

Original Article

Effects of solanine on the proliferation and growth of glioma cells

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Abstract: The aim of this study was to investigate the effects and acidification mechanisms of solanine on the proliferation and growth of glioma cells. U251 and U87 cells were treated with different concentrations of solanine. Inhibition rates were detected by the CCK8 method. Migration and invasion were checked by wound healing and Transwell assays. Apoptosis and cell cycle were measured by flow cytometry. Expression levels of Kir4.1 protein were analyzed by Western blot. It was found that solanine can inhibit the migration, invasion, and proliferation of U251 and U87 cells. Solanine can also promote apoptosis positively of U251 and U87 cells. Solanine can prevent U251 and U87 cells from proliferation and growth, causing tumor cell apoptosis through making them stop at the S phase of cell cycle. Solanine led to intracellular pH changes of the cells. Solanine is a potential drug helping to kill tumors.

Keywords: Solanine, glioma, proliferation, growth

Introduction

Gliomas which originate from neural ectoderm are the most common tumors in the central nervous system, accounting for 40%-50% of all cerebral tumors [1]. Gliomas have been classified by the World Health Organization (WHO), according to their relative malignancy, as grades I-IV, with increasing grades defining more malignant tumors. Grade I and grade II are low grade gliomas, while grade III and grade IV are malignant gliomas [2]. Patients with low-grade gliomas have better outcomes. However, malignant gliomas are incurable and long-term survival remains limited because of the characteristics of strong invasiveness and migration [3, 4]. Currently, glioma treatment predominantly includes surgical resection or radiation and chemotherapy. These are so largely ineffective that malignant gliomas have a high recurrence rate [4, 5].

Chinese medicine may have important potentiality in glioma therapy. Previous studies have shown that solanaceae and its extracts have a wide range of anti-tumor effects on liver cancer and prostate cancer, while being less toxic to

normal cells [6]. Solanine, the main component of solanaceae extracts, has been reported to play a critical role in prevention of tumor development during the processing course of tumors [7]. Whether it can inhibit the growth and proliferation of gliomas has not been reported. Accordingly, the present study explains the actions of solanine on glioma growth, providing essential evidence for clinical drug therapy.

Materials and methods

Cell lines and chemicals

Human glioma cell lines U251 and U87 (ATCC, Shanghai, China) were used. Each cell line was cultured in DMEM (HyClone, Los Angeles, USA) supplemented with 10% FBS (HyClone, Los Angeles, USA), 1% glutamine (Beyotime, Shanghai, China), and 100 U/mL of penicillin/streptomycin (Beyotime, Shanghai, China) and maintained at 37°C in an atmosphere of 5% CO₂ and 95% room air. The cells were passaged every 2-3 days and cells grown at 80% confluency were chosen for subsequent experiments.

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Solanine (purity > 95%) was purchased from Yang Lingci Edge Biological Technology Co. It was dissolved in dimethyl sulfoxide (DMSO, Beyotime, Shanghai, China) with a concentration of 1 mg/mL. After dissolution, solanine was stored at -20°C.

Cell proliferation assay

Cell Counting Kit-8 (CCK-8, Boster, Wuhan, China) was used to examine the effects of solanine on the proliferation of U251 and U87 cells. Briefly, cells were plated in 96-well plates with a density of 1×10^5 cells/mL and incubated overnight. Next, the cells were exposed to indicated concentrations of Solanine for 48 hours. Afterward, 10 μ l of the cell proliferation reagent CCK-8 was added to each well and incubated at 37°C for an additional 2.5 hours. The amount of formazan dye generated by cellular dehydrogenase activity was measured by absorbance at 450 nm with a microplate reader (Thermo Scientific, Massachusetts, USA).

Cell migration and invasion assays

To assess the effects of solanine on the migration of U251 and U87 cells, wound healing assays were carried out by scratching confluent monolayers of U251 or U87 cells in a 6-well plate with a pipette tip. U251 and U87 cells in the each well were then washed and incubated with serum-free DMEM containing indicated concentrations of Solanine for 48 hours. Pictures of fixed positions in the wound area were taken immediately after scratching, with solanine treatment for 48 hours following. The wound area of the gap between migrating edges of cells was used to quantitate the assay.

