RESEARCH ARTICLE

Scutellarin exerts anti-glioma effects by regulating STAT3 to activate the endoplasmic reticulum stress response

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Glioma is the most common brain tumor in clinical settings, and its treatment remains a tough problem to be solved urgently in modern neurosurgery. Scutellarin (SCU) exerts prominent anti-tumor activity. At present, the induction of tumor cell apoptosis has become a research focus in the field of cancer therapy. During proliferation, malignant gliomas activate proliferative pathways and inhibit apoptotic pathways, thereby leading to therapeutic resistance. As a classic oncogene, STAT3 is abnormally activated in a wide range of tumor cells and cancer tissues. Endoplasmic reticulum stress (ERS) is a vital mechanism mediating cell apoptosis, which can activate its downstream PERK, IRE1, and ATF6 signaling pathways. CHOP and Caspase 4 are ERS-associated apoptotic proteins. CHOP modulates the transcription of downstream genes and participates in the regulation of cell apoptosis. Upon activation, Caspase 4 triggers caspase family proteins to initiate caspase cascade reactions, ultimately inducing cell apoptosis. This study explored the effects and molecular mechanisms of scutellarin on inducing glioma cell apoptosis by regulating endoplasmic reticulum stress through STAT3. Graded concentrations of SCU at 0, 20, 40, 80 μg/mL were supplemented into C6 cell medium followed by 24 h continuous incubation. By using the CCK - 8 assay, cell viability was assessed to ascertain the subsequent SCU treatment concentrations. The experiment was subsequently divided into a blank control group and an SCU group with a concentration of 40 μg/mL. Cell morphology was examined using an inverted microscope. Evaluations on cell migration plus invasion relied on Transwell and wound healing detection systems, and flow cytometry served as the detecting method for cellular apoptotic status. Protein expressions were detected using Western blotting. CCK 8 results demonstrated that, within the tested concentration range, SCU effectively reduced the viability of C6 cells in a concentration - dependent manner. Observation with an inverted microscope showed a significant decrease in the number of cells in the 40 μg/mL SCU group along with a blurring of the cell boundaries. Exposure to SCU dramatically impaired the migration and invasion of C6 cells. In addition, both the late apoptosis rate and total apoptosis rate of C6 cells were obviously elevated in the 40 μg/mL SCU group. The results revealed downregulated STAT3 protein expression in SCU-treated C6 cells. CHOP and Caspase - 4 expressions were elevated in the 40 μg/mL SCU group compared with the control group. This research demonstrated that SCU effectively reduced cell viability and weakened migration and invasion abilities. Its antitumor mechanism might be linked to the downregulation of STAT3, the upregulation of CHOP and Caspase - 4, and the subsequent activation of the endoplasmic reticulum stress pathway.

Keywords: scutellarin; STAT3; apoptosis; endoplasmic reticulum stress; glioma.

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Introduction

Driven by population aging, unhealthy lifestyles, environmental pollution, and the low cure rate of conventional therapeutic strategies, the global and Chinese cancer burden has continued to rise in recent years. To cope with growing public health challenges, both China and the United States have successively launched precision medicine initiatives, in which tumor treatment is regarded as a core research direction. Among various human malignant tumors, intracranial glioma exhibits high malignancy, strong invasiveness, and poor prognosis, which severely endangers human health and brings huge economic pressure and physical suffering to patients and their families [1]. At present, conventional combined clinical treatment and temozolomide chemotherapy cannot achieve satisfactory therapeutic effects [2]. The annual incidence and postoperative recurrence rate of glioma remain at a high level, making glioma treatment a difficult and urgent problem to be solved in modern neurosurgery.

