Article

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Luteolin Reduces Temozolomide Resistance via Regulating PI3K/AKT Pathway in Glioma

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Background: Unsatisfactory prognosis of glioma partly arises from its resistance to temozolomide (TMZ). This work will focus on whether luteolin sensitize glioma cells to TMZ and explore its regulatory mechanism.
Methods: The viability, cell cycle progression and programmed death of TMZ-resistant glioma cells were evaluated by CCK-8 assay, flow cytometry, and TUNEL assay. Additionally, the cell migration and invasion were examined by Transwell assay. The expression of PI3K/AKT signaling pathway-related proteins was probed by Western blot. The target genes of luteolin, and those related to gliomas were identified by bioinformatics.
Results: *In vitro* experiments suggested that luteolin could impede the malignant biological behaviors including viability and aggressiveness of TMZ-resistant glioma cells. Bioinformatics analysis implied the target genes of luteolin and glioma were associated with the PI3K/AKT signaling pathway. Luteolin significantly inhibited p-PI3K and p-AKT proteins' expression in TMZ-resistant glioma cells.
Conclusion: Luteolin could sensitize TMZ-resistant cells to TMZ, at least partly via modulating the PI3K/AKT signaling.

Keywords: glioma; luteolin; temozolomide resistance; PI3K/AKT signaling pathway

Introduction

Glioma accounts for 81% of malignant tumors in brain [1]. As the World Health Organization defined, glioma is categorized into four grades. Glioma is a deadly disease, and Grade IV, the most malignant, also known as glioblastoma (GBM), with a two-year survival rate of only 26% [2,3]. Recently, the extensive use of temozolomide (TMZ) as a chemotherapeutic agent has extended the median survival time of glioma patients [4]. However, the resistance to TMZ has been recognized as the key contributor of treatment failure in glioma patients, especially for high grade glioma cases. In this context, to find potential drugs to reverse TMZ resistance is demanded.

Luteolin (3′, 4′, 5,7-tetrahedroflavine), a flavonoid compound, can be extracted from Chinese medicinal herb *radix Codonopsis*. It has several beneficial effects for clinical application, such as the antioxidant effects, anti-inflammatory effects, immune regulation, and anti-tumor activities [5]. Reportedly, luteolin inhibits cancer cell viability in many cancers. For example, in triple-negative breast cancer, luteolin can probably block disease progression via modulating the AKT/mTOR signaling [6]. In lung cancer, luteolin inhibits activation of focal adhesion kinase and Src, and thus the aggressiveness of lung cancer cells are repressed [7]. In prostate cancer, luteolin suppresses anoctamin 1 expressions, and the cell proliferation and migration, as a result, are impeded [8]. Notably, luteolin can reverse drug resistance in tumors. For example, in lung cancer, luteolin can inhibit PI3K/Akt/mTOR signaling, and sensitize cancer cells to erlotinib [9]; in gastric cancer, regulating the Cyt c/caspase pathway, luteolin can impede the growth of gastric cancer cells and increase the sensitivity of oxaliplatin [10]. Additionally, luteolin induces the cell apoptosis and inhibits cell migrative and invasive abilities, and alleviates the cisplatin resistance of ovarian cancer cells [11]. As research reports, luteolin also shows the potential to kill glioma cells [12]. However, how luteolin works in TMZ-resistant glioma cells has not been explicate. Here we would focus on this subject by constructing TMZ-resistant cell strains and identify the function and potential mechanism of luteolin in treating TMZ-resistant glioma.

Materials and Methods

Cell Strains

The human glioma cell strains (U251 and LN229) (ATCC) were cultured in Dulbecco's Modified Eagle's Medium/Nutrition Mixture F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) in 5% CO_2 at 37 °C. Then the two cell strains were treated with an initial concentration of 1 μM TMZ (Cat.T2577, Sigma-Aldrich) for 2 weeks. The TMZ doses were then gradually

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increased and incubated for 2 weeks at each dose until a final concentration of 400 μM. Cells that finally survived in 400 μM TMZ were considered as TMZ-resistant cells (named U251/TR cells and LN229/TR cells, respectively).