Transwell assay was performed using 8.0 μ m pore size inserts (Corning Costar Corp, Cambridge, MA, USA). Membrane inserts (upper chambers) were coated with Matrigel (Corning Costar Corp, Cambridge, MA, USA) and rehydrated with DMEM at 37°C overnight, then the serum-free medium was removed. Medium containing different concentrations of solanine and 10% FBS was applied to the lower chambers. U87 or U251 cells were re-suspended in serum-free medium, then were seeded at 2×10^4 cells/insert into the upper chamber and incubated for 48 hours in 5% CO₂ at 37°C. At the end of incubation, cells in the upper chambers were swept by cotton swabs and the attached

cells on the lower section of the membrane were fixed in 4% paraformaldehyde for 30 minutes at room temperature. The attached cells were washed twice in 0.01 M PBS (HyClone, Los Angeles, USA) and stained with 0.1% crystal violet (Sigma, USA) for 20 minutes at room temperature. Migrated and invaded cells were counted using a light microscope. The average number of cells in 8 different fields per chamber was calculated with a 100 \times lens objective. All experiments were repeated three times, independently.

Cell apoptosis and cell cycle analyses

U251 and U87 cells were detached by trypsinization, seeded with 2×10^4 cells/well in a 6-well plate, and incubated overnight. Next, U251 and U87 cells were co-cultured with different concentrations of Solanine for 48 hours, respectively. Every group of cells were then divided into two parts. One part was used to stain with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) and Vybrant Apoptosis Assay Kit Number 2 (Molecular Probes). The number of apoptotic cells was auto-calculated by flow cytometry (Becton-Dickinson, New Jersey, USA). At the same time, cells in the other part were harvested in cold PBS. They were fixed in ice-cold 75% ethanol and stored at 4°C overnight for cell cycle analysis subsequent to flow cytometry. All experiments were repeated three times, independently.

PH fluorescent probe assay

Cells were cultured in a 6-well plate with a density of 10^6 cells per square centimeter. Next, the cells were co-cultured with different concentrations of solanine for 48 hours. The cell culture solution was added with the same amount of the 3 μ mol/L AM ester (DingGuo-ChangSheng, Beijing, China), which was prepared by the serum-free medium. Cells were incubated for 60 minutes at 37°C in the cell culture solution, followed by three washes using 0.01 M PBS. Relative fluorescence intensity was assessed by confocal optical density detection and fluorescence intensity calculation was analyzed by Image J.

Western blotting assay of Kir4.1 protein

Protein expression of Kir4.1 in U251 and U87 cells was measured using Western blotting.

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Briefly, frozen samples were homogenized using a polytron homogenizer. The homogenate was kept on ice for 1 hour and centrifuged at 12,000 rpm at 4°C for 1 hour. Protein concentrations of supernatant were measured using bicinchoninic acid assay (BCA, Beyotime, Shanghai, China). Equal amounts of protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% acrylamide gel) and then transferred onto a nitrocellulose membrane. The blotted membranes were incubated overnight with mouse monoclonal Kir4.1 antibodies (1:100, Boster, Wuhan, China). The membranes were washed three times in 1 mol/L TBS (42 ml HCl, 8.5~9 g NaCl, 6.057 g Tris) of pH7.5 at room temperature. The goat anti-mouse IgG (Beyotime, Shanghai, China) with horseradish peroxidase-conjugated mark was added by incubation for 1 hour at room temperature. After washing, blotted proteins were visualized using the Western blotting detection system (ECL plus, Amersham UK) and then exposed to Hyperfilm (ECL plus, Amersham UK). Quantification of band intensity was performed using Gene Genius Bioimaging System (Bio-Rad, California, USA) and Image Lab3.0 software program.

All statistical analysis was performed with the SPSS 13.0 software package (Chicago, IL, USA). All data are presented as means \pm SD. Statistical significance was calculated by one-way ANOVA since all data showed normal distribution. *P* values lower than 0.05 are significant.

Results

Effects of solanine on cell proliferation

CCK8 assay results showed that the viability of U251 and U87 cells in solanine-treated groups was declined, compared with the control group ($P < 0.05$) (**Table 1**). U251 and U87 cell proliferation was significantly inhibited by solanine, in a dose-dependent manner ($P < 0.05$) (**Table 1**).

Effects of solanine on cell migration and invasion

According to the wound healing assay, the closure speed of wound size in solanine-treated U251 and U87 cells was slower than those of control group at 48 hours after scratching ($P <$

0.05). There was a negative correlation between the closure speed and concentrations of solanine ($P < 0.05$) (**Figure 1A-D**).

Transwell experiment assay results, in accord with the wound healing assay, showed that the number of migrated and invaded cells reduced obviously, compared to the control group ($P < 0.05$) (**Figure 2A, 2B, 2E and 2F**). In addition, the reduction became much stronger with increasing drug doses ($P < 0.05$) (**Figure 2C, 2D, 2G and 2H**). Present data demonstrated that solanine could induce the suppression of migration and invasion of U251 and U87 cells in a dose-dependent manner.

