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Original Article

Icariside II induces cell cycle arrest and apoptosis in human glioblastoma cells through suppressing Akt activation and potentiating FOXO3a activity

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Abstract: Glioblastoma multiforme (GBM) is the most common primary malignant brain tumor, and currently chemotherapeutic options for GBM are very limited. Given the poor prognosis, the development of novel anti-GBM agents is quite urgent. Using two human glioma cells (U87 and A172 cells), we demonstrated that Icariside II (ICA II), an active flavonoid compound derived from *Epimedium koreanum*, could inhibit GBM cell growth in a dose dependent manner. Wound healing data suggested that ICA II also inhibited the migration of human glioma cells. Mechanistically, ICA II induced apoptosis and cell cycle arrest, and this cytotoxic effect was dependent on the reduction of Forkhead box O3a(FOXO3a) phosphorylation mediated by Akt and the enrichment of nuclear FOXO3a, which initiated the transcription of p21/p27. Importantly, the cytotoxic effect induced by ICA II could be reversed by silencing the expression of FOXO3a, suggesting the critical role of FOXO3a in this process. Taken together, we propose ICA II as a potential novel anti-GBM candidate with a mechanism of inhibiting cell proliferation and inducing apoptosis through suppressing Akt activation and potentiating FOXO3a activity.

Keywords: Glioma, Icariside II, FOXO3a, p21, apoptosis

Introduction

Glioblastoma (GBM), is the most common and aggressive cancer that begins within the brain. The annual incidence of GBM is about 3.19 cases per 100,000 population, and average age at diagnosis is 64 years old. Without therapy, patients with GBM might die within 3-5 months. On the other hand, even with optimal therapy, most treated GBM patients only survive approximately 1 year, and only 5% live for more than 5 years [1].

Standard treatment for GBM is surgery followed by radiotherapy and chemotherapy, generally with temozolomide (TMZ). Although TMZ has been demonstrated as an effective chemotherapeutic reagent to prolong the overall survival of GBM patients, some patients do not response to it, which is mainly caused by hypermethylation of DNA repair enzyme, O(6)-methylguanine-DNA methyltransferase (MGMT) [2]. Bevacizumab, an anti-VEGFA monoclonal antibody, only benefits partial GBM patients with the proneural subtype [3-5]. Other potential investigating drugs, such as bosutinib [6], rindopepimut [7, 8], and some immunotherapy [9, 10], are still in phase I/II clinical trials. The dilemma and challenge for GBM treatment might be due to the obstruction of blood brain barrier (BBB), the instability of genomics and epigenetics, neurotoxicity, and the intratumoral heterogeneity. Thus, the prognosis of GBM still remains miserably poor, and a new therapy is urgently required to address this significant unmet medical need.

Icariside II (ICA II), is a bioactive flavonoid derived from *Epimedium koreanum*, which has been used as an anti-glioma herb in traditional

Chinese medicine. Interestingly, previous reports have suggested the anti-cancer effect of ICA II by inactivating several key signaling pathways involved in tumor progression, such as JAK2-STAT3 pathway in multiple myeloma [11] and PI3K-Akt pathway in osteosarcoma [12]. Although ICA II has been suggested to possess anticancer activities against various human cancer cell lines *in vitro* and mouse tumor models *in vivo* [13], whether it has a similar effect in GBM cells remains to be determined. Intriguingly, ICA II has been shown to have the neuroprotective activity [14, 15], suggesting that it could be a potential candidate against brain tumors.

In this study, the anti-tumor effect of ICA II in GBM cells was investigated here. Based on our data, we proposed that ICA II could serve as a potential novel anti-GBM candidate, and the mechanism underlying the apoptosis and cell cycle arrest induced by ICA II was involved with Akt-FOXO3a pathway.

Methods

Cell culture

Human glioma cells line U87MG and A172 were purchased from Chinese Academy of Sciences (Shanghai, China) and ICA II from eBioChem (Shanghai, China). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml). All cells were grown in a humidified atmosphere containing 5% CO_2 at 37°C.

Small interfering RNA interference

FOXO3a siRNA (sense, 5'UAGAAUUGGUGCGUGAACGGAAGUC-3') and the negative control siRNA (sense, 5'UUCUCCGAACGUGUCACGUAAAUGC-3') were obtained from Shanghai Generay Biotech Company. U87 and A172 cells in 6 cm dish were transfected with 250 pmol siFOXO3a or control siRNA using LipofectamineRNAiMax (Invitrogen). Forty-eight hours posttransfection, cells were treated with ICA II to determine the effect of Akt-FOXO3a signaling on cell cycle arrest.

Cell proliferation assay

Cell proliferation assay was determined by Cell Counting Kit-8 assay (Dojindo, Japan) according to the manufacture's protocol [16].

