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Original Article

Puerarin inhibits proliferation and induces apoptosis in human glioblastoma cell lines

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Abstract: Puerarin has been widely used in clinical treatment and experiment research and is considered to exert an anticancer effect recently. The present study investigated the anticancer activity of puerarin in U251 and U87 human glioblastoma cells. The cells were treated with puerarin at various concentrations for different times. Cell viability and cell proliferation were detected by cell counting kit-8 (CCK-8) assay and 5-ethynyl-2'-deoxyuridine (EdU) staining respectively. Cell cycle and apoptosis were measured separately with PI staining and Annexin V-FITC/PI double staining method by flow cytometry. DNA damage of glioblastoma cells caused by puerarin exposure was evaluated by γ-H2AX foci detection, and the expressions of p-AKT, caspase-3 and apoptosis-related proteins were detected by Western blotting after puerarin treatment. Cell viability and proliferation of glioblastoma cells treated with puerarin were significantly lower than that of the control group; the apoptosis rate increased obviously compared to the control group. Puerarin significantly decreased the proportion at G1 phase of cell cycling accompanied by increased populations at the S and G2/M phases in both cell lines. At the same time, DNA damage level of puerarin treated cells was significantly higher than that in the control cells. Moreover, puerarin treatment suppressed the expression of p-Akt and Bcl-2 and promoted the expression of Bax and cleaved caspase-3 in U251 cells. These findings indicate that puerarin exerts antitumor effects both in U251 and U87 cells.

Keywords: Glioblastoma, puerarin, proliferation, apoptosis, cell cycle, DNA damage

Introduction

Glioblastoma is the most common type of primary central nervous system tumor in adults. The highly heterogeneous tumor with high mortality and morbidity always leads to heavy social and economic burden [1]. After receiving the current standard regimen containing surgical resection followed by adjuvant radiotherapy, the median survival of newly diagnosed glioblastoma was only 14.6 moths [2]. Less than 5% of patients with glioblastoma survived more than 5 years after diagnosis [3]. Seeking for more efficient strategy to prevent and treat glioblastoma seems to be imperative.

Kudzu also named as Pueraria lobata (Willd). Ohwi is a perennial leguminous vine of the genus Pueraria native to Southeast Asia. It has been utilized in cooking and clinical practice in traditional Chinese medicine for more than two thousands of years. It has showed a wide variety of medicinal properties and been used as an antipyretic, antidiarrhetic, diaphoretic, and antiemetic agent [4]. Puerarin is the major bioactive component extracted from the root of Kudzu in the late 1950 s. Since then, puerarin has been widely used in clinical treatment and experiment research of cardia-cerebrovascular diseases [5, 6], neurodegenerative disorders [7, 8], osteoporosis [9], inhibiting alcohol intake [10], and diabetes and diabetic complications [11, 12].

Puerarin as well as daidzein and genistein are the major isoflavonoid compounds isolated from Kudzu [13]. An earlier experiment had revealed these isoflavonoids could exert an inhibition on proliferation of HSC-41E6, HSC-45M2, and SH101-P4 stomach cancer cell lines [14]. Daidzein induces apoptosis of human gastric carcinoma cells through downregulation of

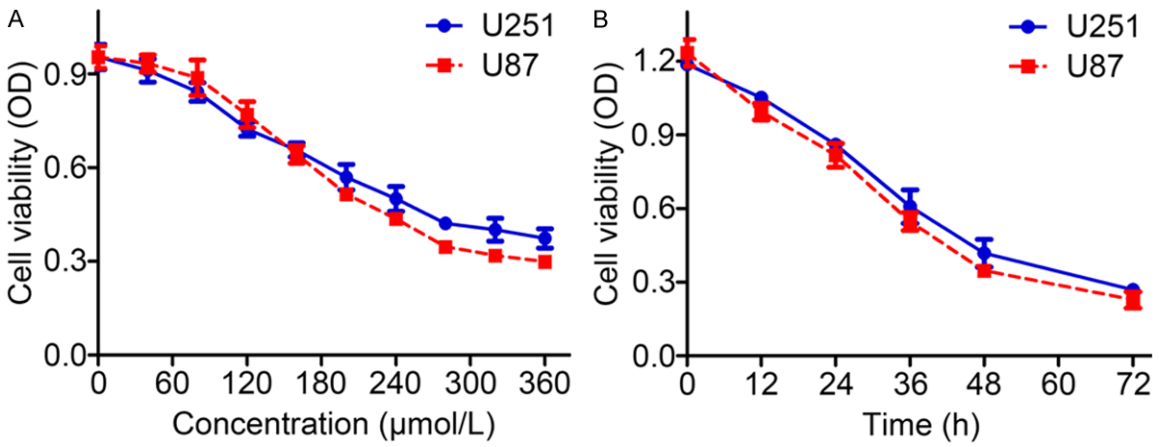

Figure 1. Puerarin suppresses the cell viability of glioblastoma cells. U251 and U87 cells were exposed to culture medium containing various concentrations of puerarin for 48 h (A) and treated with 200 μmol/L puerarin for different time periods (B), and then cell viability was measured using the CCK-8 assay. The data represent means ± SD of three experiments, and each experiment was conducted in triplicate.

