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ORIGINAL ARTICLE

Baicalein inhibits invasion and promotes apoptosis in glioma cells through the PI3K/Akt pathway

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Summary

Purpose: *The purpose of this study was to elucidate the role of Baicalein in accelerating invasiveness and inducing apoptosis of glioma cells through the phosphatidilinositol 3-kinase/protein kinase B (PI3K/Akt) pathway.*

Methods: *U251 glioma cells were treated with different doses of Baicalein (10, 20 or 40 μM) for different time periods (12, 24, 36 or 48 h). Changes in viability, clonality, cell cycle distribution and apoptosis in Baicalein-treated U251 cells were assessed. Meanwhile, relative levels of matrix metalloproteinase-2 (MMP-2) and MMP-9 in U251 cells were detected. Western blot was conducted to examine protein levels of p-Akt and Akt in Baicalein-treated U251 cells.*

Results: *Baicalein treatment attenuated dose-dependently and time-dependently the viability and clonality in U251 cells. It induced cell cycle arrest in G0/G1 phase and cell apoptosis of U251 cells. After Baicalein treatment, the relative levels of MMP-2 and MMP-9 were dose-dependently downregulated. Baicalein treatment activated the PI3K/Akt pathway. Notably, inhibitory effects of Baicalein treatment on MMP levels and invasiveness in glioma were blocked by the application of LY294002 (PI3K/Akt inhibitor), and stimulated by the application of IGF-1 (PI3K/Akt activator).*

Conclusions: *Baicalein treatment is able to suppress invasiveness and induce apoptosis of glioma cells through inactivating the PI3K/Akt pathway.*

Key words: *baicalein, glioma, invasiveness, apoptosis*

Introduction

Glioma is a common malignancy of the nervous system, and glioblastoma multiforme (GBM) is the highly prevalent subtype with an extremely high malignant behavior. The 5-year survival of GBM is as low as 5% [1]. So far, great strides have been achieved on surgical procedures, local radiation and traditional chemotherapy for glioma. Nevertheless, the overall survival of glioma is far away from satisfaction [2]. Effective chemotherapeutic agents with low toxicity for glioma treatment are urgently required.

Fruits and non-starch vegetables are beneficial in preventing cancers [3]. Plant polyphenols have been identified to regulate cancer-associated key pathways, inflammatory factors and epigenetic cofactors. These compounds exert potential chemopreventive effects [4-6], which may be applied for reducing susceptibility to glioma. Baicalein (5,6,7-trihydroxyfosone) is the main biologically active substance in Scutellaria altissima L. belonging to flavonoids. Multiple extraordinary properties of Baicalein have been identified, including

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anti-tumor effect, low toxicity, proliferation suppression, apoptosis induction and anti-metastasis ability [7-12]. Previous studies have proved the anti-tumor effects of Baicalein in many types of tumors [13-16]. It is reported that Baicalein is protective during the progression of glioma [17,18]. Nevertheless, mechanisms underlying inhibitory effects of Baicalein on invasiveness and growth of glioma remain unclear.

Tumor metastasis is a complex process involving multiple events, including tumor cell detachment, proteolysis, tumor cell infiltration, and distal colonization [19]. Extracellular matrix (ECM) and basement membrane (BM) are the most important physiological barriers for tumor cell metastasis. Once ECM degrades, tumor cells escape from primary foci, penetrate BM of capillaries and lymphatic vessels, and eventually infiltrate and migrate to distant organs or tissues [20,21]. Matrix metalloproteinases (MMPs) are a class of zinc-dependent endopeptidases that are responsible for tumor invasiveness [22,23]. MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are critical for ECM degradation. Meanwhile, abnormal activation of signaling pathways is a fundamental factor for tumorigenesis and tumor progression. The phosphatidilinositol 3-kinase/protein kinase B (PI3K/Akt) pathway has been identified to regulate the proliferative and invasive abilities of tumor cells [24].

In this study, potential regulatory effects of Baicalein on different phenotypes of U251 cells were determined, as well as the underlying mechanism.

