

Original Article

Curcumin inhibited the growth, invasion and migration of glioma U251 cells and induced apoptosis in U251 cells

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Abstract: Objective: To explore the effect of curcumin on the biological behavior of glioma U251 cells by regulating STAT3 signaling pathway. Method: Glioma U251 cells cultured in vitro were divided into a blank control group and a curcumin intervention group (20 $\mu\text{mol/L}$, 40 $\mu\text{mol/L}$ and 80 $\mu\text{mol/L}$). The cell viability of each group was detected by MTT, and the apoptosis rate of each group was detected by flow cytometry. The in vitro invasion ability of the cells was detected by Transwell chamber. The migration ability of the cells in vitro was observed by scratch-healing experiment. The expression of STAT3 protein, p-STAT3, Bax and Bcl-2 protein in each group treated with curcumin was detected by Western blot. Results: The survival rate of glioma U251 cells decreased gradually with the increase of curcumin concentration and the intervention time ($P < 0.05$). The apoptosis rate of curcumin intervention group was significantly higher than that of the blank control group ($P < 0.05$). And as the concentration of curcumin increased, the apoptotic rate increased gradually ($P < 0.05$). The number of transmembrane cells, cell migration index and expression of STAT3 protein and p-STAT3 protein in the curcumin intervention group was significantly lower than that in the blank control group ($P < 0.05$). As the concentration of curcumin increased, the cell migration index, the expression of STAT3 protein and p-STAT3 protein also decreased ($P < 0.05$). The pro-apoptotic protein Bax in the blank control group was significantly up-regulated than that in the curcumin intervention group. The expression of anti-apoptotic protein Bcl2 protein was significantly lower than that in the curcumin intervention group. Expression of Bax protein increased with the increase in curcumin concentration while expression of Bcl2 protein decreased with increase in curcumin concentration. Conclusion: Curcumin inhibited the growth, invasion and migration of glioma U251 cells and induced apoptosis in U251 cells, which may be related to an inhibition of the STAT3 signaling pathway.

Keywords: Curcumin, STAT3, glioma U251 cells, biological function

Introduction

Glioma is a common neuroepithelial tumor. Because its growth mode is mainly malignant infiltration, it is often difficult to be completely removed during surgery, and it is very easy to relapse after surgery [1]. Even with comprehensive treatments such as surgery and radiotherapy and chemotherapy, the median survival of glioma patients is only about 12 months [2]. Studies have shown that the reason why glioma is more difficult to be cured and easy to relapse is due to the resistance of glioma stem cells to chemoradiotherapy [3]. Therefore, finding a drug that has a positive effect on glioma is also a problem that needs to be solved clinically.

Curcumin is a phenolic pigment extracted from the rhizome of turmeric. In recent years, studies have shown that curcumin not only has anti-inflammatory, anti-bacterial, anti-oxidative functions, but also can be used as an anti-tumor drug to affect the biological function of tumor cells [4, 5]. For example, there have been studies [6] reported that curcumin can induce apoptosis of breast cancer cells through activation of MAPK signaling pathway. Other studies [7] found that curcumin can inhibit the colonization of many malignant tumor cells by interfering with the molecular chaperone function of HSP90. Signal transduction and activator of transcription 3 (STAT3) is a bifunctional protein in the cytoplasmic tyrosine phosphorylation sig-

Effect of curcumin on glioma U251 cells

naling pathway and is considered to be an oncogene with high expression in a variety of tumor tissues [8, 9]. Previous studies [10] found that curcumin can induce apoptosis of pancreatic cancer cells by blocking the process of STAT3 phosphorylation. Also, research [11] indicated that curcumin can inhibit the movement of ovarian cancer cells by inhibiting STAT3 phosphorylation.

Although the role of curcumin in malignant tumors has received widespread attention, there are few reports on the effects of curcumin on the biological function of glioma cells by regulating STAT3. Therefore, we explored the effect of curcumin on the regulation of STAT3 and the biological function of glioma cells, in order to provide theoretical basis and new ideas for the treatment of glioma.