the ratio of B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax) and triggering of the mitochondrial pathway [15]. In brain tumor cells, genistein has been reported to exhibit growth arrest and suppression of telomerase activity [16]. Recent studies have found that puerarin also exerts anticancer activity on Eca109 esophageal cancer cells [17]. But there is little literature material about its antitumor property on human malignant glioma cells. In the present study, we investigated the influence of puerarin on the proliferation and apoptosis of human glioblastoma cells.

Materials and methods

Cell lines and regents

The human glioblastoma cell lines U251 and U87 were purchased from State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were maintained in DMEM medium containing 10% fetal bovine serum, 100 U/m penicillin and 100 μg/mL streptomycin and incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide. The medium was replaced every 3 days. Cells were checked routinely and trypsinized until they reached 80-90% confluency. Puerarin was purchased from Enzo Life Sciences (New York, USA) and dissolved in DMSO prior to usage.

CCK-8 test

The cell viability was examined via a cell counting kit-8 (CCK-8 kit) purchased from Dojindo China CO., Ltd (Shanghai, China) according to the manufacturer's instructions. Briefly, approximately 8 × 10^3 cells were seeded in a volume of 100 μl DMEM on each well of a 96-well plate. A range of concentrations of puerarin was added to the medium, and the cells were cultured for different times. Subsequently, 100 μl of fresh medium containing 10 μl of the CCK-8 solution was added to each well and incubated at 37°C for 1 h. The absorbance at 450 nm was measured on a spectrophotometric plate reader. Each group was repeated in three wells.

5-ethynyl-2'-deoxyuridine (EdU) staining

The Cell-Light EdU DNA Cell Proliferation Kit was purchased from RiboBio Co., Ltd (Guangzhou, China) and performed according to the manufacturer's instructions. Briefly, U251 and U87 cells were seeded in 96-well plate and subsequently treated with or without puerarin for 24 h. All cells were treated with 50 μmol/L of EdU for 24 h at 37°C. After being fixed with 4% paraformaldehyde for 15 min, the cells were treated with 0.5% Triton X-100 for 20 min and rinsed with PBS three times. Thereafter, the cells were exposed to 100 μl of 1 × Apollo® reaction cocktail for 30 min and incubated with 5 μg/ml of Hoechst 33342 to stain the cell nuclei for 30 min. The images of EdU and Hoechst fluorescence in the cells were cap-

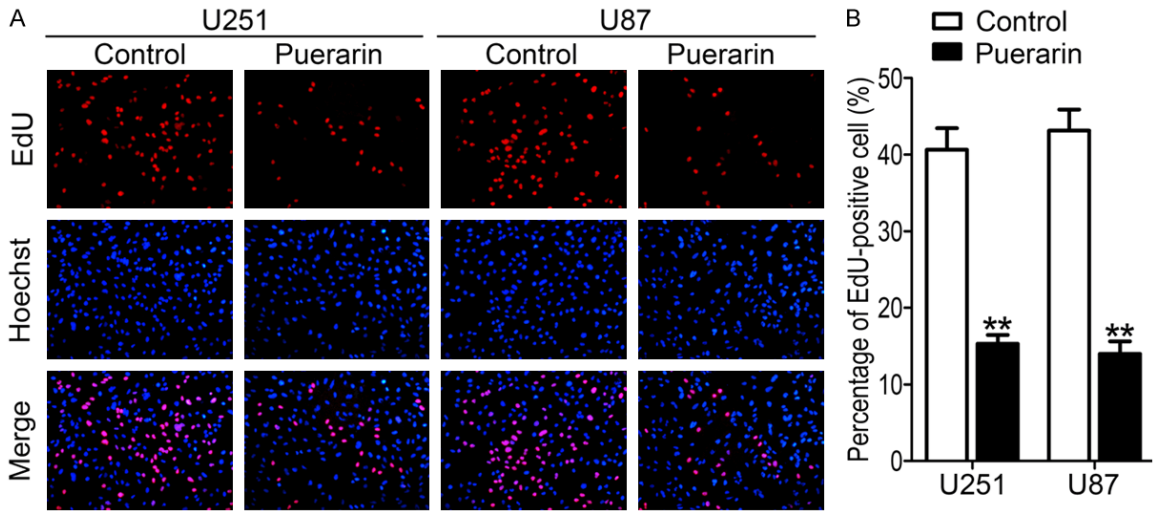

Figure 2. Puerarin inhibits the cell proliferation of glioblastoma cells. A. Proliferating U251 and U87 cells treated with puerarin or without puerarin were labeled with EdU (red). Cell nuclei were stained with Hoechst 33342 (blue). The images are representative of the results obtained by florescence microscopy (× 100). B. The percentage of EdU-positive U251 and U87 cells were quantified. $**P < 0.05$ as compared with negative control.

