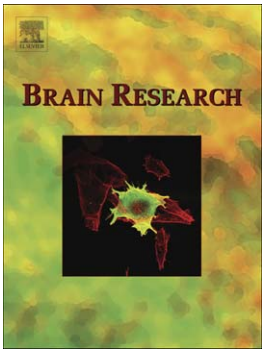

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Title Page

MiR-21 protected human glioblastoma U87MG cells from chemotherapeutic drug temozolomide induced apoptosis by decreasing Bax:Bcl-2 ratio and caspase-3 activity

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Abstract

MicroRNAs (miRNAs) are small noncoding RNA molecules that regulate protein expression by cleaving or repressing the translation of target mRNAs. In mammal animals, their function mainly represses the target mRNAs transcripts via imperfectly complementary to the 3'UTR of target mRNAs. Several miRNAs have been recently reported to be involved in modulation of glioma development, especially some up-regulated miRNAs, such as microRNA-21 (miR-21), which has been found to function as an oncogene in cultured glioblastoma multiforme cells. Temozolomide (TMZ), an alkylating agent, is a promising chemotherapeutic agent for treating glioblastoma. However, resistance develops quickly and with high frequency. To explore the mechanism of resistance, we found that miR-21 could protect human glioblastoma U87MG cells from TMZ induced apoptosis. Our studies showed that TMZ markedly enhanced apoptosis in U87MG cells compared with untreated cells ($P<0.05$). However, over-express miR-21 in U87MG cells could significantly reduce TMZ-induced apoptosis ($P<0.05$). Pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins are known to regulate the apoptosis of glioma cells. Bcl-2, resistance to induction of apoptosis, constitutes one major obstacle to chemotherapy in many cancer cells. Bax is shown to correlate with an increased survival of glioblastoma multiforme patients. Further research demonstrated that the mechanism was associated with a shift in Bax:Bcl-2 ratio and change in caspase-3 activity. Compared to control cells, cells treated with TMZ showed a significant increase in the Bax:Bcl-2 ratio and caspase-3 activity ($P<0.01$). However, such effect was partly prevented by treatment of cells with miR-21 overexpression before, which appeared to downregulate the Bax expression, upregulate the Bcl-2 expression and decrease caspase-3 activity. Taken together, these results suggested that over-express miR-21 could inhibit TMZ-induced apoptosis in U87MG cells, at least in part, by decreasing Bax:Bcl-2 ratio and caspase-3 activity, which highlighted the possibility of miR-21 overexpression in the clinical resistance to chemotherapeutic therapy of TMZ.

Keywords: MicroRNA; Temozolomide; Apoptosis; Bax; Bcl-2

1. Introduction

Malignant gliomas, the most common primary malignant tumors of the brain, are aggressive, highly invasive, and neurologically destructive. Despite the combination of surgery, chemotherapy, and radiotherapy, the median survival duration of patients with glioblastoma multiforme (GBM), the most aggressive type of malignant glioma, is only 9 to 12 months (Yung et al., 1996). Currently, first-line therapy for all GBM patients after surgery consists of the combination of temozolomide (TMZ) and regional fractionated radiation followed by TMZ alone (Agarwala and Kirkwood, 2000; Chakravarti et al., 2006; Clarke et al., 2009; Maxwell et al., 2008).

TMZ, a 3-methyl derivative of mitozolomide, is an alkylating chemotherapeutic drug that readily crosses the blood-brain-barrier in glioblastoma patients. It has shown anti-tumor activity and relatively low toxicity in Phase 2 and 3 clinical trials in patients with malignant glioblastomas. It can efficiently inhibit the proliferation of glioma cells and induce apoptosis (Athanassiou et al., 2005; Nagane et al., 2007; Groves et al., 2002; Bektas et al., 2009; Bocangel et al., 2002). However, the action of TMZ in glioblastoma cells remains largely undefined. Glioblastomas are relatively resistant to the cytotoxic effects of TMZ. Previous researches have shown that sensitivity of glioma cells to TMZ is dependent on the level of cellular O6-methylguanine-DNA methyltransferase (MGMT) repair activity (Nagane et al., 2007). Here, we reported that upregulation of miR-21 expression in human glioblastoma U87MG cells also decreased the sensitivity of glioblastoma cells to TMZ in vivo.

