

Original Article

Overexpression of miR-874 enhances chemosensitivity of glioma cells to temozolomide by the oncogenic STAT3 pathway

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Abstract: MicroRNAs, small non-coding RNAs (21-23 nt in length), are known for regulating carcinogenesis and tumor progression in glioma. MicroRNA-874 (miR-874) has shown play an important role in many human cancers as tumor suppressors. Our previous studies found that miR-874 reduced in glioma tissue and overexpression of miR-874 inhibits glioma cell proliferation and induces apoptosis. However, the biological functions of miR-874 in chemotherapy sensitivity enhancing effect have not been elucidated completely. The present study evaluated the biological function and mechanism of miR-874 in chemotherapy sensitivity enhancing effect in glioma. Our results revealed that the cell proliferation and migration were decreased, cell apoptosis was induced, and the mitochondrial membrane potential was also declined in glioma cells treated with miR-874 with temozolomide (TMZ). And miR-874 with TMZ can reduce the expression level of migration related protein MMP-2 and MMP-9, down-regulate the expression level of Bcl-2 and induce the expression level of Bax. More importantly, miR-874 with TMZ reduced the expression level of STAT3 protein and inhibited STAT3 phosphorylation. Our results demonstrated that overexpression of miR-874 potentiates chemosensitivity of glioblastoma to TMZ by the oncogenic STAT3 pathway, which might provide novel strategies for clinical treatment.

Keywords: Glioblastoma, MicroRNAs, STAT3 transcription factor, apoptosis, cell migration assays, chemotherapy, adjuvant

Introduction

Glioblastoma multiforme (GBM) is the most common primary brain tumor in adults [1]. Over the past decades, despite the improvements of neurosurgery, chemotherapy and radiotherapy, the frustrating median survival is approximately 9 months and only about 10% of patients can survive to 2 years [2]. The factors contributed to this phenomenon can be summarized as follows: invasiveness and rapidness of tumor growth, the genetic heterogeneity of tumors, poor understanding of the molecular mechanism underlying the progression and strongly resistant to chemotherapy drugs such as Temozomide [3, 4]. Owing to the distribution to all tissue of human body including CNS, TMZ can function as a broad-spectrum antitumor agent, such as melanoma, mesothelioma, lym-

phoma, sarcoma, leukemia, ovary and carcinoma of the colon [5]. Owing to these extensive effect, TMZ inevitable bring a wide range of side effects. The result of safety research showed side effects involved neutropenia, thrombocytopenia, and myelosuppression in a dose-dependent manner.

Recently, microRNA (miRNA) has become increasingly relevant in cancer research. miRNAs play important roles in the progression of gliomas, by regulating the proliferation, cell cycle, apoptosis and invasion of tumors, experientially, some miRNAs could enhances the sensitivity of TMZ [6]. miR-874 were found decline in colorectal cancer cells, non-small cell lung cancer, gastric cancer and breast cancer, which also play an important role in cell proliferation, migration and invasion [7-10]. Our team has dis-

covered that miR-874 reduced in glioma tissue and overexpression of miR-874 inhibits glioma cell proliferation and induces apoptosis [11]. Therefore, we devote to find that the effect of miR-874 to enhance the chemosensitivity in glioma. We aspire to decrease the recurrence rate through improving ability of TMZ to restrain glioma cells.

In the present study, the aim of our study was to investigate if overexpression of miR-874 could be used to increase the chemosensitivity of glioma cells to TMZ. We found that overexpression of miR-874 sensitized glioma cells to TMZ-induced apoptosis, and moreover, potentials TMZ-induced migration reduction, with increase the expression level of Bax and down-regulate the expression level of MMP-2, MMP-9, and Bcl-2.

Materials and methods

Cell culture

The human glioblastoma cell lines U87 and LN229 were purchased from Chinese Academy of Sciences Cell Bank (Beijing, China). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum and 100 U/ml penicillin, 100 µg/ml streptomycin in the appropriate atmosphere containing 5% CO₂ at 37°C.

Transfection

For miR-874 overexpression, cells were transfected with miR-874 mimics (synthesized by Shanghai GenePharma Company, China). The sequences of miR-874 mimic: 5'-CUGCCCUGG-CCCAGGGACCGA-3', negative control sequence 5'-UUCUCCGAACGUC ACGUTT-3'. Cells were trypsinized and seeded in 6-well or 96-well plates (Corning Costar, Cambridge, MA, USA) at the day before transfection. The oligonucleotides were used at concentration of 200 nM and transfected to cells by using Lipofectamine 2000 (Invitrogen Life technology, Carlsbad, CA, USA), according to the manufacturer's instructions.

