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Original Article

Scutellarin inhibits glioma cell proliferation by up-regulating miR-15a expression

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Abstract: Objective: To investigate the effect of scutellarin on the proliferation of glioma cells through microRNA (miR)-15a. Methods: Human glioma cell line T98G was cultured in vitro and divided into control group (without treatment), scutellarin group (with 10, 20, 40, 80, 160 μg/mL scutellarin, respectively), miR-15a negative control group (transfected with negative control-miR-15a + 80 μg/mL scutellarin) and miR-15a inhibitor group (transfected with miR-15a siRNA + 80 μg/mL scutellarin). The proliferation of T98G cells was detected by cell counting kit-8 (CCK-8), and the expression of miR-15a in T98G cells was detected by real-time fluorescence quantitative PCR (qRT-PCR). The apoptosis of T98G cells was assessed by flow cytometry, and the invasion of T98G cells was compared by Transwell method. The levels of proliferating cell nuclear antigen (PCNA), Bcl-2 related X protein (Bax) and matrix metalloproteinase 9 (MMP-9) in T98G cells were detected by Western blot (WB). Results: Compared with that in the control group, the OD value of T98G cells in scutellarin group was significantly lower ($P<0.05$), with the increase of scutellarin concentration, the OD value of T98G cells decreased in turn, and 80 μg/mL was used as the optimal concentration of scutellarin to treat T98G cells for subsequent experiments. Compared with those in the control group, the miR-15a expression, apoptosis rate and Bax protein expression in T98G cells of scutellarin group were higher ($P<0.05$), and the OD value, number of invasive cells, PCNA and MMP-9 protein levels were lower ($P<0.05$). Compared with scutellarin group and miR-15a negative control group, the miR-15a expression, apoptosis rate and Bax protein expression in T98G cells of miR-15a inhibitor group were lower ($P<0.05$), and the OD value, number of invasive cells, PCNA and MMP-9 protein levels were higher ($P<0.05$). Conclusions: Scutellarin can inhibit the proliferation, invasion and induce the apoptosis of glioma cells, which may be mediated by up-regulating the expression of miR-15a.

Keywords: Scutellarin, microRNA-15a, glioma cell, proliferation, apoptosis, invasion

Introduction

Brain tumor is a kind of tumor with a special location, which can easily cause serious consequences and endanger the life of patients. Glioma, the full name of neuroglioma, is the most common malignancy in brain tumors, accounting for about 50% of all brain tumors, with high invasiveness and lethality [1, 2]. Although great progress has been made in tumor treatment techniques, such as surgical resection, radiotherapy and chemotherapy, many problems remain. Surgical procedures are complicated by the particular location of gliomas, their aggressive growth patterns, and the blurred boundaries between gliomas and adjacent normal brain tissue [3]. The efficacy and prognosis of antitumor drugs such as temozolomide or neutralization therapy are not ideal [4]. Scutellarin, also known as breviscapine, is the active component of Scutellaria baicalensis, Scutellaria barbata and Erigeron breviscapus, with various pharmacological effects such as improving immunity, inhibiting platelet aggregation, antithrombotic, anti-inflammatory and antitumor [5]. In recent years, increasing attention has been paid to the antitumor effect of scutellarin, and studies have reported that scutellarin can inhibit the proliferation of glioma cells [6, 7]. The induction of

tumor cell apoptosis by scutellarin is the result of the joint action of different targets and multiple pathways.

microRNA (miR) is a popular research topic in recent years. microRNA is a non-coding RNA of approximately 18-24 nt in length and is an important member of the non-coding RNA family [8, 9]. microRNAs can serve to inhibit translation and transcription of target genes by targeting binding to the untranslated region at the 3' end of the downstream target gene, thereby altering the expression of the target gene [10]. Previous studies have shown a close association between miR and tumor development. microRNA-15a (miR-15a) is a miRNA with abnormal expression in tumors, and its over-expression inhibits the invasion and metastasis of tumor cells [11]. Studies have shown [12] that miR-15a is lowly expressed in gliomas and the growth of gliomas can be inhibited by upregulating miR-15a. Another study found that [13], Scutellarin could inhibit the metastasis of glioma cells and cisplatin resistance. However, whether Scutellarin is able to regulate the involvement of miR-15a in the development of glioma has not been reported.

This study investigated the role and mechanism of miR-15a in the inhibitor of glioma cell proliferation, invasion and apoptosis by scutellarin.