Methods

Cell culture

U251 cells were purchased from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. The medium was replaced every 2-3 days. Baicalein and LY294002 (Sigma-Aldrich, St. Louis, MO, USA) were diluted in dimethyl sulfoxide (DMSO), and preserved in the dark at -20°C and were diluted into the required doses on the day of the experiment. IGF-1 was purchased from ProteinTech TM Company (Rosemont, IL, USA). U251 cells were treated with different doses of Baicalein (10, 20 or 40μM) for different time periods (12, 24 or 48h). Cells without Baicalein treatment served as control group.

Cell counting kit-8 (CCK-8)

Cells were inoculated in 96-well plates with 2×10^3 cells/well. At the appointed time points, absorbance value at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Colony formation assay

A total of 2×10^2 cells/well were inoculated in 6-well plates, induced with different doses of Baicalein for 24 h and cultured for 15 days. Afterwards, cells were fixed with 4% polyformaldehyde for 30 min and dyed with 0.1% crystal violet for 30 min. Colonies were finally captured.

Flow cytometry

Cells were inoculated in 6-well plates with 8×10^4 cells/well. After specific treatment, cells were collected and fixed in pre-cold ethanol overnight. After reacting with PI-RNase in the dark for 4 h, cell apoptosis and cell cycle distribution were determined by flow cytometry. Cell ratios in G0/G1, S and G2/M phases were analyzed by Modfit software. Apoptotic cells were quantified using DNA content (sub-G1 peak). Approximately 1×10^4 cells per sample were recorded.

Western blot

Cellular protein was isolated using radioimmunoprecipitation assay (RIPA) and electrophoresed (Beyotime, Shanghai, China). Protein sample was loaded on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), which were immersed in phosphate buffered saline (PBS) containing 5% skim milk for 2 h, reacted with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Band exposure was achieved by electrochemiluminescence (ECL) and processed by Image Software.

Quantitative real-time polymerase chain reaction (qRT-PCR)

TRIzol method (Invitrogen, Carlsbad, CA, USA) was applied for isolating cellular RNA and its concentration was determined. After reverse transcription of RNA, the extracted complementary deoxyribose nucleic acid (cDNA) was used for PCR detection by SYBR Green method. RT-PCR was conducted at 94°C for 3 min, followed by 35 cycles at 94°C for 45 s, 58°C for 45 s and 72°C for 60 s, and finally 72°C for 10 min. The primer sequences were listed as follows: MMP-2: 5'-CTTCTTCAAGGACCGGTTCAT-3' (F), 5'-GCTGGCTGAGTAGATCCAGTA-3' (R); MMP-9: 5'-TGGGCTACGTGACCTATGACCAT-3' (F), 5'-GCCCAGCCCACCTCCACTCCTC-3' (R); GAPDH: 5'-CGGAGTCAACGGATTTGGTCGTAT-3' (F), 5'-AGCCTTCTCCATGGTGGTGAAGAC-3' (R).

Transwell assay

Cell suspension was prepared at first with 5×10^5 cells/mL. 200 μL of suspension was applied on the top of a Transwell insert placed in 24-well plates. Notably, 0.5 mg/mL Matrigel was pre-coated on the bottom of the insert. 100 μL of serum-free medium was applied in the bottom. After 24 h incubation, cells invading to the bottom were immersed in methanol for 15 min, dyed with crystal violet for 20 min and counted using a microscope. Invasive cell number was counted in 4 randomly selected fields per sample.

Statistics

SPSS 22.0 (IBM, Armonk, NY, USA) was used for statistical analyses. Data were expressed as mean ± standard deviation (SD). Comparison between multiple groups was done using one-way ANOVA test followed by *post hoc* test (least significant difference). P<0.05 indicated significant difference.

Results

Baicalein treatment suppressed the proliferative ability of U251 cells

U251 cells were treated with 0, 10, 20 or 40 μM Baicalein for 48 h, followed by viability determination. As CCK-8 assay revealed, the viability in U251 cells was reduced dose-dependently (Figure 1A). In addition, U251 cells were treated with 20 μM Baicalein for 0, 12, 24, 36 or 48 h. The viability in U251 cells presented a time-dependent decline (Figure 1B). Colony formation assay also illustrated that Baicalein dose-dependently and time-dependently suppressed the clonality in glioma cells (Figures 1C,1D). Therefore, Baicalein treatment was able to suppress glioma cells to proliferate.