tured using a fluorescence microscope (Olympus, Tokyo, Japan). Five random fields of each well were observed. The numbers of EdU- and DAPI-positive cells were quantified by IMAGEJ software, and EdU labeling index that was a ratio of the EdU-positive cell number to the DAPI-positive cell number was calculated.

Flow cytometry for cell apoptosis and cell cycle distribution analysis

The influence of puerarin on apoptosis and cell cycle distribution were detected with the Annexin V-FITC-/PI apoptosis kit and cell cycle kit separately according to the manufacturer's instructions from MultiSciences Biotech (Hangzhou, China). U251 and U87 cells were exposed to puerarin for 48 h. At the end of the treatment period, 3×10^5 or more cells were trypsinized, collected by centrifugation at 1000 rpm for 5 min and washed with cold PBS. Then corresponding regents and solution were added and incubated according to manufacturer's instructions respectively. At the end of incubation, cell apoptosis and cell cycle distribution were analyzed on a flow cytometer (Becton Dickinson, USA). The data were analyzed with Flowjo software (version 7.6).

Hochest 33258 staining and γ-H2AX foci detection

U87 cells were trypsinized and seeded in 6-well plates on 22-cm^2 coverslips and incubated for 24 hours, and then treated with or without puerarin for 48 h. The cells grew on the coverslips were fixed in 4% formaldehyde in phosphate buffered saline (PBS) for 30 min at room temperature. To examine the effect of puerarin on cell apoptosis, the cells were stained by Hoechst 33258 (Beyotime Institute of Biotechnology, Jiangsu, China) solution at room temperature for 5 min, and then washed by ice-cold PBS for three times. For γ-H2AX foci detection, the coverslips were incubated with anti-γ-H2AX rabbit monoclonal antibody (Cell Signaling Technology, USA) overnight at 4°C. After washing twice with PBS, cells were incubated with fluorescein isothiocyanate-labeled rabbit anti-mouse antibody for 1 h and washed twice with PBS. Nuclei were counterstained with DAPI (Beyotime Institute of Biotechnology, Jiangsu, China) in PBS for 30 min before cells were covered by anti-fade solution and observed with the Olympus fluorescent microscope system. For quantification of foci, clear and easily distinguished dots of certain brightness were counted as positive foci [18].

Western blot analysis

Cell proteins were extracted with RIPA lysis buffer and determined by the standard BCA method (Beyotime Institute of Biotechnology, Jiangsu, China). Equal amounts of protein (20 ug) were separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA).

