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Synergistic Inhibition of Human Glioma Cell Line by Temozolomide and PAMAM-Mediated miR-21i

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ABSTRACT: Temozolomide (TMZ) is a promising chemotherapeutic agent for treating glioblastomas. However, resistance develops quickly and with a high frequency. Efforts to overcome chemoresistance are, therefore, critically needed. In present study, a poly(amidoamine; PAMAM) dendrimer was used as a vector to deliver microRNA-21 inhibitor (miR-21i) into U87 cells and the chemosensitivity of the combination effect of miR-21i and TMZ for glioma therapy was investigated. Flow cytometry analysis showed the uptake efficiency of microRNA-21 inhibitor after complexation with PAMAM. Real-time PCR and *in situ* hybridization indicated that, compared with TMZ or miR-21i treated cells, cells simultaneously treated with miR-21i and TMZ showed a remarkable decrease in the microRNA-21 (miR-21) level. The transfection of miR-21i enhanced the chemosensitivity by significantly decreasing the IC50 value of TMZ to glioma cells. Knockdown of miR-21 promoted the cells' apoptosis, and at the same time, inhibited cell invasion. In conclusion, the combination treatment of glioma cells with TMZ and miR-21i could yield a synergistic effect in inhibition of human glioma cell line. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: MiR-21 inhibitor; temozolomide; PAMAM; glioma; chemosensitivity

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INTRODUCTION

Resistance to chemotherapy drugs is a major problem in glioma treatment.[1] Recently, the combination of gene therapy and chemotherapy has became a promising method to overcome the chemoresistance owing to their synergistic/combined effect.[2] For the highest grade form of gliomas, TMZ has been considered to be a promising chemotherapeutic agent,[3] but its effects last for only a few months and drug insensitivity or resistance develops thereafter in many cases.[4] Substantial data indicate that the oncogene miR-21, one of the noncoding RNA molecules of 21–24 nucleotides, is significantly elevated in glioblastoma multiforme (GBM) and regulates multiple genes associated with cancer cell proliferation, apoptosis, and invasiveness.[5] And most recently, it has been reported that over-express miR-21 could inhibit TMZ-induced apoptosis in U87 glioma cells.[6] Thus, the purpose of the present study is to investigate whether the down-regulation of miR-21 could enhance the chemosensitivity of glioma cells to TMZ. We used PAMAM as the vector to deliver miR-21i into glioma cells and treated the U87 cells combined with TMZ. The results showed that the combination treatment of TMZ and miR-21i could promote the apoptosis of tumor cells and at the same time inhibit the cells' invasion.

MATERIALS AND METHODS

Human glioma cell line U87 was obtained from the China Academia Sinica cell repository (Shanghai, China). Poly(amidoamine) dendrimer contained 128 amino groups (G5-PAMAM) and TMZ were purchased from Sigma-Aldrich (St. Louis, MO). Trypsin-EDTA, phosphate-buffered saline (PBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen (Gaithersburg, MD). The 2′-O-methyl (2′-OMe-) miR-21 inhibitors (miR-21i) were chemically synthesized by Shanghai GenePharma (Shanghai, China). 2′-O-Me oligos were composed entirely of 2′-O-methyl bases and had the following sequences: miR-21 inhibitor(miR-21i): 5′-GTC CAC TCT TGT CCT CAA TG-3′ and

scrambled sequences were 5′-AAG GCA AGC UGA CCC UGA AGU-3′ as negative control. The FITC-labeled miR-21 inhibitor (F-miR-21i) were also synthesized by Shanghai GenePharma (Shanghai, China).

Preparation and Characterization of the miR-21i/PAMAM Polyplex

The G5 PAMAM dendrimers were first dialyzed against PBS for 1 day and then against deionized water for another day to remove methanol. The miR-21i solution (20 μmol/L) was added and incubated with PAMAM solution at N/P ratios (ratio of the number of terminal amino groups in the dendrimer to the number of phosphate groups in the ASODN) of 16/1 for 30 min.[7] The size distribution and surface charge of complexes were determined by zeta potential analyzer (Zeta PALS, Brookhaven Instruments) at 25°C in PBS buffer. The morphologies of miR-21i/PAMAM complexes were observed by Scan Electronic Microscopy (SEM, model S-2250n, Hitachi, Japan).