Preparation of Luteolin

Luteolin ($C_{15}H_{10}O_6$, Molecular weight: 286.24, purity ≥98%) was from Sigma-Aldrich (Cat.L9283) (Fig. 4A). Luteolin was dissolved in dimethylsulfoxide (DMSO, Sigma) and prepared as a solution with 10 μM, 20 μM, 30 μM and stored at –20 °C for subsequent experiments.

CCK-8 Assay

Cell proliferative capacity was measured by the CCK-8 kit (Dojindo Molecular Technologies). Cells (5000/well) were inoculated on 96-well plates. To calculate the IC_{50} (half maximal inhibitory concentration) of luteolin, different doses of luteolin (1, 2, 5, 10, 20, 30, 40, 50, 100, 200 and 400 μM) were applied to treat the cells, and after the cells were cultured for 24 h, 10 μL CCK-8 reagent was added and incubated for 2 h at 37 °C in 5% CO_2. The OD value at 450 nm per well was measured by a microplate reader (Bio-Rad).

Cell Cycle Assay

TMZ-resistant cells, treated with TMZ and luteolin (or DMSO as control), were fixed overnight in pre-cooled 85% ethanol at 4 °C, stained in darkness at 4 °C for 30 min with propidium iodide (PI), and the cell cycle distribution was detected by flow cytometry. The percentages of each cell cycle phase were analyzed by Modfit 3.0 software (Verity Software House).

Cell Apoptosis Assay

The apoptotic rates of TMZ-resistant cells were probed by Annexin V-FITC/PI apoptosis detection kit (BD Pharmingen). After the cells were treated with TMZ and luteolin (or DMSO as control), they were treated with 0.25% trypsin and washed twice with PBS containing 5% BSA, and then the cells were incubated with 5 μL of Annexin V-FITC and 10 μL of PI solution in the dark for 20 min. The apoptotic rate was detected by a flow cytometer (BD Biosciences). Ultimately, the apoptotic rate in each group was under the analysis of FlowJo 10.0 software (Tree Star). The percentage of "Q2+Q3" cells was considered to be the apoptotic rate.

TUNEL Assay

TMZ-resistant cells were seeded at 3.5×10^5 cells/well on 6-well plates, and treated with TMZ and luteolin (or DMSO as control), fixed with 4% paraformaldehyde and permeabilized with 0.25% Triton-X 100. According to the manufacturers' instructions, cells were incubated with 50 μL of TUNEL reaction mixture (Beyotime), followed by incubation with DAPI (Beyotime) for 5min for nuclear staining (the incubation was performed in darkness). These cells were observed and counted under a fluorescence microscope (Olympus), and the apoptotic cells were with green fluorescence.

Transwell Assay

Transwell chamber (8-μm pore size; Millipore) precoated with or without Matrigel were used to test the migrative and invasive abilities of U251/TR and LN229/TR cells. 200 μL of serum-free culture solution was added to the upper room and 600 μL of culture solution with FBS was added to the lower room. 24 h later, the cells on the bottom of the porous membrane were stained with crystal violet and counted under an inverted microscope (Olympus).

Western Blot

The total protein was extracted from U251/TR and LN229/TR cells with cell lysis buffer (Boster Biological Technology). After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Millipore), which were subsequently blocked with 5% skimmed milk at ambient temperature for 2 h and subsequently incubated overnight with primary antibody at 4 °C. Anti-PI3K, anti-p-PI3K, anti-AKT, anti-p-AKT (dilution ratio: 1:1000) and anti-GAPDH (dilution ratio: 1:5000) antibodies were obtained from Abcam. The membrane was then incubated with secondary antibody (dilution ratio: 1:2000) for 2 h at room temperature and protein bands were detected by the ECL Luminance Reagent (Beyotime Biotechnology), with GAPDH as the internal reference.