Baicalein treatment induced apoptosis in U251 cells

Apoptosis initiation and cell cycle arrest are the key events during tumor progression [25-27]. Here, cell ratio in G0/G1 phase was slightly elevated after Baicalein treatment for 36 and 48 h relative to controls, suggesting that cell cycle progression was arrested (Figures 2A,2B). In the meantime, the apoptotic rate increased dose-dependently after 36 and 48 h treatment of Baicalein in glioma cells (Figures 2C,2D).

Baicalein treatment suppressed invasiveness in U251 cells

Potential influences of Baicalein on invasiveness of glioma were assessed by Transwell assay. Compared with controls, invasiveness in U251 cells was reduced dose-dependently following 10, 20 or 40 μM Baicalein treatment (Figure 3A). It is well known that MMPs are capable of degrading basement membrane components [28]. Previous studies have highlighted crucial functions of MMP-2 and MMP-9 in regulating glioma metastasis [29,30]. In this study, Baicalein treatment downregulated dose-dependently mRNA levels of MMP-2 and MMP-9 (Figure 3B).

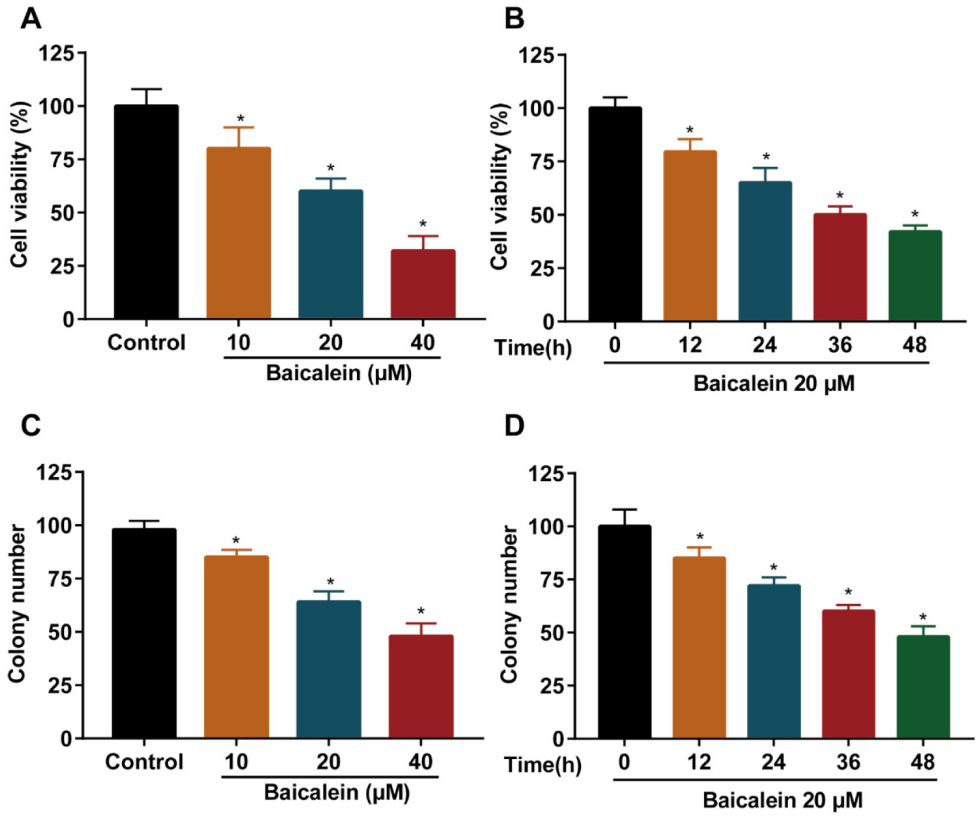

Figure 1. Baicalein treatment suppressed the proliferative ability of U251 cells. **A:** Viability of U251 cells treated with 0, 10, 20 or 40 μM Baicalein for 48 h (*p<0.05 compared with control). **B:** Viability of U251 cells treated with 20 μM Baicalein for 0, 12, 24, 36 or 48 h (*p<0.05 compared with 0 h). **C:** Colony number of U251 cells treated with 0, 10, 20 or 40 μM Baicalein for 48 h (*p<0.05 compared with control). **D:** Colony number of U251 cells treated with 20 μM Baicalein for 0, 12, 24, 36 or 48 h (*p<0.05 compared with 0 h).