Cell Culture and miR-21i Transfection

The cells were maintained in DMEM supplemented with 10% FBS (Invitrogen) 50 U/mL penicillin G, and 250 μg/mL streptomycin in an atmosphere containing 5% CO_2 at 37°C. Before transfection, the cells about 2×10^5 were seeded in 6-well plates with DMEM containing 10% FBS and cultured for 24 h, then the treatment was divided into five groups: control, scramble, miR-21i/PAMAM, TMZ, miR-21i /PAMAM, and TMZ. The five groups were added into the cells of 6-well plates and cultured for 72 h, preparing for cell proliferation and apoptosis.

miR-21i Uptake by Tumor Cells

U87 (2×10^5) cells were seeded in 6-well plates. Twenty-four hours later, F-miR-21i alone, F-miR-21i/PAMAM, or F-miR-21i/PAMAM/TMZ were added and incubated with cells for 4 h, then the cells were harvested and the FITC fluorescence was detected by flow cytometry.

miR-21 Detection by *In Situ* Hybridization

In situ hybridization detection of miR-21 in glioma cells was performed using locked nucleic acid (LNA)-modified oligonucleotides probe.[8] LNA/DNA oligos contained locked nucleic acids at eight consecutive centrally located bases (indicated by the underline) and had the following sequences: LNA-miR-21 5′-TCAA CATCAGT CTGATAAGCTA-3′. At 72 h after transfection, the U87 cells were fixed with freshly prepared 4% paraformaldehyde (containing 0.1% DEPC) according to the protocol of the manufactures. MiR-21 was labeled by Cy3-avidin at a concentration of 0.5 mg/mL and the Nuclei were dyed by DAPI (Genmed, Boston, MA). Then the fluorescence was visualized using FluoView Confocal Laser Scanning Microscopes-FV1000 (Olympus, Tokyo, Japan) and analyzed by IPP5.1 (Olympus).

RNA Extraction and Real-Time PCR

At 72 h after the treatment of U87 cells with miR-21i/PAMAM alone or concurrently with TMZ (22.5 μm), the RNA of different group was extracted using trizol reagent (Invitrogen) according to the standard protocol. Nanodrop spectrophotometer (Gene) was used to detect the concentration of total mRNA. And reverse transcription (RT) was conducted with the mir-Vana™ qRT-PCR miRNA detection kit (Ambion) in a 10 μL reaction system, comprising 2 μL mirVana™ 5×RT buffer, 1 μL mirVana™ 1×RT primer, 25 ng total miRNA, 0.4 μL ArrayScript™ enzyme mix, and DDW (Deuterium Depleted Water) up to 10 μL. MJ-real time PCR (BioRad) was used to perform the amplification reaction and the protocol was carried out for 40 cycles, comprising 95°C for 3 min, 95°C for 15 s, 60°C for 30 s. Both RT and PCR primers were purchased from Ambion. Relative quantification was conducted using amplification efficiencies derived from cDNA standard curves. Data were emerged as fold change ($2^{-\Delta\Delta Ct}$) and analyzed using Opticon Monitor Analysis Software V2.02 software (MJ Research).

MTT Assay

Cells were seeded into 96 well plates at 3000 cells/well. After the treatment of cells with different concentrations of TMZ (7.5, 15, 22.5, 40 μM), 20 μL of MTT (5 mg/mL) was added to each well and incubated at 37°C for 4 h, then the supernatant was removed and 200 μL of DMSO was added to each well to dissolve the crystallization by shaking for 15 min. Quantification was performed at a wavelength of 570 nm using spectrophotometric analysis.

Apoptosis Assays

After the treatment of cells with miR-21i, TMZ (22.5 μM) or miR-21i and TMZ(also 22.5 μM), the cells were harvested by trypsin separately and washed with cold PBS for 3 times. Then the calls were collected in tubes by centrifugation for 5 min at $800 \times g$, after which, 500 μL buffer, 5 μL of annexin V-FITC, and 5 μL of propidium iodide (PI) were added into each tube for 15 min, and immediately analyzed by FACScan Flow Cytometer (Becton Dickinson).

