

ORIGINAL ARTICLE

Resveratrol inhibits glioma cell growth via targeting LRIG1

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Summary

Purpose: To investigate the effect of resveratrol on the expression of leucine repeat immunoglobulin-like protein 1 (LRIG1) in glioma cell line U251 and the relationship between LRIG1 and U251 cell proliferation and apoptosis, so as to clarify other molecular mechanisms of resveratrol and look for possible new targets for the treatment of this condition.

Methods: U251 cells were treated with 100 μ M resveratrol for 48 hrs. Reverse transcription- polymerase chain reaction (RT-PCR) and Western blotting were used to detect the LRIG1 level in glioma cell line U251 and the expressions of related factors after resveratrol treatment. The loss-of-function assay was performed via transfection of LRIG1 small interference and the proliferation and apoptosis of U251 cells in each group were detected via MTT assay and flow cytometry.

Results: The mRNA and protein expression levels of LRIG1 in U251 cells were up-regulated after resveratrol treatment,

accompanied with decreased Epidermal Growth Factor Receptor (EGFR). MTT assay showed that the cell proliferation rate was decreased after resveratrol treatment, and flow cytometry showed that cell apoptosis was increased. The loss-of-function assay via transfection of LRIG1 small interference showed that resveratrol could reverse the increased cell proliferation and decreased apoptosis induced by LRIG1 small interference.

Conclusions: Resveratrol can inhibit the growth and proliferation of glioma and promote its apoptosis through upregulating the LRIG1 gene expression, which plays the effect of antiglioma growth, revealing that LRIG1 is a new biological target of resveratrol in antiglioma cell proliferation and growth.

Key words: glioma, LRIG1, resveratrol

Introduction

Human brain glioma is a common malignant tumor of the nervous system, which possesses higher diffuse and invasion capacity. The main clinical treatment methods include surgical resection combined with chemotherapy and radiotherapy, and although these methods have certain effects, the anticancer drugs and radiotherapy are accompanied with serious side effects. At the same time, the invasive growth of glioma leads to difficult total resection, thus contributing to future recurrence. Central Brain Tumor Registry of the United States (CBTRUS) statistics suggested that

the annual incidence of glioma was about 6.4/0.1 million [1] and showed an increasing trend year by year with a very low 5-year survival rate. Resveratrol is a natural polyphenolic compound with a variety of biological effects, such as antibacterial, antiinflammatory, antioxidative, antiheart failure, antimutation and hormone regulation, etc., and it is conducive to human metabolism [2,3]. But what really arouses the widespread concern is its role in the prevention and treatment of cancer. Studies have found that resveratrol can inhibit the invasion and metastasis of tumors through effectively

inhibiting PI3K/AKT/NFkB signaling pathway [4], and can cause lung cancer cell autophagy through regulating the Ca²⁺/AMPK-mTOR cell pathway [5]. In addition, a large number of reports has shown that resveratrol is involved in a variety of tumor cell signal transduction pathways, but its antitumor mechanism is not fully clear. Resveratrol is involved in various stages of apoptosis, but the intensity of its antitumor effect varies from cell to cell due to different sensitivity of cells. Glioma U251 cells are very sensitive to resveratrol, and experiments have shown that resveratrol causes 24% apoptosis in them [6]. However, no obvious apoptosis in LN-L8 cell line treated with resveratrol was observed [7].

LRIG1 is a multityrosine kinase receptor (RTK) inhibitor found in recent years. Previous studies have shown that LRIG1 could inhibit invasion and promote apoptosis of glioma cells [8-10].

The present study investigated the effect of resveratrol on the expression of LRIG1 in U251 glioma cell line and the molecular mechanism in its antitumor effect, so as to find a new possible therapeutic target or drug resistance mechanism of glioma.

Methods

Reagents

Resveratrol was purchased from Sigma (MO,USA), prepared with dimethylsulfoxide (DMSO) into 100 mM stock solution and stored at -20°C in the dark. The concentration of working solution was 100 µM. Rabbit antihuman LRIG1 polyclonal antibody, rabbit antihuman EGFR polyclonal antibody and GAPDH (glyceraldehyde-phosphate dehydrogenase) rabbit antihuman polyclonal antibody were purchased from Abcam (MA,USA). Lipofectamine 3000 kit was purchased from Gibco (NY, USA). LRIG1 small interference was purchased from Ribobio (Guangzhou,China). MTT kit was purchased from Beyotime Biotechnology Co., Ltd. (Shanghai,China).

