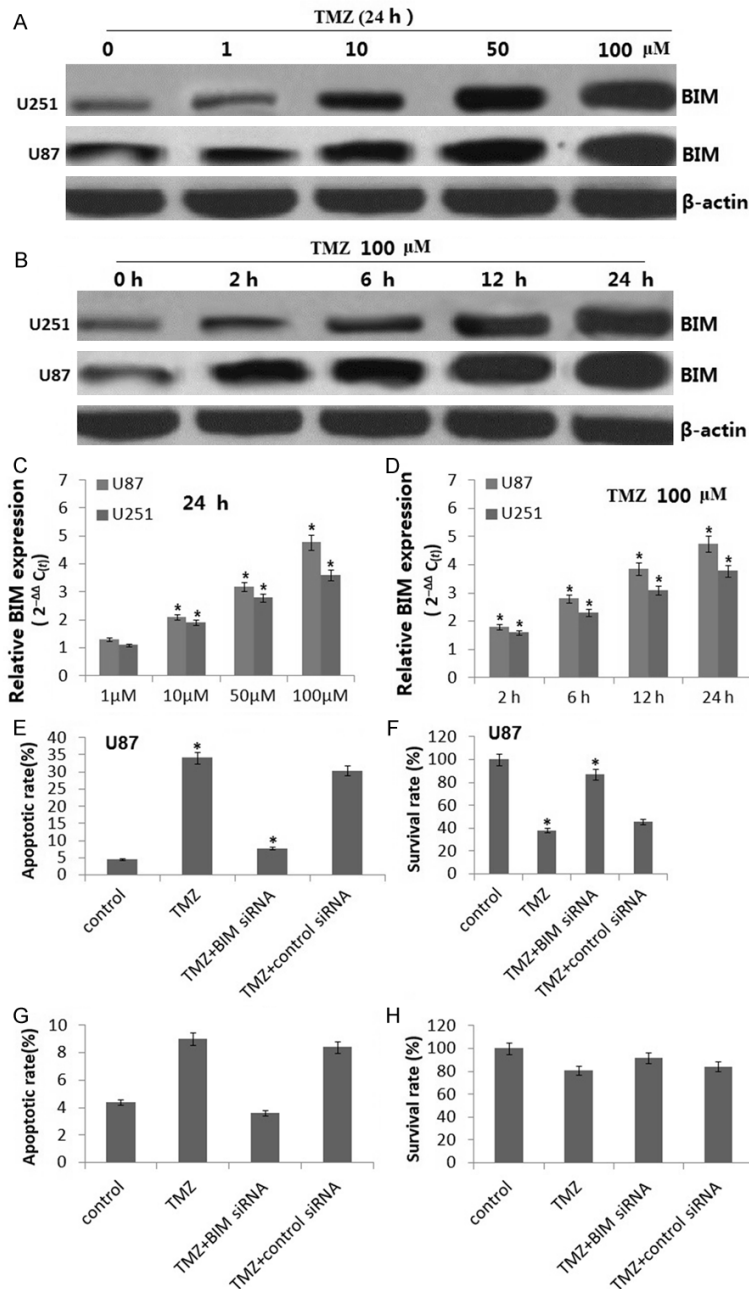


Figure 2. Effect of Temozolomide (TMZ) on proliferation, apoptosis and BIM expression of U87, U251 cells. A. Cells were treated with 1-100 μ M TMZ for 24 h. BIM protein expression was detected by western blot assay; B. Cells were treated with 100 μ M TMZ for 2-24 h. BIM protein expression was detected by Western blot assay; C, D. Cells were treated with 1-100 μ M TMZ for 24 h. BIM mRNA was detected by qRT-PCR; E, G. Cell apoptosis was detected by Flow cytometric analysis; F, H. Cell viability was detected by MTT assay. *P < 0.05.

pared with that of negative control (NC) transfected cells (**Figure 1B**). By using MTT assays, we observed that overexpression of miR-155 dramatically increased the growth rate of U87 cells as compared with that of negative control

(NC) transfected cells (**Figure 1C**). However, downregulation of miR-155 dramatically decreased the growth rate of U251 cells as compared with that of negative control (NC) transfected cells (**Figure 1C**). Moreover, ectopically expressing miR-155 in U87 cells significantly enhanced their anchorage-independent growth ability, as indicated by the increase in colony numbers (**Figure 1D**), suggesting that upregulation of miR-155 could augment the tumorigenicity of U87 cells *in vitro*. On the contrary, downregulation of miR-155 could inhibit the tumorigenicity of U251 cells *in vitro* (**Figure 1E**).

