MicroRNA-21 Inhibition Enhances *In Vitro* Chemosensitivity of Temozolomide-resistant Glioblastoma Cells

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Abstract. *Background: Glioblastoma multiforme (GBM) is a form of highly malignant brain tumour. Temozolomide (TMZ) is the standard agent for GBM, but TMZ-resistance is common and accounts for many treatment failures. MicroRNA-21 (miR-21) is a non-coding RNA that plays critical roles in many biological processes in cancer, including chemoresistance. We investigated miR-21 expression and the effect of miR-21 inhibition in GBM with acquired TMZ resistance. Materials and Methods: Human GBM cell line D54MG was treated with TMZ chronically to develop a chemoresistant subclone. MiR-21 inhibition was achieved by transfection with anti-mir-21 oligonucleotide. Results: Chronic TMZ exposure resulted in acquired TMZ-resistance and elevated miR-21 expression. Concomitant treatment with miR-21 inhibitor and TMZ resulted in a significantly higher apoptotic rate than TMZ treatment alone. Conclusion: MiR-21 may have a potential for use as a biomarker of acquired TMZ resistance. MiR-21 inhibition can be further explored as a potential chemotherapy adjunct in the treatment of TMZ-resistant GBM.*

Glioblastoma multiforme (GBM) is one of the most lethal forms of primary brain tumour. The alkylating agent temozolomide (TMZ) has been shown to provide significant survival benefits for patients with GBM (1). The standard treatment for GBM consists of maximal surgical resection, concomitant TMZ therapy and irradiation, and six-cycles of adjuvant TMZ (2). The overall prognosis, however, remains unsatisfactory due to the tumour's intrinsic or acquired resistance to TMZ (3). The molecular mechanisms underlying TMZ resistance are incompletely understood, and therapies aimed at overcoming it have attracted considerable research effort (4, 5). An area under investigation concerns the roles of microRNAs (miRNAs) (6). microRNAs belong to a class of small (~21 nucleotides) non-coding RNAs that regulate gene expression post-transcriptionally (7). miRNAs play many important roles in normal biological processes such as cell growth, proliferation, differentiation and apoptosis (8). Their roles in determining the biological behaviour of brain tumours have been described (9, 10). Amongst the oncogenic miRNAs, miRNA-21 *(miR-21)* was found to be consistently overexpressed in GBM cells (11). miR-21 is anti-apoptotic (12), and may promote glioma invasion (13) and proliferation (14). The role of miR-21 in modulating tumour response to TMZ has been described (15, 16). In this study, we further investigated the effects of specific *miR-21* inhibition on a subclone of TMZ-resistant GBM cells. Our hypothesis was that *miR-21* inhibition would re-sensitize chemoresistant GBM cells to TMZ.

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Materials and Methods

TMZ-resistant GBM cells. We have previously described the development of isogenic subclones of TMZ-resistant GBM cells by means of chronic TMZ exposure (17). Briefly, human GBM cell line D54MG (Duke University Medical Center, Durham, NC, USA) was maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen, Carlsbad, CA, USA), supplemented with 10% foetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37˚C in 5% carbon dioxide and 95% air atmosphere. The parental TMZ-sensitive cells (designated as P-D54MG) were exposed to 100 μM TMZ (TEMODAL®, Schering-Plough, NJ, USA) for two weeks, and then repeatedly to TMZ at the 50% inhibitory concentration (IC_{50}) of 500 μM for 12 months. The TMZ-resistant subclone produced (designated as R-D54MG) was isolated and maintained in DMEM/F12 with low-dose TMZ (100 μM).

miRNA expression. Total RNA was obtained using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA concentrations were measured with a NanoDrop ND1000 Spectrophotometer (Thermo Scientific, MA, USA). Quantitative reverse transcription-PCR (qRT-PCR) of mature miRNAs was conducted. The quantities of mature miRNAs were

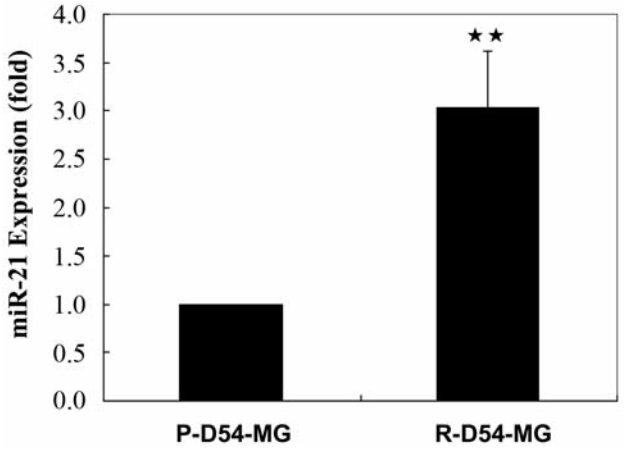


Figure 1. *Levels (mean±SD) of miR-21 expression in R-D54MG cells and their parental control cells P-D54MG were assessed by TaqMan-based quantitative reverse transcription-PCR (qRT-PCR). The former showed significantly up-regulated miR-21 expression (**p<0.01).*