Materials and methods

Experimental materials and reagents

Glioma U251 cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. Curcumin was purchased from Sigma Bioreagents, USA. The microplate reader SpectraMax M5 was purchased from Shanghai Meigu Molecular Co., Ltd. DMEM medium was purchased from Gibco, USA. Fetal bovine serum (FBS) and trypsin were purchased from Hyclone, USA. The MTT cell proliferation and cytotoxicity assay kit was purchased from Sigma. Annexin V-FITC/PI double stained cell apoptosis assay kit was purchased from Shanghai Shengsheng Biotechnology Co., Ltd. The Transwell chamber was purchased from Corning. The RIPA protein lysate was purchased from the Biyuntian Biotechnology Research Institute. STAT3 monoclonal antibody and GAPDH antibody were purchased from Beijing Zhongshan Jinqiao Biological Company.

Cell culture and passage

U251 cells were taken out and placed in a medium containing 10% fetal bovine serum, and cultured in an incubator at 37°C in a 5% CO₂ condition. After adherent growth of the cells reached 85%, the cells were washed with PBS, and were then digested with 1 ml of 25% trypsin. After the digestion was completed, 10% of the culture solution was added, and incubation was continued for 48 hours in 5% CO₂ at 37°C

for passage. The cells growing in the log phase were selected for subsequent experiments.

MTT assay for cell proliferation

The frozen logarithmic U251 cells were resuscitated, and the cells were inoculated in a 96-well plate for culture, and the number of cells per well was about 5×10^4 cells. The cells were divided into a blank control group and a curcumin intervention group. The blank control group only added culture medium. According to the different concentration of curcumin intervention, the cells in the curcumin intervention group were divided into three groups, 20 μmol/L, 40 μmol/L and 80 μmol/L, respectively. After the cells were cultured for 48 h, 20 μl of MTT solution was added to the cells, and the reaction was incubated at 37°C for 4 h. The supernatant was discarded and 100 μl of dimethyl sulfoxide (DMSO) solution was added. The absorbance in each well was detected at 570 nm with a microplate reader, and the viability of the cells was calculated at 24 h, 48 h and 72 h after the drug was added.

Detection of apoptosis by flow cytometry

Apoptosis was detected by Annexin V-FITC/PI double staining combined with flow cytometry. The frozen logarithmic U251 cells were inoculated at a density of 3×10^5 /well in a 6-well plate and grouped according to the above method. Single cell suspension was prepared 48 h after drug was added, then washed with PBS, and centrifuged at 2000 rpm for 5 min. The reaction was repeated twice, the supernatant was removed, and the cells were resuspended. 5 μL of Annexin V-FITC and 5 ml of PI were added and incubated for 20 min at room temperature in the dark. Apoptosis detection was performed using a CytoFLEX LX flow cytometer. The experiment was repeated three times.

The in vitro invasion ability of cells after drug intervention was detected in Transwell chamber

Cells in the logarithmic phase were inoculated and grouped in 24 well plates with approximately 3000 cells in each well according to the above method. After 48 h of drug intervention, the cells were diluted to a density of 3×10^4 cells/ml in serum-free DMEM medium. 200 μl of the diluted cells was added to the upper

Effect of curcumin on glioma U251 cells

Table 1. Effect of curcumin on survival rate of U251 cells (%)

Time	Blank control group	20 μmol/L	40 μmol/L	80 μmol/L	F	P
24 h	92.5 ± 2.5	74.2 ± 2.8	61.4 ± 3.2	41.9 ± 3.1	160.7	< 0.001
48 h	84.9 ± 2.2	61.5 ± 3.2	52.3 ± 3.3	29.9 ± 3.4	165.7	< 0.001
72 h	78.3 ± 3.1	48.7 ± 2.3	41.5 ± 2.9	15.8 ± 3.2	236.4	< 0.001
F	21.95	62.60	30.24	48.90	-	-
P	< 0.001	< 0.001	< 0.001	< 0.001	-	-

chamber, and 600 μl of DMEM-F12 medium containing 20% FBS was added to the lower chamber. After 24 hours, the removed chamber was washed with PBS. The chamber was fixed with a 4% paraformaldehyde solution for 10 min, taken out and rinsed again with PBS. It was then stained with 0.5% crystal violet for 10 min, removed and washed with PBS until clear. Finally, the invasion of 5 field cells was calculated randomly with microscope, and the average value was calculated. The experiment was repeated for 3 times.