Materials and methods

Experimental methods

Cell culture: T98G cells were cultured in a DMEM medium (Cat. No.: KL-P0032, purchased from Merck/Sigma, Germany) containing 10% fetal bovine serum (Cat. No.: FBS500-S, purchased from AusGeneX, Australia) and 100 U/mL of penicillin and streptomycin at 37°C in a 5% CO_2 incubator (constant temperature incubator model: MIR-162-PC/MIR-262-PC, purchased from Japan Panasonic), and the cell fusion rate reached 70-80%, digested with trypsin and sub-cultured. Human glioma cells and T98G cells (Cat. No.: CL1449) were purchased from ATCC cell bank in the United States.

T98G cells were transfected using Lipofectamine 2000 (Invitrogen, MA, USA) transfection reagent and the cells were divided into: Control group, scutellarin (Item No.: BP1124-BVN was purchased from Beijing Biolab Technology Co., Ltd.) group, miR-15a negative control group and miR-15a inhibitor group (negative control-miR-15a and miR-15a-5p inhibitor were synthesized by Sangon Bioengineering Shanghai Co., Ltd.). T98G cells in the control group were not treated, and the scutellarin group was added with final concentrations of 10 μg/mL, 20 μg/mL, 40 μg/mL, 80 μg/mL, and 160 μg/mL, respectively. T98G cells for the miR-15a negative control group and miR-15a inhibitor group were transfected with negative control-miR-15a and miR-15a siRNA using Lipofectamine 2000 transfection reagent (Item No.: 11668019, purchased from Invitrogen, USA), respectively, and the optimal concentration of scutellarin was added. miR-15a-5p inhibitor sequence was: 5'-CACAAACCATTATGTGCTGCTA-3', miR-15a negative sequence was: 5'-CAGTACTTTTGTGTAGTACAA-3'.

Proliferation of T98G cells in each group was detected by CCK-8 assay: Collect each group of fine particles 48 h after transfection and adjust to 1×10^5/well, add to 96 well plate, add CCK-8 reagent, and continue to culture for 2 hours (product No.: CK-04, purchased from Dujin Chemical Research Institute of Japan). Measure the absorbance (optical density, OD) value of each hole at the wavelength of 450 nm using a full-automatic micro tablet reader. In addition, 10, 20, 40, 80, 160 μg/mL scutellarin was used to treat T98G cells, and the optimal inhibitory concentration of scutellarin on T98G cells was observed. Microboard reader (model: MODEL550) and chemiluminescent imaging system (model: ChemiDocXRS) were purchased from Bio Rad in the United States.

Expression of miR-15a in each group of T98G cells was detected by qRT-PCR: After 48 hours of transfection, cells in each group were collected, total RNA was extracted with TRIzol reagent, reverse transcription, and the expression of miR-15a was detected by qRT-PCR. The internal reference gene was U6. Real time quantitative polymerase chain reaction kit (Cat. No. K1002S) was purchased from Promega, USA. Reaction program: pre-denaturation at 95°C for 1 min; denaturation at 95°C for 15 s, annealing at 55°C for 30 s, extension at 72°C

for 30 s, 40 cycles. miR-15a upstream primer: 5'-GCGGTAGCAGCACATAATG-3', downstream primer: 5'-GTGCAGGGTCCGAGGT-3'; internal reference U6 upstream primer: 5'-CTCGCTTCGGCAGCACA-3', downstream primer: 5'-AACGCTTCACGAATTTGCGT-3'. The Ct value is the cycle number when the fluorescence signal of the amplified product reaches the critical threshold, and the relative expression of miR-15a is expressed by the $2^{-\Delta\Delta Ct}$ method. Fluorescence quantitative PCR instrument (model: ABI 7500) was purchased from Applied Biosystems, USA.

Apoptosis of T98G cells in each group was detected by Flow cytometry: The cells were collected, digested with trypsin, washed with PBS, and added with 400 μL of 1 × binding buffer. The concentration of cells was adjusted to 1 × 10^6 cells/mL, followed by Annexin V-FITC/PI apoptosis detection kit (AnnexinV-FITC/PI Apoptosis Detection Kit Item No.: S0185, purchased from Harbin Xinhai Genetic Testing Co., Ltd.). 5 μL each of Annexin V-FITC and PI were added, and incubated in the dark for 1 h. The apoptosis rate was detected by flow cytometry. Flow cytometer (model: BD FACSCanto II) was purchased from BD, USA.