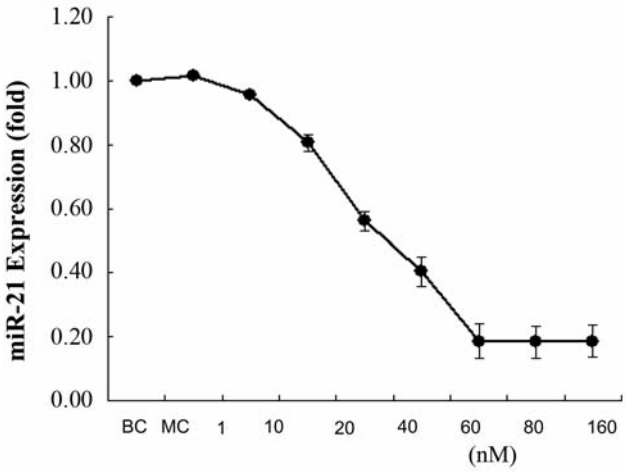


Figure 2. *Transfection with miR-21 inhibitor reduced miR-21 expression in R-D54MG cells in a dose-dependent manner. In comparative CT fold-change analysis, blank transfection control (BC) was used as the control. MC, Mock transfection control (RNAiMax).*

evaluated using specific TaqMan real-time PCR primers and probes (Applied Biosystems, CA, USA). Real-time PCR was performed using GeneAmp Fast PCR Master Mix (Applied Biosystems) and an ABI 7900HT real-time PCR instrument. The expression levels of mature miRNAs were evaluated using the comparative CT method ($2^{-\Delta\Delta CT}$). All experiments were performed in triplicates.

miR-21 inhibition. Transfection with *anti-miR-21* inhibitor (AMI17000, ID No. AM10206) (Applied Biosystems, CA, USA) was performed using Lipofectamine™ RNAi Max Transfection Agent (Invitrogen, Carlsbad, CA, USA). Briefly, cells were seeded at 5000 cells/well in 96-well plates, and different amounts of the inhibitor (1 nM to 160 nM) were tested. The lowest possible concentration that achieved the most significant inhibition was chosen as the optimal dosage. Cells transfected with this optimal dosage (60 nM) were subjected to miRNA expression confirmation assay and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis. To study the concomitant effects of miR-21 inhibition and TMZ, cells were seeded onto 8-well Chamber Slide System (Thermo Scientific, MA, USA) at 10,000 cells/well, and then transfected with *anti-miR-21* at 60 nM, with or without TMZ treatment at IC_{30} for 72 hours.