Bioinformatics Analysis

We screened out the target genes for luteolin using the Traditional Chinese Medicine Systems Pharmacology (TCMSP) database. The targets associated with gliomas were analyzed and identified using the Genecards database. The intersecting target genes in TCMSP database and Genecards database were analyzed using an online tool (Venny 2.1.0) and the Venn diagram was drawn. DAVID database is an online site for gene function annotation and analysis. GO analysis and KEGG enrichment analysis were accomplished on the intersecting target genes, with "$p < 0.05$" as the threshold value.

Statistical Analysis

GraphPad Prism 7.0 was used. The results were expressed as mean ± standard deviation. Besides, student's t test was adopted for comparison between groups, and $p < 0.05$ indicated significant difference.

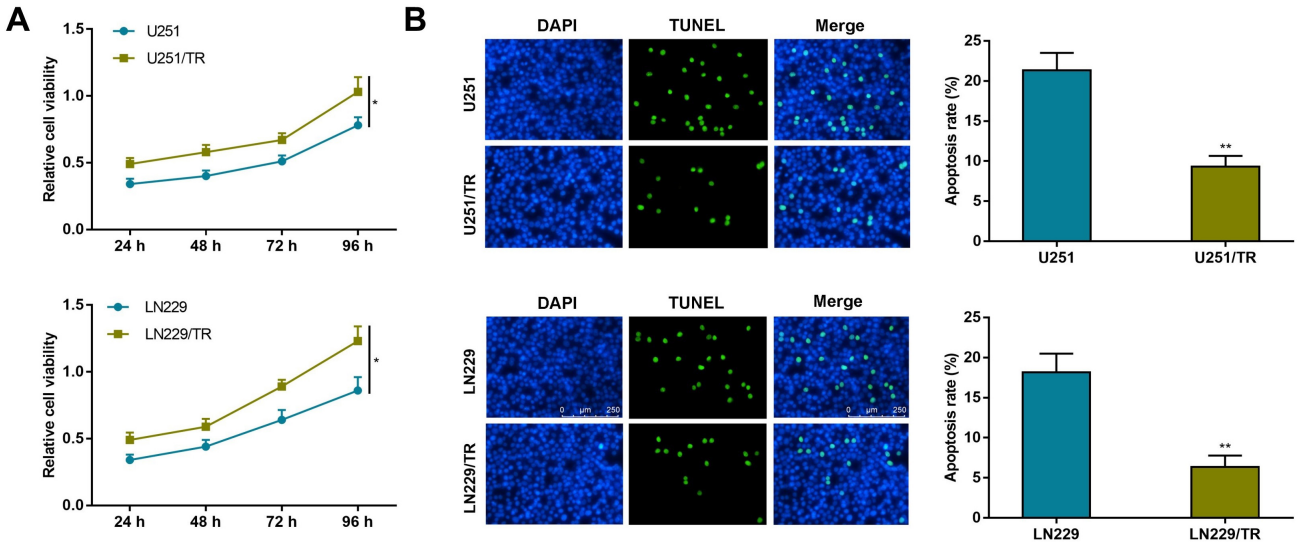

Fig. 1. The construction of temozolomide (TMZ)-resistant cells. (A) CCK-8 assay was applied for testing the cell viability of wild type and TMZ-resistant glioma cell strains. (B) TUNEL staining was used to detect the apoptosis of wild type and TMZ-resistant glioma cell strains. *$p < 0.05$; and **$p < 0.01$.

Results

The Construction of TMZ-Resistant Glioma Cells

U251/TR and LN229/TR were constructed with increasing concentration of TMZ. Compared with U251 and LN229 cells, as CCK-8 assay displayed, the proliferation rates of TMZ-resistant cells were remarkably higher (Fig. 1A). Under the analysis of TUNEL assay, the apoptotic rates of TMZ-resistant cells were greatly lower than those of wild type cells (Fig. 1B). These demonstrations indicated that TMZ-resistant cells strains were successfully modeled.