MicroRNAs (miRNAs) are small regulatory RNA molecules that in recent years have been identified in the progression of various cancers and proposed as novel targets for anticancer therapies (Zeng et al., 2003). Recently, microRNA-21 (miR-21) has been reported to be overexpression in glioblastomas, and function as an oncogene involved in the pathogenesis of glioblastoma cell lines. Inhibiting miR-21 expression leads to proliferation inhibition and apoptotic cell death in multiple glioblastoma cell lines (Silber et al., 2009; Conti et al., 2009; Corsten et al., 2007; Shi et al., 2008; Gabriely et al., 2008; Chan et al., 2005). On the other hand, previous studies have shown that correction of altered expression of miRNA has significant

implications for therapeutic strategies aiming to overcome cancer cell resistance. It is evidenced by that inhibition of miR-21 and miR-200b sensitizes cholangiocytes to gemcitabine; downregulation of miR-451 leads to the increased metabolism of DOX; downregulation of miR-328 results in increased mitoxantrone sensitivity; and overexpression of miR-221 and miR-222 in MCF-7 cells confers resistance to tamoxifen (Ren et al., 2010; Kovalchuk et al., 2008; Miller et al., 2008; Zhao et al., 2008; Meng et al., 2006). Thus, we infer that miR-21 somehow favors tumor growth by impeding apoptosis，and is a key factor in resistance to chemotherapeutic therapy of TMZ.

To prove this hypothesis, we further improved the levels of miR-21 expression in U87MG cells to observe its affect of the sensitivity of glioma cells to TMZ. Our results showed that compared to control (CTL) cells, TMZ caused apoptosis in U87MG cells at a rate of approximate 53%, however, this effect was partly abolished by upregulation of miR-21 expression in U87MG cells, and over-express miR-21 decreased the TMZ-induced apoptosis to a rate of approximate 39%. Then, we sought to investigate the mechanism of miR-21 overexpression on inhibiting TMZ-induced glioma cell apoptosis. Pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins are known to regulate the apoptosis of glioma cells (Manero et al., 2006). Bcl-2, resistance to induction of apoptosis, constitutes one major obstacle to radiotherapy and chemotherapy in many cancer cells (Wick et al., 2001). Bax plays a major role in the apoptotic response of glioblastoma multiforme cells, the high expression of which is shown to correlate with an increased survival of glioblastoma multiforme patients (Cartron et al., 2002). And a low Bax:Bcl-2 ratio was usually observed in glioblastoma multiforme patients (Shinoura et al., 1999). Therefore, we investigated the levels of these apoptosis regulatory proteins following treatment of U87MG cells with miR-21 overexpression and TMZ. We found that a treatment of U87MG cells with miR-21 overexpression before TMZ decreased the level of Bax protein and increased the level of Bcl-2 protein, compared with treatment with TMZ alone. The caspases, especially caspase-3, are known to act downstream of Bax/Bcl-2 and play a key role in the execution of apoptosis (Salakou et al., 2007). We also found that TMZ effectively increased the activity of caspase-3; however, a treatment with miR-21 overexpression before TMZ decreased the caspase-3 activity induced by TMZ. Taken together, this study demonstrated that the overexpression of miR-21 in U87MG cells protected cells from TMZ

induced apoptosis by decreasing Bax:Bcl-2 ratio and caspase-3 activity.

2. Results

2.1. Evaluation of miR-21 expression in human glioma tissues, normal brain tissues and U87 glioma cells

In previous research, Chan et al. showed markedly elevated miR-21 levels in human glioblastoma tumor tissues and in six established glioblastoma cell lines (A172, U87, U373, LN229, LN428, and LN308) (Chan et al., 2005). To investigate whether miR-21 was also elevated in glioma tissues from Chinese patients and U87MG cells, we performed the TaqMan-based real-time stem-loop RT-PCR analyses. Our data showed similar results that miR-21 were strongly elevated in all grade glioma samples (WHO-III and WHO-IV glioma tissues) and U87MG cells versus normal brain tissues（$P < 0.01$）(Fig. 1A).

Then to construct miR-21 overexpression model, U87MG cells were transfected with miR-21 overexpression by FuGENE HD6 (Fig. 1B). As shown in Fig. 1C, the miR-21 expression was increased about 30-fold in miR-21 overexpression model compared with control（$P < 0.01$）.