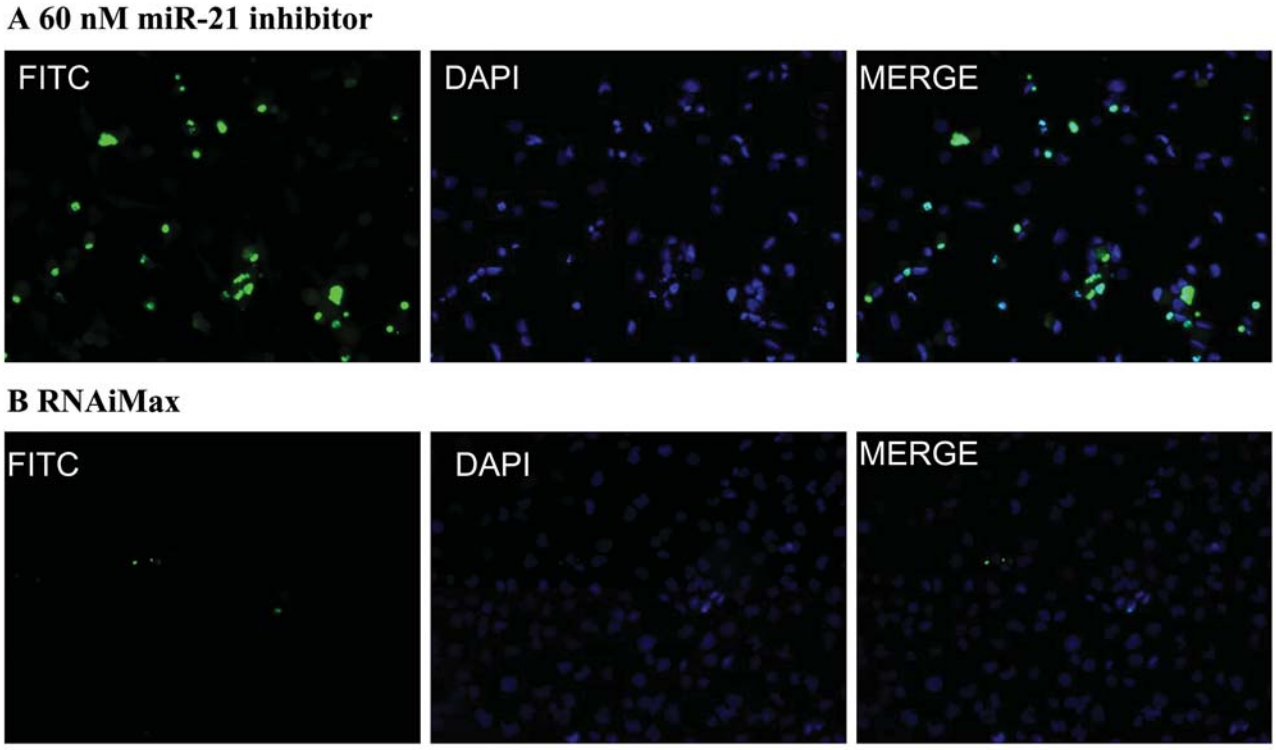
TUNEL analysis. Fluorescent in situ hybridization was performed using the In situ Cell Death Detection Kit, Fluorescein, (Roche, Schweiz, Germany) according to the manufacturer's instructions. Omission of the terminal deoxynucleotidyl transferase enzyme during processing was used as a negative control. The chamber slides were observed with fluorescent microscope (Nikon Eclipse 80i, Nikon Intenselight c-HGFI, Kanagawa, Japan). Three high-power fields (×200) were randomly selected to count both the apoptotic cells [Fluorescein Isothiocyanate (FITC) stained] and the total cell population [counterstained by 4',6-diamidino-2-phenylindole (DAPI)]. At least 100 cells in total were counted in each field. The apoptotic rate was calculated by dividing the total cell number by the number of apoptotic cells.

Annexin V assay. Cells were seeded at a density of 1×10^5 cells/ml in 6-well plates, and harvested after treatments, as described above. Annexin V-FITIC/PI kit (BD Biosciences, San Jose, USA) was used to label apoptotic cells. In analyzing the results of flow cytometery (BD FACSCalibur, CA, USA), annexin V-positive cells were considered as apoptotic regardless of propidine iodide (PI) staining. Cells stained positively for PI were considered as dead cells regardless of annexin V staining.

Statistical analysis. Data were expressed as mean±standard error. Student's *t*-test was used to examine statistical correlation. A *p*-value <0.05 was considered to be statistically significant. Statistical analysis was performed with SPSS 16.0 software (SPSS Inc., IL, USA).

Results

miR-21 in TMZ-resistant cells. We measured the expression of *miR-21* using TaqMan-based qRT-PCR (Figure 1). GBM cells with acquired TMZ-resistance (R-D54MG) exhibited significantly higher levels of *miR-21* expression than their parental control cells (mean fold change=3.2, $p<0.01$). We then proceeded to knock-down endogenous miR-21 expression. The inhibition effect was assessed by qRT-PCR at 72 hours after transfection. To achieve the optimal effect of silencing with minimal off-target effects, different inhibitor concentrations (from 1 nM to 160 nM) were tested. We found that *miR-21* inhibition suppressed miR-21 expression in R-D54MG cells in a dose-dependent manner. The effect was maximal at the optimal concentration of 60 nM, which resulted in around an 80% decrease in miR-21 expression (Figure 2). Mock control transfection did not have the same effect. Moreover, miR-21 inhibition alone led to increased apoptosis on TUNEL analysis (Figure 3).



Concomitant use of TMZ and miR-21 inhibition. We then investigated whether miR-21 inhibition would increase the response of TMZ-resistant cells to TMZ re-challenge. R-D54MG cells were transfected with miR-21 inhibitor for 24 hours and then treated with TMZ. TUNEL analysis was performed after 72 hours of TMZ treatment. For cells pretreated with *miR-21* inhibitor, TMZ treatment at IC_{30} resulted in a 52.9% apoptosis rate; cells treated with TMZ only without prior *miR-21* inhibition exhibited only a 10.8% rate. Few apoptotic cells were observed in the corresponding mock controls (Figure 4 A to C). We also used annexin V-PI staining to quantify apoptosis. Treatment with miR-21 inhibitor (96 hours) plus TMZ (72 hours) resulted in a significant increase in the apoptosis rate when compared with TMZ treatment alone. The corresponding mock control cells again exhibited low levels of apoptosis (Figure 4E).