Luteolin Inhibits the Proliferative, Migrative and Invasive Phenotypes of TMZ-Resistant Cell Strains

Next, we calculated the IC_{50} of luteolin to TMZ-resistant cells, and the IC_{50} values for U251/TR and LN229/TR cells were 14.5 ± 2.6 μM, and 18.4 ± 3.4 μM, respectively. We treated the TMZ-resistant cell lines with 10 μM, 20 μM and 30 μM luteolin for 48 h and tested the cell proliferation through the CCK-8 experiment, with the findings showing that, the cell proliferation of the cells were greatly inhibited by 20 μM and 30 μM Luteolin (Fig. 2A). As the difference was not obvious between 20 μM and 30 μM, we selected 20 μM luteolin for subsequent experiments. Under the analysis of flow cytometry, after TMZ treatment, the proportion of cells in the G0/G1 phase was dramatically raised and that in the S phase was greatly declined in the luteolin-treated group (Fig. 2B). Transwell assay showed that treatment with 20 μM luteolin caused the decreased migrative and invasive abilities of U251/TR and LN229/TR cells (Fig. 2C). Collectively, luteolin significantly repressed the proliferative, migrative and invasive capabilities of TMZ-resistant glioma cell strains and blocked cell cycle process.

Luteolin Induces Apoptosis of TMZ-Resistant Cells

We next focus on the impact of luteolin on the programmed death of TMZ-resistant cells through TUNEL staining experiment and flow cytometry. After TMZ treatment, TUNEL staining results showed significantly higher apoptotic rates of TMZ-resistant cells in the luteolin-treated group compared with the vehicle group (Fig. 3A). Flow cytometry took on the same results (Fig. 3B). These results indicated that luteolin and TMZ synergistically kill TMZ-resistant glioma cell strains.

The Downstream Targets of Luteolin were Identified

To expound how luteolin functions in TMZ drug-resistant cells, we screened out the target genes of luteolin through TCMSP database and searched for those related to glioma in Genecards database. As the Venn diagram displayed, there were 18 overlapping genes (Fig. 4B,C). They were then subjected to GO and KEGG Enrichment Analysis. GO analysis results uncovered that the biological process of these genes was greatly enriched in glial cell apoptosis and cell cycle; cellular components were mainly concentrated in cytoplasm, protein complex, cytosol and nucleus; molecular function was mainly displayed on protein binding, enzyme binding, protein kinase activity, etc. As KEGG enrichment analysis revealed, these genes were pertinent to the PI3K/AKT signaling and cell cycle (Fig. 4D,E).

Luteolin Regulates PI3K/AKT Signaling Pathway

As Western blot exhibited, there was no significant difference in the expression of total PI3K and AKT between