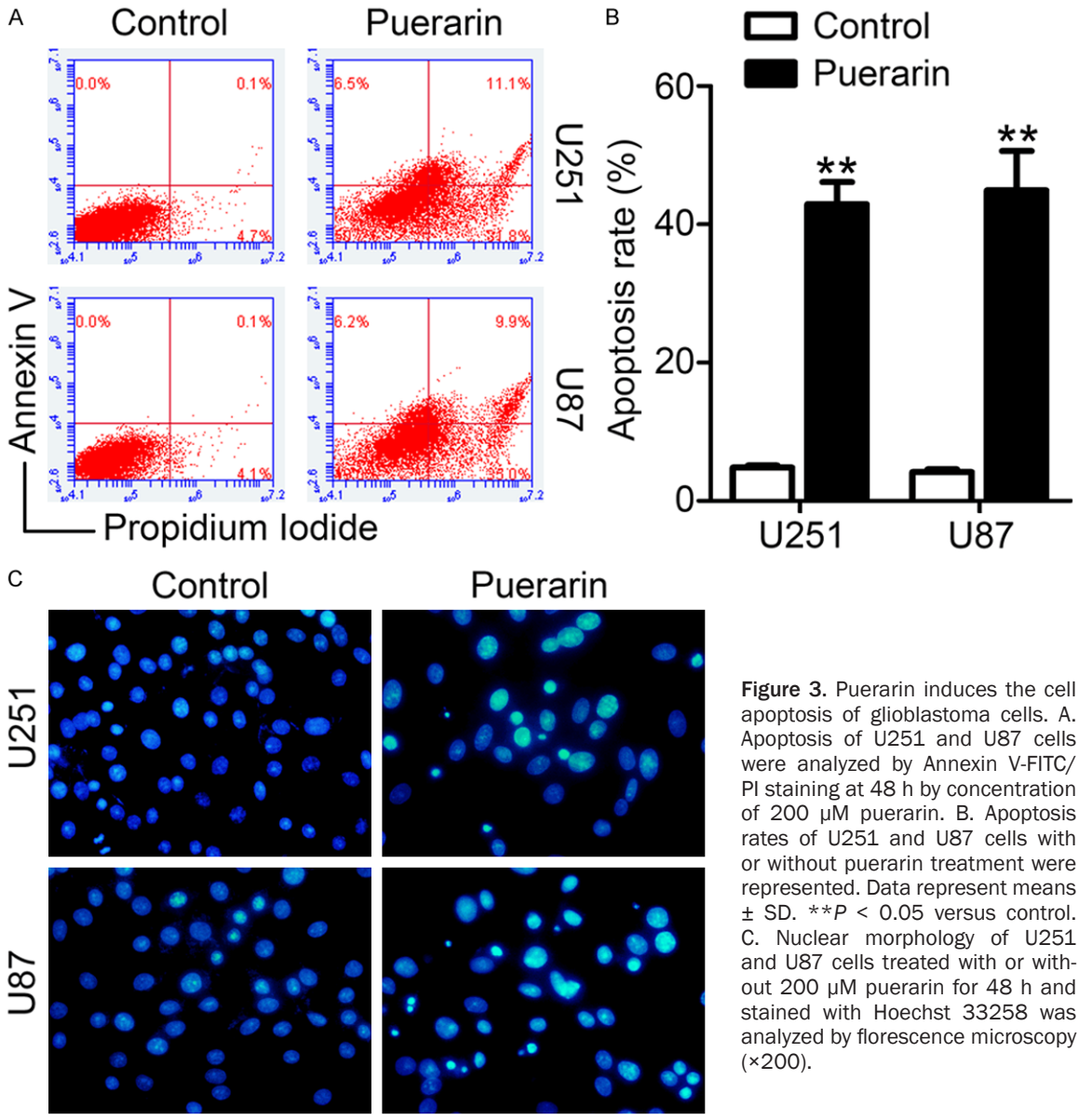

Figure 3. Puerarin induces the cell apoptosis of glioblastoma cells. A. Apoptosis of U251 and U87 cells were analyzed by Annexin V-FITC/PI staining at 48 h by concentration of 200 μM puerarin. B. Apoptosis rates of U251 and U87 cells with or without puerarin treatment were represented. Data represent means ± SD. $^{**}P < 0.05$ versus control. C. Nuclear morphology of U251 and U87 cells treated with or without 200 μM puerarin for 48 h and stained with Hoechst 33258 was analyzed by florescence microscopy (×200).

Membranes were blocked in TBS containing 0.1% Tween-20 and 5% powdered milk, and probed with primary antibody. Primary antibody directed against cleaved caspase-3, caspase-3, bcl-2, p-Akt, Akt (all form Cell Signaling Technology, USA), Bax and GAPDH (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at a dilution of 1:1000 or 1:100. Blots were visualized by LI-COR Odyssey Infrared Imaging System with Alex Fluor 680/790 labeled goat anti-rabbit IgG (LI-COR Biosciences, USA) used as second antibody.

Statistical analysis

All experimental results are expressed as means ± SD. The Student's *t*-test was used to determine the significances between two mean values, and *p* values < 0.05 were considered statistically significant.

Results

Puerarin suppressed the cell viability of glioblastoma cells

In order to investigate whether puerarin treatment affects cell viability, U251 and U87 cells treated with various concentrations (0-400 μM) of puerarin were tested using CCK-8 at several different time points. As shown in **Figure 1**, puerarin significantly reduced cell viability in U251 and U87 cells in a time and dose-depen-