Cell apoptosis was determined using PI staining followed by flow cytometry analysis. Cells transfected with anti-miR-155 for 72 hrs. showed significant apoptosis in U251 cells, and the non-transfected cells showed less apoptosis, suggesting miR-155 targeting induced cell apoptosis (**Figure 1F**). However, miR-155 did not affect apoptosis in the U87 cells (data not show).

Temozolomide induces apoptosis by Bim-dependent way in glioma cells in vitro

To identify whether Bim modulates the response to temozolomide (TMZ), we treated the U87 and U251 cells with TMZ (1, 10, 50 and 100 μ M) for 24 h or TMZ (100 μ M) for 2, 6, 12 and 24 h. The results showed that Bim protein (**Figure 2A, 2B**) and mRNA (**Figure 2C, 2D**) was significantly induced within 24 h in U251 and U87 cells and the induction occurs in a time and concentration-dependent manner.

Treatment of 100 μ M TMZ for 24 h leads to a significant increase in U87 cell apoptosis

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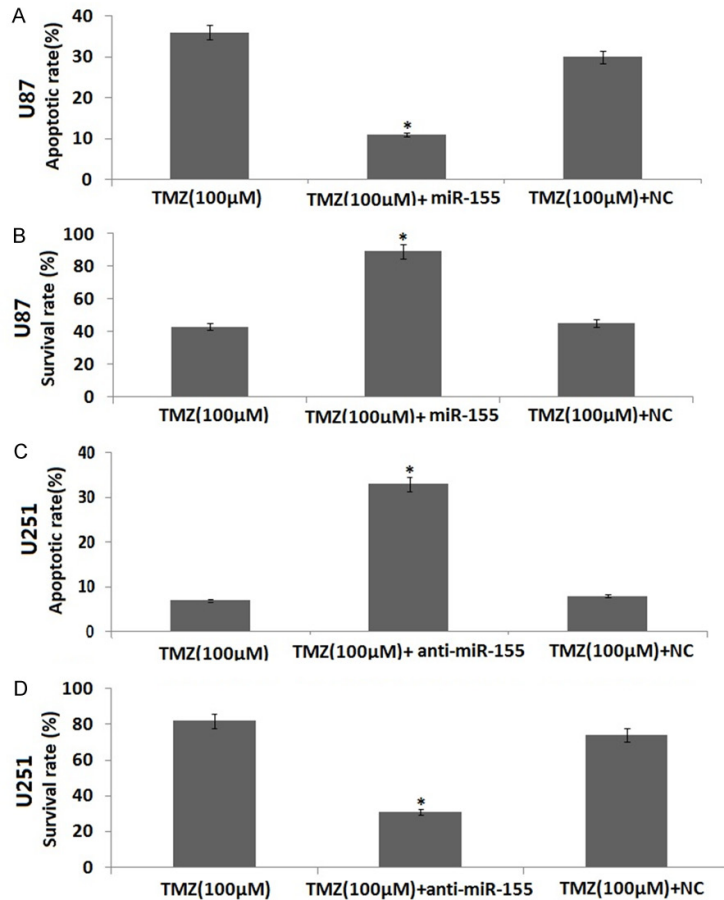


Figure 3. *miR-155 induces gliomas cell chemoresistance.* U251 cells or U87 cells were transfected with anti-*miR-155* or *miR-155* and NC for 6 h. Then the cells were treated with and without TMZ (100 μM) for 24 h. A, C. Apoptosis was assayed with Flow cytometric analysis. B, D. Cell survival was assayed by MTT. Data represent three independent experiments with four replicates each. *, $P < 0.05$. Error bars, S.E.