Cell Invasion Assessment

Cell invasion abilities were examined using a 24-well invasion chamber system, (BD Biosciences, Bedford, MA) employing a polycarbonic membrane (diameter 6.5 mm, pore size 8 μm). The cell invasion chambers were prepared by placing 100 μL of a 1:5 dilution of matrigel onto the filter, and incubating the filter at 37°C for 30 min to allow matrigel polymerization. Cells with different drug treatment were transferred on the top of matrigel-coated invasion chambers with serum-free DMEM. The lower compartment of the chamber was added DMEM containing 20% of FBS. Then the cells were incubated for 48 h at 37°C. The filters were then fixed in 95% ethanol and stained with crystal violet. After staining, cells were counted under a microscope in four random fields (magnification ×100) and the experiments were repeated in triplicate wells.

Statistical Analysis

All results were expressed as the mean ± standard deviation. Statistical evaluation for data analysis was determined by *t*-test. Statistical significance was determined as $P < 0.05$ (*).

RESULTS

Characterization of the miR-21i/PAMAM Complexes

The morphology of miR-21i/PAMAM was observed by SEM, as shown in Figure 1(a), after incubating miR-21i with PAMAM for 30 min, the complexes with homogeneous size about 50 nm were formed. The average hydration diameter of the complexes was 55 nm [Figure 1(b)] measured by the dynamic light scattering (DLS). The zeta potential of PAMAM was 29 mV. And after mixed with miR-21i, the zeta potential was slightly decreased to 20 mV, demonstrating the binding of miR-21i with PAMAM.

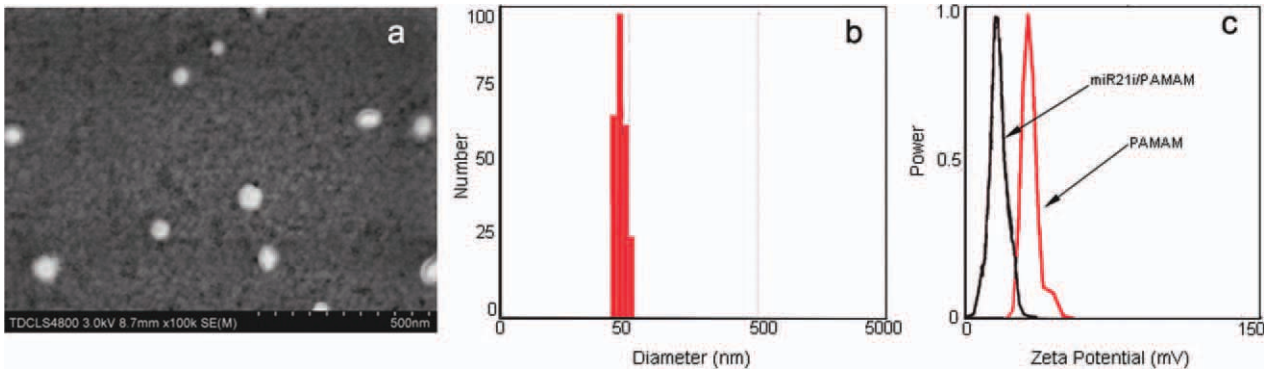

Figure 1. The morphologies (a), size distribution (b), and zeta potential (c) of miR-21i/PAMAM complexes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The Uptake and Transfection of miR-21i

The uptake of miR-21i by U87 cells were 0.5%, but after complexed with PAMAM, the uptake efficiency extremely increased to 56.1% [Figure 2(a,b)]. Interestingly, the treatment of tumor cells with TMZ had no influence on their uptake of miR-21i (58.2%), because there was no static difference between the two groups.