The ability of cell migration in vitro was observed by scratch-healing assay

The log phase cells were taken out and made into a single cell suspension, and the cells were diluted to 3×10^5 cells/ml as described above. The cells were added to a 6-well plate and grouped as above, and culture medium and drug were added for culture. After 48 h, the cells were delimited to form a cell-free zone using 200 μl sterile sample nozzle in the culture plate. The cells were washed with PBS and the new medium was added to culture. At 0 h (W0) and 24 h (W24) after the cells were delimited, the widths of the cell-free regions of the scratches at three different positions were calculated by microscopy. Cell migration index (M1) = $(W_0 - W_{24}) / W_0 \times 100\%$.

The expression of STAT3 protein, p-STAT3 protein, Bax protein and Bcl-2 protein in each group of cells was detected by Western blot after curcumin treatment

The transfected cells of each group were collected and added to the RIPA cell lysate. The cells were lysed and the total protein of each group of cells was collected, and then the protein was separated by 10% SDS-PAGE, transferred to a PVDF membrane, and 5% skim milk was added. It was then blocked overnight at a

temperature of 4°C. After blocking, primary antibodies (STAT3 1:1000), p-STAT3 (1:500), Bax (1:500) and Bcl-2 (1:500) were added and allowed to stand overnight at 4°C. Then, the secondary antibody GAPDH (1:500) was added and incubated in an incubator at 37°C. Finally, the color was developed with ECL developer.

Statistical methods

In this study, SPSS18.0 software (Bizinsight Information Technology Co., Ltd., Beijing) was used for statistical analysis of experimental data. Measurement data were expressed using mean ± standard deviation. Variance analysis was used to analyze comparisons between groups and different time points within groups. LSD/t test was used for pairwise comparison, and t test was used for analysis between the two groups. GraphPad Prism 6 software was used to draw all the images in this experiment. When $P < 0.05$, there was a statistical difference.

Results

Curcumin decreased the survival rate of U251 cells

The cell viability of the blank control group at 24 h, 48 h and 72 h after drug intervention were $92.5 \pm 2.5\%$, $84.9 \pm 2.2\%$ and $78.3 \pm 3.1\%$, respectively. The cell viability of curcumin intervention in the 20 μmol/L group at 24 h, 48 h and 72 h after drug intervention was $74.2 \pm 2.8\%$, $61.5 \pm 3.2\%$ and $48.7 \pm 2.3\%$, respectively. The cell viability of curcumin intervention in the 40 μmol/L group at 24 h, 48 h and 72 h after drug intervention was $61.4 \pm 3.2\%$, $52.3 \pm 3.3\%$ and $41.5 \pm 2.9\%$, respectively. The cell viability of curcumin intervention in the 80 μmol/L group at 24 h, 48 h and 72 h after drug intervention was $41.9 \pm 3.1\%$, $29.9 \pm 3.4\%$ and $15.8 \pm 3.2\%$, respectively. The survival rate of glioma cell U251 decreased with the increase of curcumin intervention concentration and the intervention time ($P < 0.05$) (**Table 1** and **Figure 1**).

Effect of curcumin on glioma U251 cells

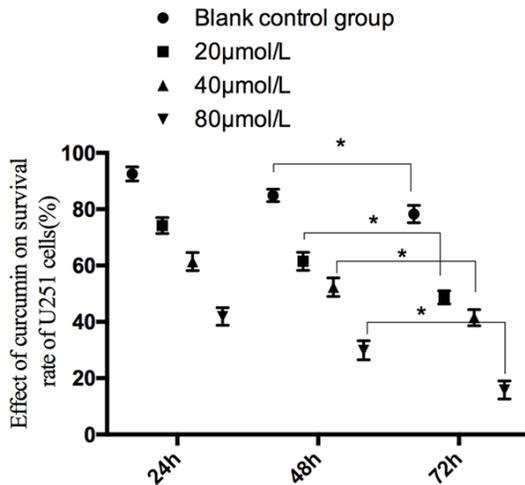


Figure 1. Effect of curcumin on survival rate of U251 cells. Note: *expression $P < 0.05$; *compared with the blank control group, $P < 0.05$.