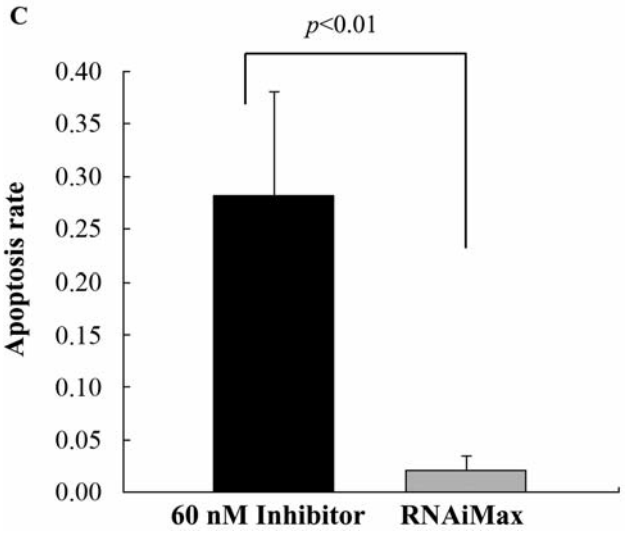


Figure 3. *Treatment with miR-21 inhibitor alone increased the apoptosis rate in R-D54MG cells. Apoptotic nuclei are shown in green on the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Magnification: ×200. A: Transfection with miR-21 inhibitor; B: transfection with RNAiMax (mock control) alone. C: Bar chart showing the percentage (mean±SD) of apoptotic cells counted from at least three randomly selected fields of view. (p<0.01).*

Discussion

GBM is characterized by its resistance towards a broad spectrum of structurally unrelated cytotoxic drugs with different modes of action (18). Despite the positive impact of TMZ on survival, the majority of patients with GBM continue to suffer from progressive or recurrent disease (1, 3). The mechanisms underlying TMZ resistance may include contributions from cancer stem cells and genetic mutations, as well as epigenetic phenomena such as the silencing of *O*-6-methylguanine-DNA-methyltransferase (*MGMT*) (4, 19, 20).