Chemotherapy treatment with TMZ and group

U87 and LN229 cells were seed into 6 or 96-well plates and then transfected with NC, miR-874 mimics or not. Then the U87 and LN229 cells were exposed with different concentration or at different points under TMZ

(100 µM). The group of experiments were described as follow: (1) Scramble group (cells were transfected with scramble sequences) (2) miR-874 mimics group (cells were transfected with miR-874 mimics) (3) TMZ group (cells were treatment with TMZ only) (4) miR-874 + TMZ group (cells were transfected with miR-874 mimics and then swap with medium contain TMZ).

Western blotting

U87 and LN229 cells were subject to lysis buffer (CWBio, China) containing protease inhibitor cocktails (Roche, Basel, Switzerland). The cells were scraped into EP tube, incubated for 30 min, centrifuged at 12,000×g for 15 min at 4°C. Proteins were resolved on an SDS denatured polyacrylamide gel and then electrotransferred onto Immun-Blot PVDF membranes (BioRad, CA, USA) and blocked with 5% milk in TBST buffer for 2 hours at room temperature, and incubated overnight at 4°C with primary antibodies. After washed 5 times in TBST buffer, the membranes reacted with HRP-conjugated secondary antibody and developed with the ECL kit (Millipore, Billerica, USA). MMP-2, MMP-9, Bcl-2, Bax (Santa Cruz, CA, USA), STAT3 and p-STAT3 (SAB, USA) were used.

MTT assay

U87 and LN229 cells (5×10³) were incubated in 96-well plates contain 100 µl medium each well. After cells were seed into 96-wells for 12 hours, the transfection was performed, and then exchanges the medium with or without TMZ. The proliferation rate was measured 24 and 48 hours after exchange the media. The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) 20 µl of 5 mg/ml was add into supernatant. After incubated for 4 hours, exchanged the medium with 150 µl DMSO and determined the absorption at 490 nm by SPECTERA MAX 190. Each experiment was performed in triplicate.

In vitro migration and invasion assays

To investigate the cell invasive and migratory abilities, the transwell plates (24-well, 8-µm pore size, Milipore, USA) with or without a Matrigel (BD Bioscience, USA) coating layer were used. The cells were suspended at 1×10⁵ cells/ml add into the well with or without TMZ in