Curcumin inhibits apoptosis, invasion and migration of glioma cell U251

The apoptosis rates of the blank control group, 20 $\mu\text{mol/L}$ group, 40 $\mu\text{mol/L}$ group and 80 $\mu\text{mol/L}$ group were $4.31 \pm 1.09\%$, $14.56 \pm 2.78\%$, $23.85 \pm 2.67\%$ and $33.25 \pm 2.49\%$, respectively. The apoptosis rate of 20 $\mu\text{mol/L}$ group, 40 $\mu\text{mol/L}$ group and 80 $\mu\text{mol/L}$ group was significantly higher than that of the blank control group, and the difference was statistically significant ($P < 0.05$). With the increase of the concentration of curcumin intervention, the apoptotic rate also increased gradually ($P < 0.05$). The number of transmembrane cells in the blank control group, 20 $\mu\text{mol/L}$ group, 40 $\mu\text{mol/L}$ group and 80 $\mu\text{mol/L}$ group were $68.55 \pm 5.19\%$, $41.27 \pm 6.19\%$, $30.46 \pm 6.69\%$, and $21.33 \pm 1.35\%$, respectively. The number of transmembrane cells in the 20 $\mu\text{mol/L}$ group, 40 $\mu\text{mol/L}$ group and 80 $\mu\text{mol/L}$ group was significantly lower than that in the blank control group, and the difference was statistically significant ($P < 0.05$). And with the increase of the concentration of curcumin intervention, the number of transmembrane cells also gradually decreased ($P < 0.05$). The cell migration index of the blank control group, 20 $\mu\text{mol/L}$ group, 40 $\mu\text{mol/L}$ group and 80 $\mu\text{mol/L}$ group were $79.18 \pm 1.39\%$, $58.26 \pm 1.54\%$, $43.66 \pm 2.29\%$, and 31.43 ± 2.29 , respectively. The cell migration index of 20 $\mu\text{mol/L}$ group, 40 $\mu\text{mol/L}$ group and 80 $\mu\text{mol/L}$ group was significantly lower than that of the blank control group, and the differ-

ence was statistically significant ($P < 0.05$). As the concentration of curcumin intervention increased, the cell migration index also gradually decreased ($P < 0.05$) (Tables 2 and 3; Figures 2-4).

Curcumin inhibit STAT3 protein and p-STAT3, Bax and Bcl-2 protein expression

The expressions of STAT3 protein in blank control group, 20 $\mu\text{mol/L}$ group, 40 $\mu\text{mol/L}$ group and 80 $\mu\text{mol/L}$ group were $0.97 \pm 0.19\%$, $0.75 \pm 0.13\%$, $0.64 \pm 0.14\%$ and $0.52 \pm 0.12\%$, respectively. The expression of STAT3 protein in the 20 $\mu\text{mol/L}$ group, 40 $\mu\text{mol/L}$ group and 80 $\mu\text{mol/L}$ group was significantly lower than that in the blank control group, and the difference was statistically significant ($P < 0.05$). And as the concentration of curcumin increased, the expression of STAT3 protein gradually decreased ($P < 0.05$). The expressions of p-STAT3 protein in blank control group, 20 $\mu\text{mol/L}$ group, 40 $\mu\text{mol/L}$ group and 80 $\mu\text{mol/L}$ group were $1.31 \pm 0.23\%$, $0.82 \pm 0.24\%$, $0.62 \pm 0.19\%$, and $0.50 \pm 0.18\%$. The expression of p-STAT3 protein in the 20 $\mu\text{mol/L}$ group, 40 $\mu\text{mol/L}$ group and 80 $\mu\text{mol/L}$ group was significantly lower than that of the blank control group, and the difference was statistically significant ($P < 0.05$). With the increase of the concentration of curcumin, the expression of p-STAT3 protein was gradually decreased ($P < 0.05$). The pro-apoptotic protein Bax in the blank control group was significantly up-regulated than that in the curcumin intervention group. The expression of anti-apoptotic protein Bcl2 protein was significantly lower than that in the curcumin intervention group. Expression of Bax protein increased with the increase in curcumin concentration while expression of Bcl2 protein decreased with increase in curcumin concentration (Tables 4 and 5; Figures 5 and 6).