Effects of solanine on cell apoptosis and cell cycle

Flow cytometry, analyzing cell apoptosis and cell cycle, was carried out, as described previously. More and more apoptotic cells of U251 and U87 were detected along with an increase of solanine concentrations, compared to the control group ($P < 0.05$). Moreover, there were significant differences among different concentrations of solanine treatment groups ($P < 0.05$) (**Figure 3A-D**). This phenomenon suggests that solanine was able to cause the apoptosis of U251 and U87 cells, playing a positive role in a dose-dependent manner.

To investigate the effects of solanine on the cell cycle of U251 and U87 cells, flow cytometry was performed. As shown in **Figure 3E-H**, the percentage of U251 and U87 cells in the S phase was significantly enhanced after exposure to Solanine for 48 hours, compared with controls ($P < 0.05$). The number of S phase cell was positive to the enhance solanine concentration. Hence, solanine can curb cells by inhibiting the S phase during its growing process in both U251 and U87 cells.

Effects of solanine on intracellular pH

Based on BCECF (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein) under different pH values of the emission spectrum of reference, the excitation wave length of 488 nm can correspond to the different pH values. When the fluorescence intensity becomes stronger, the pH value becomes higher. In this experiment, U251 and U87 cells fluorescence intensity became more weakened with increasing concen-

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Table 1. Effects of different solanine concentrations on U251 and U87 cells proliferation by CCK8 assay, n=3

| Inhibition rate (%)/ Concentration (µg/µL) | Control | 2.5 | 5 | 10 | 15 | 20 | 25 | 30 | 35 |
|---|-----------|-----------|------------|--------------|--------------|--------------|--------------|--------------|--------------|
| U251 | 0.35±0.20 | 0.57±0.43 | 1.21±0.25* | 17.90±0.18** | 29.35±3.20** | 50.39±0.16** | 78.30±0.17** | 88.06±0.17** | 93.44±0.21** |
| U87 | 0.26±0.30 | 0.25±0.68 | 6.48±2.85* | 12.78±5.63* | 38.61±0.95** | 45.61±1.25** | 60.23±6.75** | 72.35±2.55** | 91.53±0.96** |

* $P < 0.01$ vs Control group; ** $P < 0.001$ vs Control groups.

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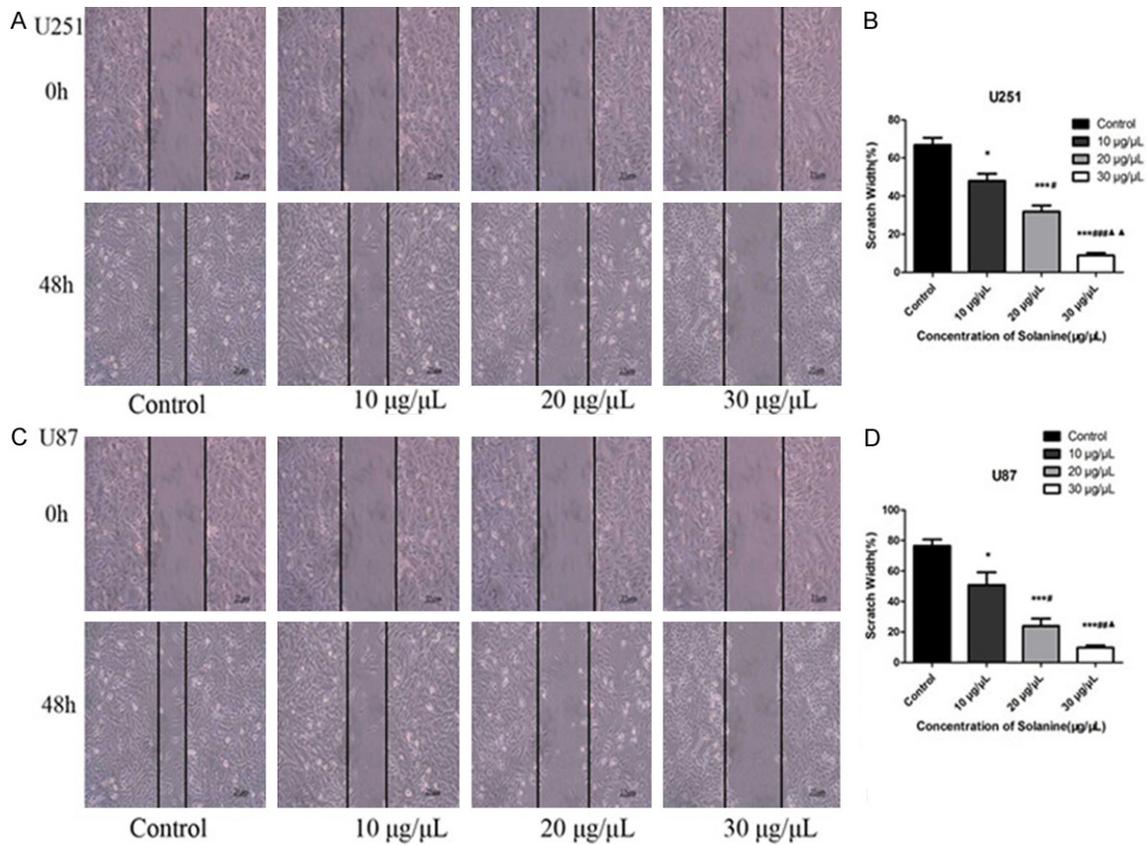