2.2. Evaluation of viability and apoptotic death morphologically

As shown in Fig. 2A, it seemed that 100 μM TMZ could not only inhibit the growth of U87MG cells, but also induce some U87MG cells apoptosis or necrosis from the morphological observation. And a treatment of cells with miR-21 overexpression before TMZ seemed to decrease this trend in U87MG cells treated with 100 μM TMZ. To identify the cell viability changes, the trypan blue dye exclusion assay was evaluated in U87MG cells using a hemocytometer after all treatments. Trypan blue is the most commonly used test the integrity of the biological membrane staining reagents. Healthy normal cells exclude trypan blue, while only nonviable cells take up trypan blue. In this study, results showed cell viability of U87MG cells was significantly decreased by the clinically used methylating agent 100 μM TMZ. However, a treatment with miR-21 overexpression before TMZ prevented the decrease of cell viability induced by 100 μM TMZ (Fig. 2B).

Morphological features of apoptosis were observed in TMZ, and miR-21 overexpression+TMZ by Hoechst 33258 staining after all treatments, and counted to determine the amount of apoptotic cell death based on characteristic morphological features (Fig. 2C). All treatment groups were examined under the fluorescence microscopy and cells were counted to determine the percentage of apoptotic cells (Fig. 2D). Morphological observation showed that TMZ could significantly induced apoptosis in U87MG cells compared with control ($P < 0.05$), however, as shown in Fig. 2C, upregulating the level of miR-21 expression could partly decreased the percentage of apoptotic cells induced by TMZ ($P < 0.05$).

2.3. Explicit evaluation of the apoptosis rate by FACS Analysis

Fluorophore-labeled Annexin V (a protein that exhibits nanomolar affinity for phosphatidylserine) binding to externalized phosphatidylserine has been extensively employed as a reliable marker of apoptosis (Narayan et al., 2001). After cell apoptosis was evaluated by morphological analysis, we used Annexin V-propidium iodide staining to improve and quantify apoptosis detection. As shown in Fig. 3, the control, negative control or miR-21 overexpression treated cells showed little or no apoptotic cells. Apoptosis was obviously induced by TMZ compared with untreated controls ($P < 0.01$). Apoptosis rate was increased from 4% in medium controls to approximate 53% in treatment with TMZ ($P < 0.01$). However, when cells were pre-transfected with miR-21 overexpression, the apoptosis effect was partly prevented, and apoptosis rate of U87MG cells induced by TMZ decreased to approximately 39%. Further statistical analysis indicated that reduction of apoptosis by miR-21 overexpression treatment before TMZ was significant different from treatment with TMZ alone ($P < 0.05$). Collectively, these data suggested markedly elevated miR-21 levels in U87MG cells decrease the sensitivity of glioma cells to TMZ and protected cells from TMZ induced apoptosis.

2.4. MiR-21 blocked TMZ induced apoptosis via an decrease in Bax:Bcl-2 ratio

Previous study showed the disorders of the expression of the Bcl-2 family of proteins in human glioblastoma and cell lines enhanced cell survival by inhibiting apoptosis (Bojes et al., 1998). Bcl-2 family, including Bcl-2, Bcl-X_L, Bax, and Bad, regulates various steps in apoptosis. Bcl-2

and Bcl-X_L block cell death whereas Bax and Bad promote programmed cell death (Zhai et al., 2008; Simonian et al., 1997; Terrano et al., 2010). The decrease in Bax:Bcl-2 ratio is thought to contribute to the resistance of glioma cells to anticancer therapy by modulating the apoptotic cascade (Weller et al., 1997). To confirm that the inconsequence of miR-21 overexpression on the decrease of sensitivity to TMZ relied on the alterations of Bax:Bcl-2 ratio, the levels of Bax and Bcl-2 were measured by western blot experiments in all treatment groups. In this study, we observed that there was no significant difference (P>0.05) in Bax:Bcl-2 ratio between control group or negative control group and miR-21 overexpression group (Fig. 4A and 4B). Compared to control cells, a increase in Bax:Bcl-2 ratio was observed in cells exposed to TMZ (P<0.01). However, a treatment of cells with miR-21 overexpression before TMZ decreased TMZ-induced Bax:Bcl-2 ratio, compared to treatment of cells with TMZ alone (P<0.01) (Fig. 4A and 4B). Taken together, these results showed that the sensitizing activity of U87MG cells to TMZ is critically determined by the cellular Bax:Bcl-2 ratio, and the intrinsic pathway of apoptosis was involved in altered miR-21 levels.