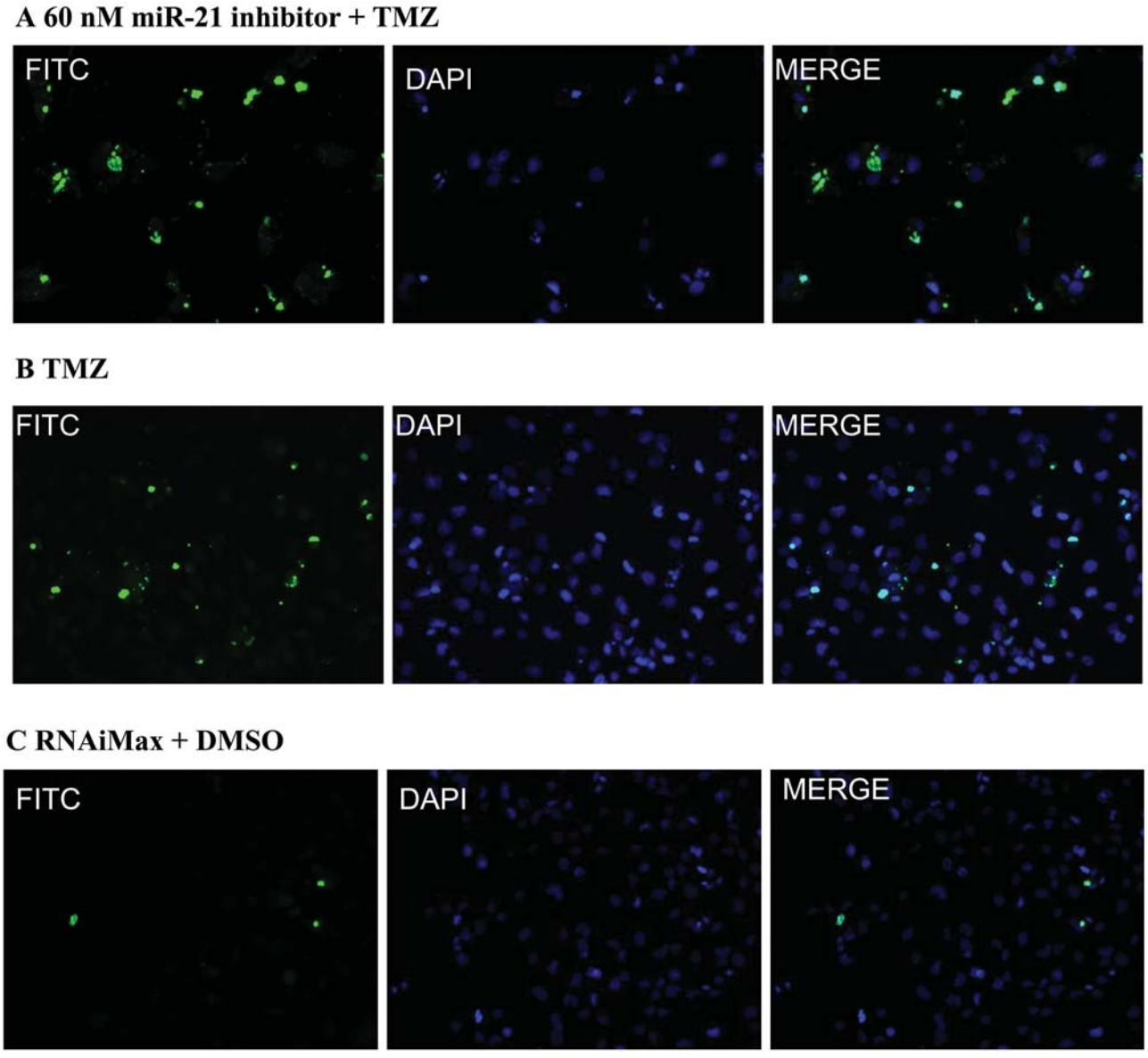


Figure 4. *Continued*

Recently, miRNAs have been shown to play critical roles in determining the biological behavior of GBM. One of the candidates, miR-21, has been identified as a potential target for anticancer therapies (14). Overexpression of miR-21 is implicated in many malignant conditions, including cancer of the breast (21), lung (22), and colorectum (23), as well as in hepatocellular carcinoma (24). In malignant glioma, *in vitro* studies demonstrated that miR-21 may down-regulate the expression of important tumour suppressor genes such as programmed cell death protein 4 (*PDCD4*) (23), tropomyosin alpha-1 (*TPM1*) (25), and phosphatase and tensin homolog (*PTEN*) (24). Clinically, miR-21 expression has been shown to correlate with tumour grading (13), as well as patient survival (26). Shi *et al*. reported that overexpression of miR-21 inhibited TMZ-induced apoptosis by reducing the BCL-2–associated X protein (BAX)/B-cell lymphoma 2 (BCL-2) ratio and caspase-3 activity in treatment-naive GBM cells (15). Our findings provide additional information on GBM cells that have previously been subjected to chronic TMZ treatment. Firstly, chronic TMZ exposure and the acquisition of TMZ resistance were associated with up-regulation of miR-21 expression. Secondly, *miR-21* knock-down increased the cytotoxic effect of TMZ in resistant cells. To our knowledge, this is the first report that demonstrates the effect of *miR-21* inhibition in TMZ-resistant GBM. Our findings suggest two potential