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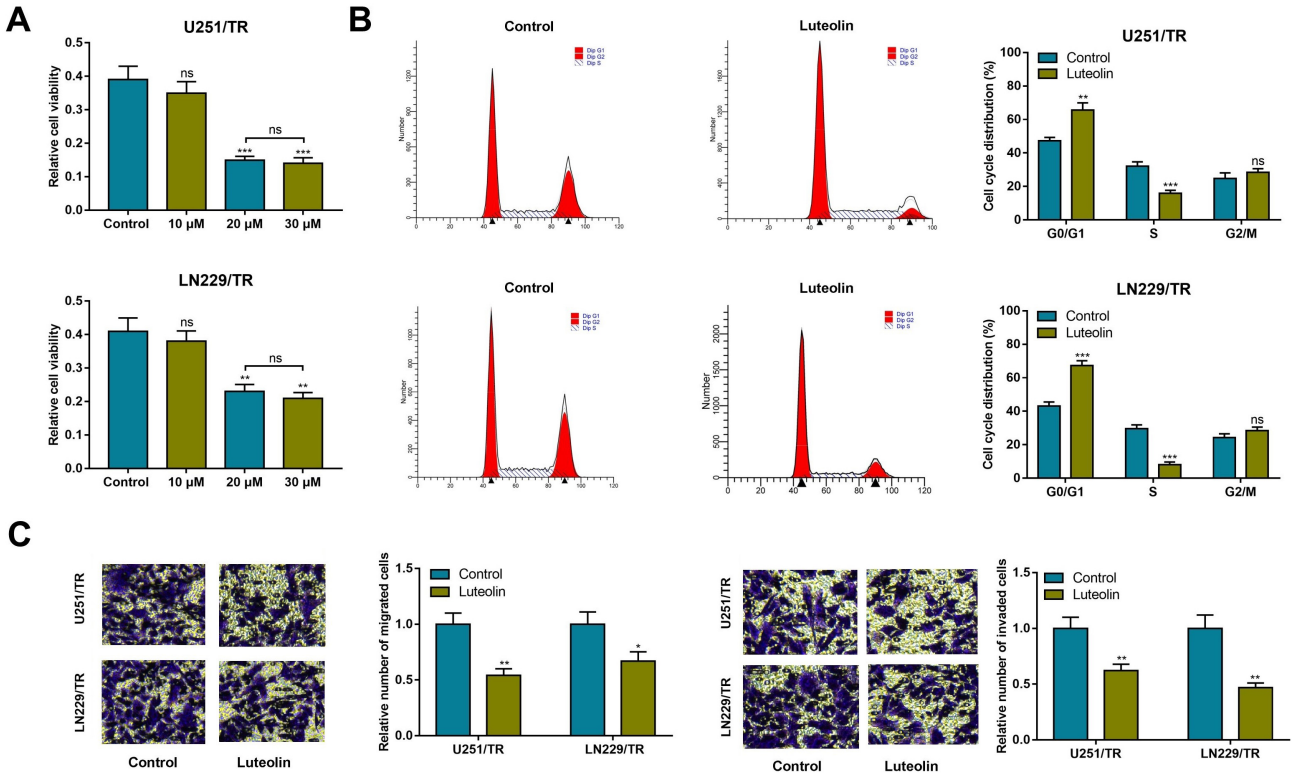

Fig. 2. Luteolin synergize with TMZ, to inhibit the malignant phenotypes of TMZ-resistant cell strains. (A) U251/TR and LN229/TR cells were treated with different doses of luteolin and the cell viability was measured by CCK-8. (B) Flow cytometry was used to analyze the cell cycle distribution of U251/TR and LN229/TR cells treated with luteolin and TMZ. (C) Transwell assay was used to test the cell migration and invasion ability of U251/TR and LN229/TR cells after treatment with luteolin and TMZ. ns, $p > 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$.