Discussion

As a common central nervous system tumor, glioma has the highest incidence in intracranial malignant tumors [12]. The young and middle-aged population is a high-risk population of gliomas, and once a glioma is diagnosed, most patients often die within 2 years. Due to its high mortality and recurrence rate, gliomas pose a major threat to human health [13]. With the advancement of social and medical level, although the treatment of glioma has been

Effect of curcumin on glioma U251 cells

Table 2. Apoptosis of cells under different concentrations of curcumin (%)

Cell state	Blank control group	20 $\mu\text{mol/L}$	40 $\mu\text{mol/L}$	80 $\mu\text{mol/L}$	F	P
Necrotic cells	1.81 \pm 0.12	1.69 \pm 0.11	3.55 \pm 0.41	4.24 \pm 0.78	24.14	< 0.001
Late apoptotic cells	3.51 \pm 0.56	5.93 \pm 0.71	6.88 \pm 1.21	7.86 \pm 1.17	11.94	< 0.001
Early apoptotic cells	4.31 \pm 1.09	14.56 \pm 2.78	23.85 \pm 2.67	33.25 \pm 2.49	83.09	< 0.001
Living cells	90.37 \pm 4.87	77.82 \pm 3.92	65.72 \pm 3.12	54.65 \pm 3.56	46.30	< 0.001

Table 3. Apoptotic rate, number of transmembrane cells and cell migration index of U251 cells after 48 h of curcumin intervention

	Blank control group	20 $\mu\text{mol/L}$	40 $\mu\text{mol/L}$	80 $\mu\text{mol/L}$	F	P
Number of cell penetration	68.55 \pm 5.19	41.27 \pm 6.19	30.46 \pm 6.69	21.33 \pm 1.35	44.91	< 0.001
Cell migration index	79.18 \pm 1.39	58.26 \pm 1.54	43.66 \pm 2.29	31.43 \pm 2.29	342.2	< 0.001