(Figure 2E) and decrease in U87 cell viability (Figure 2F). After Bim siRNA transfection, cell apoptosis and cell viability was reversed following TMZ treatment as assessed by flow cytometry analysis and MTT assay (Figure 2E, 2F). These data indicate that TMZ induces apoptosis by Bim-dependent way. Treatment of 100 μM TMZ for 24 h leads to a less increase in U251 cell apoptosis. Cell apoptosis and cell viability was also inhibited following Bim siRNA transfection as assessed by flow cytometry analysis and MTT assay (Figure 2G, 2H).

miR-155 is a determinant in chemosensitivity of gliomas in vitro

To examine the effect of *miR-155* on chemosensitivity, we ectopically expressed *miR-155* in U87 (low *miR-155*) and knocked down *miR-155* in U251 (high *miR-155*) cells. We found that

expression of *miR-155* renders U87 cells resistant to Temozolomide (TMZ) (Figure 3A, 3B). On the other hand, knockdown of *miR-155* sensitizes U251 cells to growth inhibition and apoptosis induced by TMZ (Figure 3C, 3D). These data indicate that *miR-155* is a determinant of chemosensitivity in gliomas cells.

Targeting miR-155 induces apoptosis by Bim-dependent way in glioma cells

We co-transfected Bim siRNA or control siRNA and anti-*miR-155* into U251 cells for 48 h. We found that Bim siRNA transfection blocked anti-*miR-155* induced Bim expression (Figure 4A, 4B), and inhibited apoptosis and promoted cell viability (Figure 4C, 4D) in the anti-*miR-155* transfected U251 cells. In contrast, Bim cDNA transfection reversed *miR-155*-induced the Bim expression (Figure 4E) and reversed *miR-155*-induced apoptosis and cell viability in the U87 cells (Figure 4F, 4G). These results provide mechanistic support in favor of our claim that the apoptosis-inducing effect by anti-*miR-155* is mediated through the activation of Bim pathway.

Targeting miR-155 enhances the sensitivity to Temozolomide by Bim-dependent way in glioma cells in vitro

The stably *miR-155* transfected U87 cells were transiently expressed Bim cDNA for 24 h, then treated with 100 μM TMZ for 48 h. The results showed that introduction of *Bim* cDNA abrogates *miR-155*-induced cell survival and chemoresistance by flow cytometry analysis (Figure 5A) and MTT assay (Figure 5B).

The stably anti-*miR-155* transfected U251 cells were transiently expressed Bim siRNA for 24 h, then treated with 100 μM TMZ for 48 h. The results showed that introduction of *Bim* siRNA abrogates anti-*miR-155*-induced cell apoptosis and chemosensitivity by flow cytometry analysis (Figure 5C) and MTT assay (Figure 5D).