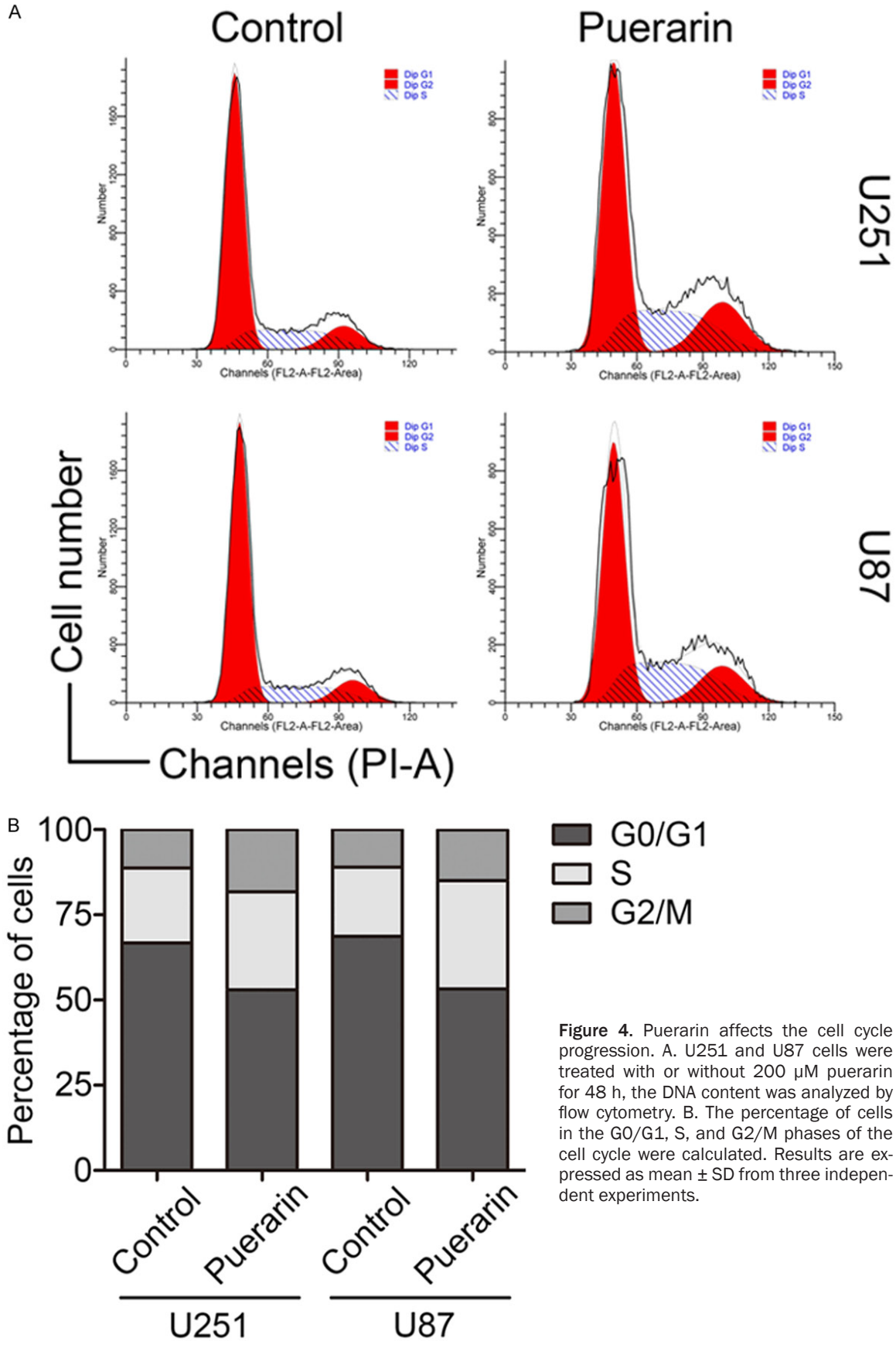

Figure 4. Puerarin affects the cell cycle progression. A. U251 and U87 cells were treated with or without 200 μM puerarin for 48 h, the DNA content was analyzed by flow cytometry. B. The percentage of cells in the G0/G1, S, and G2/M phases of the cell cycle were calculated. Results are expressed as mean ± SD from three independent experiments.