In situ hybridization was employed to test the expression level of miR-21. Cy-3 with red fluorescence indicated the miR-21 expression level. As shown in Figure 2(c), compared to control group (with nothing added), the red fluorescence significantly reduced after treating the cells with either TMZ or miR-21i. What's more, the lowest fluorescence level was found in the case of combination of TMZ with miR-21i. These results were confirmed by PCR experiments, in which either the TMZ or miR-21 caused a decrease expression of miR-21 in U87 cells(46.4% and 20.1%, respectively, $P^* < 0.05$). Paralleled with the results of *in situ* hybridization, the lowest miR-21 level was found when the tumor cells were treated with TMZ and miR-21i simultaneously (9.4%, $P^* < 0.05$ compared to the treatment of cells with TMZ or miR-21i alone).

Combination Effect on Cell Proliferation and Apoptosis

MTT assay was performed *in vitro* and the results were shown in Figure 3(a). At all drug level, the viable cells were dramatically decreased by the combined treatment of TMZ and miR-21i, compared with those treated by TMZ alone. The IC50 value

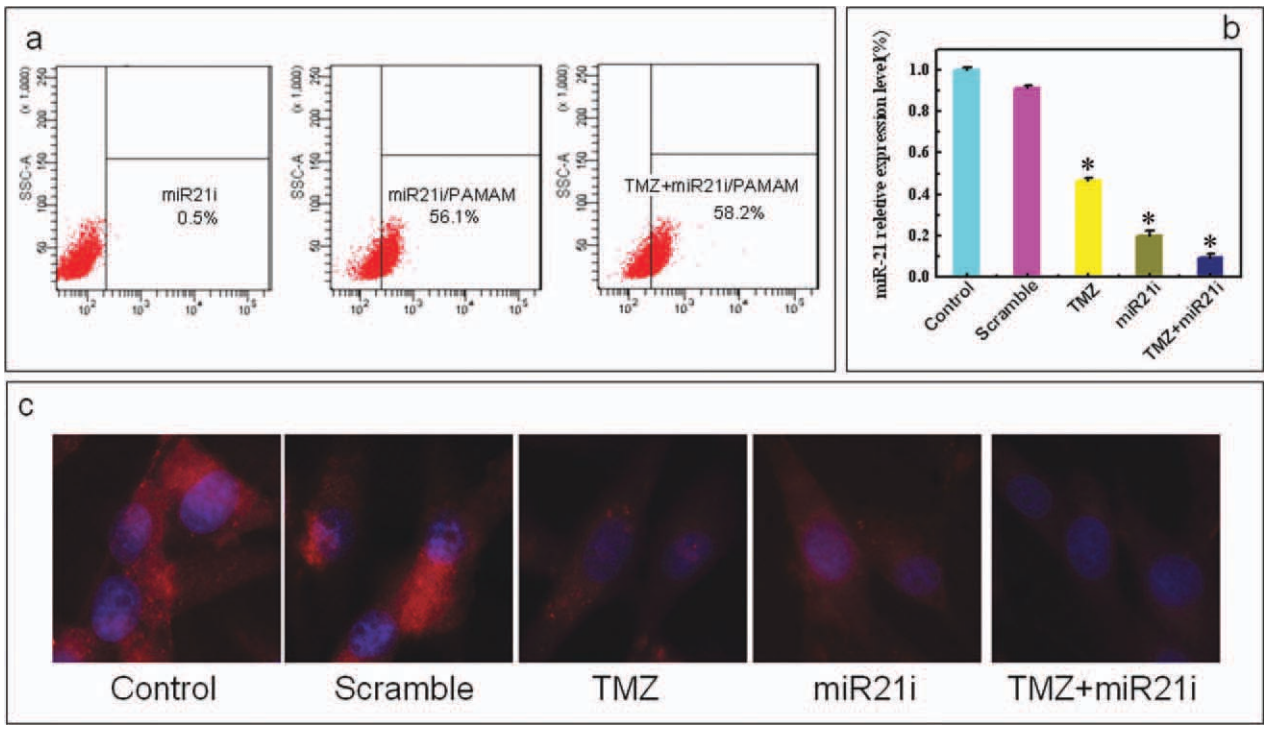

Figure 2. Uptake of miR-21i by U87 cells detected with flow cyctometry (a). Cells were treated by miR-21i, miR-21i/PAMAM or combination with TMZ. MiR-21 expression level detected by Real time PCR analysis (b) and *in situ* hybridization (c). The *t*-test was performed ($^*P < 0.05$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