Induction of tumor cell apoptosis has been universally recognized as a core and promising therapeutic strategy for modern oncotherapy [3-5]. Malignant glioma cells often obtain therapeutic resistance by activating proliferative signaling pathways and inhibiting endogenous apoptotic cascades, which is the key cause of clinical treatment failure [6]. As a classic oncogenic signaling molecule, signal transducer and activator of transcription 3 (STAT3) is abnormally hyperactivated in a variety of tumor cells. Persistent STAT3 phosphorylation and nuclear translocation can continuously initiate the transcription of downstream oncogenes, thereby promoting tumor proliferation and inhibiting cell apoptosis [7, 8]. Endoplasmic reticulum stress (ERS) is a critical adaptive cellular stress response. Moderate ERS helps cells resist external damage, while excessive and sustained ERS can trigger irreversible apoptotic procedures [9, 10]. The classical PERK, IRE1, and ATF6 signaling pathways are involved in ERS-mediated apoptosis [11]. Multiple key proteins including CHOP, JNK, and Caspase-4 serve as core regulatory factors in ERS-related apoptotic cascades, which can effectively initiate and amplify tumor cell apoptosis [12-15]. Traditional Chinese medicine (TCM) monomers have become popular anti-tumor research candidates due to their low toxicity, high safety, and stable pharmacological activity. As a natural flavonoid compound, Scutellarin (SCU) is extracted from multiple TCM materials. Existing studies have confirmed that SCU possesses multiple biological activities such as anti-inflammation, immunomodulation, and anti-tumor effects [16], and can regulate multitarget apoptotic signaling in various tumor cells [3, 17, 18]. Although the anti-tumor effect of SCU has been reported in multiple tumors, its specific regulatory mechanism against glioma remains unclear. At present, it is still unknown whether SCU can exert anti-glioma activity by regulating the crosstalk between STAT3 signaling and ERS apoptotic pathways. In addition, the specific changes of key ERS apoptotic proteins (CHOP and Caspase-4) after SCU intervention in glioma cells lack systematic verification. Clarifying the above molecular regulatory mechanism is essential to fill the research gap of SCU in glioma treatment.

This study clarified the anti-glioma effect of SCU and explored its underlying molecular mechanism by verifying the inhibition of SCU on the proliferation, migration, and invasion of glioma cells and the induction of cell apoptosis. Furthermore, this study focused on confirming whether SCU regulated the expression of STAT3, thereby activating the ERS apoptotic pathway and upregulating the levels of CHOP and Caspase-4 to mediate glioma cell apoptosis. The results of this study systematically clarified the molecular mechanism of SCU against glioma based on the STAT3/ERS apoptotic pathway, which not only enriched the anti-tumor pharmacological mechanism of SCU, but also provided new experimental evidence and theoretical support for the clinical application of natural TCM monomers in glioma treatment. Furthermore, this research offered a novel targeted therapeutic idea for improving the poor

prognosis and high recurrence rate of glioma, which had certain reference value for the development of new anti-glioma drugs and the optimization of clinical treatment strategies.

Materials and methods

Cell culture

Rat C6 glioma cell line was provided by the Scientific Research Platform of Basic Medical College, Zhangzhou Health Vocational College, Zhangzhou, Fujian, China. Complete culture medium was prepared by mixing Dulbecco's Modified Eagle Medium (DMEM) with fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA). Frozen cells were thawed rapidly in a 37°C water bath. The recovered cell suspension was centrifuged for 5 min and resuspended in 1 mL of fresh culture medium before being inoculated into a culture flask containing 9 mL of medium and incubated in a humidified incubator at 37°C with 5% CO_2. Cell adherence was observed 24 h after resuscitation, and cellular morphology and growth density were examined microscopically on the following day. Cell subculture was conducted when stable cell adherence and approximately 80% cell confluence were achieved.

Drug preparation

Scutellarin was purchased from Adamas, Shanghai, China. 0.8 mg of Scutellarin powder was precisely weighed and completely solubilized with minimal DMSO. PBS was supplemented to reach a final volume of 1 mL, yielding 8,000 mg/L SCU stock solution to be stored at 4℃. The SCU stock solution should be used within 1 – 2 weeks to avoid loss of pharmacological activity. Corresponding working concentrations were prepared *via* serial dilution based on the preset experimental dosages in later tests.