Baicalein treatment induced dysfunction of the PI3K/Akt pathway

In breast cancer, the PI3K/Akt pathway contributes to changes of MMPs levels and metastatic ability [31]. Western blot analysis revealed that Baicalein treatment downregulated p-Akt in glioma cells, and decreased p-Akt/Akt ratio (Figures 4A,4B). Furthermore, the PI3K/Akt pathway inhibitor (LY294002) or activator (IGF-1) was applied to analyze the involvement of this pathway in glioma cells. Relative levels of MMP-2 and MMP-9 in Baicalein-treated U251 cells were partially inhibited after applying 20 μM LY294002, which

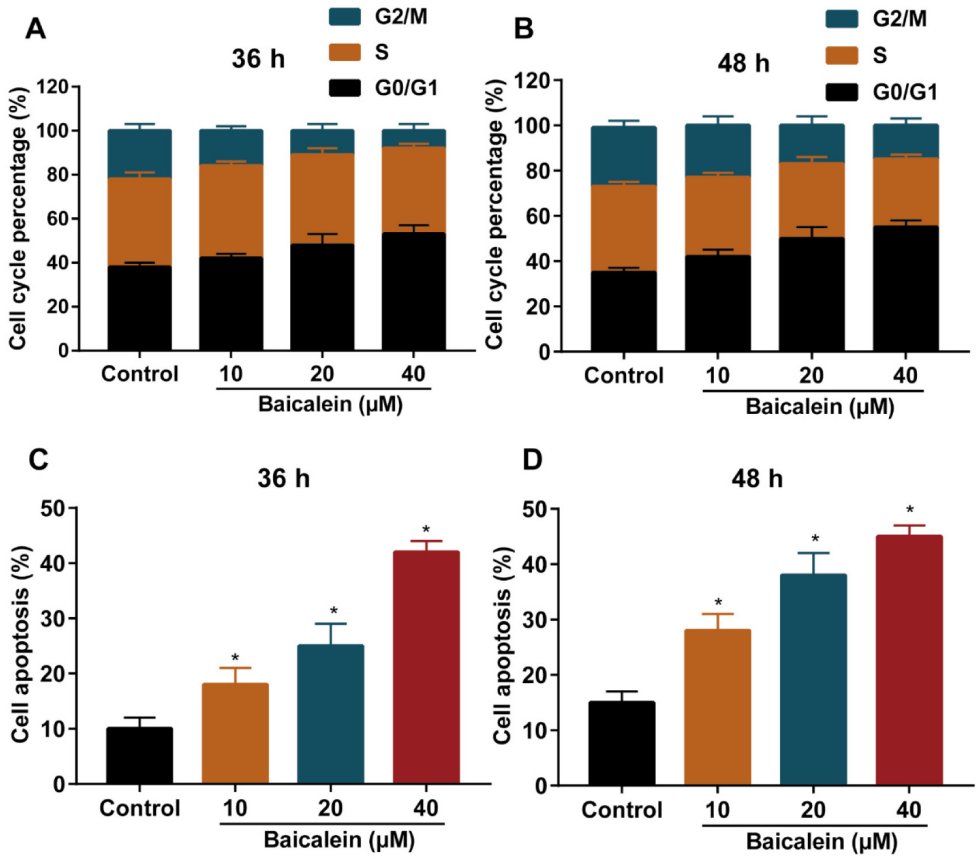

Figure 2. Baicalein treatment induced the apoptosis of U251 cells. **A,B:** Cell cycle distribution in U251 cells treated with 0, 10, 20 or 40 μM Baicalein for 36 h **(A)** and 48 h **(B)**. **C,D:** Apoptosis of U251 cells treated with 0, 10, 20 or 40 μM Baicalein for 36 h **(C)** and 48 h **(D)** (*p<0.05 compared with control).