2.5. Determination of Caspase-3 Activity

Caspase proteins are cysteine proteases that act downstream of the Bcl-2 family by initiating cellular breakdown during apoptosis. Among the effector caspases, caspase-3 is most frequently involved in neuronal apoptosis (Jarskog et al., 2004; Kobayashi et al., 2007). To determine whether caspase-3 is activated after TMZ and/or miR-21 overexpression treatment, caspase-3 activity was measured by the Caspase-3 activity kit. In this study, results showed that there was no significant difference (P>0.05) in caspase-3 activity between miR-21 overexpression group and control group or negative control group. Compared to control cells, treatment of cells with TMZ alone caused a significant increase in caspase-3 activation (P<0.05). Caspase-3 activity in cells treated with TMZ+miR-21 overexpression was more than 30% decrease (P<0.05), compared to cells treated with TMZ alone. These results suggested that the overexpression of miR-21 could significantly decrease the caspase-3 activity activated by TMZ in U87MG cells.

3. Discussion

Temozolomide (TMZ) is a DNA-methylating agent that has recently been introduced into Phase 2 and 3 trials for the treatment of gliomas (Hirose et al., 2001). Previous studies showed TMZ could effectively inhibit glioma cell growth and induce apoptosis (Kim et al., 2006; Son et al, 2006). MiR-21, which was significantly elevated in glioblastoma and regulated multiple genes associated with cancer cell proliferation, apoptosis, and invasiveness, functioned as an antiapoptotic factor in cultured glioblastoma multiforme cells (Papagiannakopoulos et al., 2008). Thus, we speculated that miR-21 might become a target to regulate the chemotherapeutic effect in cancer therapy. So far, the effect and mechanism of miR-21 on regulating the chemotherapeutic effect of TMZ has not been studied in human glioblastoma multiforme cells. In this study, we first evaluated the apoptotic effect of miR-21 overexpression and TMZ on the glioblastoma U87MG cell, and showed that increased miR-21 expression could reduce TMZ induced apoptosis rate of U87MG cells. Next, we found that treatment of miR-21 overexpression before TMZ leaded to reduction of Bax:Bcl-2 ratio and caspase-3 activity. In this report, we provide data indicating the importance of miR-21 dysregulation in the acquisition of glioma cell resistance to TMZ, and report for the first time increased expression of miR-21 is as a potential signature of TMZ resistance in glioma.

At present, cancer drug resistance is considered as a multifactorial phenomenon involving several major mechanisms, such as decreased uptake of water-soluble drugs, increased repair of DNA damage, reduced apoptosis, altered metabolism of drugs, and increased energy-dependent efflux of chemotherapeutic drugs that diminish the ability of cytotoxic agents to kill cancer cells (Prokopenko and Mirochnitchenko, 2009; Chekhun et al., 2007; Roberti et al., 2006). MiRNA expression affecting multiple genes simultaneously provided support for this hypothesis (Mendes et al., 2009; Pradervand et al., 2009). Recent findings have confirmed a critical role of miR-21 as powerful diagnostic and prognostic indicators of human gliomas, resulting in the development of novel approaches to gliomas management (Krichevsky and Gabriely, 2009; Shi et al., 2008). Despite the well-established role of miR-21 in gliomas and the dedication of research on the elucidation of the molecular mechanisms involved in the development of resistance gliomas cells to chemotherapy, the role of miR-21 in gliomas drug resistance remains largely unexplored. In this study, our findings supported a relationship between miR-21 and TMZ-induced apoptosis of U87MG cells. We showed that a treatment of human glioblastoma U87MG cells with miR-21

overexpression before TMZ decreased the chemotherapeutic action of TMZ. The results strongly suggested that miR-21 overexpression treatment could well interfere with chemotherapy efficacy of TMZ in human glioma cells.