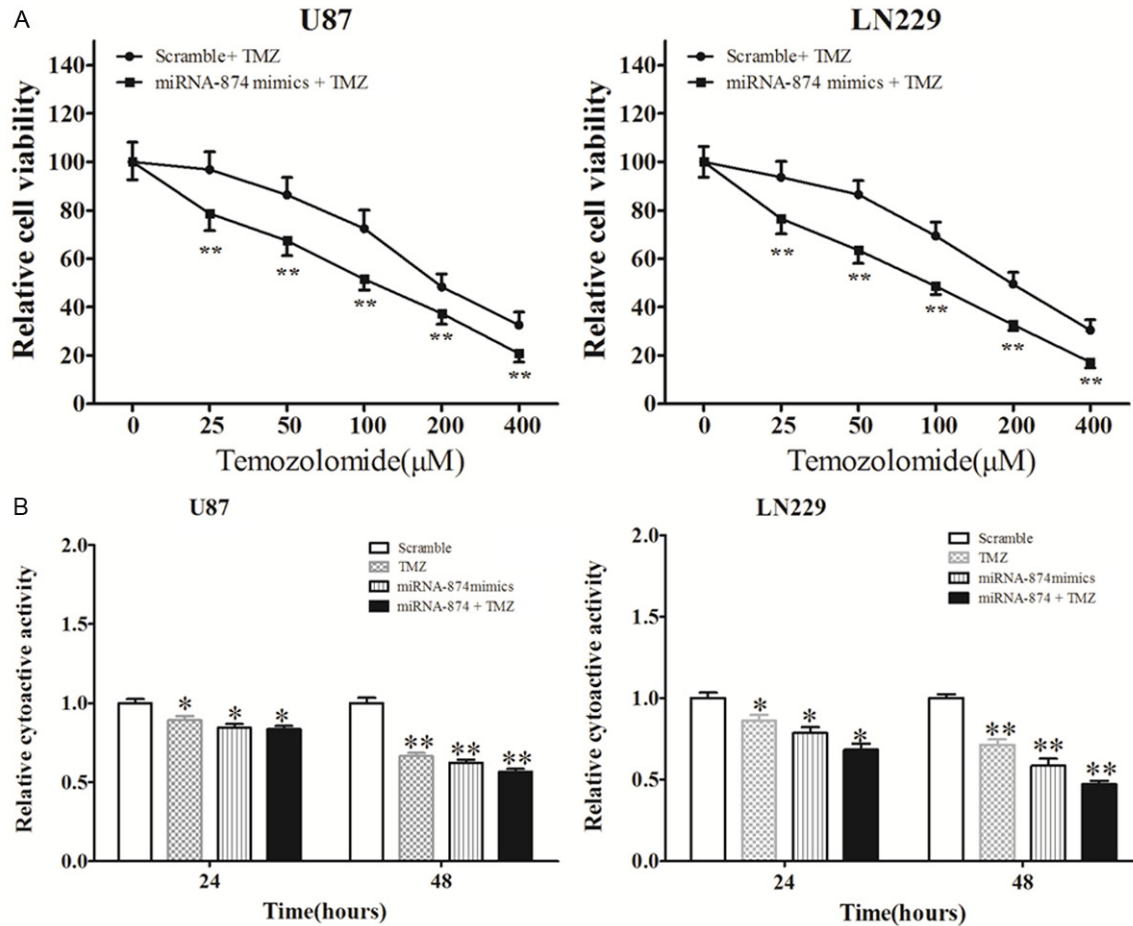


Figure 1. Over-expression of miR-874 inhibit glioma cell U87 and LN229 proliferation. A. Cell proliferation was evaluated in glioma cells stably expressing scramble or miR-874, with or without the TMZ treatments at different doses. MTT assay was performed 48 h after treatment. * $P < 0.05$ in comparison with Scramble. B. Cell proliferation was evaluated in glioma cells stably expressing scramble or miR-874, with or without the TMZ (100 μ M) treatments at different times. MTT assay was performed 24 h and 48 h after treatment. * $P < 0.05$ in comparison with Scramble.

serum-free medium and seeded in the upper well. The lower well were filled with culture media containing 10% FBS as a chemoattractant. The cells that had migrated to the lower chamber were fixed by methanol for 15 minutes, stained with 0.2% crystal violet for 30 minutes, and counted under a light microscope.

Flow cytometric analysis of apoptosis

U87 and LN229 cells were transfection with NC or miR-874 mimics for 6 hours, and then exchange medium with or without TMZ. At 48 hours post-treatment with TMZ, cells were harvest by trypsin. Cells were washed in PBS, and resuspended in 100 μ l of binding buffer. After incubation with 5 μ l Annexin V-FITC and 5 μ l propidium iodide (PI) (BD Pharmingen, San

Diego, CA, USA) for 15 min in dark environment, another 400 μ l binding buffer to each tube. The samples were detected and analyzed by FACS Canto II (BD Pharmingen, San Diego, CA, USA).

Measurement of mitochondrial membrane potential

U87 and LN229 cells were seeded in a 6-well plate. After incubation for 12 h, the medium was replaced with a fresh medium and cells were transfected with miR-874 or scramble sequences. After incubation for 24 h, cells were treated with 100 μ M TMZ for 24 h. Next, the cells were harvested and incubated with 5 μ M JC-1 (Beyotime Biotechnology, China) in PBS for 20 min at 37°C. Then, the samples were detected and analyzed by FACS Canto II (BD Pharmingen, San Diego, CA, USA).