Cell culture

Human malignant glioma cell line U251 (purchased from China Center for Type Culture Collection of Wuhan University, Wuhan, China) was cultured in DMEM culture solution containing 10% fetal bovine serum (FBS), an appropriate amount of Hepes and 3% glutamine.

Protein extraction and western blotting

After cleavage, U251 cells were centrifuged at 12000g for 15min and protein concentration was quantified using bicinchoninic acid (BCA) kit. Cells were separated by sodium dodecyl sulfonate polyacrylamide gel electrophoresis (SDS-PAGE), followed by polyvinylidene fluoride (PVDF) membrane transfer and sealing at room temperature using 5% skim milk for 1 hr. LRIG1 rabbit antihuman monoclonal antibody (1:1000) and goat anti-

rabbit secondary antibody (1:2000) were used to identify LRIG1, and EGFR rabbit antihuman monoclonal antibody (1:1000) and goat antirabbit secondary antibody (1:2000) were used to identify LRIG1; GAPDH was used as control. After incubation using primary antibody for 4 hrs they were washed for 6 min×5 times with tris-buffered saline (TBS) and incubated for 1 min. After incubation with primary antibody for 1 hr, the membrane was washed with tris-buffered saline tween (TBST) for 6 min×5 times, and after incubation with secondary antibodies for 1 hr the membrane was washed again with TBST for 6 min×5 times. The water was sucked clean using filter paper, and the enhanced chemiluminescence (ECL) reagent (Tanen, Changhai, China) was added for color development.

Total RNA extraction and RT-PCR

Trizol reagent was used to extract the total RNA of U251 cells, and the concentration and purity were determined via ultraviolet spectrophotometer. Reverse transcriptase (purchased from Bio-Rad, CA, USA) was used for reverse transcription, and LRIG1 and EGFR were amplified via PCR. Amplified GAPDH was used as internal reference (ABI-7900, MA, USA). LRIG1 primer sequence: sense: 5'-CACGCAGTTGGGCACTTTTG-3', antisense: 5'-TTGGG ACAGCTTGGATCAC-3'; GAPDH primer sequence: sense: 5'-ACGGATTTGGTCTATTGGG-3', antisense: 5'-TGATTTTGGAGGGATCTCGC-3'.

Cell transfection

U251 cells in the logarithmic growth phase were digested using trypsin (0.25%) containing EDTA and counted, and then inoculated onto a 6-well plate (2×10⁵ cells/well) overnight. The medium was discarded and cells were washed with phosphate buffer solution (PBS) for 3 times. LRIG1 small interference was added into the blank Opti-MEM and mixed, and then lipofectamine 3000 was added and mixed, placed at room temperature for 15 min and added into the culture plate. U251 cells added with blank lipofectamine 3000 were used as negative control (SiNC). After 6 hrs, the cell culture medium was replaced with complete DMEM. After 48 hrs, resveratrol was added for 48 hrs to detect the cell proliferation rate and apoptosis.

Detection of cell apoptosis via flow cytometry

A quantitative analysis of apoptotic cell death was performed using a FITC Annexin V Apoptosis Detection kit II staining (Becton Dickinson, Franklin Lakes, NJ, USA) following the manufacturer's protocol. Briefly, U251 cells were cultured in incubator (CO₂ 5%, 37°C). After digestion with trypsin (0.25%) containing EDTA and centrifugation, cells were washed twice with cold PBS and then 1× binding buffer was added to resuspend cells and the cell concentration was adjusted to 1×10⁶/mL. 100 µL solution (1×10⁶ cells) was placed into 5 mL culture tube. Then 5 µL Annexin V-FITC or 5 µL propidium iodide (PI) was added and cells were slightly shaken. After incubation in the dark (25°C) for 20 min, 400 µL 1×binding buffer was added to each tube, followed by detection via flow cytometer within 1 hr.

Detection of cell proliferation rate

MTT assay was used to detect the cell proliferation rate. U251 cells in the logarithmic growth phase were digested with trypsin and inoculated in a 96-well plate (5000 cells (200 μ L)/well). Twenty μ L MTT were added for incubation, 4 hrs before detection in the dark. Then the supernatant was discarded and 150 μ L DMSO was added, followed by vibration in the dark for 10 min. The absorbance (A_{492}) at 492 nm was measured with the fully automatic microplate reader. Cell growth rate (%) = $[(A_{\text{experimental group}} - A_{\text{blank control}}) - (A_0 - A_{\text{blank control}})] / (A_0 - A_{\text{blank control}}) \times 100\%$, where A_0 is the A_{492} value of the experimental group at the beginning of the experiment.