Invasion of T98G cells in each group was detected by Transwell assay: Dilute the matrix gel with a serum-free medium (1:8) and add 50% 50 μl to each Transwell chamber. It was cured at 37°C for 1 h. Then 200 μL transfected cells were added to the upper chamber and cultured for 48 hours. 500 μL 10% fetal bovine serum medium was added in the lower chamber and cultured in 37°C, 5% carbon dioxide incubator for 24 hours. The cells in the lower layer were fixed with 4% paraformaldehyde, washed with PBS after 10 minutes, stained with 0.5% crystal violet (catalog number 0528-100 g, purchased from Amresco, USA), and washed with PBS for 20 minutes. The number of invasive cells was calculated under inverted microscope (CX31, purchased from Olympus,Japan). Matrix gel (project number: 356-234) was purchased from BD Company of the United States.

Protein levels of PCNA, Bax and MMP-9 in T98G cells in each group were detected by western blot: The cells were collected, the total protein of each group of cells was extracted using a protein extraction kit, and the protein concentration was determined and quantified using a BCA protein assay kit. Equal amount of protein was separated by electrophoresis and transferred to PDVF membrane and blocked with TBST containing 5% nonfat milk powder for 1 h. PCNA antibody, Bax antibody, MMP-9 antibody, and GAPDH antibody (1:500) were added and incubated at 4°C overnight. After washed with TBST, horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:5000) was added, and incubated at room temperature for 1 h. Immunoreactive chemiluminescence was used for color development. The Tanon 600 image analysis system took images and analyzed the grayscale of the bands. Relative expression of target protein = gray value of target protein/gray value of internal reference GAPDH.

Statistical analysis

Data processing was performed using SPSS 24.0 statistical software. The measurement data were expressed as mean ± standard deviation, and the comparison among multiple groups was performed by one-way ANOVA, and the further pairwise comparison was performed by SNK-q test. $P<0.05$ denotes the difference is statistically significant.

Results

Proliferation of T98G cells in scutellarin group treated with different concentrations of scutellarin

Compared with the control group, the OD value of T98G cells in the scutellarin group treated with different concentrations of scutellarin were decreased ($P<0.05$). In the range of 10-80 μg/mL, with the increase of scutellarin concentration, the OD value of T98G cells decreased sequentially, and the difference was statistically remarkable ($P<0.05$). There was no marked difference in OD value of T98G cells between 80 μg/mL and 160 μg/mL scutellarin concentration ($P>0.05$), so 80 μg/mL was selected as the optimal concentration of scutellarin for subsequent experiments (**Figure 1**).

Expression of miR-15a in T98G cells in each group

Before the study, we first determined the transfection efficiency of miR-15a inhibitor, and we

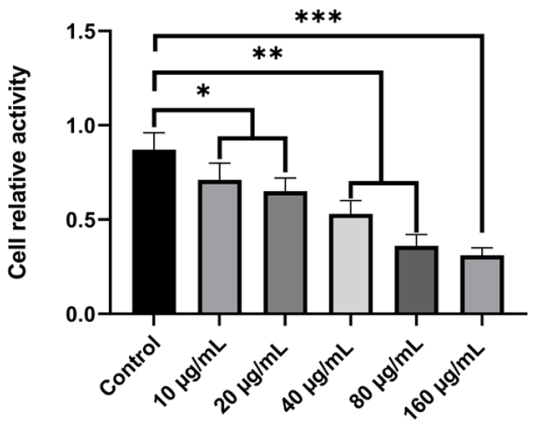

Figure 1. OD values of T98G cells in each group. Note: *P<0.05, **P<0.01, ***P<0.001. The experiment was repeated 6 times.

found that the relative expression of miR-15a in cells treated with miR-15a inhibitor plasmid was lower compared with the control, blank group (**Figure 2A**, P<0.001). Compared with the control group, the expression of miR-15a in T98G cells in the scutellarin group was increased (P<0.001). There was no significant difference in the expression of miR-15a in T98G cells between the control group and the miR-15a negative control group (P>0.05). Compared with the scutellarin group and the miR-15a negative control group, the expression of miR-15a in T98G cells of the miR-15a inhibitor group was decreased (P<0.01, **Figure 2**).