Several in vitro studies have documented a role for miR-21 in apoptosis of human glioma cells (Ren et al., 2010). However, the mechanisms of miR-21 mediated TMZ-induced cell death are not yet fully understood. Apoptosis is regulated by several protein families, including the upstream Bcl-2 family (e.g., the antiapoptotic Bcl-2 and proapoptotic Bax) and the downstream caspase family (e.g., caspase-3) (Jarskog et al., 2004; Kobayashi et al., 2007). Previous studies have shown that treatment with TMZ changes the expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 involved in the mitochondrial pathway of apoptosis (Ma et al., 2002; Das et al., 2004). Si et al. and Li et al. recently showed the knockdown of miR-21 inhibited tumor cell growth in vitro and in vivo by affecting an increase in apoptosis associated with downregulation of Bcl-2 expression and upregulation of Bax expression (Si et al., 2007; Li et al., 2009). Thus, we examined the levels of expression of Bax and Bcl-2 proteins in U87MG cells following treatment with TMZ and miR-21 overexpression. Results demonstrated that treatment of cells with miR-21 overexpression prior to treatment with TMZ appeared to decrease the upregulation of Bax expression and increase the downregulation of Bcl-2 expression in treatment with TMZ alone, which indicated an efficient inhibition effect of miR-21 overexpression on TMZ mediated regulation in Bax:Bcl-2 ratios.

Previous research has demonstrated that Bcl-2 acts to prevent the release of cytochrome c and caspase activation, while Bax has the opposite function, which in turn promotes the release of cytochrome c into the cytosol from mitochondria and activates caspase 3 (Kluck et al., 1997; Ryan et al., 2001). In this study, caspase-3 activity was measured in all treatment groups. We showed that caspase-3 activity increased more than 2-fold in TMZ treatment alone, compared to control group or negative control group. However, this effect of caspase-3 activity induced by TMZ treatment was partly prevented by miR-21 overexpression. These results indicated that miR-21 overexpression could, at least in part, inhibit TMZ induced the activation of mitochondria-related apoptosis.

In conclusion, this study demonstrates that in vivo miR-21 overexpression protected human

glioblastoma U87MG cells from TMZ induced apoptosis by decreasing Bax:Bcl-2 ratio and the downstream caspase-3 activity.

4. Materials and Methods

4.1. Cell culture and treatments

Human glioma cell line, U87MG, was purchased from the Chinese Academy of Sciences Cell Bank. U87MG glioma cells were maintained in a 37°C, 5% CO_2 incubator in DMEM supplemented with 10% fetal bovine serum (FBS) and were routinely passaged at 2- to 3-day intervals. And experiments were divided into five groups as control group, negative control group, miR-21 overexpression group, TMZ group, and miR-21 overexpression + TMZ group. U87MG were treated with miR-21 overexpression vectors before TMZ for 48 h. Then cells were subsequently treated with 100 μM TMZ for 6 h. After all treatments, cells were washed with drug-free medium and allowed to grow for 48 h.

4.2 Plasmids transfection

Expression vectors for pre-miR-21 and negative vectors (Genesil, Wuhan, China) were constructed by technical support from Wuhan Genesil. U87MG cells were grown to 70~80% confluence in 12-well plates (BD Biosciences). And then miR-21 and negative vectors were transfected into U87MG cells with FuGENE HD6 (Roche) at a ratio of 3 μl FuGENE HD6 per 1 μg DNA according to the manufacturer's instructions. Cells were screened by the aminoglycoside G418 after 24 h transfection. The aminoglycoside G418 resistant U87MG glioma cells with high levels of mature miR-21 were identified by TaqMan-based real-time quantification RT-PCR after 48 h.

4.3. RNA isolation

Human glioma tissue samples were obtained from the first affiliated hospital of Nanjing Medical University after informed consent from adult patients diagnosed with glioma, three WHO-II, three WHO-III and three WHO-IV glioma tissues, freshly resected during surgery and immediately

frozen in liquid nitrogen for subsequent total RNA extraction. Two normal adult brains were obtained after informed consent from the patients with severe traumatic brain injury (TBI) who needed post-trauma surgery. RNA was extracted from tissues and U87MG glioma cell line using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions.