Figure 1. Effects of solanine on the migration of U251 cell and U87 cell by wound healing assay, $\times 40$, $n=3$. A and C: Migration of U251 cell and U87 cells. B and D: Analysis of migration in U251 cell and U87 cells, * $P<0.05$ vs Control group, *** $P<0.001$ vs Control group, # $P<0.05$ vs 10 $\mu\text{g}/\mu\text{L}$ group, ## $P<0.01$ vs 10 $\mu\text{g}/\mu\text{L}$ group, ### $P<0.001$ vs 10 $\mu\text{g}/\mu\text{L}$ group, ▲ $P<0.05$ vs 20 $\mu\text{g}/\mu\text{L}$ group; ▲▲ $P<0.01$ vs 20 $\mu\text{g}/\mu\text{L}$ group.

trations of solanine, compared to the control group ($P<0.05$) (Figure 4A-D). Results suggest that pH values were negatively correlated with concentrations of solanine in U251 and U87 cells.

Effects of solanine on expression of Kir4.1 in glioma cells

The present study assessed changes of Kir4.1 protein mass in U251 and U87 cells by Western blot analysis. Expression of Kir4.1 protein mass of cells in solanine-treated groups was lower than the control group. Expression was decreased in a dose-dependent manner in both U251 and U87 cells (Figure 4E-H), indicating that suppression of solanine to the migration and invasion of U251 and U87 cells may be related to reduced expression of Kir4.1 total mass, resulting in the acidification stage of glioma cells.

Discussion

The effects of solanine are not only anti-inflammatory, anti-bacterial, and anti-viral, but also anti-tumor growth [7]. Many studies have shown that solanine can inhibit the development of cervical cancer, lung cancer, and breast cancer [8, 9]. In recent years, increasing evidence has shown that the mechanisms of solanine inhibition in tumor growth are mainly reflected in these aspects: affecting the structure and function of cell membranes, inducing apoptosis of tumor cell, inhibiting proliferation, affecting blood vessels of tumor, and so forth [8]. Solanine is widely found in nature and available as one of the major constituents of *Solanum nigrum* [10]. In view of this, solanine needs to be exploited as a potential drug for the treatment of tumors. Since malignant tumors have the same characteristics of migration and invasion, the following question has been propo-