Apoptosis analysis

After washing cells three times with ice-cold phosphate-buffered saline (PBS), they were resuspended in binding buffer (BD Biosciences, Franklin Lakes, USA), stained with APC conjugated Annexin V (BD Biosciences, Franklin Lakes, USA) and propidium iodide (PI; Sigma-Aldrich), and analyzed by flow cytometry (BD Biosciences, Franklin Lakes, USA).

Cell cycle assay

Cells were seeded 16 h before analysis at 20% of confluence, collected and stained with 200 $\mu g \cdot ml^{-1}$ of propidium iodide, 0.1% sodium azide, 0.1% Triton-X100 and 10 $\mu g \cdot ml^{-1}$ RNAses for 2-4 h. Single cell events were collected for G1, S and G2 phases quantification by fluorescence-activated cell sorting array (BD Bioscience) and data were analyzed using FlowJo software.

Migration assay

Cell migration was measured by counting the number of cells that migrated through transwell inserts with 3 μm pores, as described previously [17]. Briefly, after 24 h incubation, cells that migrated through the filter into the lower wells were quantitated by gentian violet assay, and presented as the total cell numbers in the lower wells.

Wound healing assay

Cells were cultured to 90% confluency in six-well plates, then a thin scratch (wound) was introduced in the central area using a 200 μl pipette tip. Detached and damaged cells were carefully removed by PBS and the medium was replaced with serum-free medium. Wound closure was observed by light microscopy and images were captured at the indicated time points.

Real-time PCR

Total RNA from U87 and A172 cells was extracted using Trizol reagent (Invitrogen, Carlsbad, CA), and 4 μg RNA was reverse-transcribed using the Superscript First Strand synthesis system (Invitrogen, Carlsbad, CA). The primer sequences were listed below: p21 (forward primer 5'-AGTCAGTTCCTTGTGGAGCC-3'; reverse primer 5'-CATTAGCGCATCACAGTCGC-3'), p27 (forward primer 5'-GGCAAGTACGAGTGGCAAGA-3'; reverse primer 5'-CGTGTCCTCAGAG-