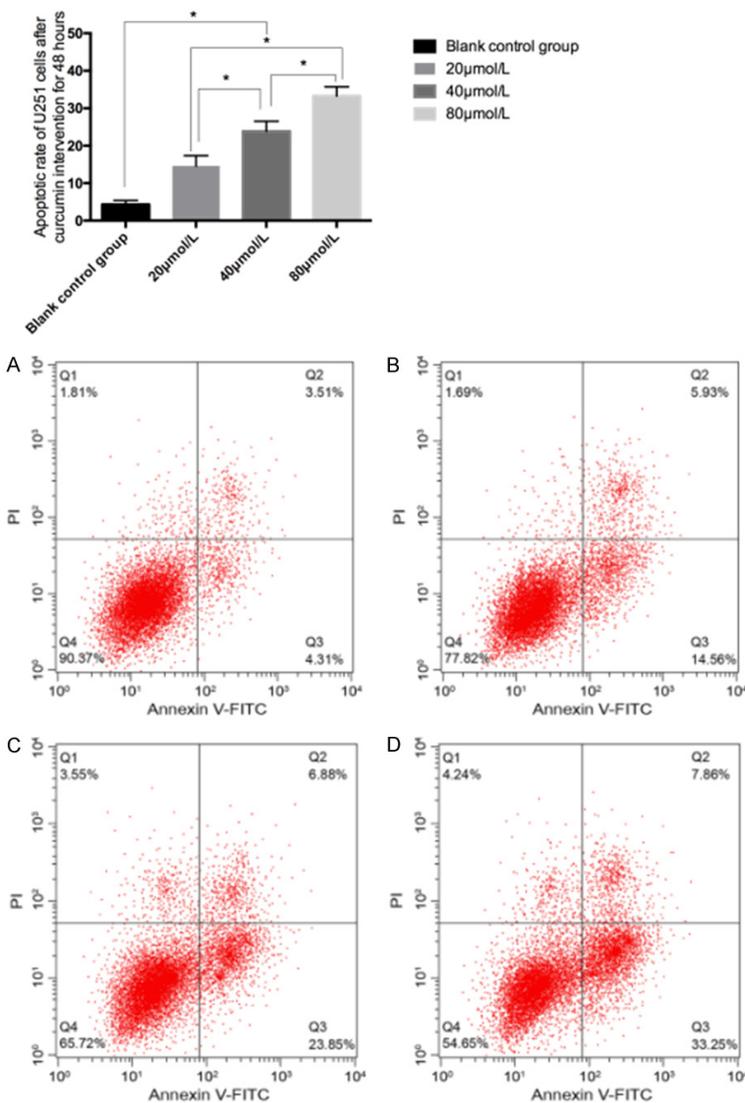


Figure 2. Flow cytometry results for apoptotic rate of glioma cell U251. A. Blank group. B. 20 $\mu\text{mol/L}$. C. 40 $\mu\text{mol/L}$. D. 80 $\mu\text{mol/L}$. The apoptosis rate increased with the increase of curcumin concentration ($P < 0.05$).

improved, the survival rate of glioma patients is still low, so the effective treatment of finding a glioma is clinically urgent question [14]. Glioma cells have the characteristics of immortal proliferation and anti-apoptotic malignant expression as well as abnormally active invasive growth. Therefore, the evaluation of the malignant biological behavior of glioma is also an important direction for the prevention and treatment of glioma [15].

The effect of curcumin on the biological function of glioma U251 cells was explored in our study. With the increase of curcumin concentration and the increase of intervention time, the survival rate of glioma cell U251 decreased gradually ($P < 0.05$). With the increase of curcumin concentration, the apoptosis rate and cell permeation rate increased. The cell migration index increased and the cell migration index decreased gradually ($P < 0.05$), which was confirmed by the fact that expression of Bax protein increased with the increase in curcumin concentration while expression of Bcl2 protein decreased with the increase in curcumin concentration. As an

Effect of curcumin on glioma U251 cells

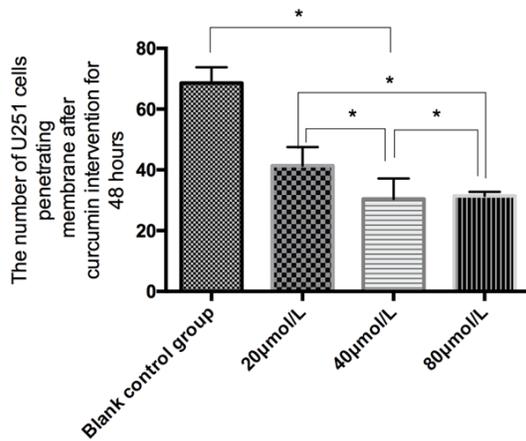


Figure 3. Effect of curcumin on cell transmembrane of glioma cell U251. The number of transmembrane cells in the curcumin intervention group was significantly lower than that in the blank control group ($P < 0.05$). Note: *indicated $P < 0.05$.

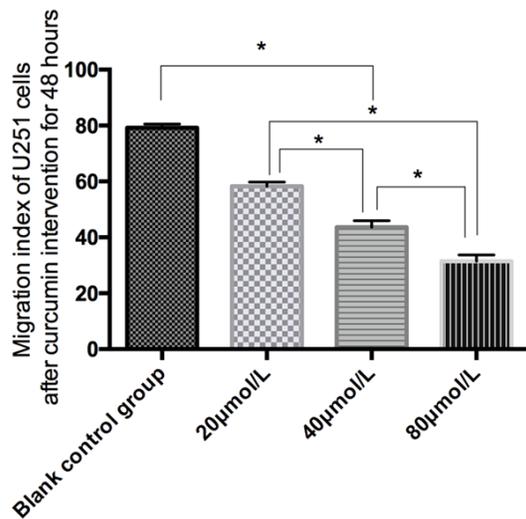


Figure 4. Effect of curcumin on cell migration index of glioma cell U251. The cell migration index of the curcumin intervention group was significantly lower than that of the blank control group, and the difference was statistically significant ($P < 0.05$). As the concentration of curcumin increased, the cell migration index also increased ($P < 0.05$). Note: *indicated $P < 0.05$.