Statistics

All quantitative data were presented as mean \pm standard deviation. Group differences were analyzed by a two-tailed Student's t-test. For multiple comparisons, one-way ANOVA with Bonferroni post hoc test were used. All statistical analyses were conducted using SPSS 15.0 or GraphPad Prism 5 and $p < 0.05$ was considered statistically significant.

Results

Changes in LRIG1 and EGFR expressions in U251 cell lines after resveratrol treatment

First, U251 cells were treated with resveratrol (100 μ M) for 48 hrs. The changes in mRNA expression of LRIG1 and its downstream molecule EGFR were detected via PCR. The changes in protein expression of LRIG1 and EGFR were detected via Western blotting. The results showed that the LRIG1 level in U251 cell lines in the resveratrol

treatment group was significantly upregulated, and the EGFR level was significantly downregulated (Figure 1).

Resveratrol inhibited the growth of U251 cell line by upregulating LRIG1

After confirming that resveratrol could upregulate LRIG1, the possible inhibition of growth of glioma U251 cell line through upregulating LRIG1 was investigated. First, U251 cell lines were transfected with LRIG1 small interference (100 nmol) for 48 hrs, and the total RNA and protein were collected for relevant detection. The mRNA and protein expressions of LRIG1 in the LRIG1 small interference group were significantly decreased compared with those in the control group, and the differences were statistically significant (Figure 2A-B); however the mRNA and protein expressions of EGFR were significantly increased compared with those in the control group, and the differences were statistically significant (Figure 2A-B). The detection of proliferation rate via MTT assay showed that the proliferation capacity of U251 cells transfected with LRIG1 small interference was significantly higher than that in the control group, and such a difference was also greater over time (Figure 2C).

After the transfection efficiency and function of LRIG1 were clarified, whether resveratrol could play a role in inhibiting tumor growth through LRIG1-EGFR pathway was further verified. Resveratrol (100 μ M) was added for 48 hrs after