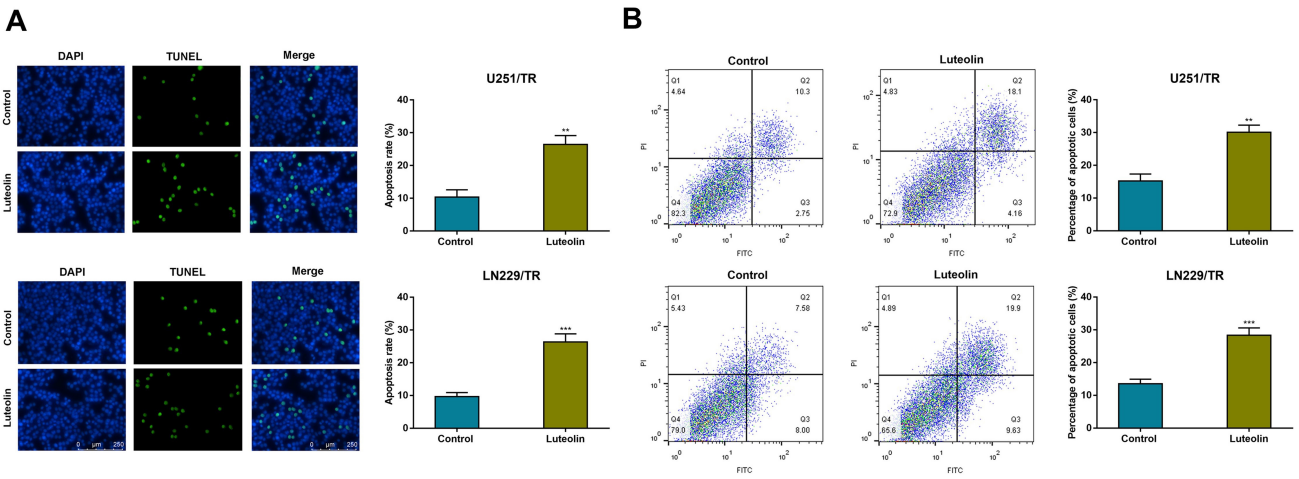

Fig. 3. Luteolin induces apoptosis of TMZ-resistant cells. (A) TUNEL staining experiment was conducted to detect the apoptosis of TMZ-resistant glioma cell strains treated with luteolin and TMZ. (B) Flow cytometry was used to analyze the apoptosis of TMZ-resistant glioma cell strains treated with luteolin and TMZ. $^{**}p < 0.01$; and $^{***}p < 0.001$.

luteolin treatment group and the vehicle group, but those of p-PI3K and p-AKT were greatly declined in TMZ-resistant cells treated with luteolin (Fig. 5A,B). The above results indicated that luteolin could inactivate PI3K/AKT pathway in TMZ-resistant cell strains.

Discussion

Given that glioma has high aggressiveness, maximum surgical resection combined with chemoradiotherapy is regarded as the standard treatment at present for high-grade