Cell proliferation assay

The C6 cells were harvested and resuspended in fresh cultural medium at 1×10^5 cells/mL before inoculating to 96 well plates. The cells were divided into control, 20 mg/L SCU, 40 mg/L SCU, 80 mg/L SCU groups with three replicate wells for each group. The plates were shaken gently to ensure sufficient contact between drugs and cells and incubated for 24 h. Subsequently, 10 µL of CCK-8 reagent (Biosharp, Hefei, Anhui, China) was added to each well followed by further incubation for 4 hours in the dark before the absorbance of each well was measured at 450 nm using ReadMax 1900 microplate reader (Shanghai Shanpu Biotechnology Co., Ltd., Shanghai, China). Cell viability was calculated, and the IC_{50} value was determined.

Observation of cell morphology

C6 cells were harvested at approximately 80% confluence. Cells were seeded in 24 well plates at 1×10^5 cells/well. Cells with favorable adherence and normal growth status were then divided into a blank control group and a 40 mg/L SCU treatment group. After 24 h of continuous drug incubation, cellular morphological changes were observed, and cell images were captured using an inverted microscope.

Invasion assays

Matrigel (Corning, Corning, NY, USA) was diluted with serum-free medium at a ratio of 1:8 and coated plates at 4℃ by putting 60 µl of Matrigel evenly on the upper surface of the polycarbonate membrane at the bottom of 8 µm pore size Transwell inserts and incubating at 37℃ for 1 h to allow Matrigel polymerization to form a thin film. After incubation, the liquid in the upper chamber was aspirated followed by adding 100 µL of serum-free medium and incubating for 30 min to achieve basement membrane hydration. After 24 h, serum-free medium and serum-supplemented medium were dispensed into upper and lower chambers separately with the latter as chemoattractant. Cells in blank control and SCU groups were serum - starved for 24 h before harvesting and resuspending to 1×10^5 cells/mL. 100 µL cell suspension was loaded to each chamber and then incubated at 37℃ overnight. Residual medium inside insert was aspirated followed by repeated gentle PBS rinsing. 4% paraformaldehyde was applied for cell fixation followed by crystal violet staining.

Cells without invasive capacity were carefully wiped off. The morphology and cell count of stained specimens were analyzed, and microscopic images were collected *via* an inverted microscope.

Scratch assay

C6 cells were evenly mixed with serum-free medium, inoculated into 6-well plates, and incubated until cells fully covered the bottom before scratches were created with a 10 µL pipette tip. Suspended cells and cellular debris were thoroughly rinsed away with serum-free medium. Two experimental cohorts including control and SCU administration groups were established in the study. The wound healing status was observed and photographed at the same visual field at 0 and 24 h after incubation. The scratch width was measured for migration rate calculation.

Flow cytometry with Annexin V-FITC/PI double staining

The cells were incubated overnight on 6-well plates to reach approximately 80 % confluence before the test. The control group was supplemented with 2 mL of routine medium, while the SCU group was treated with 2 mL of SCU-containing medium. After additional 24 h incubation, Annexin V FITC/PI Apoptosis Detection Kit (Adamas life, Shanghai Titan Scientific Co., Ltd., Shanghai, China) was employed to assess the cellular apoptosis following manufacturer's instructions through EXFLOW 2000 flow cytometer (Dakewe Biotech Co., Ltd., Shenzhen, Guangdong, China).