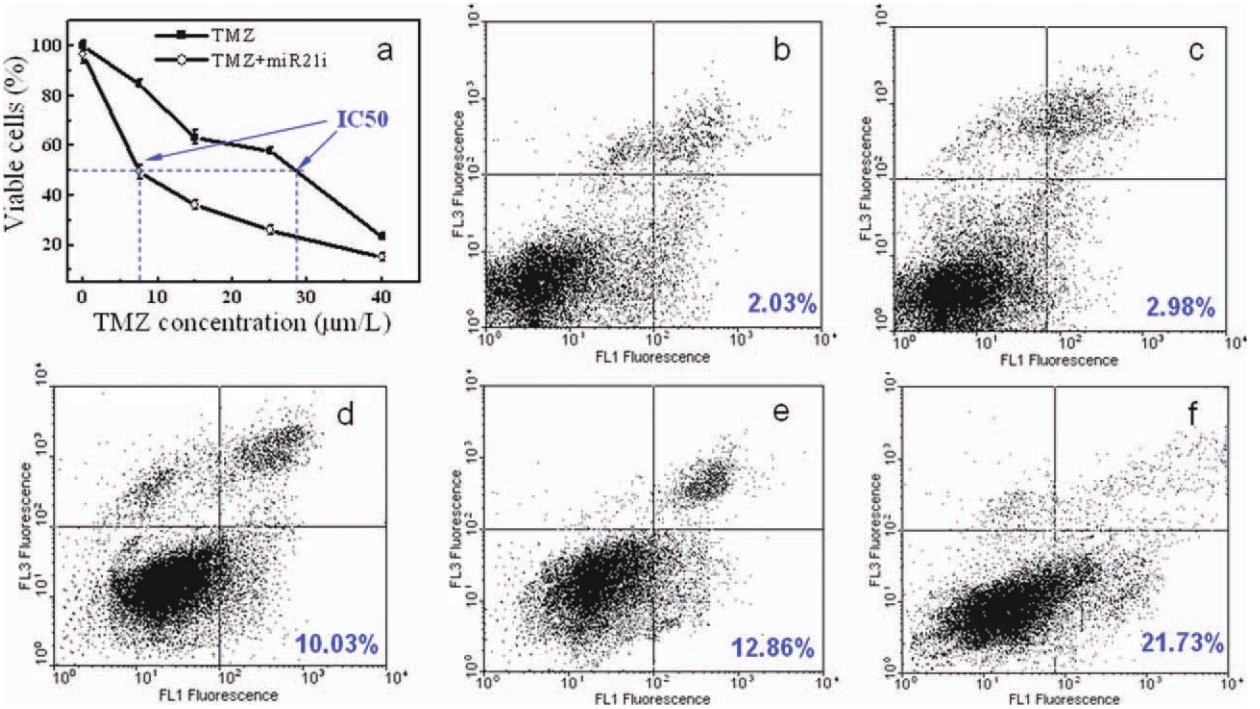

Figure 3. The cell viability (a) determined by MTT assay. U87 cell line was treated with various concentrations of TMZ or TMZ and miR-21i. Cell apoptosis analyzed by flow cytometric: (b) Control, (c) Scramble, (d) TMZ alone, (e) miR-21i/PAMAM alone, and (f) TMZ + miR-21i/PAMAM. Each value represents the mean ± SD from triplicate determinations. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

for cells treated with TMZ alone was 29 μM. This value decreased to 7.5 μM after exposure of cells to TMZ and miR-21i simultaneously (about 4-fold lower than TMZ). In contrast with proliferation, the cell apoptosis was greatly increased when treated with TMZ and miR-21i [Figure 3(b–f)], the apoptosis rate increased from 2.03% to 21.73%. These findings indicated that the ability of inducing apoptosis was strengthened in the cells combination of TMZ and miR-21 inhibitor.