Proliferation of T98G cells in each group

Compared with the control group, the OD value of T98G cells in the scutellarin group was lower (P<0.05). There was no marked difference in the OD value of T98G cells between the control group and the miR-15a negative control group (P>0.05). Compared with the scutellarin group and the miR-15a negative control group, the OD value of T98G cells in the miR-15a inhibitor group was increased (P<0.05, **Figure 3**).

Apoptosis of T98G cells in each group

Compared with the control group, the apoptosis rate of T98G cells in the scutellarin group was increased (P<0.05). There was no significant difference in the apoptosis rate of T98G cells between the control group and the miR-15a negative control group (P>0.05). Compared with the scutellarin group and the miR-15a negative control group, the apoptosis rate of T98G cells in the miR-15a inhibitor group was decreased (P<0.05, **Figure 4A**, **4B**).

Invasion of T98G cells in each group

Compared with the control group, the number of invasive T98G cells in the scutellarin group was decreased (P<0.05). There was no marked difference in the number of invasive cells between the control group and the miR-15a negative control group (P>0.05). Compared with the scutellarin group and the miR-15a negative control group, the number of invasive T98G cells in the miR-15a inhibitor group was increased (P<0.05, **Figure 5A**, **5B**).

Expression of PCNA, Bax and MMP-9 in T98G cells in each group

Compared with the control group, the protein levels of PCNA and MMP-9 in T98G cells of the scutellarin group were decreased, and the protein expression of Bax was increased (P<0.05). There was no obvious difference in the levels of PCNA, MMP-9 and Bax proteins in T98G cells between the control group and the miR-15a negative control group (P>0.05). Compared with the scutellarin group and the miR-15a negative control group, the protein levels of PCNA and MMP-9 in T98G cells of the miR-15a inhibitor group were increased, and the protein expression of Bax was decreased (P<0.05, **Figure 6**).

Discussion

Scutellarin has toxic effects on a variety of tumor cells, and its main anti-tumor mechanisms include inhibiting cell cycle, inhibiting tumor cell proliferation and inducing apoptosis, resisting invasion, and enhancing adhesion. Liu et al. [14] showed that scutellarin can inhibit the proliferation and invasion of liver cancer cells by down-regulating the JAK2/STAT3 pathway. Yang et al. [15] showed that scutellarin can induce apoptosis of HCT-16 colon cancer cells in vitro by regulating the expression of Bcl-2/Bax protein and increasing the phosphorylation of p53. He et al. [16] showed that the combination of C18H17NO6 and scutellarin can inhibit the proliferation of human glioma cells and induce their apoptosis by up-regulating the

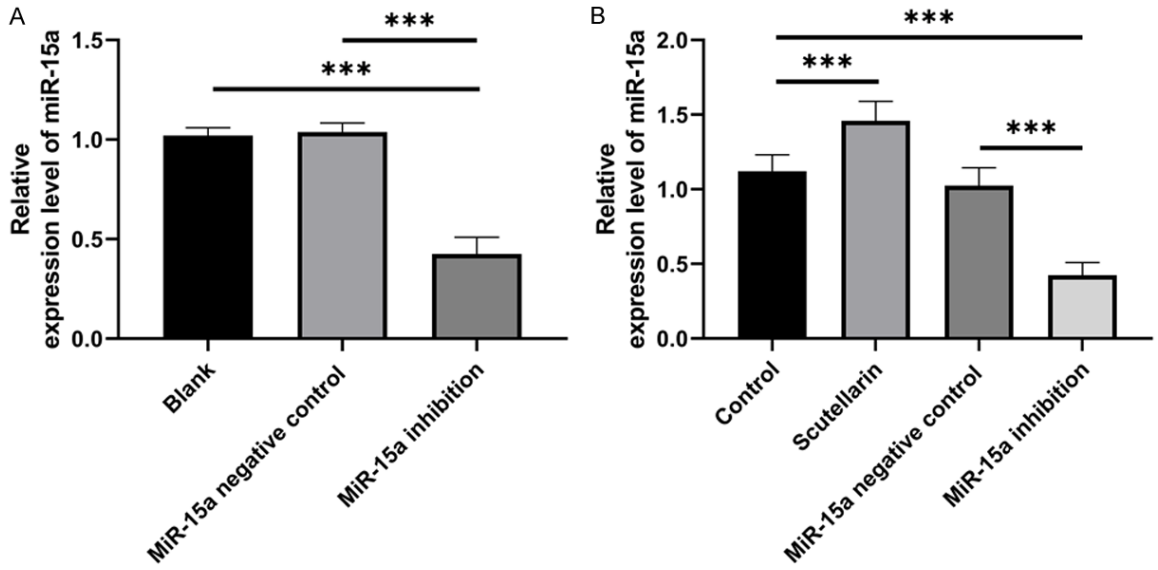

Figure 2. Expression of miR-15a in T98G cells in each group. A. The transfection efficiency of miR-15a in plasmids was detected by qRT-PCR. B. qRT-PCR was used to detect the relative expression of miR-15a in cells after miR-15a inhibitor transfection into cells. Note: **P<0.01, ***P<0.001. The experiment was repeated 6 times. miR: microRNA.