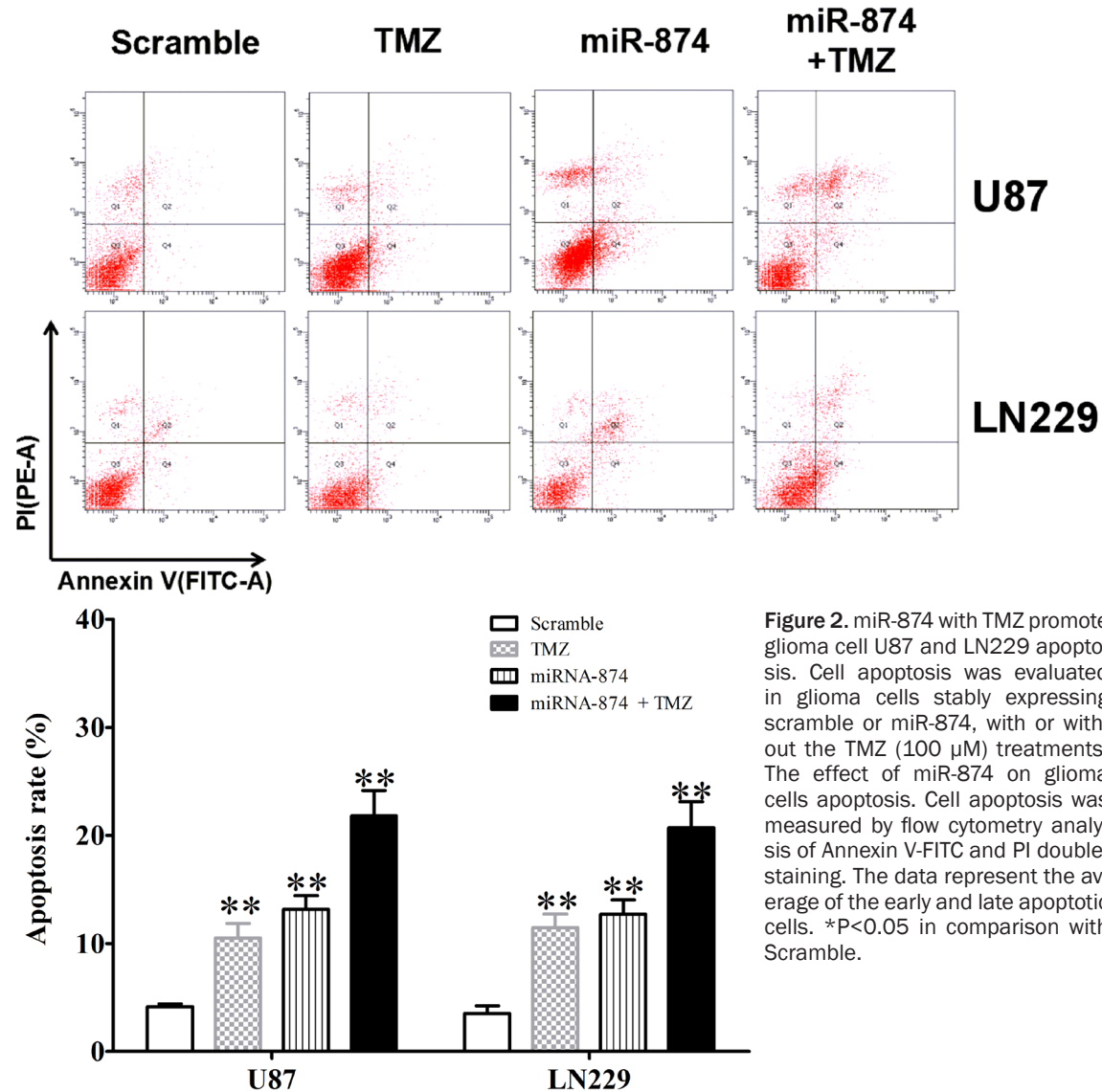


Figure 2. miR-874 with TMZ promote glioma cell U87 and LN229 apoptosis. Cell apoptosis was evaluated in glioma cells stably expressing scramble or miR-874, with or without the TMZ (100 μ M) treatments. The effect of miR-874 on glioma cells apoptosis. Cell apoptosis was measured by flow cytometry analysis of Annexin V-FITC and PI double-staining. The data represent the average of the early and late apoptotic cells. * $P < 0.05$ in comparison with Scramble.

Statistical analysis

Statistical analyses were performed using Graphpad 5.0, and compare through one-way analysis of variance (ANOVA). The data are showed as mean \pm standard deviation (SD) of three independent experiments. The P values less than were considered to be statistically significant.