Combination Effect on Cell Invasion

The transwell assay was used to determine cell invasiveness when the glioma cells were treated with different groups. As shown in Figure 4(a–e), for the control or scramble groups, a large number of cells invaded the lower chamber, and there was no statistic difference between these two groups [Figure 4(f)]. As for TMZ or miR-21 inhibitor alone, the number of invaded cells obviously decreased. However, when the cells were treated with TMZ and miR-21i, much fewer glioma cells went through the matrigel ($P^* < 0.01$) compared with either single TMZ or miR-21i alone (about 2.3-fold lower than TMZ alone).

DISCUSSION

Malignant gliomas are the most frequent and aggressive brain tumor. Adjuvant chemotherapy is quite necessary followed by the surgical resection.[9] However, only a moderate increase was found in survival, due to the infiltrative feature GBM which led to their resistant to treatment.

The combination of two or more anti-tumor agents has been investigated to overcome the current limitations in the treatment of patients with GBM and results in a better prognosis for these patients. For instance, the combination of paclitaxel and topotecan with filgrastim has resulted in modest activity in adults with recurrent or refractory GBM and anaplastic astrocytoma in phase II trials.[10] And taking enzyme-inducing antiepileptic drugs (EIAED) substantially decreases plasma exposure of imatinib and achieves adequate plasma concentrations, leading to a higher therapeutic efficacy for malignant gliomas patients.[11] Besides, various small-molecule inhibitors, such as gefitinib and erlotinib (inhibitor for EGFR), PTK787/ZK222584 (inhibitor for VEGFR), and thalidomide have demonstrated the improving of patient outcomes in combination with chemotherapy in recurrent GBM.[12]

Recent therapies are also focusing on antisense technology used alone or combined with pharmacological treatment. Trojan indicated that treatment of glioblastoma by antisense anti-IGF-I cellular therapy resulted in a prolonged survival time (19 and 24 months, respectively) compared to the range of 12–15 months in conventional therapy.[13] Also clinical trials are under way assessing transforming growth factor-beta2 antisense oligonucleotides and regulatory T cell depletion. Combination of any of the above approaches with chemotherapy or radiotherapy is strongly supported by animal and clinical observations.[14] Now actual applications of growth factor inhibitors and antisense approaches have become a promising treatment of glioblastoma multiforme: using inhibitors and antisense targeting growth factors, including IGF-I, EGF, TGF, TGF-β2, their receptors, and their downstream steps of signal transduction pathways (IRS-1, PI3K, AKT, PKC, Bcl-2, GSK3, glycogen synthase GS, PTEN).[15–17] The inhibition of