Western blot analysis

Cultured C6 cells in control and SCU treatment groups were harvested after a 24 h intervention. Proteins were extracted by using the Whole Cell Extraction Kit (Abcam, Shanghai, China) following manufacturer's instructions and separated using SDS-PAGE (Beyotime Biotechnology, Shanghai, China). After electrophoretically blotted proteins onto PVDF membranes (Thermo Fisher Scientific, Waltham, MA, USA), 5% bovine serum albumin was used for blocking at room temperature followed by incubating with primary antibodies against STAT3, CHOP, and Caspase 4 (Abcam, Shanghai, China) at 4°C overnight. After PBS washing, the membrane was incubated with the secondary antibody before chromogenic reaction and band scanning to quantify the proteins.

Statistical analysis

GraphPad Prism 5 (GraphPad Software, LLC, Boston, MA, USA) was used for statistical analysis of this study. All resulting data were presented as mean ± SD from triplicate independent assays. Student's t-test was used for the pairwise comparisons between the two groups with *P* value less than 0.05 as statistically significant.

Results and discussion

Regulation of C6 glioma cell viability by SCU

The results demonstrated that the control group showed superior cell viability compared with other groups. All SCU-exposed groups exhibited obvious declines in cellular viability relative to the controls ($P < 0.05$ or $P < 0.001$). The group with 80 µg/mL SCU produced the most prominent suppressive impact on the viability of C6 cells (Figure 1). The results confirmed that SCU reduced the viability of tumor cells. The IC_{50} of SCU was calculated to be 38.06 µg/mL, and therefore, 40 µg/mL was selected as the intervention concentration for subsequent experiments.

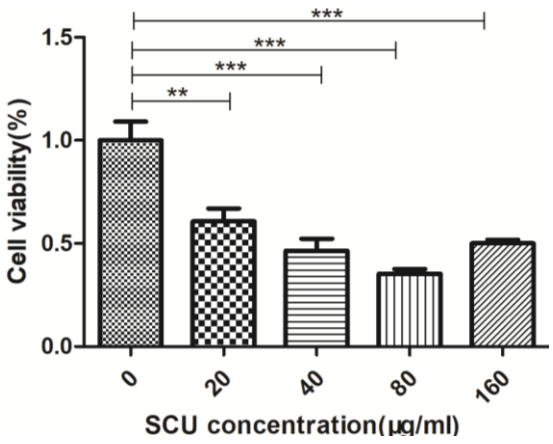


Figure 1. Inhibitory activity of SCU on C6 glioma cell viability. **: $P < 0.01$. ***: $P < 0.001$.

Consistent with the CCK-8 detection results, SCU exerted a dose-dependent inhibitory effect on the viability of C6 glioma cells, and its tumor-suppressive potency was gradually enhanced with the increase of drug concentration. As a natural active compound, SCU presented low toxic side effects and excellent anti-tumor activity, which laid a reliable experimental foundation for its subsequent application in glioma research.

Inhibitory Activity of SCU Against the Growth of Rat C6 Glioma Cells

Representative cellular morphology from control and SCU groups showed that the control cells grew vigorously and arranged compactly. In contrast, the total number of C6 cells in the SCU group was markedly reduced. Meanwhile, cells in the SCU group presented obvious shrinkage, the intercellular spacing was increased, the boundary of adherent cells became blurred, and partial cell detachment was observed (Figure 2). According to morphological observations, SCU effectively inhibited the proliferation of C6 cells. Meanwhile, SCU triggered detrimental morphological alterations in C6 cells.

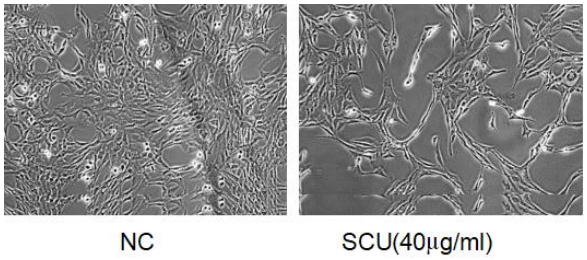


Figure 2. Phenotypic changes in C6 glioma cells across experimental groups (Inverted microscope, magnification ×100) (NC: control).