Results

Overexpression of miR-874 coincides with increased TMZ cytotoxicity

To detect whether overexpression of miR-874 can enhanced the inhibition ability of TMZ to

glioma cells, we examined the cell proliferation with different treatment. After cells transfected with miR-874 mimics and treated by TMZ, we determined cells numbers by MTT assay after treatment. As shown in **Figure 1A**, overexpression of miR-874 in U87 and LN229 cells significantly increased chemosensitivity to TMZ treatment, and cell viability was significantly suppressed by TMZ treatment with correlation of the drug concentrations compared with scramble group cells. Furthermore, cell viability in the presence of TMZ (100 μ M) was assayed by MTT at different time points. The results showed that overexpression of miR-874 significantly inhibited cell survival of both U87 and LN229 cells in the presence of TMZ (**Figure 1B**). Combining with the result of MTT, we can con-

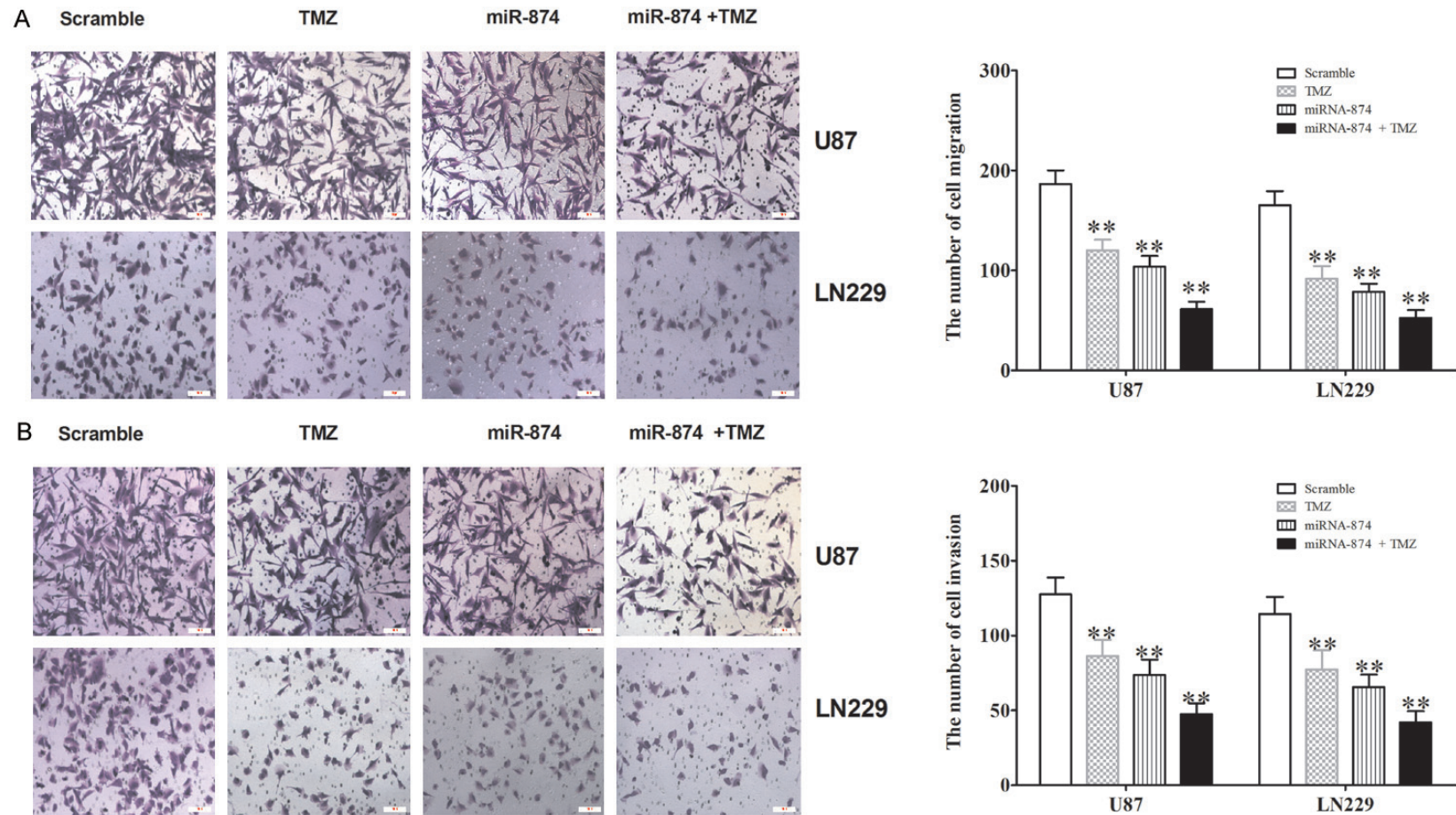