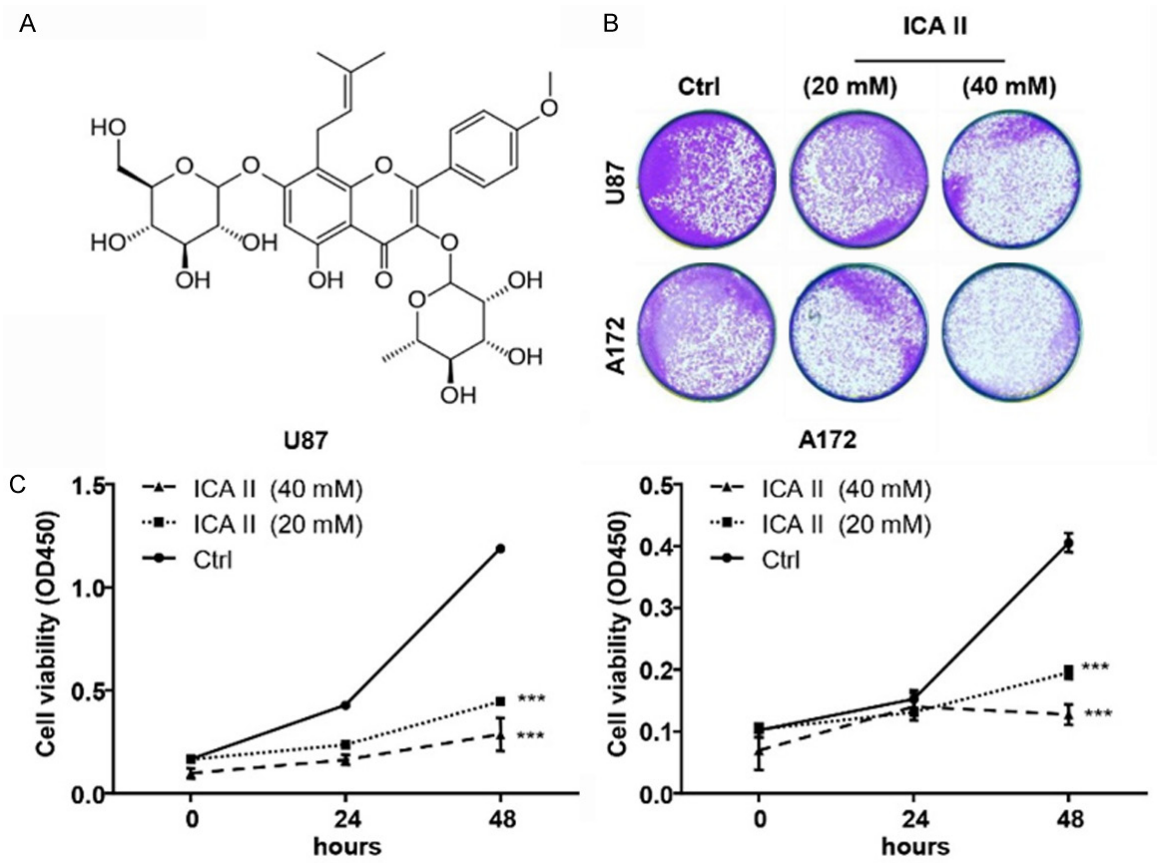


Figure 1. *In vitro* effect of ICA II on cell viability in glioma cells. A: Chemical structure of ICA II. B: Colony formation assay was performed after U87 and A172 glioblastoma cells were treated with ICA II (20 and 40 μM) or control for 24 h. C: Cell viability of U87 and A172 cells was analyzed using CCK-8 assay after indicated treatment. **P*<0.05, ***P*<0.01, ****P*<0.001 vs. control group.

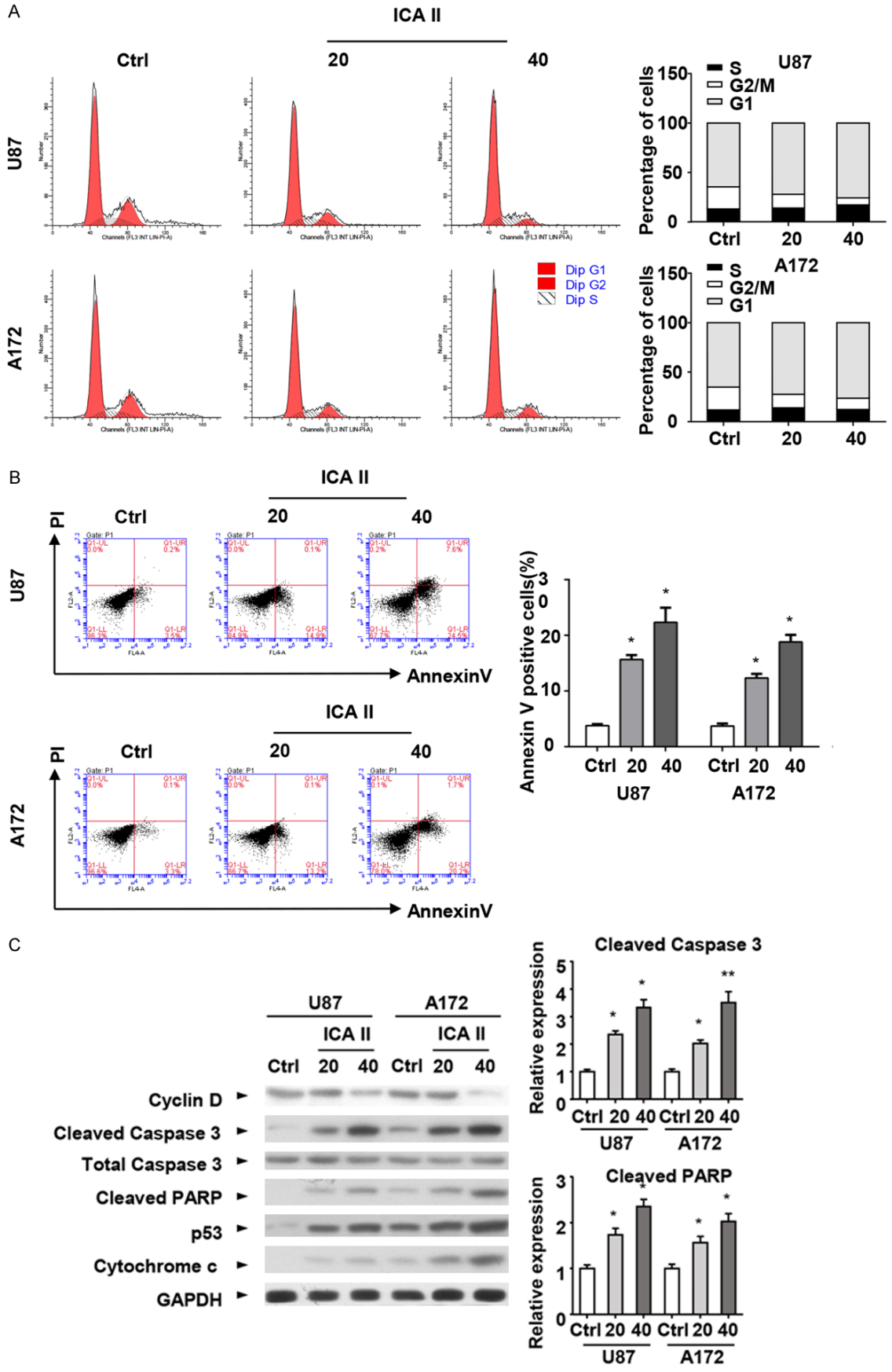
TTAGCCG-3') and GAPDH (forward primer 5'-TGACTTCAACAGCGACACCCA-3'; reverse primer 5'-CACCCTGTTGCTGTAGCCAAA-3'). Primers and probes for amplification and detection were from the Universal Probes Library (Roche, UK) with an initial heating at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 10 s, and 72°C for 1 s. Histone 3 and GAPDH were served as internal controls.