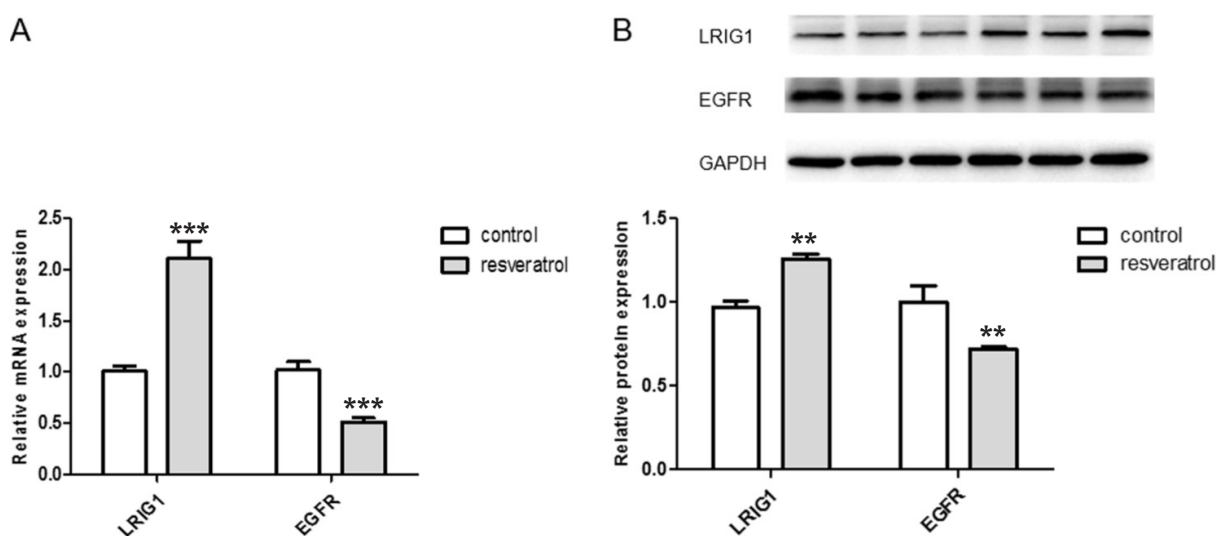


Figure 1. Changes in LRIG1 and EGFR expressions in U251 cell line after resveratrol treatment. U251 cells were treated with resveratrol (100 μ M) for 48 hrs. **(A):** The mRNA levels of LRIG1 and EGFR were detected by RT-PCR; **(B):** Cell lysates were subjected to Western blotting analysis to detect LRIG1 and EGFR expressions. GAPDH was used as internal control. The results showed that the LRIG1 level in U251 cell line in the resveratrol treatment group was significantly upregulated, and the EGFR level was significantly downregulated. The data represent mean \pm SD of three independent experiments. ** $p < 0.01$, *** $p < 0.001$.

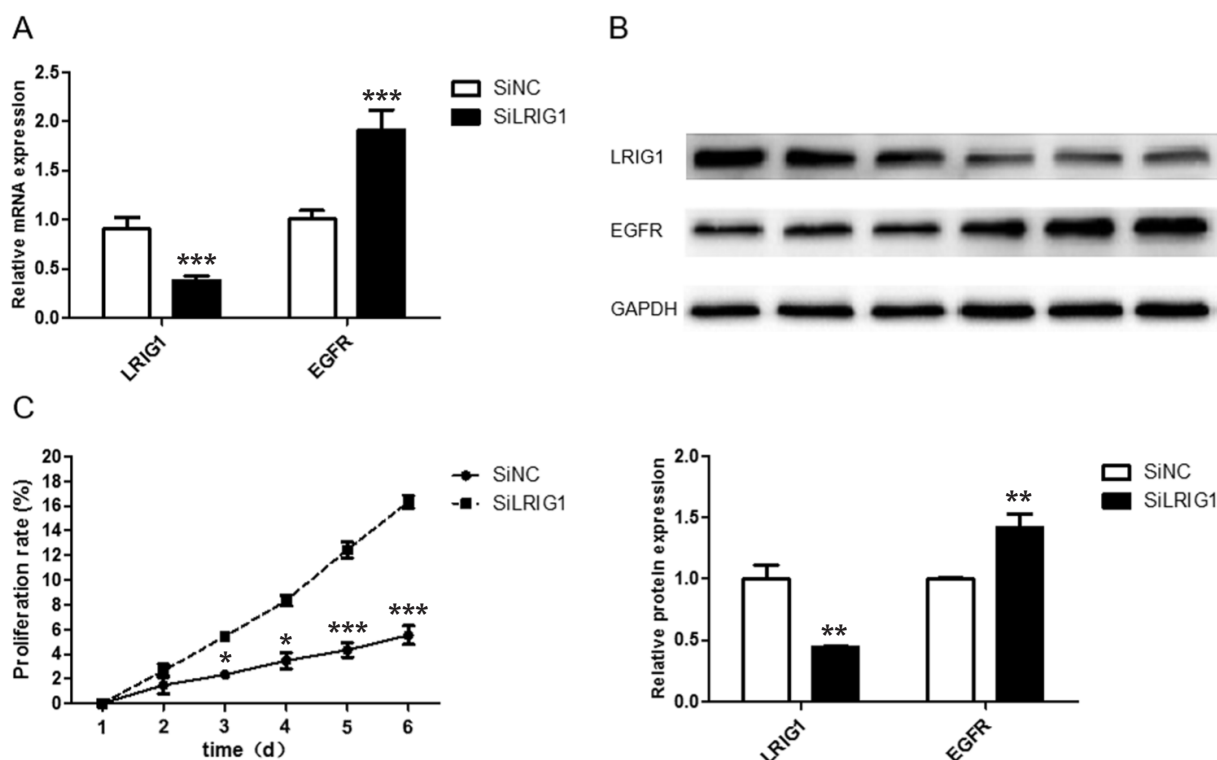


Figure 2. U251 cell line was transfected with LRIG1 small interference (100 nmol) for 48 hrs. **(A):** The mRNA levels of LRIG1 and EGFR were detected via RT-PCR; **(B):** The protein levels of LRIG1 and EGFR were detected by Western blotting. The mRNA and protein expressions of LRIG1 in the LRIG1 small interference group were significantly decreased compared with those in the control group. **(C):** The proliferation rate of U251 cells transfected with LRIG1 small interference was detected via MMT assay. The proliferation capacity of U251 cells transfected with LRIG1 small interference was significantly higher than that in the control group. GAPDH was used as internal control. The data represent the mean \pm SD of three independent experiments. * p <0.05, ** p <0.01, *** p <0.001.