4.4. Real-time quantification of miRNAs by stem-loop RT-PCR

For the TaqMan-based real-time reverse transcription-polymerase chain reaction (RT-PCR) assays, the ABI 7300 HT Sequence Detection system (Applied Biosystems, Foster City, CA) was used. All the primers and probes of the miR-21 (P/N: 4373090) and RNU6B endogenous controls (P/N: 4373381) for TaqMan miRNA assays were purchased from Applied Biosystems. Real-time PCR was performed as described in Chen et al. (Chen et al., 2005). Relative gene expression was calculated via a $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

4.5. Trypan blue dye exclusion test for cell viability

Following all treatments the viability of attached and detached cell populations was evaluated by trypan blue dye exclusion test. Cells were harvested with trypsin/EDTA, suspended in PBS and mixed with an equal amount of 0.4% trypan blue stain (Invitrogen) after all treatments. The number of cells excluding trypan blue, representing viable cells, was then counted. Viable cells maintained membrane integrity and did not take up trypan blue. Cells with compromised cell membranes took up trypan blue, and were counted as dead. Cells were counted in four different fields and the number of viable cells was calculated as percentage of the total cell population. The count for non-treated cells was considered 100%.

4.6. Hoechst 33258 staining for morphological analysis of apoptosis

U87MG cells were seeded on sterile cover glasses placed in the 6-well plates. After all treatments, cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min, and then incubated with 50 μM Hoechst33258 staining solution for 10 min. Apoptotic morphological changes in the nuclear chromatin of cells were detected, and then counted under a fluorescence microscope. The percentage of apoptotic cells was calculated from three separate

experiments.

4.7. Apoptosis assays

U87MG glioma cells were plated in 12-well plates, transfected with miR-21 for 48 h, and the treated with 100 μM TMZ for 6 h, after which the cells were washed and placed in TMZ-free medium and allowed to grow for 48 h. The apoptosis ratio was analyzed after all treatments via using Annexin V FITC Apoptosis Detection Kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. Annexin V/FITC and propidium iodide double stain was used to evaluate the percentages of apoptosis. Annexin V^- and PI^- cells were used as controls. Annexin V^+ and PI^- cells were designated as apoptotic and Annexin V^+ and PI^+ cells displayed necrotic. Tests were repeated in triplicate.

4.8. Western blot analysis

To determine the levels of protein expression, soluble proteins were isolated by lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM MgCl2, 0.1% Triton X-100, 25 mM MOPS, 100 μM phenylmethylsulfonyl fluoride and 20 μM leupeptin, adjusted to pH 7.2). One-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed with a corresponding gel concentration using the discontinuous buffer system of Laemmli (Bio-Rad Laboratories, Richmond, CA). The electrophoresed proteins were transferred to a polyvinylidene difluoride membrane and subjected to immunoblot analysis with antibodies to Bax and Bcl-2 (used at a 1/200 dilution, Santa Cruz Biotechnology). The reaction was detected with enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL). The membranes were reblotted with a β-actin antibody (1/2000, Santa Cruz Biotechnology) after washing to check for equal loading of the gel.

4.9. Caspase-3 Activity Assay

The activity of caspase-3 was determined using the Caspase-3 activity kit (Beyotime Institute of Biotechnology, Haimen, China). To evaluate the activity of caspase-3, cells were homogenized in 100 mL reaction buffer (1% NP-40, 20 mM Tris-HCl (pH 7.5), 137 mM Nad and 10% glycerol)

containing 10 mL caspase-3 substrate (Ac-DEVD-pNA) (2 mM) after all treatments. Lysates were incubated at 37℃ for 2 h. Samples were measured with an ELISA reader at an absorbance of 405nm.

5.0. Statistical analysis

All tests were done using SPSS Graduate Pack 11.0 statistical software (SPSS, Chicago, IL). Descriptive statistics including mean and SE along with one-way ANOVAs were used to determine significant differences. $P<0.05$ was considered significant.

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Figure Legends

FIG. 1. miR-21 expression in glioma progression and U87MG cells. TaqMan-based real-time stem-loop RT-PCRs for miR-21 have been performed with primers specific for mature miRNAs

among control brain tissues, glioblastoma tumor tissues and glioblastoma cells. (A) Levels of miR-21 expression in glioma tissues, normal brain tissues and U87MG cells. Significant difference between normal brain tissues and WHO-III, WHO-IV glioma tissues or U87MG cells was indicated by ** ($P < 0.01$). (B) U87MG cells were transfected with negative vectors. Significant difference between CTL and transfection group was indicated by ** ($P < 0.01$). (C) Levels of miR-21 expression in U87MG cells after transfection with miR-21 overexpression and negative vectors. Significant difference between miR-21 overexpression and CTL or NC was indicated by ** ($P < 0.01$). The reactions were performed in duplicates, and the data are represented as means ± SEM.CTL indicates control. NC indicates negative control.