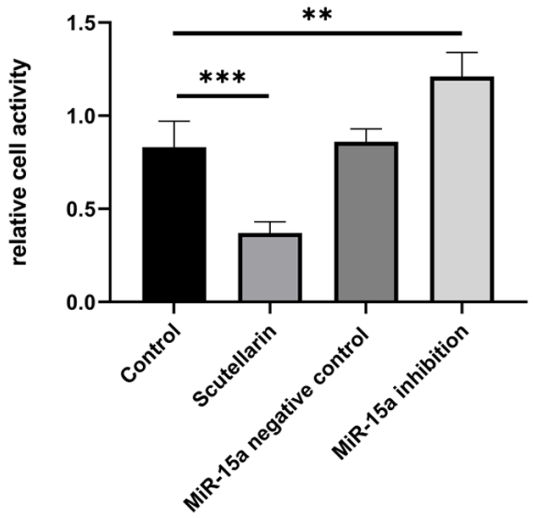

Figure 3. OD values of T98G cells in each group. Note: **P<0.01, ***P<0.001. The experiment was repeated 6 times. miR: microRNA.

expression of Fas-related factors. This study showed that the T98G cells in the scutellarin group treated with different concentrations of scutellarin were lower than those in the control group, and within the range of 10 μg/mL-80 μg/mL, with the increase of scutellarin concentration, the OD of T98G cells decreased. The OD value of T98G cells decreased in turn, and there was no significant difference in the OD value of T98G cells after treatment with 80 μg/mL and 160 μg/mL scutellarin, suggesting that scutellarin can inhibit the proliferation of T98G cells, and the OD value was in the range of 10 μg/mL-80 μg/mL. It was dose-dependent, so 80 μg/mL scutellarin was selected as the scutellarin group for subsequent experiments. Compared with the control group, the apoptosis rate of T98G cells in the scutellarin group was increased, and the number of invasive cells was decreased, suggesting that scutellarin can inhibit the invasion of T98G cells and induce their apoptosis.

microRNAs are a class of non-coding RNAs that widely exist in life. They mainly regulate the expression level of genes after transcription by two mechanisms: cutting mRNA or inhibiting mRNA translation, and participating in the regulation of various complex activities such as cell proliferation and apoptosis in life. It is closely related to the occurrence and development of tumors [17]. Studies have found that miR-15a/16 is lowly expressed in non-small cell lung cancer tissues, and curcumin can inhibit the proliferation of non-small cell lung cancer cells and promote cell apoptosis by up-regulating the expression of miR-15a/16 [18, 19]. Liu et al. [20] found that miR-15a could inhibit the migration and invasion of liver cancer cells by directly targeting cMyb. Sriharikrishnaa et al. [21] found that miR-15a, as a tumor suppressor