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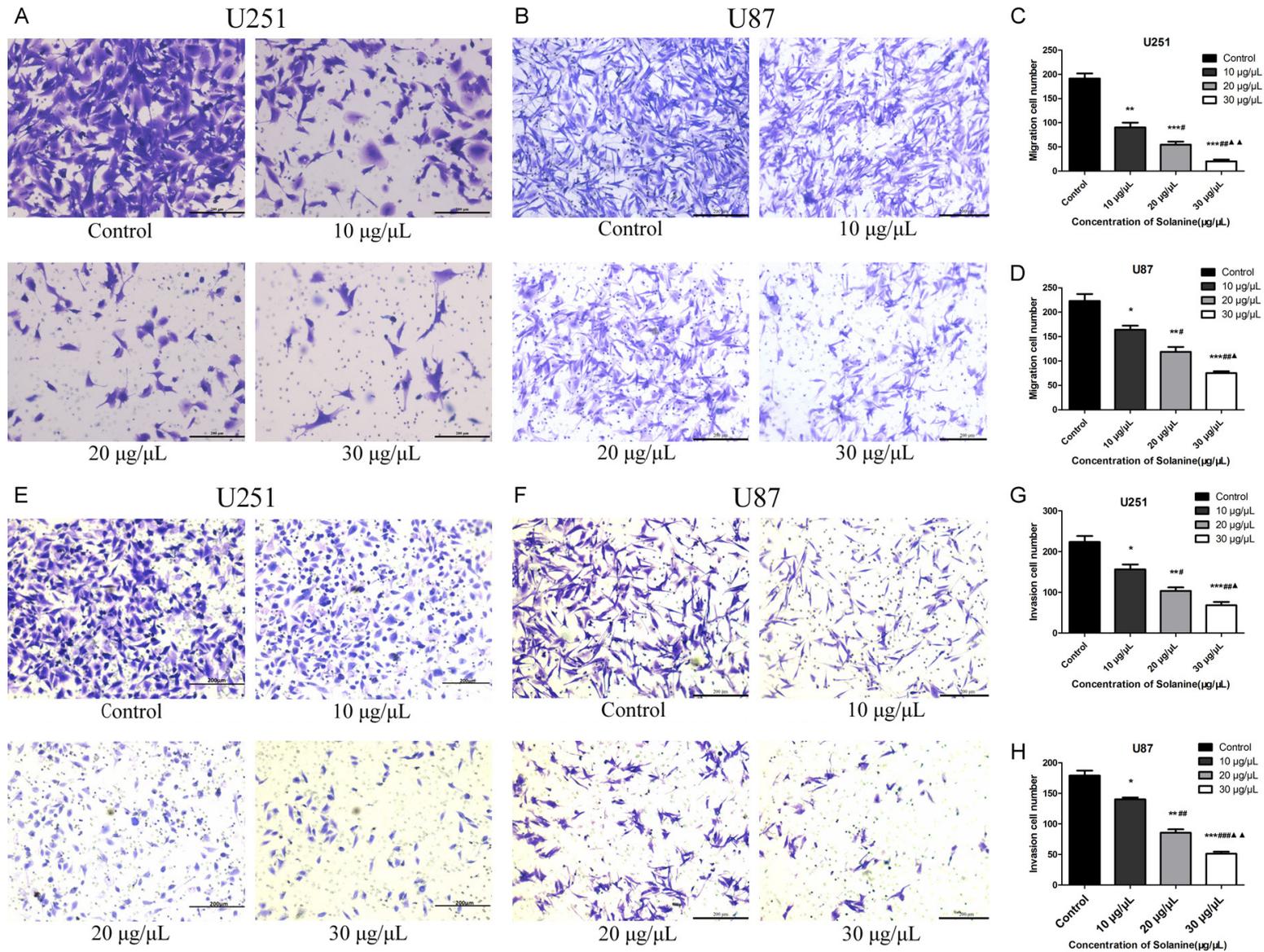
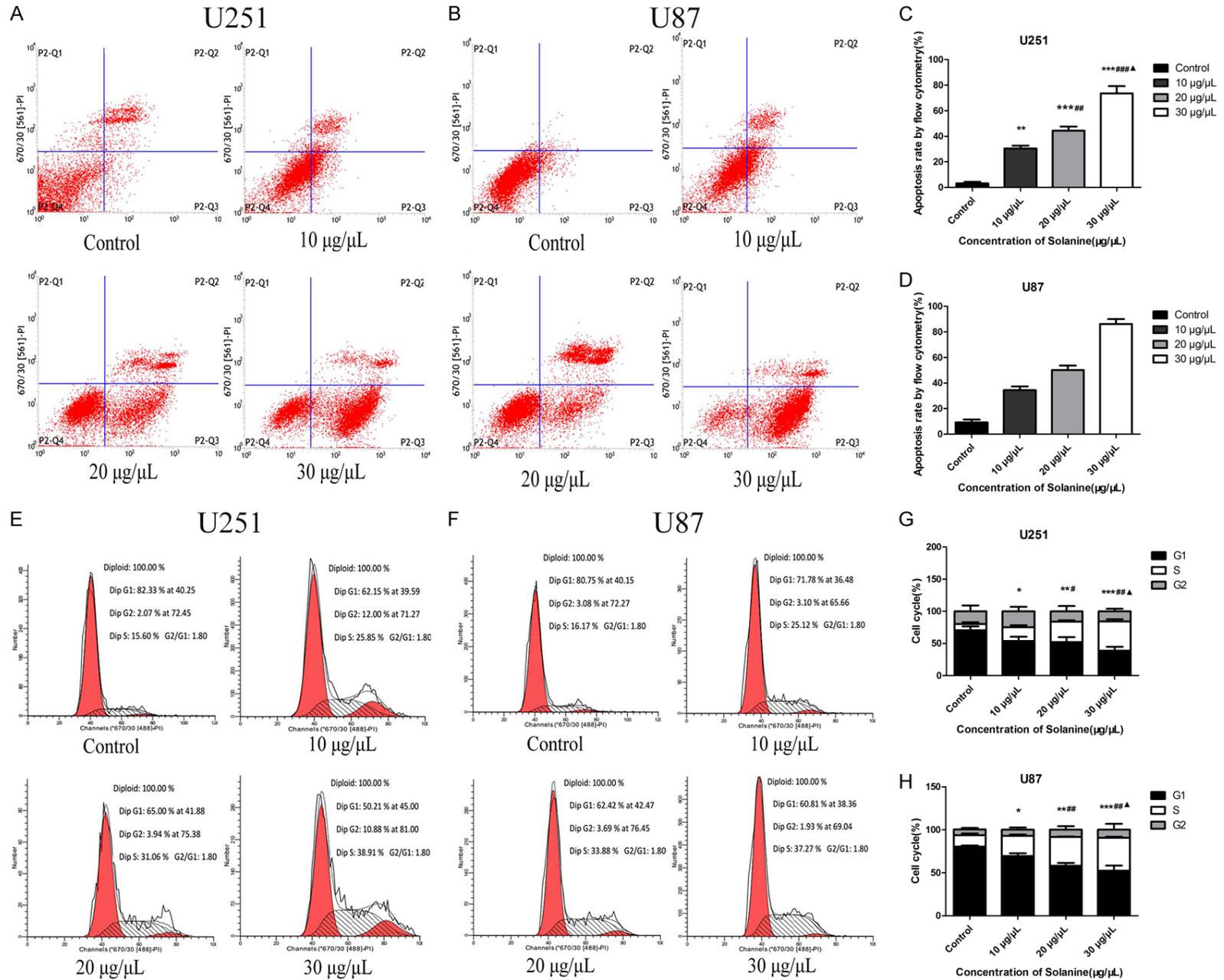