FIG. 2. Effects of miR-21 overexpression on TMZ-treated U87MG cells.(A) After exposure to 100 μM TMZ for 6 h showed morphological changes U87MG cells. A treatment of cells with miR-21 overexpression before TMZ decreased this trend of morphological changes. Arrows indicate apoptotic or necrotic cells. (B) The trypan blue exclusion assay was used to assess cell viability in U87MG cells. MiR-21 overexpression prevented TMZ mediated decrease in U87MG cell viability. (C) Hoechst 33258 staining assay was used to assess cell apoptosis in U87MG cells. Photomicrographs showed miR-21 overexpression prevented TMZ mediated increase in U87MG cell apoptosis. Arrows indicate apoptotic cells. (D) Bar graphs represent the percentage of apoptotic cells counted from each group. Data are presented as the means of triplicate experiments. Significant difference between TMZ and CTL, NC or miR-21 overexpression treated cells was indicated by * ($P < 0.05$) and significant difference between TMZ and miR-21+TMZ treated cells was indicated by ^ ($P < 0.05$).

FIG. 3. Effects of miR-21 overexpression on TMZ-induced U87MG cells apoptosis. U87MG cells treatment with miR-21 overexpression before TMZ for 48 h followed by 100 μM TMZ for 6 h. After all treatments, cells were harvested and double stained for Annexin V and propidium iodide and analyzed by flow cytometry. Early apoptotic cells are Annexin V^+/PI^-, late apoptotic cells are Annexin V^+/PI^+, necrotic cells are Annexin V^-/PI^+ and healthy cells are Annexin V^-/PI^-. (A) A representative experiment of three performed was shown. MiR-21 overexpression significantly prevented TMZ mediated increase in U87MG cell apoptosis. (B) Bar graphs represent the

percentage of apoptotic cells calculated from each group. Data are presented as the means of triplicate experiments. Significant difference between TMZ and CTL, NC or miR-21 overexpression treated cells was indicated by ** ($P < 0.01$) and significant difference between TMZ and miR-21+TMZ treated cells was indicated by ^ ($P < 0.05$).

FIG. 4. Effects of miR-21 overexpression on Bax:Bcl-2 ratio in TMZ-treated U87MG cells. U87MG cells treatment with miR-21 overexpression before TMZ for 48 h followed by 100 μM TMZ for 6 h. After all treatments, cells were harvested and measured by Western blot analysis.(A) Compared with control and negative control group, a rise in Bax:Bcl-2 ratio was found in TMZ-treated U87MG cells, while cells pretreated with miR-21 overexpression showed a significant decrease in the Bax:Bcl-2 ratio. (B) Bar graphs represent the relative expression of Bax:Bcl-2 ratio calculated from each group. Data are presented as the means of triplicate experiments. Significant difference between TMZ and CTL, NC or miR-21 overexpression treated cells was indicated by ** ($P < 0.01$) and significant difference between TMZ and miR-21+TMZ treated cells was indicated by ^ ($P < 0.01$).

FIG. 5. Effects of miR-21 overexpression on caspase-3 activity in TMZ-treated U87MG cells. U87MG cells treatment with miR-21 overexpression before TMZ for 48 h followed by 100 μM TMZ for 6 h. After all treatments, cells were harvested, and caspase-3 activity was measured by the Caspase-3 activity kit. Compared with control and negative control group, a rise in caspase-3 activity was found in TMZ-treated U87MG cells, while cells pretreated with miR-21 overexpression showed a significant decrease in the caspase-3 activity. (B) Bar graphs represent the relative expression of caspase-3 activity calculated from each group. Data are presented as the means of triplicate experiments. Significant difference between TMZ and CTL, NC or miR-21 overexpression treated cells was indicated by * ($P < 0.05$) and significant difference between TMZ and miR-21+TMZ treated cells was indicated by ^ ($P < 0.05$).