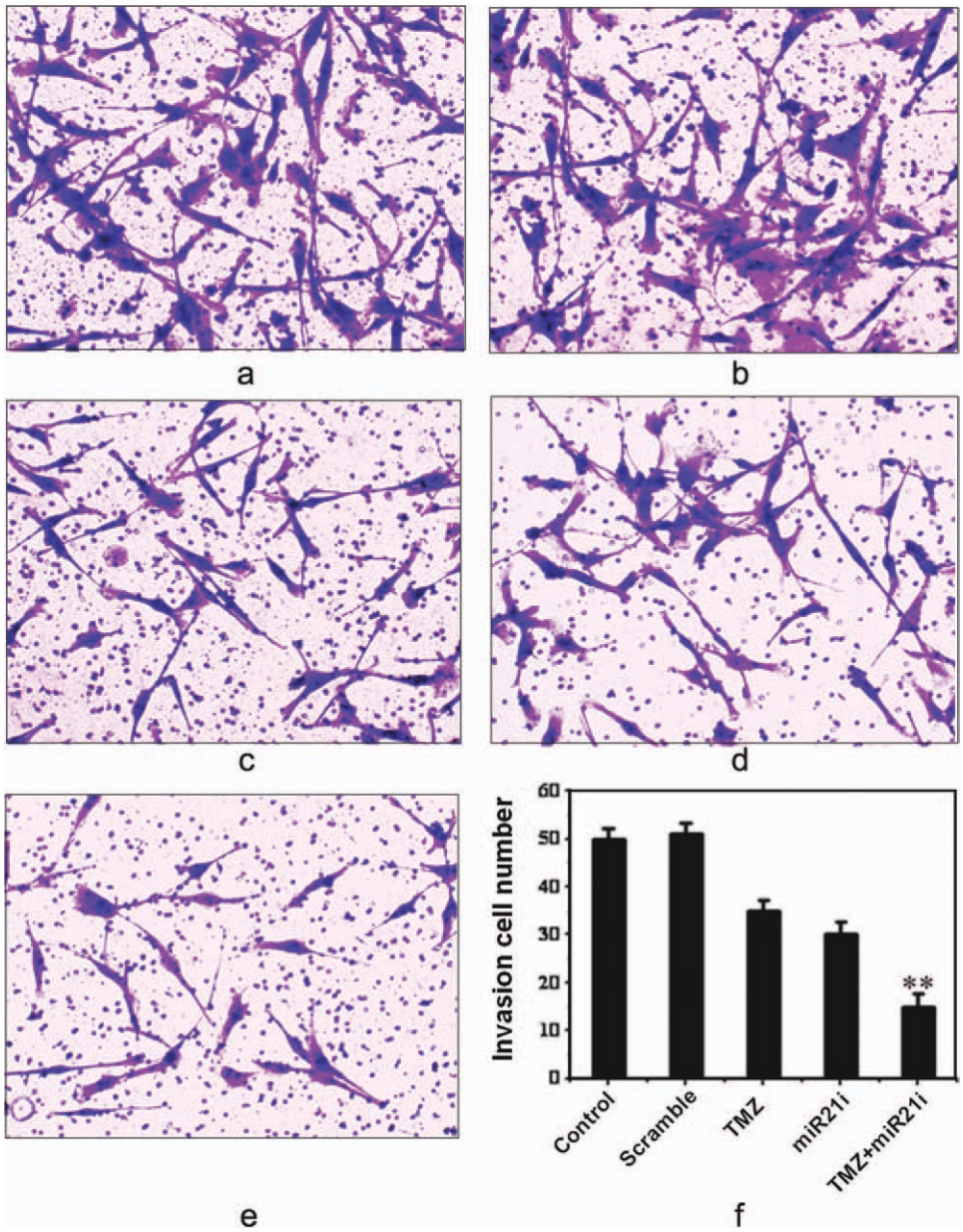

Figure 4. Cell invasion ability assessed by a transwell assay at 72 h after the treatment of U87 cells with: (a) Control, (b) Scramble, (c) TMZ alone, (d) miR-21i/PAMAM alone, and (e) TMZ+miR-21i/PAMAM. The number of cells that could invade via the membrane were shown as a histogram underside (**$P < 0.01$) (f). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

signal transduction pathways common to growth factors and glycogen synthesis by antisense glycogen synthase validates a putative target and a new approach for further study to advance the much-needed efficacy of intervention strategies for malignant gliomas.

Most recent technologies introduced for glioma treatment including those of triple helix, TH,[18] as well as potentially useful siRNA[19] and miRNA.[20] The role of 21-23 mer double-stranded RNA (siRNA) in the silencing of genes is strongly similar to that of the TH DNA mechanism, which also involves 23 mer RNA.[18] The silencing of critical gene products by RNAi technology has generated significant antiproliferative and proapoptotic effects in cell-culture systems or in preclinical animal models. Nevertheless, current limitations of RNAi therapy such as *in vivo* delivery, incomplete suppression of target genes, nonspecific immune responses and resistance, need to be overcome.[19,21]

It has reported recently that in various cancer malignancies, approximately 200 miRNAs that have been identified are dysregulated significantly. Up-regulated or down-regulated miRNAs could modulate sensitivity and resistance to anticancer drugs in substantial ways.[22] MiR-21 has been identified as one of the most overexpressed microRNAs in a number of human cancers,[23] including gliomas.[24] The knockdown of miR-21

significantly altered expression of 169 genes involved in nine cell-cycle and signaling pathways.[25,26] So, suppressing the expression level of miR-21 has been considered as promising approach to cancer treatment.[27,28] Previous studies had demonstrated that combining LNA-antimiR-21 oligonucleotides with S-TRAIL leads to synergistic cytotoxicity *in vitro* and *in vivo* in human gliomas.[5] Our previous study indicated the enhancement of chemo-sensitivity of glioma cells to taxol by miR-21 inhibitor.[29]