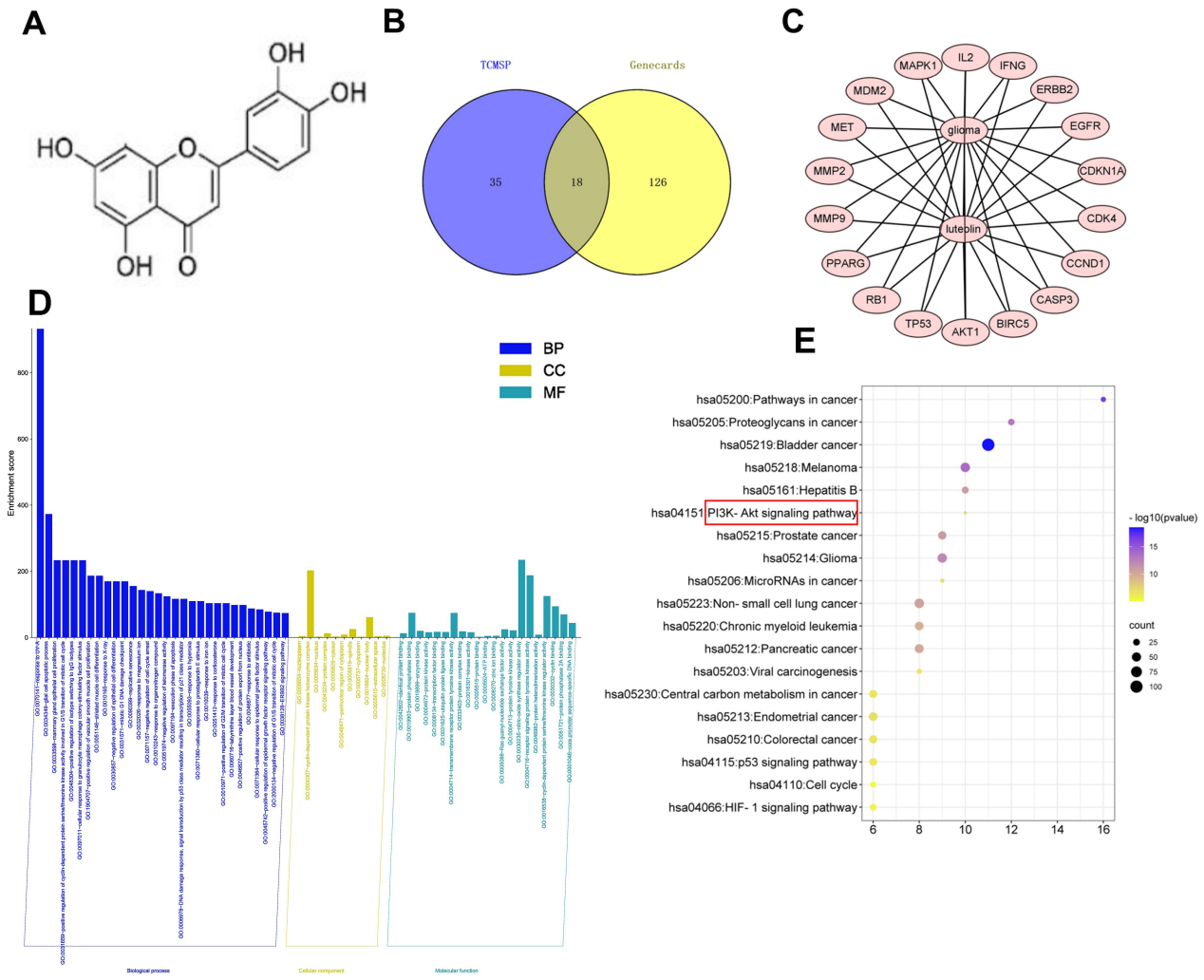

Fig. 4. The identification of the targets of luteolin. (A) The chemical structure of luteolin. (B,C) The target genes of luteolin screened out from the TCMSP database and those genes related to glioma in the Genecards database were plotted in the Venn's map. (D,E) The GO and KEGG nrichment analysis of 18 target genes.

glioma [13]. TMZ, a first-line oral alkylating agent for glioma therapy, is the optimal choice for treating high-grade gliomas. TMZ, reportedly, triggers DNA damage and promotes cell cycle arrest, and thus the apoptosis of glioma cell is induced [14]. However, the chemotherapeutic efficacy is greatly hampered by the resistance to TMZ. In this context, the strategy to sensitize glioma cells to TMZ is urgently required.

As reported, some pharmaceutical active ingredients extracted from Chinese herbal medicine exert an inhibitory effect on glioma progression [15]. Luteolin is a natural antioxidant, and its properties of scavenging free radicals and cell protection, as well as anti-tumor activity have been reported [16]. In glioma, luteolin can interfere with AKT and MAPK pathways, and consequently, the EGFR-mediated GBM cell proliferation is inhibited [17]. Additionally, in GBM, luteolin induces apoptosis of cancer cells through ROS/ER stress and mitochondrial dysfunction [18,19]; luteolin also suppresses Cdc42 expression and inhibits PI3K/AKT signaling, and thus the aggressiveness of GBM cells are restrained [20]. Reportedly, luteolin enables GBM cells sensitive to radiation and olaparine (a kind of PARP inhibitor) [12]. Importantly, another work reports that, by modulating MAPK pathway, luteolin represses the aggressiveness of glioma cells via inducing apoptosis and autophagy [18]. Nevertheless, little is known about the role of luteolin in modulating TMZ resistance of gliomas. Here we constructed TMZ-resistant cells and testified through functional experiments that Luteolin inhibits the malignancy of TMZ-resistant cells. Our data suggest that luteolin may by an optional natural drug benefit glioma patients with TMZ resistant, as a chemosensitizer.

PI3K/AKT pathway contributes to modulating diverse cellular biological processes, of which, PI3K is a lipid kinase, and AKT is an important downstream effector of PI3K signaling to exert multiple biological effects such as apoptosis, cell growth, and metabolism [21]. As reported, this pathway is one of the determinants in glioma progres-