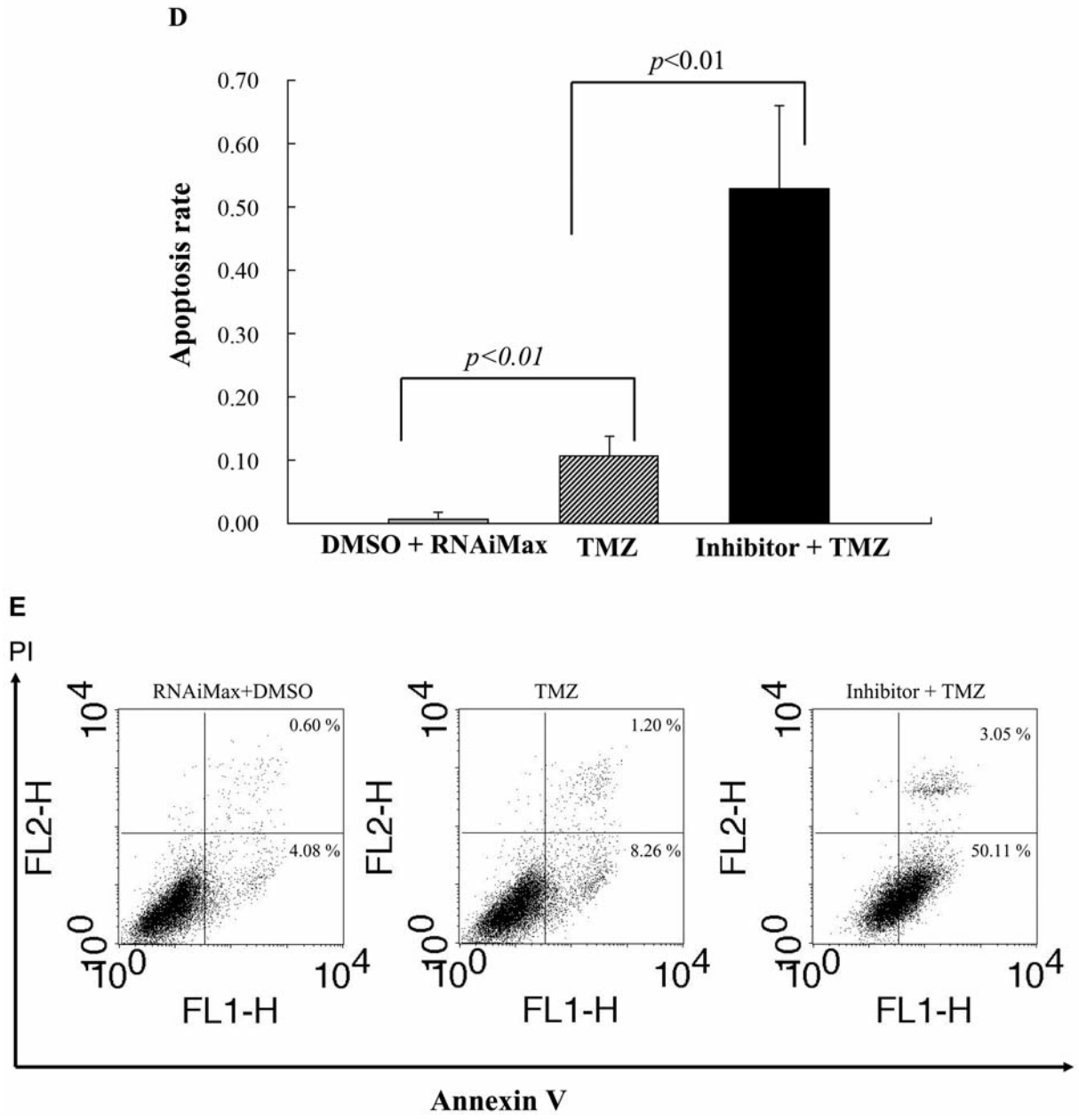


Figure 4. *Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay showing increased apoptosis after concomitant treatment with miR-21 inhibitor and temozolomide (TMZ) at 30% inhibitory concentration (IC_{30}). Apoptotic nuclei are shown in green on TUNEL assay. Magnification: ×200. A: miR-21 inhibitor plus TMZ; B: TMZ alone; C: RNAiMax plus DMSO (mock control). D: Bar chart showing the percentage (mean±SD) of apoptotic cells counted from at least three randomly selected fields of view (p<0.01). E: Apoptotic quantification using annexin V-propidine iodide (PI) flow cytometric analysis, showing increased apoptosis after concomitant treatment with miR-21 inhibitor and TMZ (right), when compared with control (left) and TMZ alone (centre).*

clinical uses of miR-21 in the treatment of GBM. miR-21 is detectable in serum and has been shown to be a potential biomarker for oesophageal cancer (27). The elevated expression of miR-21 in our chemoresistant subclone may be exploited similarly as a biomarker of TMZ resistance during treatment or upon disease recurrence. miR-21 inhibition has been shown to enhance the chemosensitivity of human GBM to taxol (28), to activate caspase-3 and -9 (29), and to suppress growth in glioma cells independently of PTEN regulation (30). Our findings therefore indicate the potential

use of *miR-21* inhibition as a chemotherapy adjunct, for example, for patients who have elevated miR-21 levels serologically or within their tumours. Other miRNA candidates that are reportedly associated with TMZ resistance in GBM include miR-195, miR-455-3p, miR-10a, miR-181b and miR-181c (31, 32). These may be further explored for similar clinical applications alone or in combination with miR-21. A major obstacle to miRNA-based therapy is finding an effective mode of delivery for miRNAs and their inhibitors. Systemic administration is unlikely to be successful for glioma due to the blood-brain barrier. Although this barrier is disrupted in GBM, it is intact in the adjacent brain into which GBM cells may have infiltrated. Interestingly, miRNAs may be transferred between glioma cells, which can potentially reduce the threshold efficiency required (33). Other approaches such as convection-enhanced delivery with nanoparticles (34), and the use of viral vectors (35) can be explored. miRNA inhibition may carry the risk of undesirable side-effects in normal cells. But since the endogenous expression of targeted miRNAs may be normal within normal cells, the impact of off-target effects is likely to be small (36).