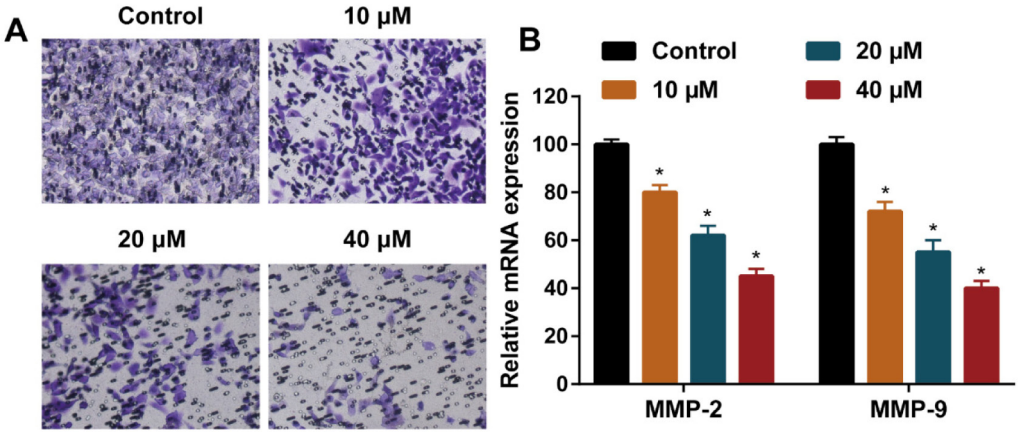

Figure 3. Baicalein treatment suppressed invasiveness of U251 cells. **A:** Invasiveness of U251 cells treated with 0, 10, 20 or 40 μM Baicalein for 48 h (magnification: 40×). **B:** Relative levels of MMP-2 and MMP-9 in U251 cells treated with 0, 10, 20 or 40 μM Baicalein for 48 h (*p<0.05 compared with control).