Temozolomide (TMZ) has been accepted by the US Food and Drug Administration (FDA) as a standard antitumor drug for glioma in recent two years.[30] However, insensitivity or resistance of glioma cells to TMZ is a major problem in the treatment of glioma.[31] Recent studies have reported that over-express miR-21 could inhibit TMZ-induced apoptosis in U87MG cells by decreasing Bax/Bcl-2 ratio and caspase-3 activity.[6] Thus here we used the miR-21i and TMZ to investigate the combination effect of the two reagents in glioma cell line U87 with high miR-21expression.

To ensure an efficiously transfection of miR-21i, PAMAM was chosen as the vector because of its high gene transfer ability.[32,33] The flow cytometry analysis [Figure 2(a)] confirmed the high cell uptake efficiencies of miR-21i mediated by PAMAM, probably due to their small size [Figure 1(a,b)] and the positive charge of the polyplexes [Figure 1(c)]. The "proton sponge effect"[34] also ensured the high transfection efficiency of miR-21i, which was indicated by the expression level of the miR-21 as shown in [Figure 2(b,c)]. Interestingly, simultaneously using TMZ had no significant effect on the uptake of miR-21i, but the expression level of miR-21 was much lower than those treated by miR-21i alone, implying the synergistic regulation effect by TMZ and the miR-21i.

To comprehensively study the combination effect when treated with TMZ and miR-21i, the cell proliferation [Figure 3(a)], apoptosis [Figure 3(b–f)], and invasion (Figure 4) were administered. On the whole, the therapy effect was significantly improved when combined treatment of TMZ and miR-21i to glioma cell lines. The reasons for the two drugs achieved synergistic effects could be analyzed from two aspects. Firstly, as shown in Figure 3(a), the IC50 value of TMZ was decreased from 29 μM to7.5 μM when cells were exposed to miR-21i, suggesting chemosensitization of miR-21i to TMZ.[35] These results demonstrated that miR-21i could definitely increase the sensitivity of glioma cells to TMZ, further proving Shi's speculation that the overexpression of miR-21 on inhibiting TMZ-induced glioma cell apoptosis.[6] Second, when the cells were treated with TMZ, the miR-21 expression level was significantly decreased [Figure 2(b,c)], leading to the enhancement in apoptosis and inhibition of proliferation and invasion. Though the exact mechanism for the decreased miR-21 expression by TMZ was unknown, we could speculate that signal transducer and activator of transcription 3 (STAT3), an oncogenic transcription factor,[36,37] maybe the key for TMZ to decrease miR-21 expression. It has reported that STAT3 contributed to TMZ-resistance in gliomas[38] and it was a potential target for the reversal of TMZ-resistance in patients with a recurrent glioma.[39] Furthermore, there were 2 phylogenetically conserved STAT3 binding sites in the upstream region of miR-21 and Stat3 could directly regulate the expression of miR-21.[40,41] So we inferred that TMZ reduced miR-21 expression through STAT3. Naturally, further research would be done to prove this speculation. Also the synergistic activity of miR21i and TMZ together on glioma *in vivo* would be further studied.

CONCLUSIONS

The combination of miR-21i with TMZ not only induced more tumor suppression but also returned chemo-sensitivity of TMZ. TMZ could knockdown the expression level of miR-21, and treatment of glioma cells with TMZ and miR-21 inhibitor delivered by PAMAM extremely promoted the apoptosis, and meanwhile inhibited the invasion of tumor cells. Collectively, our discovery provided a novel therapeutic strategy for the clinic treatment of glioma therapy in future. Although the miRNA knockdown technology has not yet been used in clinical, its molecular mechanisms of underlying targets therapy and sensitizing chemotherapy for gliomas could be promising tumor therapies.

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