Conclusion

Using an *in vitro* model of acquired TMZ resistance, we have demonstrated that chronic TMZ exposure up-regulates the expression of miR-21 in GBM, and that inhibition of miR-21 can resensitize chemoresistant GBM cells to TMZ re-challenge. Further *in vivo* studies are required to demonstrate the roles of miR-21 as a biomarker and as a novel therapeutic target for the treatment of TMZ-resistant GBM.

Declaration of Interest

The Authors have no potential conflict of interest to report.

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References

1 Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E and Mirimanoff RO: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med *352*: 987-996, 2005.

2 Koukourakis GV, Kouloulias V, Zacharias G, Papadimitriou C, Pantelakos P, Maravelis G, Fotineas A, Beli I, Chaldeopoulos D and Kouvaris J: Temozolomide with radiation therapy in high-grade brain gliomas: pharmaceuticals considerations and efficacy; a review article. Molecules *14*: 1561-1577, 2009.

3 Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, Ludwin SK, Allgeier A, Fisher B, Belanger K, Hau P, Brandes AA, Gijtenbeek J, Marosi C, Vecht CJ, Mokhtari K, Wesseling P, Villa S, Eisenhauer E, Gorlia T, Weller M, Lacombe D, Cairncross JG and Mirimanoff RO: Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol *10*: 459-466, 2009.

4 Bocangel DB, Finkelstein S, Schold SC, Bhakat KK, Mitra S and Kokkinakis DM: Multifaceted resistance of gliomas to temozolomide. Clin Cancer Res *8*: 2725-2734, 2002.

5 Tentori L and Graziani G: Recent approaches to improve the antitumor efficacy of temozolomide. Curr Med Chem *16*: 245-257, 2009.

6 Moore LM and Zhang W: Targeting miR-21 in glioma: a small RNA with big potential. Expert Opin Ther Targets *14*: 1247-1257, 2010.

7 Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell *116*: 281-297, 2004.

8 Fernandez LA, Northcott PA, Taylor MD and Kenney AM: Normal and oncogenic roles for microRNAs in the developing brain. Cell Cycle *8*: 4049-4054, 2009.

9 Malzkorn B, Wolter M, Liesenberg F, Grzendowski M, Stuhler K, Meyer HE and Reifenberger G: Identification and functional characterization of microRNAs involved in the malignant progression of gliomas. Brain Pathol *20*: 539-550, 2010.

10 Ciafre SA, Galardi S, Mangiola A, Ferracin M, Liu CG, Sabatino G, Negrini M, Maira G, Croce CM and Farace MG: Extensive modulation of a set of microRNAs in primary glioblastoma. Biochem Biophys Res Commun *334*: 1351-1358, 2005.

11 Conti A, Aguennouz M, La Torre D, Tomasello C, Cardali S, Angileri FF, Maio F, Cama A, Germano A, Vita G and Tomasello F: miR-21 and 221 upregulation and miR-181b down-regulation in human grade II-IV astrocytic tumors. J Neurooncol *93*: 325-332, 2009.

12 Chan JA, Krichevsky AM and Kosik KS: MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. Cancer Res *65*: 6029-6033, 2005.

13 Gabriely G, Wurdinger T, Kesari S, Esau CC, Burchard J, Linsley PS and Krichevsky AM: MicroRNA 21 promotes glioma invasion by targeting matrix metalloproteinase regulators. Mol Cell Biol *28*: 5369-5380, 2008.

14 Papagiannakopoulos T, Shapiro A and Kosik KS: MicroRNA-21 targets a network of key tumor-suppressive pathways in glioblastoma cells. Cancer Res *68*: 8164-8172, 2008.

15 Shi L, Chen J, Yang J, Pan T, Zhang S and Wang Z: miR-21 protected human glioblastoma U87MG cells from chemotherapeutic drug temozolomide induced apoptosis by decreasing BAX/BCL-2 ratio and caspase-3 activity. Brain Res *1352*: 255-264, 2010.

16 Shi L, Zhang S, Feng K, Wu F, Wan Y, Wang Z, Zhang J, Wang Y, Yan W, Fu Z and You Y: MicroRNA-125b-2 confers human glioblastoma stem cells resistance to temozolomide through the mitochondrial pathway of apoptosis. Int J Oncol *40*: 119-129, 2012.