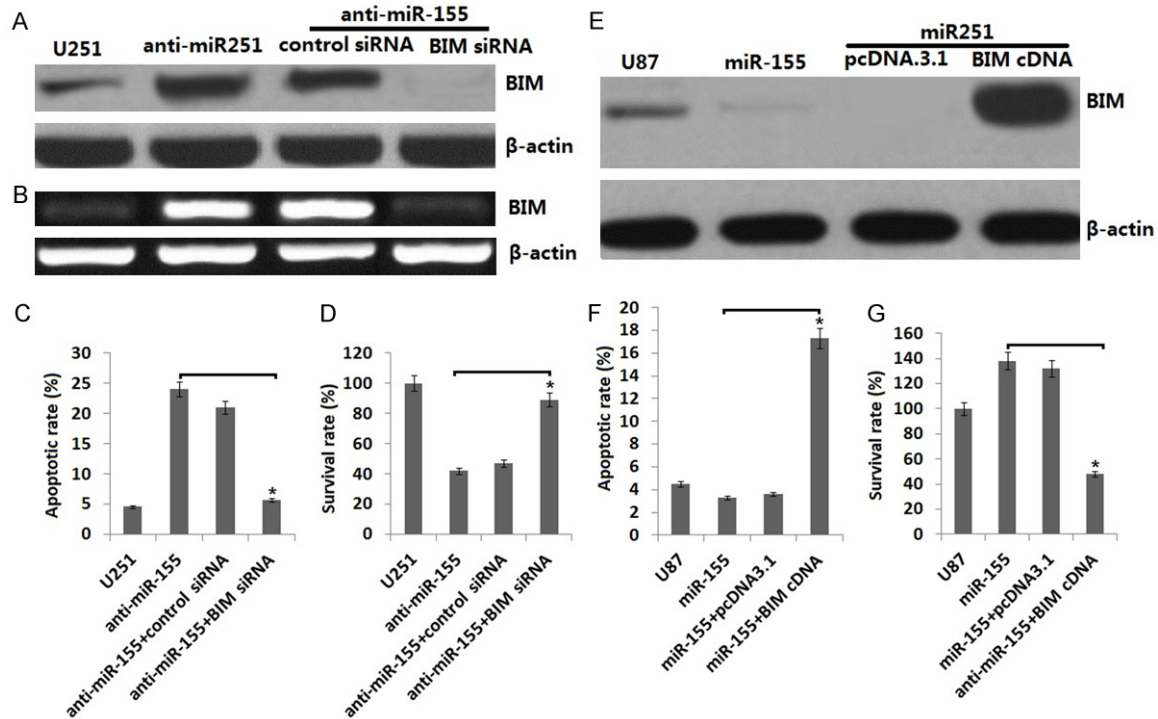


Figure 4. miR-155 regulates apoptosis by Bim-dependent way in glioma cells. Bim siRNA or control siRNA and anti-miR-155 was co-transfected into U251 cells for 48 h. BIM protein was detected by western blot assay (A); qRT-PCR assay (B); Cell apoptosis was detected by Flow cytometric analysis (C); Cell viability was detected by MTT assay (D); Bim cDNA or control pcDNA3.1 and miR-155 was co-transfected into U87 cells for 48 h. BIM protein was detected by Western blot assay (E); Cell apoptosis was detected by Flow cytometric analysis (F); Cell viability was detected by MTT assay (G); *, $P < 0.05$. Error bars, S.E.

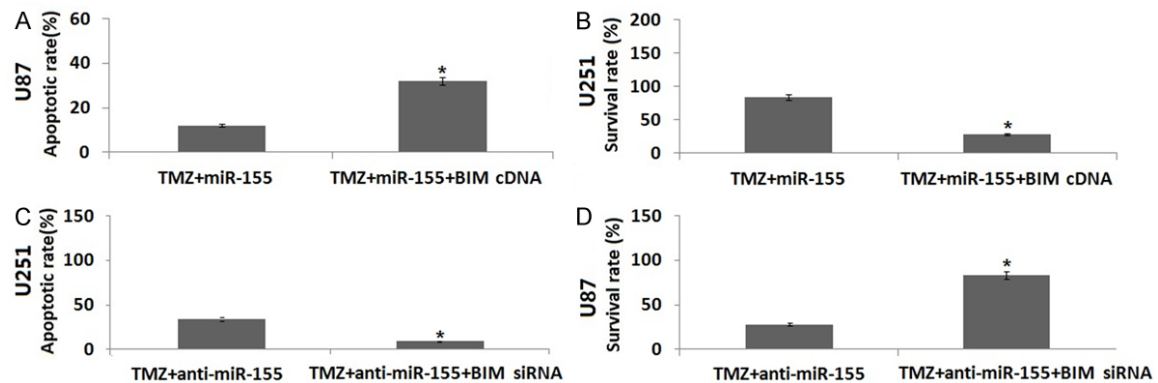


Figure 5. miR-155 regulates the sensitivity to Temozolomide by Bim-dependent way in glioma cells in vitro. The stably miR-155 transfected U87 cells were transiently expressed BIM cDNA for 24 h, then treated with 100 μ M TMZ for 48 h. Cell apoptosis was detected by Flow cytometric analysis (A); Cell viability was detected by MTT assay (B); The stably anti-miR-155 transfected U251 cells were transiently expressed BIM siRNA for 24 h, then treated with 100 μ M TMZ for 48 h. Cell apoptosis was detected by flow cytometric analysis (C); Cell viability was detected by MTT assay (D); *, $P < 0.05$. Error bars, S.E.