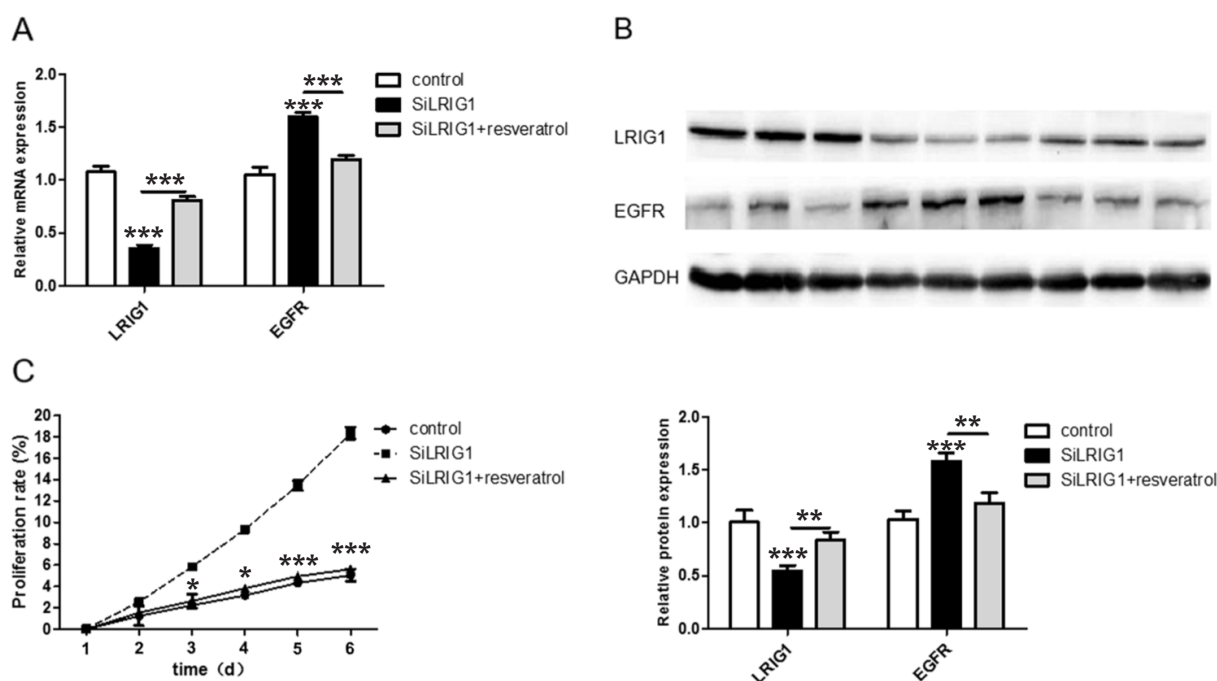


Figure 3. Resveratrol (100 μ M) was added for 48 hrs after transfection with LRIG1. **(A):** The mRNA expressions of LRIG1 and EGFR were detected via RT-PCR; **(B):** The protein levels of LRIG1 and EGFR were detected via Western blotting. The expression of LRIG1 in small interference-transfected group was upregulated after resveratrol was added. **(C):** The proliferation rate of each group was detected via MMT assay. Resveratrol reduced the proliferation rate of U251 cells in LRIG1 small interference-transfected group. GAPDH was used as internal control. The data represent the mean \pm SD of three independent experiments. * p <0.05, ** p <0.01, *** p <0.001.

transfection of U251 cells with LRIG1 for 48 hrs. The result of RT-PCR and Western blotting showed that the expression of LRIG1 in small interference-transfected group could be upregulated after resveratrol was added (Figure 3A-B), and MTT assay results suggested that resveratrol could reduce the proliferation rate of U251 cells in LRIG1 small interference-transfected group, thereby inhibiting tumor cell growth (Figure 3C).

Resveratrol promoted apoptosis of glioma U251 cell line by upregulating LRIG1

Previous studies have shown that resveratrol has a definite effect on promoting the apoptosis of glioma U251 cells. In this study, whether LRIG1 was one of the biological targets of resveratrol-mediated U251 cell apoptosis was directly investigated. Therefore, resveratrol (100 μ M) was added for 48 hrs after transfection of glioma U251 cell line with LRIG1 small interference for 48 hrs. The apoptosis of each group was detected via flow cytometry and the results showed that the apoptotic rate in LRIG1 small interference-transfected group was lower than that in the control group, and such an effect could be reversed by resveratrol (Figure 4A-B). The difference was statistically significant.