Figure 2. Effects of solanine on the migration and invasion of U251 cell and U87 cells by Transwell assay, × 200, n=3. A and E: Migration and invasion in U251 cell; B and F: Migration and invasion in U87 cell; C, D, G and H: Analysis of migration and invasion, *P<0.05 vs Control group, **P<0.01 vs Control group, ***P<0.001 vs Control group, #P<0.05 vs 10 µg/µL group, ##P<0.01 vs 10 µg/µL group, ###P<0.001 vs 10 µg/µL group, ▲P<0.05 vs 20 µg/µL group, ▲▲P<0.01 vs 20 µg/µL group.

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Figure 3. Effects of solanine on apoptosis and cell cycle in U251 cell and U87 cells. (A and B) Apoptosis in U251 cell (A) and U87 cells (B) by flow cytometry, Mean \pm SD, n = 3; (C and G) Analysis of apoptotic rates (C) and S cycle (G) in U251 cell by flow cytometry, Mean \pm SD, n = 3; (E and F) S cycle of U251 cell (E) and U87 cell (F) by flow cytometry, Mean \pm SD, n = 3; (C and G) Analysis of apoptotic rates (C) and S cycle (G) in U251 cell by flow cytometry, *P<0.05 vs Control group, **P<0.01 vs Control group, ***P<0.001 vs Control group, #P<0.05 vs 10 μ g/ μ L group, ##P<0.01 vs 10 μ g/ μ L group, ###P<0.001 vs 10 μ g/ μ L group, ▲P<0.05 vs 20 μ g/ μ L group; (D and H) Analysis of apoptotic rates (D) and S cycle (H) in U87 cell by flow cytometry, *P<0.05 vs Control group, **P<0.01 vs Control group, ***P<0.001 vs Control group, #P<0.05 vs 10 μ g/ μ L group, ##P<0.01 vs 10 μ g/ μ L group, ###P<0.001 vs 10 μ g/ μ L group, ▲P<0.05 vs 20 μ g/ μ L group.

sed: Can solanine be used for broad-spectrum anti-tumor treatment? Therefore, in this experiment, U251 and U87 cells were taken as the objects of study to observe the effects of solanine on proliferation and growth of glioma cells. Results showed that solanine can inhibit the proliferation, invasion, and migration of gliomas. This change is more pronounced with increasing drug concentrations.