Discussion

The mechanisms of miR-155 functions as an oncomiR are largely unknown. In cancer, dereg-

ulation of *miR-155* is implicated in a wide range of malignancies, including various forms of lymphoma and carcinomas of breast, lung, pancreas, head and neck, and kidney [22-26]. In

immunology and lymphoma, *miR-155* has been extensively investigated; however, it is only evident that *miR-155* expression is elevated in gliomas [16], and detailed function remains elusive. Our study here was to investigate the effects of miR-155 on cell proliferation, cell apoptosis and chemosensitivity of gliomas cells and tried to explore the mechanisms of miR-155 in this process. We observed that up-regulation of miR-155 significantly stimulated cell growth and induced chemoresistance in gliomas cells *in vitro*, which was consistent with results that blocking miR-155 using miR-155 inhibitors inhibited cell growth, promoted cell apoptosis and induced chemosensitivity *in vitro*.

The BH3-only Bim protein is a major determinant for initiating the intrinsic apoptotic pathway under both physiological and pathophysiological conditions. Several studies suggest that Bim functions as a tumor suppressor. In mice, inactivation of one allele of Bim accelerates Myc-induced B cell leukemia [27]. Similarly, Bim deficiency in mice overexpressing the E_v-Abl oncogene accelerated the development of plasmacytomas [28]. These authors [29] also showed that Bim-deficiency led to paclitaxel-resistant tumor cells. Tumor cells have evolved different mechanisms to suppress Bim expression and/or activity thereby overcoming the apoptotic barrier that else would have led to their eradication. The efficacy of many anti-cancer drugs depend on Bim, and insufficient Bim induction or Bim function is often an underlying cause of therapy failure [30]. Many cancer cells have developed one or more mechanisms for preventing Bim from acting, intervention of which may result in the reactivation of the apoptotic process.

Translation of Bim mRNA can be regulated through the 3'-untranslated region (3'-UTR) [31, 32]. This region binds microRNAs and RNA-binding proteins (RBPs) that regulate mRNA stability and/or translation [33]. Of note, miR-17~92 targets Bim [34], making it a potential therapeutic target for increasing the response of anti-cancer drugs. miR-32 was also found to target Bim in prostate cancer [35]. miR-301a promotes pancreatic cancer cell proliferation by inhibiting Bim expression [36], while miR-363 supports human glioma stem cell survival for the same reason [37]. Our data showed that overexpression of miR-155 not only led to the

down-regulation of BIM but also inversed the effect of BIM by promoting cell proliferation, suppressing cell apoptosis and increasing chemoresistance *in vitro*. Restoration of BIM by knockdown of miR-155 using miR-155 inhibitors was accompanied by decreased cell proliferation, enhanced cell apoptosis and increased chemosensitivity. These findings demonstrate for the first time that deregulation of *miR-155* in gliomas is associated with chemosensitivity and that *BIM* is a target of *miR-155*. The conclusion that miR-155 negatively regulated the protein expression of BIM was further supported by data showing that miR-155 significantly decreased the relative BIM mRNA expression. MiR-155 can directly repress BIM expression, thereby negatively regulating BIM function. Thus, we have reasons to believe that BIM is possible the target gene of miR-155 in gliomas cells.

In conclusion, our researches suggest that miR-155 functions as an oncomiR by targeting BIM and contributes to the control of cell survival, growth and chemosensitivity in gliomas cells. Pro-apoptotic gene BIM is negatively regulated by miR-155 and mediates miR-155 functions in inducing cell proliferation and inhibiting cell apoptosis. Therefore, BIM is identified to be the direct target of miR-155. The significantly enhanced antitumor activity that results from the combination of TMZ and targeting miR-155 offers promise as a novel treatment for glioma patients.

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Disclosure of conflict of interest

None.

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