The morphological differences between the two groups further verified the anti-proliferative effect of SCU on C6 glioma cells. C6 glioma cells in control group featured dense arrangement, regular morphology, and vigorous growth status, while SCU intervention significantly changed cell morphology and growth state, weakened cell adhesion ability, and inhibited cell proliferation, which was consistent with the cell viability detection results.

Regulatory Effects of SCU on Invasive Behavior of Rat C6 Glioma Cells

Results of trans-well invasion tests demonstrated that SCU exposure led to a dramatic decline in the invasive potential of C6 cells compared to the cells in control group. SCU treatment greatly cut down the count of C6 cells penetrating trans-well filters relative to untreated group ($P < 0.01$) (Figure 3). Collectively, experimental findings confirmed that SCU exerted potent inhibitory effects on tumor cell invasion. Combining the results of cell proliferation and morphological observation, the results indicated that SCU not only inhibited the proliferation of C6 glioma cells, but also effectively suppressed the invasive ability of tumor cells, showing a multi-dimensional antitumor effect on glioma cells.

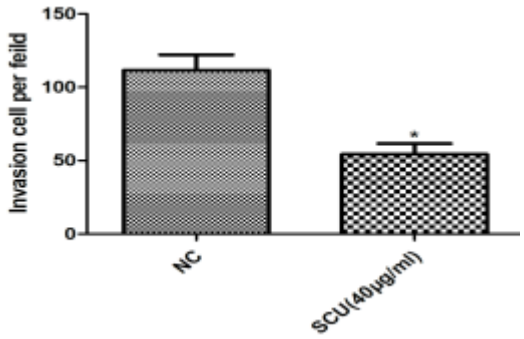


Figure 3. Variations in relative invasive potential of C6 cells between groups. *: $P < 0.05$.

Suppressive influence of SCU on the migration potential of C6 glioma cells

The results of wound-healing assay showed that, at 24 h after scratching, the wound gap was markedly narrowed in the NC group. However, obvious wound closure was not observed in cells treated with 40 μg/mL SCU (Figure 4). The quantitative analysis of relative cell migration showed that the relative migration rate of C6 cells was significantly decreased following 40 μg/mL SCU treatment compared to the NC group ($P < 0.05$) (Figure 5). The results indicated

that SCU significantly inhibited the migration of C6 glioma cells. Wound-healing assay results further supplemented and confirmed the inhibitory effect of SCU on the metastatic phenotype of C6 glioma cells. Combined with trans-well invasion results, it fully demonstrated that SCU could effectively block the migration and invasion processes of glioma cells, thereby inhibiting the malignant progression of tumors.

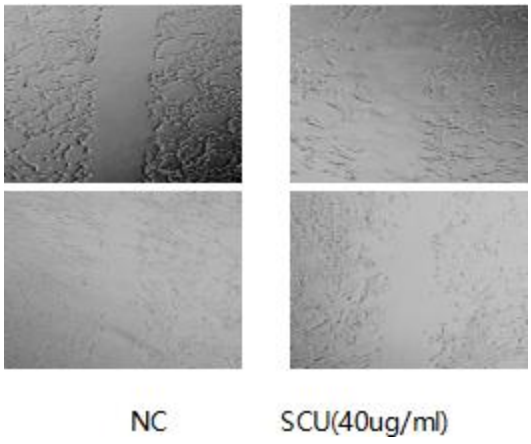


Figure 4. C6 cell migration across experimental groups.