In addition, apoptosis assays showed that solanine promoted the occurrence of apoptosis in U251 and U87 cells. It has been established that apoptosis is a very important mechanism in maintaining the stability of the body environment. The process is accurate, tight, and complex. The main pathways of apoptosis include endogenous mitochondrial pathways, death receptor pathways, and endoplasmic reticulum stress pathways [11, 12]. In addition, this process is regulated by many genes. It has been reported that solanine can inhibit the proliferation of melanoma cell line B16 cells and reduce its invasion and migration. PKC and PI3/Akt signaling pathways may be destroyed to induce tumor cell apoptosis [13, 14]. Because apoptosis does not cause damage to the surrounding tissue easily, the best way to eliminate a tumor may be to promote its apoptosis. Many studies have indicated that solanine can also inhibit the expression of oncogenes, affecting the signal transduction of apoptosis pathways directly or indirectly or affecting the cell cycle of tumor cells and cause apoptosis. This achieves the purpose of eliminating the growth and proliferation of tumor cells [15-18]. However, how solanine induces apoptosis of glioma cells requires evidence. Results of flow cytometry in this study showed that solanine can block glioma cells to stagnate in the S phase of the cell cycle, the synthesis of DNA and synthase required for DNA copying in the S phase. These results further demonstrate that solanine may affect the growth cycle of tumor cells, achieving the effect of promoting apoptosis.

Present results also showed that pH values within the tumor cells become acidic as the concentration increases when solanine acts on U251 and U87 cells, suggesting intracellular acidification. Intracellular acid-base balance and cell growth are closely related [19, 20]. Intracellular alkalinization contributes to the proliferation of cells [21]. It was predicted that solanine affects the proliferation and growth of tumor cells by regulating the pH of tumor cells. Previous experimental results have shown that Kir4.1 is highly expressed in human glioblastoma tissue cells and has a positive correlation with the grade of glioma [23]. Kir4.1 protein and glioma proliferation and growth are closely related. Kir4.1, a member of the Kir family, can deliver extracellular potassium ions into cells with the characteristics of inwardly rectifying potassium ions [24, 25]. It was surmised that increased expression of Kir4.1 may be related to changes in intracellular pH and ultimately maintain the cells alkaline state, contributing to the proliferation of tumor cells. Lina Lin [26] also pointed out that Kir4.1 is sensitive to pH values. The alkalization of intracellular environment and increased pH values can activate amounts of Kir4.1 channels and promote the proliferation of tumor cells [26]. Present results showed that solanine can reduce the expression of Kir4.1 protein in U251 and U87 cells, consistent with the above evidence combined with pH results. However, the causal relationship between Kir4.1 and intracellular pH is not known yet. Furthermore, there are other signaling pathways and related proteins involved in intracellular acidification mechanisms, such as members of the (MCT) family, sodium-potassium ATPase, and sodium-H exchanger. Some analysts believe that the increased intracellular acid metabolites activate the Na⁺/H⁺ exchanger and pump out the redundant H⁺ intracellular. At the same time, Na⁺ enters the cell and activates the Na⁺-K⁺-ATP enzyme, thus the excessive Na⁺ is pumped out and K⁺ enters the