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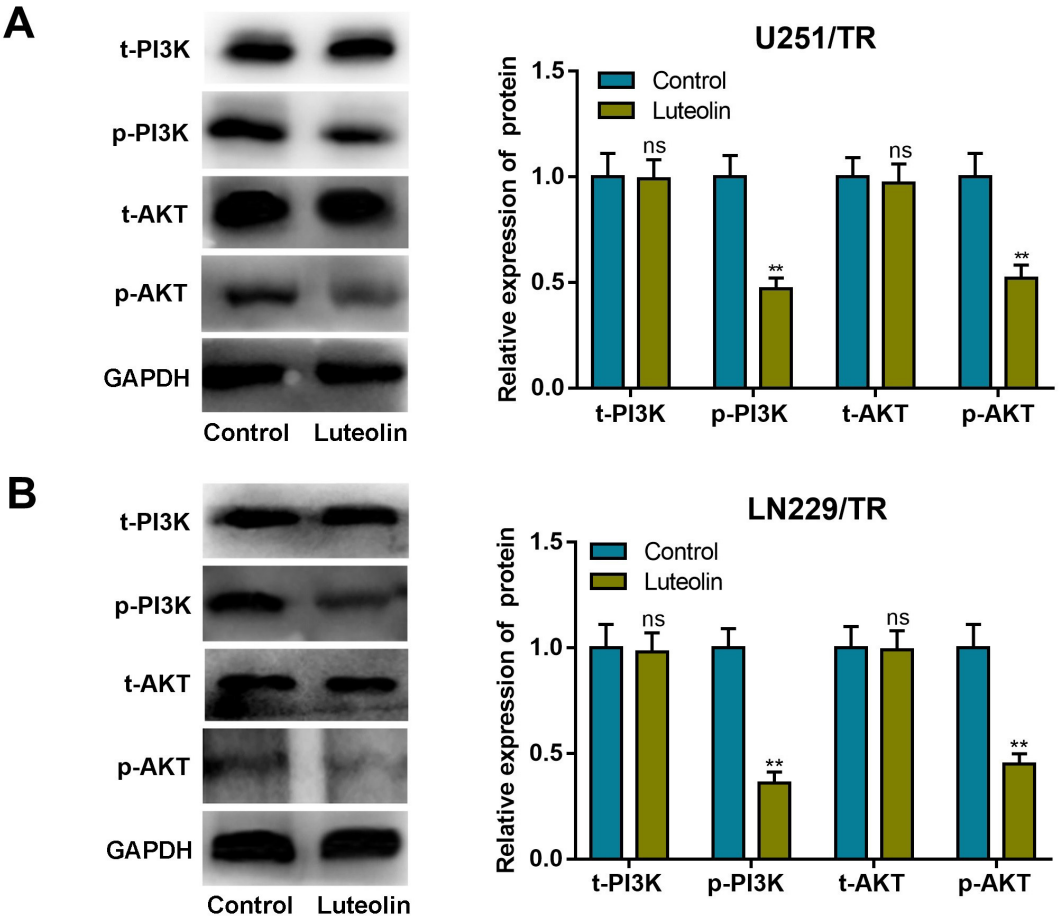

Fig. 5. Luteolin regulates PI3K/AKT pathway. (A,B) Western blot assay was conducted to detect the expressions of PI3K (total), p-PI3K, AKT (total) and p-AKT proteins after treatment with luteolin in TMZ-resistant cell strains. ns, $p > 0.05$; and $^{**}p < 0.01$.

sion glioma [22]. For example, EEF1D can increase the phosphorylation of PI3K and AKT, and thus facilitating glioma development [23]. In glioma, SNHG1 decoys miR-140-5p to activates PI3K/AKT pathway to promote disease progression [24]. Shikonin and evodiamine inactive PI3K/AKT, and therefore they are candidates for glioma treatment [25,26]. In addition, some previous studies report luteolin can suppress the activation of PI3K/Akt axis in choroidal melanoma cells, breast cancer cells and pulmonary artery smooth muscle cells [27–29]. Here we found that in TMZ-resistant glioma cells, luteolin could down-regulate p-PI3K and p-AKT expressions, and our data are consistent with the reports from other groups [27–29].

In conclusion, luteolin represses the malignancy of TMZ-resistant glioma cells and induces cell cycle arrest and apoptosis; in terms of mechanism, luteolin may be vital in reversing the TMZ resistance of glioma via inhibiting the PI3K/AKT signaling pathway. In short, luteolin may be a candidate for glioma therapy. However, animal models are required to further validate the role of luteolin to sensitize glioma cells to TMZ, and before its clinical application, the side effects and optimal dose are necessary to be carefully evaluated.

Availability of Data and Materials

The data used to support the findings of this study are available from the corresponding author upon request.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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