Discussion

The antitumor mechanism of resveratrol is more complex, owing to the result of combined action of several mechanisms or there may be other unknown pathways. Resveratrol-induced apoptosis mechanisms include p53 pathway, affecting Bcl-2 (B-cell lymphoma 2) family, mitochondria mediator, Fas pathway, cyclin and cyclin-dependent kinases, etc [11,12]. It has been reported in previous studies that resveratrol can cause cell cycle G0/G1 blockade through inhibiting the expression of cyclin D1 in human glioma U251 cells [13], and it was also found that LRIG1 and EGFR are involved in the regulation of glioma U251 cell proliferation and apoptosis [14]. Therefore, this study aimed to investigate whether LRIG1 is one of the targets of antitumor activity of resveratrol.

LRIG1 gene is a newly-discovered tumor suppressor gene associated with tumor development and progression, which is a frequently deleted gene in human malignancies, including breast and lung cancer [15]. The gene is expressed almost in all tissues in the human body in the same degree, which is higher in the brain, lung and kidney. The transmembrane protein encoded by it identifies and binds to the extracellular fragment of EGFR

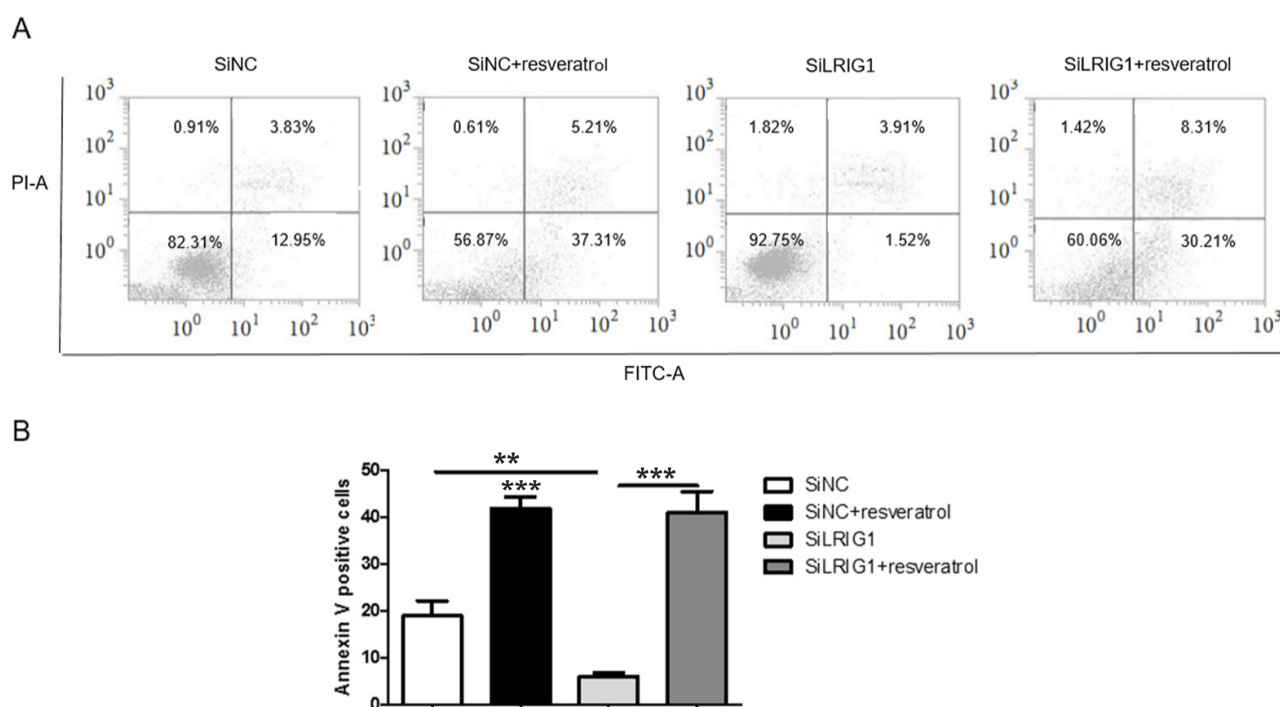


Figure 4. Resveratrol promoted the apoptosis of U251 glioma cell line by upregulating LRIG1. **(A):** The percentage of apoptotic cells in each group was detected via flow cytometer (annexin V-FITC and PI staining). **(B):** The percentage of apoptosis in each fraction is expressed as a graph. The results showed that the apoptotic rate in LRIG1 small interference-transfected group was lower than that in the control group, and such an effect could be reversed by resveratrol. The data represent mean \pm SD of three independent experiments. ** $p < 0.01$, *** $p < 0.001$. V-FITC: V-fluorescein isothiocyanate; PI: propidium iodide; SiNC: negative control; SiLRIG1: LRIG1 small interference.

through the extracellular LRR domain and Ig-like domain. The intracellular fragment promotes the activation of the E₃ ubiquitin ligase c-Cbl, thus leading to degradation of EGFR and inhibition of tumor cell growth. Some results have confirmed that LRIG1 overexpression can significantly promote glioma cell apoptosis [16,17]. In addition, LRIG1 can also inhibit the functions of gliocyte-derived nerve growth factor (GDNF) [18], the latter of which can reduce the sensitivity of neuroblastoma to chemotherapeutic agents [19]. In terms of mechanism of glioma, Feng et al. studied the role of LRIG1 in glioma by PCR and immunohistochemistry and found that the high expression of LRIG1 in cells changed the expression pattern of cyclinD1, leading to the G₀/G₁ cell cycle arrest and inhibiting cell proliferation [8]. He et al. [20] cultured U87 glioma cells and found