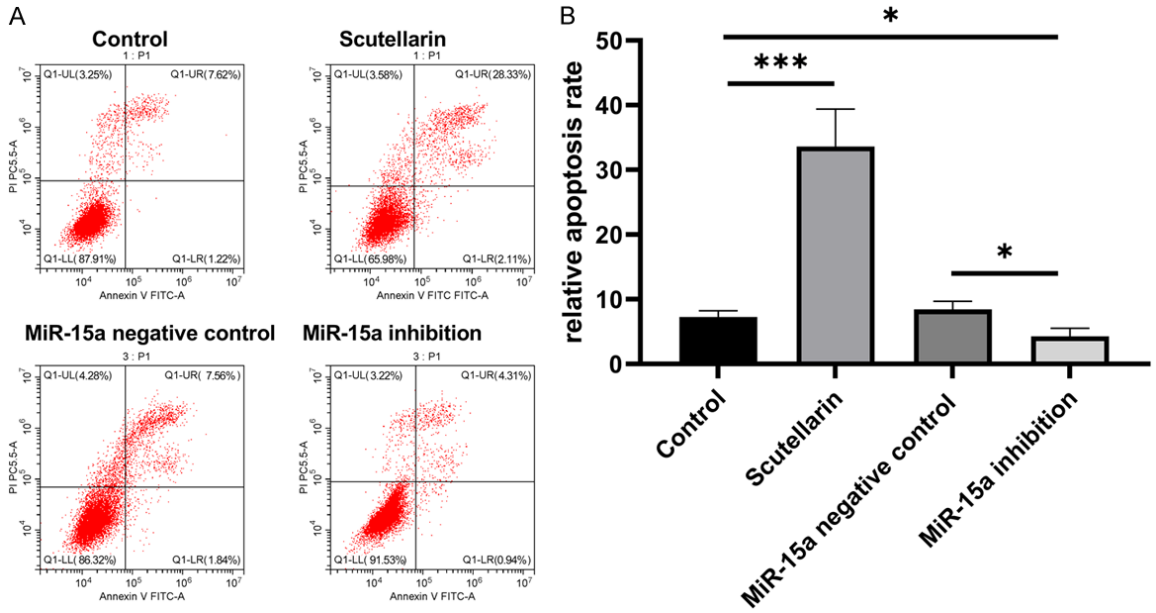

Figure 4. Apoptotic cell death after intervention. A. Flow cytometry original pictures. B. Apoptosis of T98G cells in each group. Note: *P<0.05, ***P<0.001. The experiment was repeated 6 times. miR: microRNA.

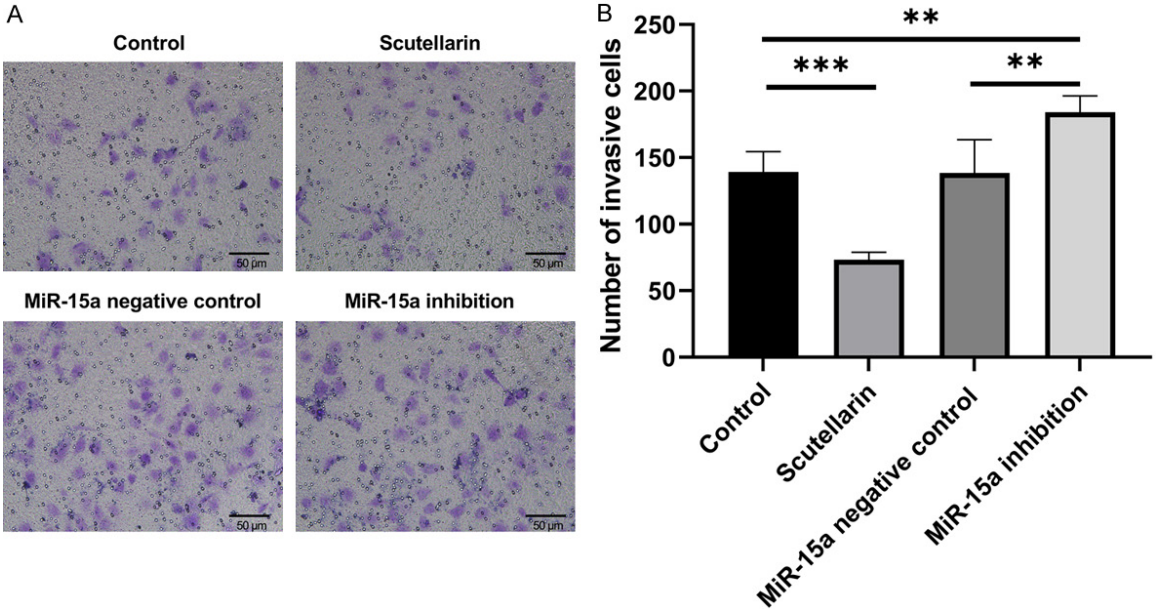

Figure 5. Cell invasion after intervention. A. Original pictures of Transwell experiment. B. Apoptosis of T98G cells in each group Note: **P<0.01, ***P<0.001. The experiment was repeated 6 times. miR: microRNA.

gene in endometrial cancer tissue, may play a role in inhibiting cancer cell metastasis and invasion by inhibiting the expression of MMP-2 protein. Shi et al. [22] discovered that scutellarin could inhibit the proliferation of human breast cancer cell line MCF-7, and up-regulate the expression of miR-15a and miR-16. This study showed that compared with the control group, the expression of miR-15a in T98G cells of the scutellarin group was increased, suggesting that scutellarin may affect the expression of miR-15a in T98G cells. After inhibiting the expression of miR-15a, the OD value and the number of invasive cells in T98G cells were