active ingredient extracted from turmeric, curcumin is shown to have anti-inflammatory, anti-rheumatic and anti-tumor functions in many studies [16, 17]. More studies have shown that curcumin as an anti-tumor drug has an effective inhibitory effect on tumor cell apoptosis, and can be used as an ideal adjuvant for chemoradiotherapy [18]. For example, studies

on the role of curcumin in nasopharyngeal carcinoma cells have shown that the turmeric group can block tumor cells in the S phase and G2/M phase of the cell cycle, thereby inhibiting the proliferation of nasopharyngeal carcinoma cells [19]. This verifies our conclusions from the side. Other studies have shown that high concentrations of curcumin can also block the cycle of breast cancer cells in the S phase and G2/M phase, in order to inhibit the proliferation of breast cancer cells. It also promotes apoptosis in breast cancer cells by down-regulating bc1.2 levels and up-regulating bax levels [20]. Other studies [21] reported that curcumin can inhibit the proliferation of promyelocytic leukemia HL-60 cells and promote their apoptosis. All of the above studies are similar to our conclusions. Although some studies have previously discussed the effects of curcumin on glioma cells, the specific mechanism remains unclear. Previous studies [22, 23] have shown that the STAT3 signaling pathway is overexpressed in both primary and tumor cells. And the activation of STAT3 plays an important role in the regulation of biological functions such as proliferation and apoptosis of tumor cells. This may be related to the activation of a series of target genes after STAT3 activation. To further explore the mechanism of curcumin on glioma cells, we studied the expression of STAT3 protein and p-STAT3 protein in cells after drug intervention. The results showed that the expression of STAT3 protein and p-STAT3 protein in curcumin intervention group were significantly lower than that in the blank control group ($P < 0.05$). And as the concentration of curcumin increased, the expression of STAT3 protein and p-STAT3 protein decreased gradually ($P < 0.05$). This result suggests that curcumin can affect the expression of STAT3 signaling pathway-associated proteins. Studies [24] have explored the effect of tanshinone on glioma cells, indicating that tanshinone can inhibit the proliferation of glioma cells by inhibiting the activation of STAT3. However, there were also studies on the regulation of curcumin on STAT3 signaling pathway [25], which indicated that curcumin can induce apoptosis of lung cancer cells by regulating STAT3 signaling pathway. This conclusion is similar to our results. This is why we hypothesized that curcumin may affect the biological function of glioma U251 cells through regulation of the STAT3 signaling pathway.

Effect of curcumin on glioma U251 cells

Table 4. Western blot detection of STAT3 protein and p-STAT3 protein expression

Factor	Blank control group	20 μmol/L	40 μmol/L	80 μmol/L	F	P
STAT3	0.97 ± 0.19	0.75 ± 0.13	0.64 ± 0.14	0.52 ± 0.12	5.048	< 0.050
p-STAT3	1.31 ± 0.23	0.82 ± 0.24	0.62 ± 0.19	0.50 ± 0.18	8.542	< 0.050

Table 5. Western blot analysis of the expression of apoptosis-related proteins Bax and Bcl-2

Factor	Blank control group	20 μmol/L	40 μmol/L	80 μmol/L	F	P
Bax	0.86 ± 0.09	1.03 ± 0.11	1.13 ± 0.10	1.24 ± 0.12	7.004	0.013
Bcl-2	1.21 ± 0.18	1.08 ± 0.15	0.92 ± 0.13	0.81 ± 0.14	4.066	0.049