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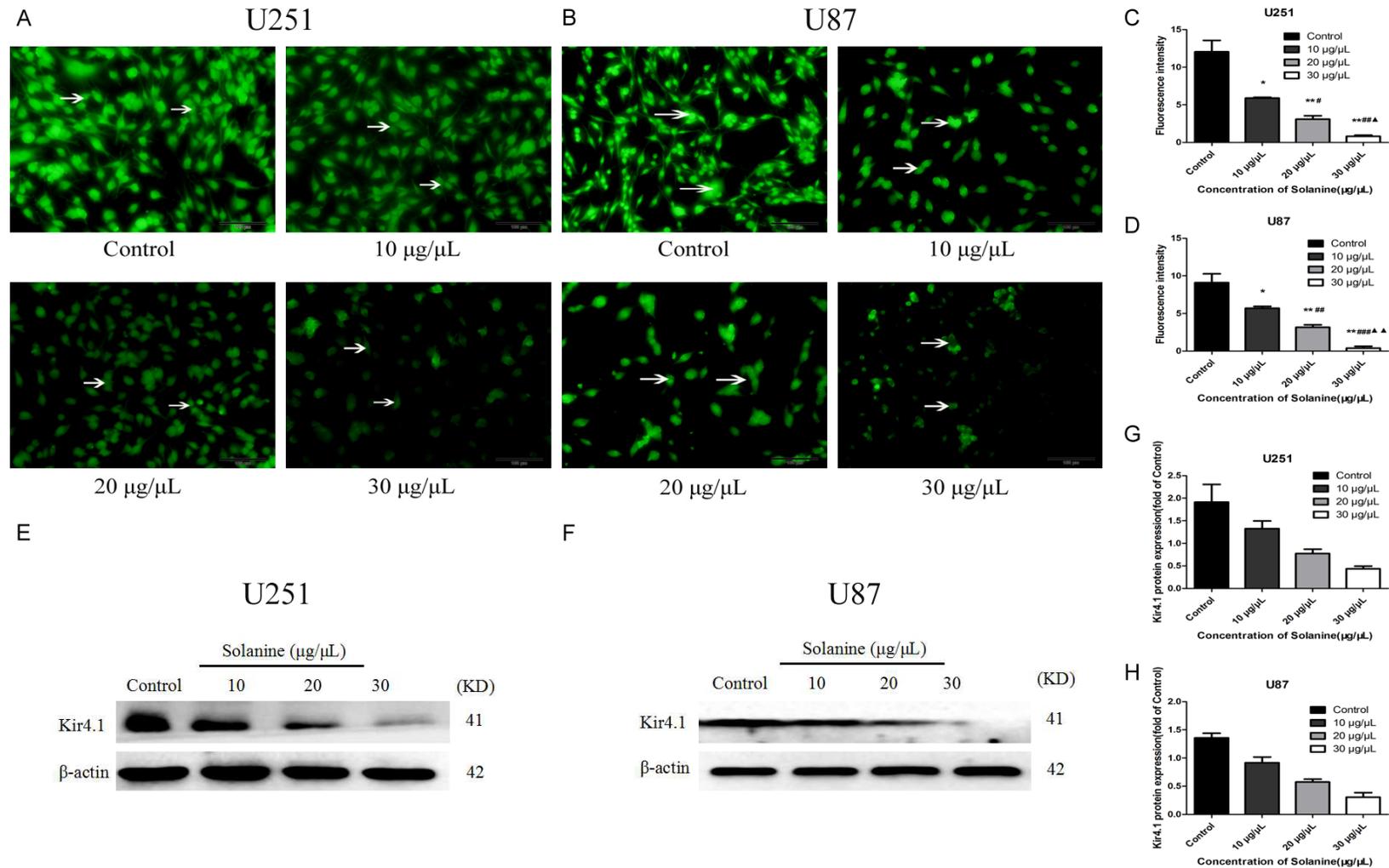


Figure 4. Effects of solanine on pH and protein expression of Kir4.1. (A and B) The arrow indicates fluorescence intensity of cell, $\times 200$, $n = 3$; (A and B) pH of U251 cell (A) and U87 cell (B), the arrow indicates fluorescence intensity of cell, $\times 200$, $n = 3$; (E and F) Expression of Kir4.1 in U251 cell (E) and U87 cell (F), Mean \pm SD, $n = 3$; (C and G) Analysis of fluorescence intensity (C) and expression of Kir4.1 (G) in U251 cell, * $P < 0.05$ vs Control group, ** $P < 0.01$ vs Control group, # $P < 0.05$ vs 10 $\mu\text{g}/\mu\text{L}$ group, ## $P < 0.01$ vs 10 $\mu\text{g}/\mu\text{L}$ group, ▲ $P < 0.05$ vs 20 $\mu\text{g}/\mu\text{L}$ group; (D and H) Analysis of fluorescence intensity (D) and expression of Kir4.1 (H) in U87 cell, * $P < 0.05$ vs Control group, ** $P < 0.01$ vs Control group, # $P < 0.05$ vs 10 $\mu\text{g}/\mu\text{L}$ group, ## $P < 0.01$ vs 10 $\mu\text{g}/\mu\text{L}$ group, ### $P < 0.001$ vs 10 $\mu\text{g}/\mu\text{L}$ group, ▲ $P < 0.05$ vs 20 $\mu\text{g}/\mu\text{L}$ group, ▲▲ $P < 0.01$ vs 20 $\mu\text{g}/\mu\text{L}$ group.

cell at the same time. Excessive K^+ in the cell activates the potassium channel and prompts the outlet of K^+ to achieve this “cascade process” [27, 28]. MCT1 and MCT4, members of the MCT family, have unique roles in intracellular lactate transport mechanisms that may also be involved in the maintenance of intracellular acid-base balance [20-30]. How do these proteins affect the acidification of tumor cells? What kind of relationship is there between them? The mechanisms of solanine blocking the growth of tumor cells also require further study.

In conclusion, the present study provides evidence that solanine inhibits the proliferation of U251 and U87 cells and reduces their invasion and migration capacity. Results showed that solanine can promote their apoptosis. This may, on the one hand, affect the intracellular acid-base balance through the regulation of Kir4.1 protein expression. On the other hand, it may be achieved by inhibiting the growth cycle of tumor cells.

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Disclosure of conflict of interest

None.

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