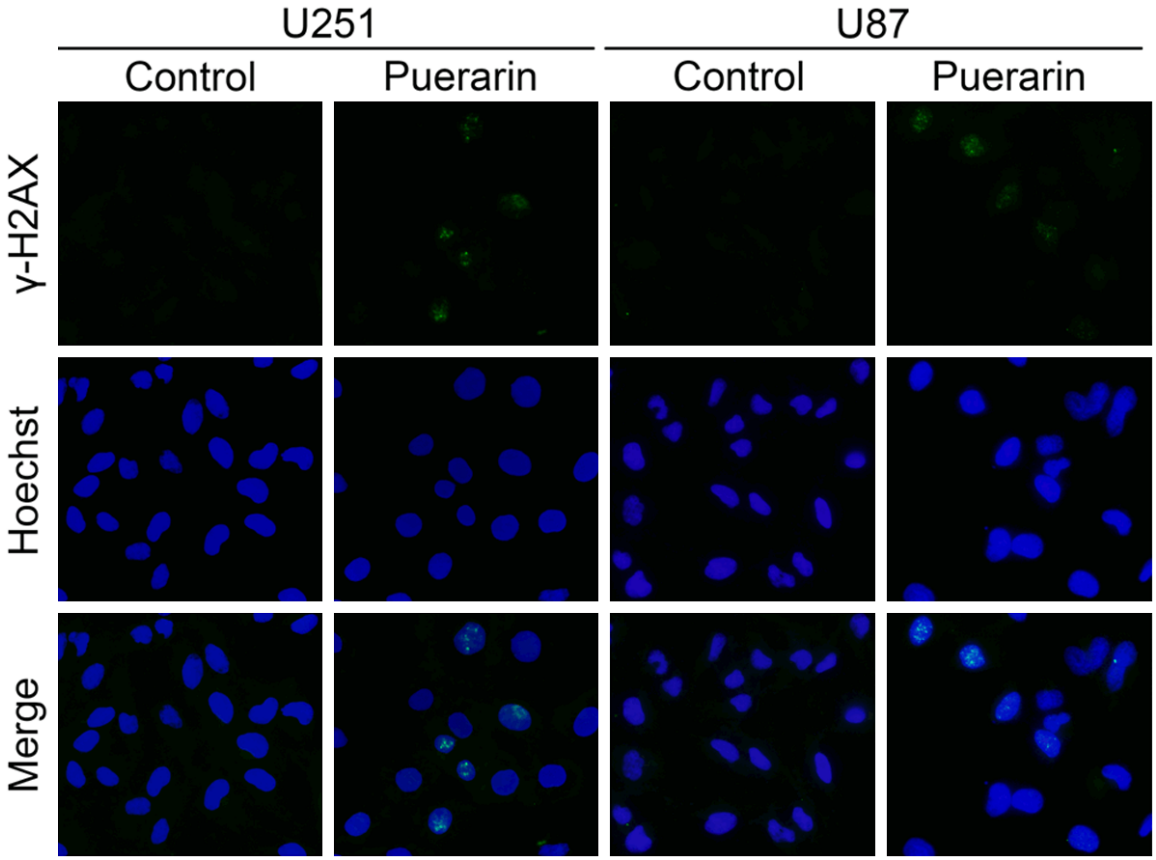

Figure 5. Puerarin induces DNA damage in glioblastoma cells. U251 and U87 cells treated with or without puerarin for 48 h were stained with γ-H2AX anti-body (green). Cell nuclei were stained with Hoechst 33342 (blue) followed by immunofluorescence (× 400).

dent manner. After 48 h incubation, the IC50 values of puerarin against cell viability of U251 and U87 cells were 197.1 μM and 190.7 μM respectively.

Puerarin inhibited the proliferation of glioblastoma cells

Previous studies had shown that puerarin exerted anticancer activity mainly involved inhibiting the proliferation of cancer cells [19-22]. We explored the effect of puerarin on proliferation of glioblastoma cells by EdU assay. A significant inhibition of cell proliferation was observed in both U251 and U87 cells treated with 200 μM of puerarin at 48 h (**Figure 2**). More specifically, the number of cell nucleus with thymidine analog incorporated into newly synthesized DNA significantly decreased after treatment with puerarin. The percentages of stained nucleus in total cells treated with puerarin were lower than the control group ($P < 0.05$).

Puerarin induced the cell apoptosis of glioblastoma cells

The effect of puerarin on cell apoptosis was investigated by flow cytometry. The apoptosis rates at 48 hours after treatment with and without puerarin are shown in **Figure 3A**. Puerarin exposure increased the apoptosis rate of U251 and U87 cells to 42.9% and 44.9% separately with a dose of 200 μM. At the same time, the nucleuses of U251 and U87 cells were stained with Hoechst 33258. Puerarin treatment leads to heterogeneous staining, nucleus condensation, and fragmentation (**Figure 3B**). The results indicated that puerarin induced apoptosis in both glioblastoma cell lines and U87 cells were slightly more sensitive to puerarin than U251 cells.

Puerarin affected the cell cycle progression of glioblastoma cells

In order to examine the possible mechanism of anti-proliferation and pro-apoptosis activity of