Figure 3. miR-874 with TMZ reduce the invasion and migration capability of U87 and LN229. A. Transwell migration assays were performed in U87 and LN229 cells evaluated in glioma cells stably expressing scramble or miR-874, with or without the TMZ (100 μ M) treatments. Migratory cells were stained and the average number of cells was counted. * $P < 0.05$ in comparison with Scramble. B. Transwell invasion assays were performed in U87 and LN229 cells evaluated in glioma cells stably expressing scramble or miR-874, with or without the TMZ (100 μ M) treatments. The invasive cells were stained and the average number of cells was counted. * $P < 0.05$ in comparison with Scramble.

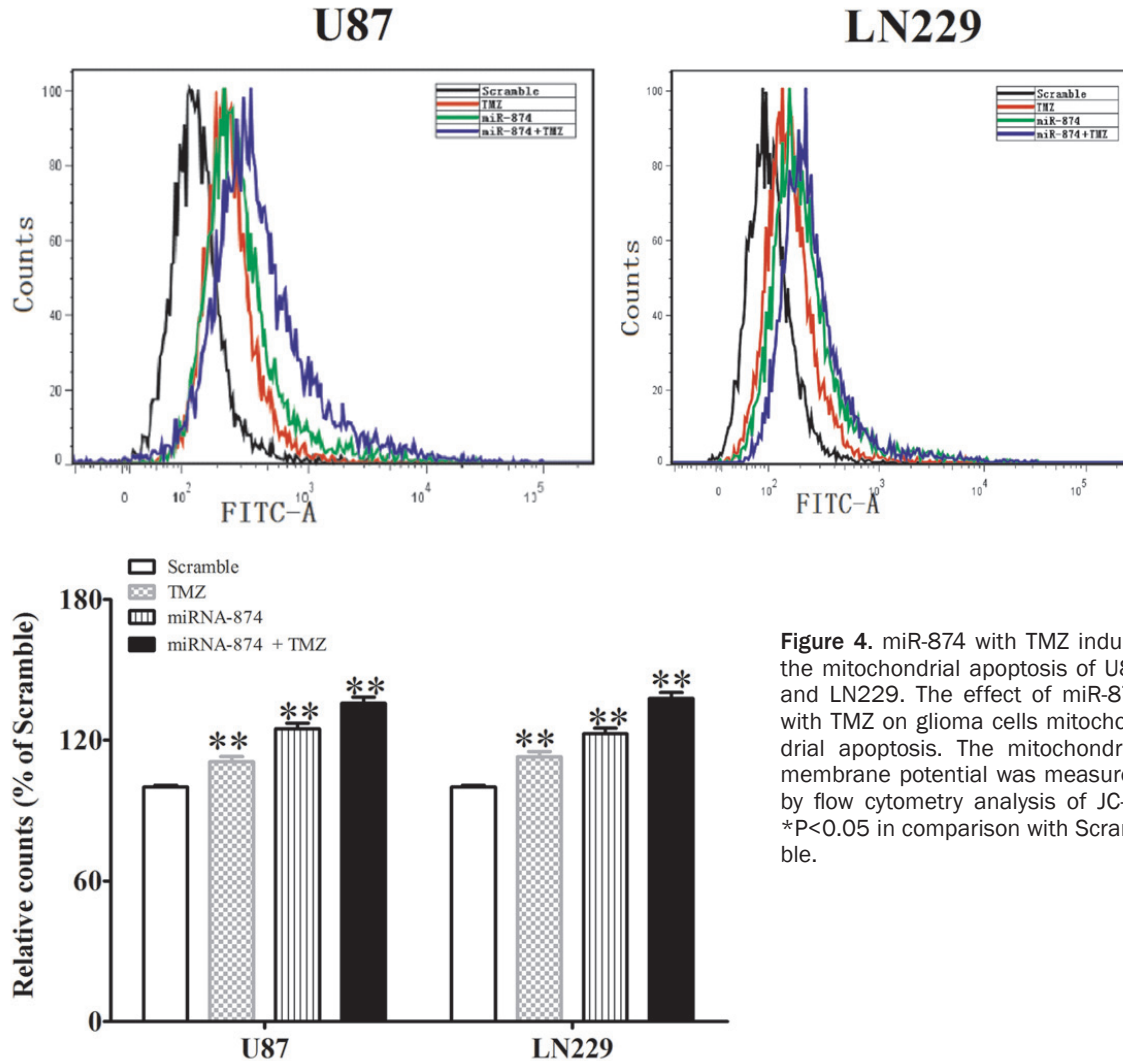


Figure 4. miR-874 with TMZ induce the mitochondrial apoptosis of U87 and LN229. The effect of miR-874 with TMZ on glioma cells mitochondrial apoptosis. The mitochondrial membrane potential was measured by flow cytometry analysis of JC-1. *P<0.05 in comparison with Scramble.