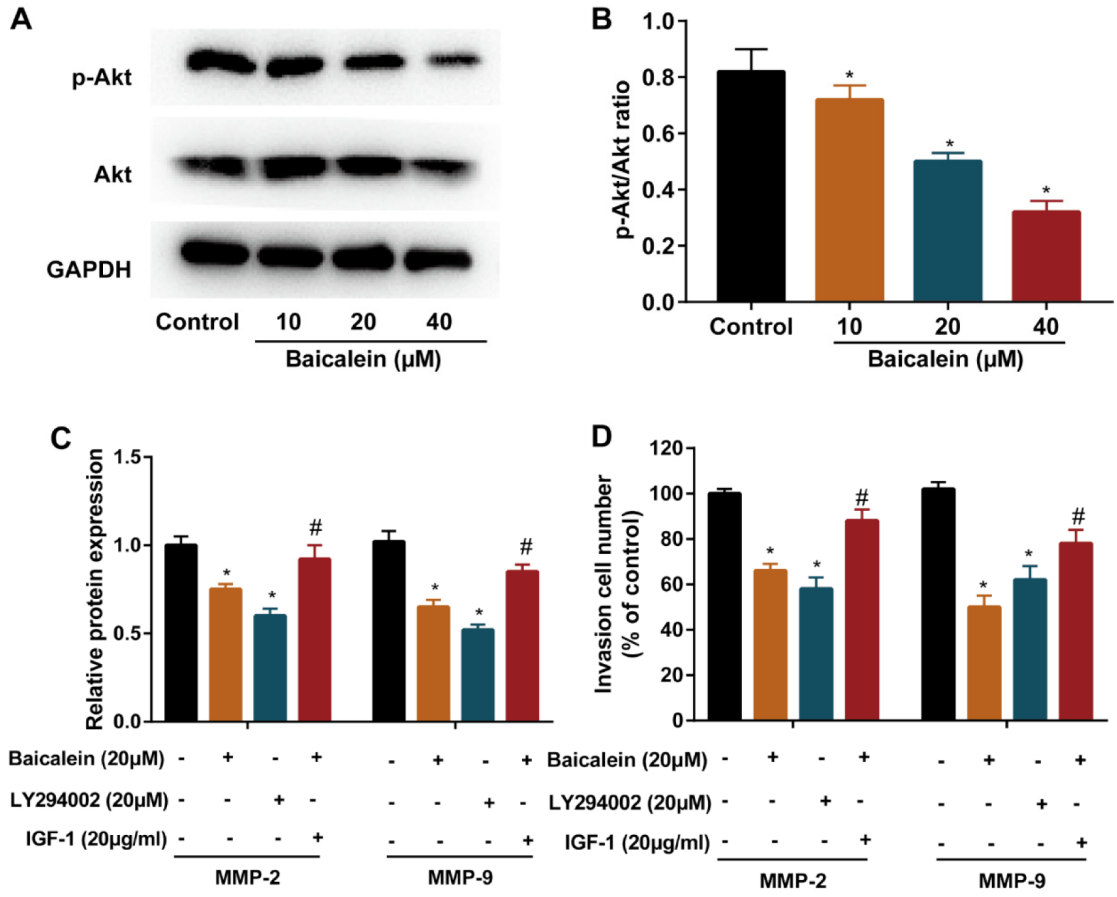

Figure 4. Baicalein treatment induced dysfunction of the PI3K/Akt pathway. **A:** Protein levels of Akt and p-Akt in U251 cells treated with 0, 10, 20 or 40 μM Baicalein for 48 h. **B:** p-Akt/Akt ratio in U251 cells treated with 0, 10, 20 or 40 μM Baicalein for 48 h (*p<0.05 compared with control). **C:** Relative levels of MMP-2 and MMP-9 in U251 cells treated with Baicalein, LY294002 or IGF-1. **D:** Invasiveness of U251 cells treated with Baicalein, LY294002 or IGF-1 (*p<0.05 compared with control; #p<0.05 compared with the group with 20μM Baicalein).

were elevated by administration of 20 μg/mL IGF-1 (Figure 4C). Baicalein-induced invasiveness inhibition became much more pronounced by LY294002 administration, and it was partially reversed by IGF-1 application (Figure 4D).

Discussion

High rates of incidence, recurrence and mortality and low cure rate are the major characteristics of glioma. Glioma is a common primary brain tumor in adults [32]. It is estimated that the median survival of glioma is only about one year even after active treatment [33]. Strong invasiveness of glioma to surrounding normal brain tissues is the leading cause of its high recurrence rate [34]. Clinical efficacy of postoperative radiation on killing glioma cells is limited. Nowadays, traditional Chinese medicines have been well concerned because of their low toxicity and high safety. Baicalein, the Scutellaria altissima L. extract, has been extensively applied owing to its multiple biological functions [35,36]. Its administration in malignant tumors looks promising. Our findings uncovered that Baicalein treatment reduced dose-dependently and time-dependently the viability and clonality in U251 cells. It also induced apoptosis and arrested cell cycle progression of glioma cells.

Tumor invasion is complicated involving the interaction among tumor cells, stromal cells and ECM [9]. Invasion of glioma into normal brain parenchyma requires the participation of MMP secretion [37]. MMPs are key regulators in influencing the invasive and migratory capacities of tumor cells, especially MMP-2 and MMP-9 [38,39]. Here, Baicalein treatment in U251 cells downregulated mRNA levels of MMP-2 and MMP-9, which may be the explanations for glioma progression regulated by Baicalein.

As a critical pathway, tumorigenesis-associated biological activities are affected by the PI3K/Akt pathway [40]. Through regulating MMPs, tumor angiogenesis and metastasis are regulated by the activated PI3K/Akt pathway [41,42]. In this

analysis, we speculated that the PI3K/Akt pathway was involved in the anti-metastatic ability of Baicalein in glioma. Our results demonstrated that Baicalein downregulated dose-dependently p-Akt level, suggesting the activated PI3K/Akt pathway. It is reported that expression levels of MMP-2 and MMP-9 are downregulated owing to the activated PI3K/Akt pathway [43]. Here, treatment of PI3K/Akt pathway inhibitor, LY294002 remarkably downregulated MMP-2 and MMP-9, and attenuated invasiveness in Baicalein-treated U251 cells. Conversely, IGF-1 treatment, the PI3K/Akt pathway activator, partially reversed the regulatory effects of Baicalein on U251 cells. As a result, Baicalein treatment regulated glioma progression *via* the PI3K/Akt pathway.

Conclusions

Baicalein treatment is able to suppress invasiveness and induce apoptosis of glioma cells by inactivating the PI3K/Akt pathway.

Conflict of interests

The authors declare no conflict of interests.

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