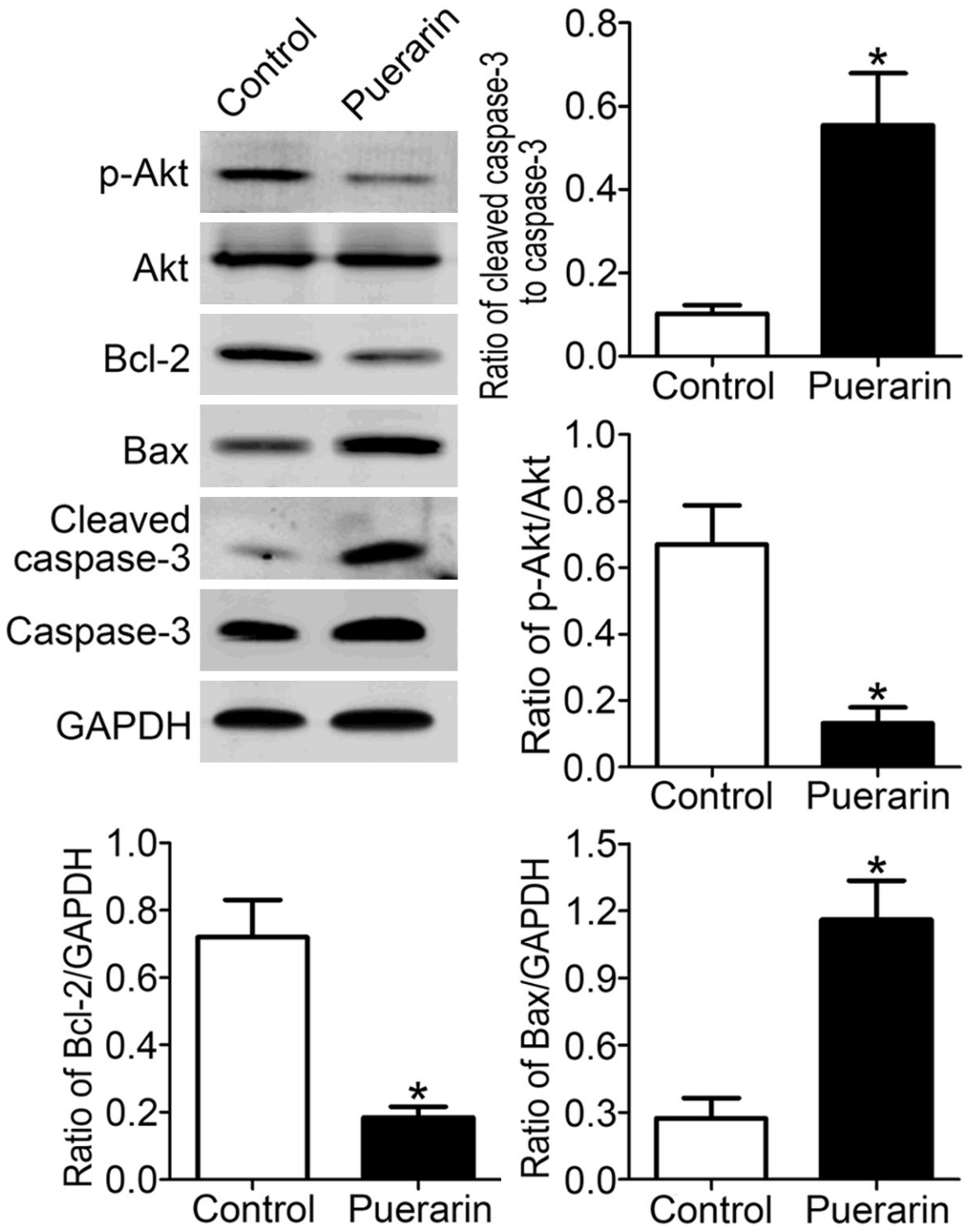

Figure 6. Puerarin regulates p-Akt and apoptosis-related proteins in glioblastoma cells. Expression of Akt, p-Akt, cleaved caspase-3, caspase-3, Bax, and Bcl-2 in U251 cells treated with or without puerarin at 200 μM for 48 h. GAPDH was used as a loading control. *$P < 0.05$ versus control.

puerarin, the cell cycle distribution of both cell lines was evaluated by flow cytometry in presence and absence of puerarin. As shown in **Figure 4**, cultivating U251 and U87 cells with puerarin respectively for 48 h resulted in 13.65% and 14.54% decreases separately in the percentage of cells in the G1 phase compared with the control cells. The decrease in

percentage of cells in the G1 phase was accompanied by a concomitant increase in the percentage of cells in the S and G2/M phases, which suggested that puerarin induces cell cycle arrest at S and G2/M phases in glioblastoma cells.

Puerarin led to DNA damage in glioblastoma cells

Many chemical regents always lead to cell DNA damage, which results in cell apoptosis even necrosis with the failure to repair. The double-strand breaks (DSBs) induced by chemical regents or ionizing radiation rapidly recruits a large amount of phosphorylated histone H2AX named γ-H2AX which could be visualized by anti-γ-H2AX antibody and detected by fluorescence microscope. Therefore, in this set of experiments, we explored the effects of puerarin on DNA structure in U251 and U87 cells. As exhibited in **Figure 5**, the DSBs and sequent γ-H2AX foci spots induced by puerarin were significantly increased compared with control cells at 48 h. The results demonstrated that high concentration of puerarin could lead to the lethality DSBs in glioblastoma cells.

Puerarin affected cell apoptotic and survival signaling in glioblastoma cells

Changes in the expression of proteins related to cell apoptotic and survival signaling were analyzed by Western blotting in order to determine the mechanism of puerarin-induced inhibitory effects. As shown in **Figure 6**, GAPDH was used as loading control. After treatment with puerarin, the expression levels of total caspase 3 and Akt in U251 cells did not show significant changes. However, U251 cells exposure to puerarin exhibited increase of protein levels of cleaved caspase-3, Bax accompanied with reduced protein level of Bcl-2. In addition, the expression level of p-Akt as well as the ratio of p-Akt (p-Akt/Akt) was decreased in response to the treatment of puerarin. Therefore, puerarin exerted its inhibitory effects in glioblastoma cells through affecting cell survival and apoptotic signaling.