clude the synergies between miR-874 and TMZ possess more power to inhibit glioma.

Overexpression of miR-874 enhances TMZ-induced apoptosis

We next detected whether the reason decreased cell viability is caused through increasing apoptosis. The number of apoptosis cells in every group was count by flow cytometry by labeling with Annexin V-FITC and PI. As shown in **Figure 2**, the rate of apoptotic cells number was significantly higher of miR-874 combined with TMZ group than other groups.

Overexpression of miR-874 leads to decrease migration in response to TMZ

As we observed that the capability of U87 and LN229 cells to migration and invasion was decreased under miR-874 mimics or treating

with TMZ. Transwell assay without Matrigel demonstrated that forced-expression of miR-874 reduced migration in U87 and LN229 cells respectively when compared with negative control (**Figure 3A**). Furthermore, overexpression of miR-874 also significantly decreased the number of invasive U87 and LN229 cells in the inserts covered with Matrigel (**Figure 3B**).

miR-874 combined with TMZ significantly decreased the mitochondrial membrane potential of glioma cells

The main change of endogenous apoptotic pathway is mitochondrial membrane potential collapse. The mitochondrial membrane potential was detected through JC-1, which is a lipophilic and cationic dye accumulation in a potential mitochondrial dependent. JC-1 emits red fluorescence in normal cells, but the fluorescence will disappear if the mitochondrial mem-

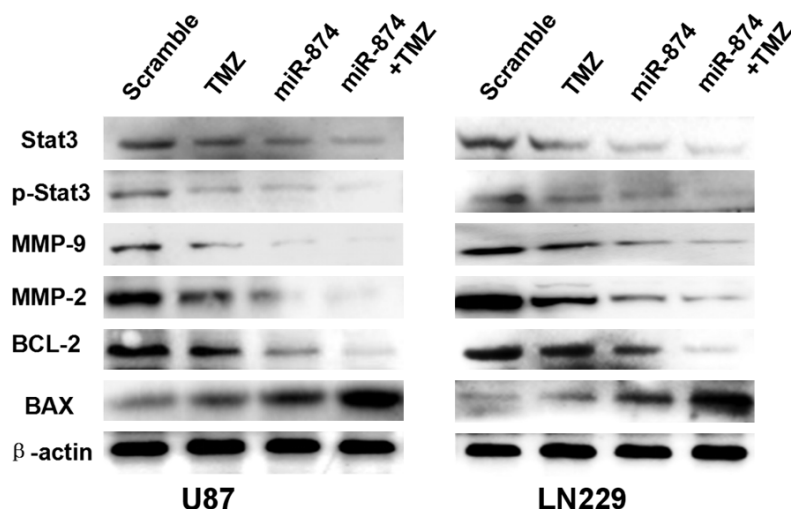


Figure 5. miR-874 with TMZ altered related protein expression. The of apoptosis related proteins Bcl-2 and Bax, migration/invasion marker protein expression of MMP2 and MMP9 in U87 and LN229 cells were determined by western blotting. Also, the oncogenic STAT3 pathway related proteins STAT3 and p-STAT3 in U87 and LN229 cells were determined by western blotting.

brane potential decline. As shown in **Figure 4**, the negative controls and TMZ group displayed similar fluorescen intensity of JC-1. In contrast, an obvious increase in the proportion of cells with loss of mitochondrial membrane potential was shown in miR-874 combined with TzM group compared

miR-874 enhanced chemotherapy sensitivity to TMZ by inhibiting the STAT3 pathway

To investigate the molecular mechanism of this synergies, we found that miR-874 combined with TMZ reduce the expression level of STAT3 protein and inhibit STAT3 phosphorylation. Also, we measured the level of apoptosis related proteins Bcl-2 and Bax, migration related proteins matrix metalloproteinases (MMPs). As shown in **Figure 5**, the result shows that the translation of Bcl-2, MMP-2 and MMP-9 were block in U87 and LN229 cells. The expression of Bax significantly increased in U87 and LN229 cells. Similarly, the expression of miR-874 combined with TMZ group was least.