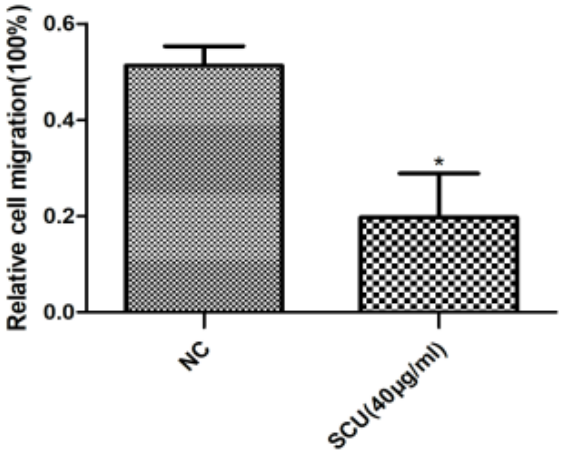


Figure 5. Intergroup disparities in relative migratory rates of C6 glioma cells.

Influences of SCU on C6 cell apoptosis

Changes in the apoptotic ratio of SCU treated C6 cells were quantitatively determined by flow cytometry. After 24 h of SCU treatment, the late apoptotic rate (16.45%) and total apoptotic rate (21.99%) were significantly increased in the 40 μg/mL SCU group compared to the control (8.12% and 9.27%) ($P < 0.001$) (Figure 6). The results verified that SCU intervention could significantly induce the apoptosis of C6 glioma cells. The remarkable increase in total apoptotic rate and late apoptotic rate indicated that SCU exerted a pro-apoptotic effect on glioma cells, which was one of the key mechanisms for SCU to inhibit the growth and malignant progression of C6 glioma cells.

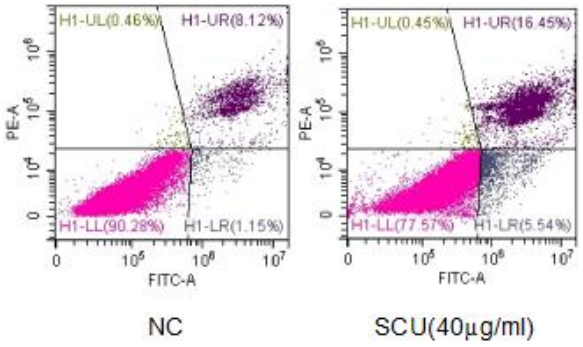


Figure 6. Analysis of C6 cell apoptosis in distinct groups using flow cytometry.

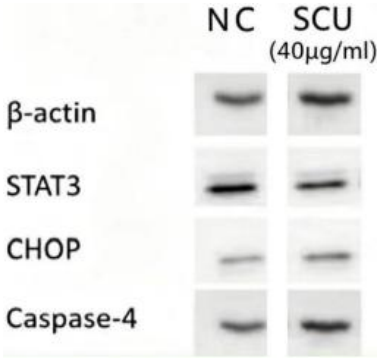


Figure 7. Quantitative changes in STAT3 and ERS protein levels in different C6 cell groups.

Expression of STAT3 and ERS-Associated Proteins in SCU-Treated C6 Cells via Western Blotting

Relative to the control group, the SCU group exhibited reduced STAT3 protein levels and elevated CHOP and Caspase-4 levels (Figure 7).

Western blotting results revealed the potential molecular mechanism of SCU against C6 glioma cells. SCU could downregulate the expression of STAT3 protein and further upregulate the expression of endoplasmic reticulum stress (ERS) marker proteins CHOP and Caspase-4, which activated the ERS signaling pathway in C6 glioma cells, thereby inhibiting cell proliferation, blocking cell migration and invasion, and ultimately inducing tumor cell apoptosis.

Conclusion

SCU exerted robust inhibitory effects on the proliferation, migration, and invasion of rat C6 glioma cells, and effectively induced tumor cell apoptosis. As a natural compound with low toxicity and high antitumor activity, SCU regulated the ERS signaling pathway *via* mediating the STAT3/CHOP/Caspase-4 molecular axis, thereby playing a comprehensive anti-glioma role. This study clarified the antitumor mechanism of SCU in C6 glioma cells, providing a novel research basis and potential therapeutic direction for clinical glioma treatment. Further *in vivo* research and in-depth molecular mechanism exploration should be carried out to promote the clinical transformation and application of SCU as a natural anti-glioma agent.

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