Discussion

Since puerarin isolated from the root of Kudzu half of a century ago, the investigators have been increasingly attracted by the pharmacological properties of puerarin [23]. Several reports revealed that puerarin exerted an intriguing role in inducing cell apoptosis and suppressing cell proliferation especially in tumor cells. Apoptosis in colon cancer HT-29 cells was increased after treatment with puerarin at concentrations of 25, 50, 75 and 100 μM [19]. Combined with or without 5-fluorouracil (5-FU), puerarin induced significant proliferation suppression and remarkable apoptosis in Eca-109 esophageal cancer cells *in vitro* and *in vivo* [17]. SMMC-7721 hepatocellular carcinoma cells were sensitive to high concentrations of puerarin with significant proliferative inhibition and apoptotic promotion [22]. However, the effects of puerarin on human malignant glioma have not been investigated yet. In the present study, we treated the human glioblastoma U251 and U87 cell lines with puerarin. The results firstly demonstrated that puerarin exhibited an inhibitory effect on cell viability and proliferation of human malignant glioma cells in a time-and dose-dependent manner.

Previous studies had shown that cell cycle arrest was associated with the inhibition of cancer cell proliferation. Puerarin inhibited cell growth of four breast cancer cell lines through inducing cell cycle arrest in the G2/M phase and cell apoptosis [21]. In mantle cell lymphoma (MCL), puerarin treatment leads to cell proliferation inhibition via inducing cell cycle arrest [24]. DNA double-strand breaks caused by chemical regents or ionizing radiation triggers DNA damage repair. The failure of damage repair usually results in cell apoptosis. The phosphorylation of H2AX on S139 site, γ-H2AX, exerts an essential role in DNA double-strand breaks repairmen. High density nuclear-wide γ-H2AX is related with S-phage apoptosis [18, 25]. In accordance with these previous studies, after administration of puerarin, we observed cell cycle redistribution in both glioblastoma cell lines. At the same time, the increased γ-H2AX foci spots at 48 h indicated that puerarin lead to DNA damage and yielded DSBs in glioblastoma cells. Therefore, the cell cycle arrest and DNA damage induced by puerarin putatively contributed to those inhibitory consequences induced by puerarin.

The Akt signaling pathway exerts a critical role in promotion of cell survival and inhibition of cell apoptosis in cancer cells especially the glioblastoma cells [26, 27]. Akt enhances the cell survival by direct or indirect interaction with

proteins associated with apoptosis, such as anti-apoptotic factor Bcl-2 and pro-apoptotic factors Bax and caspase-3. Activated Akt suppresses the activity of Bax which promotes mitochondrial permeability leading to apoptosis [28]. Caspase-3 is activated by exposure to puerarin, contributing to cell apoptosis in SMMC-7721 hepatocellular carcinoma cells and HT-29 colon cancer cells [19, 22]. In the present study, the results demonstrated that puerarin promoted the expression level of Bax and cleaved caspase 3. After puerarin treatment, the protein expression levels of Bcl-2 and p-Akt were downregulated. These results further verified the anticancer activity of puerarin and classified puerarin into a novel potential treatment to human malignant glioma cells.

Although puerarin has already been widely utilized in experimental research and clinical trials with low toxicity and high efficiency, there are several limitations even notable side-effect of puerarin deserved with caution. The low aqueous solubility and intestinal permeability values lead to lower blood concentration after oral administration of puerarin [29]. Recent studies revealed several side-effects including disturbing pregnancy [30], penetrating the placental barrier [31], inducing immune hemolytic anemia [32] and remarkable inhibiting cytochrome P450 (CYP) enzymes and affecting hepatic drug metabolism [33]. In order to acquire better therapeutic effects of puerarin, investigators are attempting to design nanoparticle or other puerarin encapsulations and delivery systems to improve penetration and bioactivity even easily cross the blood-brain barrier [34, 35].

To sum up, in this *in vitro* experiment, we demonstrated that puerarin inhibited the proliferation and induced apoptosis of human glioblastoma cell lines. The cell cycle arrest and DNA damage caused by puerarin could be involved in mechanism of anticancer effects of puerarin on these human brain malignant tumor cells. However, the specific molecular mechanism and genetic and epigenetic alternations were absent in this research. In addition, the evidences of puerarin negatively regulating the invasiveness and growth of glioblastoma cells in xenograft models in the future will provide more convincing basis to support that puerarin may be a candidate to treat malignant brain tumors.

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Disclosure of conflict of interest

None.

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