Discussion

In recent years, the molecular mechanism of the occurrence and development in glioma is closely related to be studied [12, 13]. Also, the hundreds of miRNAs have been shown to involve in regulating gene expression at post-

transcriptional level. Micro-RNAs, mostly worked as a tumor-suppressor agent in carcinoma, is commonly abrogated due to the frequently hypermethylated of CpG islands of its primary transcripts [14, 15]. MiRNAs are involved in glioma tumorigenesis, metastasis or angiogenesis, including that in glioma cells biological processes in vitro. Recent researches have proclaimed that the decreased expression of miR-874 may play an important role in the development of various cancers, such as colorectal cancer cells, non-small cell lung cancer, gastric cancer and breast cancer [7-10]. In our

previous studies, we discovered that miR-874 reduced in glioma tissue and miR-874 inhibited cell proliferation and induced apoptosis in human glioma [11]. Ordinary treatment is lack of specificity and do damage normal tissue. Even treated with surgery, chemotherapy and radiotherapy, the clinical outcome of patients is poor. So we want to found an effective strategy that miR-mediated inhibition of proteins to enhance glioma cells TMZ sensitivity.

In the present study, the cells were transfected with miR-874 mimics to overexpression level of miR-874, and then exposed to TMZ. Combine treatment with miR-874 and TMZ significantly decreased the proliferation and increased apoptosis than miR-874 or TMZ treated alone. To investigate the mechanism of this synergism, we found the expression of Bcl-2 was down-regulated and the expression of Bax was up-regulated. Previous study show Bcl-2 plays crucial roles in the mitochondrial apoptosis pathway, we examined whether introduction of miR-874 combined with TMZ triggered a mitochondrial pathway, based on the assays of mitochondrial membrane potential [16, 17]. We found that an obvious increase in the proportion of cells with loss of mitochondrial membrane potential was shown in miR-874 combined with TMZ group compared to both miR-874 and TMZ-alone treatment group.

Besides, as an important feature of tumor, migration frequently contributes to the failure of treatment [18]. We performed Transwell assay to study the combined effect on migration and invasion of glioma cells. The result revealed that pre-treat with miR-874 and then combine with TMZ decreased migration and invasion. MMPs are the family than can degrade extracellular matrix. But MMPs are synthesized as inactive zymogens requiring the dissociation of a propeptide by proteinase cleavage [19, 20]. In our research, the western blot result show that MMP2 and MMP9 were decline in miR-874 combine with TMZ group.

Some researchers revealed that miR-874 promoted migration, inhibited cell growth and induced apoptosis by targeting STAT3 in human cancer cells. In glioblastoma, the abnormal expression of STAT3 was related to the poor prognosis [21, 22], and it is an effective strategy that inhibition of the STAT3 signaling pathway by miRNAs for glioma therapy [23]. And some studies showed that STAT3 contributes to TMZ-resistance in gliomas and the inhibition of STAT3 expression could enhance glioma cells TMZ sensitivity. In this research, pre-treated with miR-874 enhance the effect of TMZ on proliferation, apoptosis, and migration. Moreover, we detected the protein expression of STAT3 by western blot and found miR-874 upregulation combined with TMZ in U87 and LN229 cells inhibited STAT3 and p-STAT3 expression. Constitutive STAT3 activation is involved in cell proliferation, apoptosis, angiogenesis, migration, and invasion in glioma [24]. STAT3 activation requires phosphorylation of its tyrosine residue [25, 26], resulting in p-STAT3 forming dimers with partner proteins in order to translocate into the nucleus to activate expression of downstream targets, including Bcl-2, Bax, MMP9 and MMP2.

To the glioma, it is urgently need to change conventional strategies instead by complexing agent to enhance cytotoxicity of chemotherapeutic drugs. We found overexpression of miR-874 can enhance cytotoxicity of TMZ through oncogenic STAT3 pathway. These results suggest that targeting miR-874 combine with TMZ could be a promising therapy strategy for glioma.

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Disclosure of conflict of interest

None.

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