that the expressions of EGFR and its downstream signal transduction pathway Akt/mTORC1 in U87 cells with upregulated LRIG1 was downregulated, while the Akt activation achieved by knockout of LRIG1 gene via RANi could cause the proliferation of U87 cells, indicating that LRIG1 induces cell growth inhibition and apoptosis via inhibition of EGFR and Akt/mTORC1. In addition, in glioma U251 cells, the expression of LRIG1 was negatively correlated with EGFR expression, which inhibited the expression of EGFR and activation of its downstream signaling pathway, thus interfering with the expression of Bcl-2/Topo-2 and enhancing the sensitivity of cells to the chemotherapeutic drug temozolomide [21].

EGFR, as a downstream molecule of LRIG1, is located at the beginning of cell signaling pathway, playing an important role in cell survival, apoptosis, adhesion and proliferation. EGFR belongs to tyrosine protein kinase receptors, and its overactivation contributes to the development and progression of glioma, leading to therapeutic resistance and decreased survival rate [17]. It can be seen that LRIG1 negatively regulates the glioma

cell proliferation, migration and invasion by inhibiting the activation of EGFR and excitation of its downstream signaling pathway and promotes cell apoptosis, thus playing a role as tumor suppressor gene.

A large number of reports shows that the common chemotherapeutic drug resveratrol is involved in a number of tumor cell signal transduction pathways, but its antitumor mechanism is not fully clear yet, which may be the result of combined action of several mechanisms, and there may be other unknown pathways. This study found that resveratrol could upregulate the mRNA and protein expressions of LRIG1 in glioma U251 cell line, and decrease the expression level of EGFR. In view of the previous studies on the biological functions of LRIG1 and EGFR, it is speculated that LRIG1 may be one of the biological targets of resveratrol in antiglioma cell growth. So we transfected LRIG1 small interfering for 48 hrs and added resveratrol for treatment, finding that the cell apoptosis in LRIG1 small interference-transfected group was significantly lower than that in the control group, and such an effect could be reversed by resveratrol. Comprehensive results showed that resveratrol plays a role in inhibiting glioma growth and promoting apoptosis through upregulating LRIG1 expression.

Conclusion

In conclusion, this study found at the cellular level that resveratrol can inhibit the growth and proliferation of glioma cells and promote their apoptosis by upregulating the expression of LRIG1 gene, exerting antiglioma properties and revealing that LRIG1 is a new biological target of resveratrol.

Conflict of interests

The authors declare no conflict of interests.