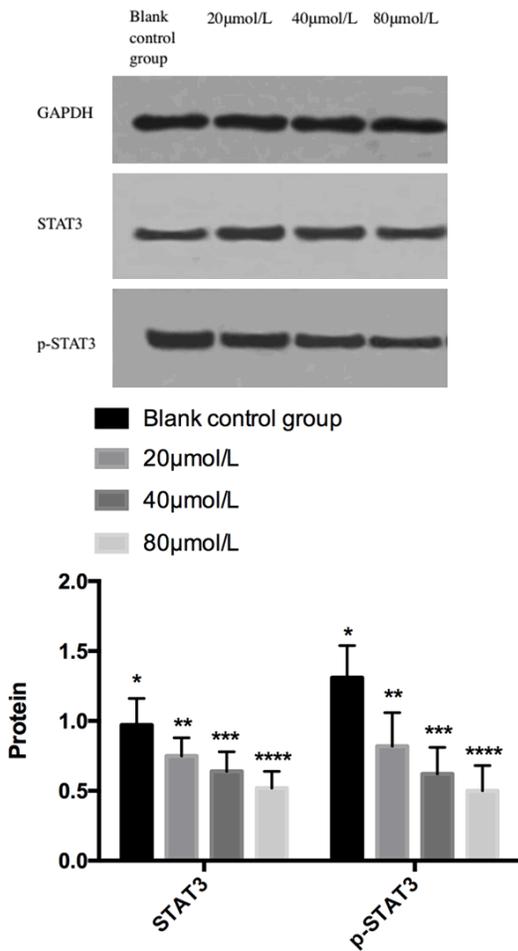


Figure 5. Western blot detection of STAT3 protein and p-STAT3 protein expression. Note: * compared with **, ***, ****, $P < 0.05$; ** compared with ***, ****, $P < 0.05$; *** compared with ****, $P < 0.05$.

In summary, curcumin inhibited the growth, invasion and migration of glioma cell U251 and induced apoptosis in U251 cells. Its mechanism may be related to inhibition of the STAT3 signaling pathway, which provided new ideas

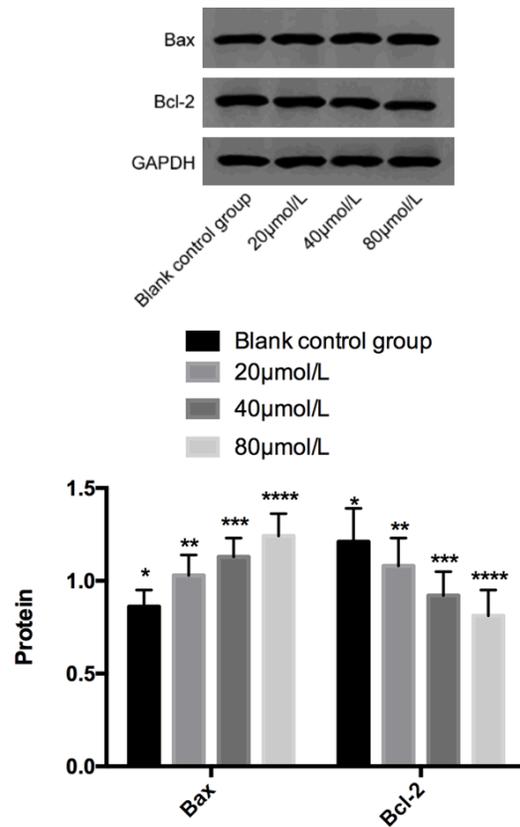


Figure 6. Western blot detection of bax protein and bcl2 protein expression. Note: * compared with **, ***, ****, $P < 0.05$; ** compared with ***, ****, $P < 0.05$; *** compared with ****, $P < 0.05$.

and directions for the treatment of glioma. However, in this study, no experiments were performed on multiple glioma cells, and no further investigation of the target genes of STAT3 was conducted. The study also did not explore why expression of STAT3 protein and p-STAT3 protein was changed and how did curcumin activate p-STAT3. These will be the focus of our curcumin treatment of gliomas in the future,

and it is hoped that scholars can conduct further research.

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Disclosure of conflict of interest

None.

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