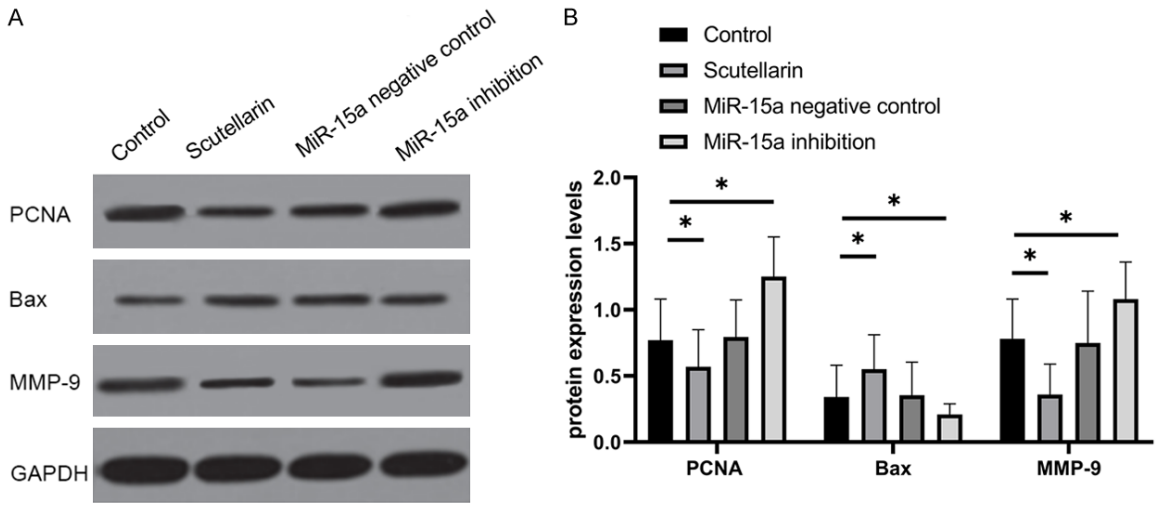

Figure 6. Effect of scutellarin on glioma cell proteins. A. Western blot pictures. B. Expression of PCNA, Bax and MMP-9 proteins in T98G cells in each group. Note: *P<0.05. The experiment was repeated 6 times. microRNA (miR), proliferating cell nuclear antigen (PCNA) antibody, Bcl-2 associated X protein (Bcl-2 associated X protein, Bax) antibody, matrix metalloproteinase-9 (MMP-9).

higher than those in the scutellarin group, and the apoptosis rate was lower than that in the scutellarin group, suggesting that inhibiting the expression of miR-15a could reduce the effect of scutellarin on T98G cells proliferation, apoptosis, and invasion. PCNA, Bax, and MMP-9 are factors related to proliferation, apoptosis, and invasion signaling pathways, respectively [23, 24]. Compared with the control group, the protein levels of PCNA and MMP-9 in T98G cells of the scutellarin group were decreased, and the protein expression of Bax was increased. After the expression of miR-15a was inhibited, the protein levels of PCNA and MMP-9 in T98G cells were decreased, while the expression of Bax protein was significantly decreased. It suggested that PCNA, Bax, and MMP-9 were involved in the process of scutellarin on the proliferation, apoptosis, and invasion of T98G cells, and were related to the expression of miR-15a, indicating that scutellarin may exert great effects. Up-regulating miR-15a expression can adjust the levels of PCNA, Bax, and MMP-9, thereby affecting the proliferation, apoptosis and invasion of T98G cells.

Nevertheless, the present study still has some limitations. First, in the current study, we only analyzed miR-15a inhibitor in glioma, and it remains unclear whether the inhibitor of glioma invasion and apoptosis is enhanced by upregulating miR-15a in combination with scutellarin. Second, whether scutellarin has the same effect in animal models requires further experiments. Finally, whether scutellarin can be used clinically needs to be verified in more clinical trials. Thus, we hope to conduct more experiments in future studies to refine our findings.

In summary, scutellarin can inhibit the proliferation, invasion and promote apoptosis of glioma cells, and up-regulation of miR-15a expression may be one of the mechanisms. Other mechanisms still need to be explored.

Disclosure of conflict of interest

None.

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