References

1. Chien LN, Gittleman H, Ostrom QT et al. Comparative brain and central nervous system tumor incidence and survival between the United States and Taiwan based on population-based registry. *Front Publ Health* 2016;4:151.
2. Raj P, Louis XL, Thandapilly SJ, Movahed A, Zieroth S, Netticadan T. Potential of resveratrol in the treatment of heart failure. *Life Sci* 2014;95:63-71.
3. Feng Y, Zhou J, Jiang Y. Resveratrol in lung cancer- a systematic review. *JBUON* 2016;21:950-3.
4. Li W, Ma J, Ma Q et al. Resveratrol inhibits the epithelial-mesenchymal transition of pancreatic cancer cells via suppression of the PI-3K/Akt/NF-kappaB pathway. *Curr Med Chem* 2013;20:4185-94.
5. Zhang J, Chiu J, Zhang H et al. Autophagic cell death induced by resveratrol depends on the Ca⁽²⁺⁾/AMPK/mTOR pathway in A549 cells. *Biochem Pharmacol* 2013;86:317-28.

6. Derissen EJ, Beijnen JH, Schellens JH. Concise drug review: Azacytidine and decitabine. *Oncologist* 2013;18:619-24.
7. Turcan S, Fabius AW, Borodovsky A et al. Efficient induction of differentiation and growth inhibition in IDH1 mutant glioma cells by the DNMT Inhibitor Decitabine. *Oncotarget* 2013;4:1729-36.
8. Mao F, Wang B, Xiao Q et al. A role for LRIG1 in the regulation of malignant glioma aggressiveness. *Int J Oncol* 2013;42:1081-7.
9. Ding J, Liu B, He Y et al. LRIG1 improves chemosensitivity through inhibition of BCL-2 and MnSOD in glioblastoma. *Cell Biochem Biophys* 2015;71:27-33.
10. Wang X, Xiao Q, Xing X et al. LRIG1 enhances cisplatin sensitivity of glioma cell lines. *Oncol Res* 2012;20:205-11.
11. George J, Singh M, Srivastava AK et al. Resveratrol and black tea polyphenol combination synergistically suppress mouse skin tumors growth by inhibition of activated MAPKs and p53. *PLoS One* 2011;6:e23395.
12. Park JW, Choi YJ, Suh SI et al. Bcl-2 overexpression attenuates resveratrol-induced apoptosis in U937 cells by inhibition of caspase-3 activity. *Carcinogenesis* 2001;22:1633-9.
13. Wang Q, Li H, Wang XW, Wu DC, Chen XY, Liu J. Resveratrol promotes differentiation and induces Fas-independent apoptosis of human medulloblastoma cells. *Neurosci Lett* 2003;351:83-6.
14. Yang JA, Liu BH, Shao LM et al. LRIG1 enhances the radiosensitivity of radioresistant human glioblastoma U251 cells via attenuation of the EGFR/Akt signaling pathway. *Int J Clin Exp Pathol* 2015;8:3580-90.
15. Wang Y, Poulin EJ, Coffey RJ. LRIG1 is a triple threat: ERBB negative regulator, intestinal stem cell marker and tumour suppressor. *Br J Cancer* 2013;108:1765-70.
16. Gur G, Rubin C, Katz M et al. LRIG1 restricts growth factor signaling by enhancing receptor ubiquitylation and degradation. *EMBO J* 2004;23:3270-81.
17. Yarden Y. The EGFR family and its ligands in human cancer. Signalling mechanisms and therapeutic opportunities. *Eur J Cancer* 2001;37 (Suppl 4):S3-S8.
18. Ledda F, Bieraugel O, Fard SS, Vilar M, Paratcha G. Lrig1 is an endogenous inhibitor of Ret receptor tyrosine kinase activation, downstream signaling, and biological responses to GDNF. *J Neurosci* 2008;28:39-49.
19. Hansford LM, Marshall GM. Glial cell line-derived neurotrophic factor (GDNF) family ligands reduce the sensitivity of neuroblastoma cells to pharmacologically induced cell death, growth arrest and differentiation. *Neurosci Lett* 2005;389:77-82.
20. He XY, Liu XJ, Chen X et al. Gambogic acid induces EGFR degradation and Akt/mTORC1 inhibition through AMPK dependent-LRIG1 upregulation in cultured U87 glioma cells. *Biochem Biophys Res Commun* 2013;435:397-402.
21. Qi XC, Xie DJ, Yan QF et al. LRIG1 dictates the chemosensitivity of temozolomide (TMZ) in U251 glioblastoma cells via down-regulation of EGFR/topoisomerase-2/Bcl-2. *Biochem Biophys Res Commun* 2013;437:565-72.