17 Sun S, Wong TS, Zhang XQ, Pu JK, Lee NP, Day PJ, Ng GK, Lui WM and Leung GK: Protein alterations associated with temozolomide resistance in subclones of human glioblastoma cell lines. J Neurooncol *107*: 89-100, 2012.
18 Lu C and Shervington A: Chemoresistance in gliomas. Mol Cell Biochem *312*: 71-80, 2008.
19 Chakravarti A and Palanichamy K: Overcoming therapeutic resistance in malignant gliomas: current practices and future directions. Cancer Treat Res *139*: 173-189, 2008.
20 Cheng L, Bao S and Rich JN: Potential therapeutic implications of cancer stem cells in glioblastoma. Biochem Pharmacol *80*: 654-665, 2010.
21 Huang GL, Zhang XH, Guo GL, Huang KT, Yang KY, Shen X, You J and Hu XQ: Clinical significance of miR-21 expression in breast cancer: SYBR-Green I-based real-time RT-PCR study of invasive ductal carcinoma. Oncol Rep *21*: 673-679, 2009.
22 Markou A, Tsaroucha EG, Kaklamanis L, Fotinou M, Georgoulias V and Lianidou ES: Prognostic value of mature microRNA-21 and microRNA-205 overexpression in non-small cell lung cancer by quantitative real-time RT-PCR. Clin Chem *54*: 1696-1704, 2008.
23 Asangani IA, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S and Allgayer H: MicroRNA-21 (miR-21) post-transcriptionally down-regulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene *27*: 2128-2136, 2008.
24 Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST and Patel T: MicroRNA-21 regulates expression of the *PTEN* tumor suppressor gene in human hepatocellular cancer. Gastroenterology *133*: 647-658, 2007.
25 Zhu S, Si ML, Wu H and Mo YY: MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (*TPM1*). J Biol Chem *282*: 14328-14336, 2007.
26 Zhi F, Chen X, Wang S, Xia X, Shi Y, Guan W, Shao N, Qu H, Yang C, Zhang Y, Wang Q, Wang R, Zen K, Zhang CY, Zhang J and Yang Y: The use of hsa-miR-21, hsa-miR-181b and hsa-miR-106a as prognostic indicators of astrocytoma. Eur J Cancer *46*: 1640-1649, 2010.
27 Kurashige J, Kamohara H, Watanabe M, Tanaka Y, Kinoshita K, Saito S, Hiyoshi Y, Iwatsuki M, Baba Y and Baba H: Serum microRNA-21 is a novel biomarker in patients with esophageal squamous cell carcinoma. J Surg Oncol, 2012 Feb 21. doi: 10.1002/jso.23064. [Epub ahead of print].
28 Ren Y, Zhou X, Mei M, Yuan XB, Han L, Wang GX, Jia ZF, Xu P, Pu PY and Kang CS: MicroRNA-21 inhibitor sensitizes human glioblastoma cells U251 (PTEN-mutant) and LN229 (PTEN-wild type) to taxol. BMC Cancer *10*: 27, 2010.
29 Zhou X, Zhang J, Jia Q, Ren Y, Wang Y, Shi L, Liu N, Wang G, Pu P, You Y and Kang C: Reduction of miR-21 induces glioma cell apoptosis via activating caspase 9 and 3. Oncol Rep *24*: 195-201, 2010.
30 Zhou X, Ren Y, Moore L, Mei M, You Y, Xu P, Wang B, Wang G, Jia Z, Pu P, Zhang W and Kang C: Down-regulation of miR-21 inhibits EGFR pathway and suppresses the growth of human glioblastoma cells independent of PTEN status. Lab Invest *90*: 144-155, 2010.
31 Slaby O, Lakomy R, Fadrus P, Hrstka R, Kren L, Lzicarova E, Smrcka M, Svoboda M, Dolezalova H, Novakova J, Valik D, Vyzula R and Michalek J: MicroRNA-181 family predicts response to concomitant chemoradiotherapy with temozolomide in glioblastoma patients. Neoplasma *57*: 264-269, 2010.
32 Ujifuku K, Mitsutake N, Takakura S, Matsuse M, Saenko V, Suzuki K, Hayashi K, Matsuo T, Kamada K, Nagata I and Yamashita S: miR-195, miR-455-3p and miR-10a(*) are implicated in acquired temozolomide resistance in glioblastoma multiforme cells. Cancer Lett *296*: 241-248, 2010.
33 Katakowski M, Buller B, Wang X, Rogers T and Chopp M: Functional microRNA is transferred between glioma cells. Cancer Res *70*: 8259-8263, 2010.
34 Allard E, Passirani C and Benoit JP: Convection-enhanced delivery of nanocarriers for the treatment of brain tumors. Biomaterials *30*: 2302-2318, 2009.
35 Klinghoffer RA, Magnus J, Schelter J, Mehaffey M, Coleman C and Cleary MA: Reduced seed region-based off-target activity with lentivirus-mediated RNAi. RNA *16*: 879-884, 2010.
36 Purow B: The elephant in the room: Do microRNA-based therapies have a realistic chance of succeeding for brain tumors such as glioblastoma? J Neurooncol *103*: 429-436, 2011.

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