Western blot

Whole cell lysates were obtained using an ice-cold Mammalian Protein Extraction Reagent (M-PER, Pierce). Nuclear and cytoplasmic fractionations were separated by the Nuclear and Cytoplasmic Extraction Kit (Pierce). Proteins (20 μg) were resolved by 10% SDS-PAGE, and electro-transferred onto a polyvinylidenedifluoride membrane. Western blot was performed according to standard methods. Anti-cleaved-PARP, anti-p53, anti-cytochrome c, anti-Caspase3, anti-Akt, anti-phosphorylated-Akt (Ser-473), anti-FOXO3a, anti-phosphorylated FOXO3a (Ser318/321), anti-Histone 3 antibodies were from Cell Signaling Technology (USA), while anti-p21, anti-p27, and anti-GAPDH antibodies were from Abcam (USA). The membranes were developed using an enhanced chemiluminescence detection system (Millipore) and an Image Quant LAS-4000 Chemiluminescence and Fluorescence Imaging System (FujiFilm).

Luciferase assay

The p21 and p27-luciferase constructs were generated by inserting their promoter sequences into the pGL4-BASIC-luciferase plasmid (Promega, Tokyo, Japan). A commercial plasmid containing a CMV-driven Renilla reporter system was used as an internal control (Promega). Cells were plated in 6-well plates at 50-70% confluence and were co-transfected with the


A
ICA II
Ctrl
20
40
U87
A172
Dip G1
Dip G2
Dip S
S
G2/M
G1
Percentage of cells
B
PI
AnnexinV
Annexin V positive cells(%)
C
Cyclin D
Cleaved Caspase 3
Total Caspase 3
Cleaved PARP
p53
Cytochrome c
GAPDH
Relative expression

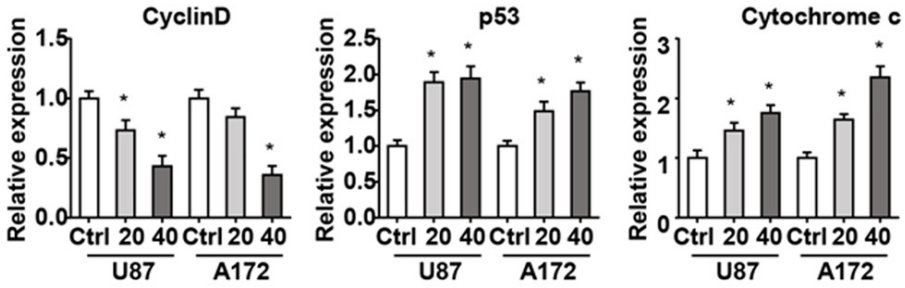


Figure 2. ICA II induces cell cycle arrest and apoptosis in U87 and A172 glioblastoma cells. A: U87 and A172 cells were treated with ICA II for 24 h prior to cell cycle analysis by flow cytometry (left panel). The percentage of cells in each phase of the cell cycle (sub G1, G0/G1, G2/-M and S) is shown in the right panel. B: U87 and A172 cells were treated with ICA II for 24 h prior to apoptosis rate analysis by flow cytometry (left panel). The percentage of early apoptosis cells is shown in the right panel. C: U87 and A172 cells were treated with ICA II (20 and 40 μM) for 24 h, and western blot was performed to examine the effect of ICA II on the protein levels of cyclin D, Caspase3, cleaved-PARP, p53, and cytochrome c.

pCMV-p21/p27 construct or with an equimolar amount of the pCMV empty vector and the pGL4-p21/p27 construct utilizing PureFection-TM reagents (System Biosciences). Twenty-four hours post transfection, the luciferase assay was performed in triplicate using the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega).

Statistical analyses

All the experiments were repeated at least three times, and the data were presented as means ± S.D., unless otherwise indicated. Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by Student's t-test, and *P* values less than 0.05 were considered significant.

Result

ICA II inhibits glioma cell proliferation in a dose-dependent manner

ICA II is a bioactive flavonoid derived from *Epimedium koreanum*, and its structure is shown in **Figure 1A**. To test its effect on GBM cells, we chose two human glioma cells (U87 and A172 cells) as models, and treated them with ICA-II (20 or 40 μM) or vehicle control for 24 hours. As shown in **Figure 1B**, we found that ICA II treatment reduced tumor cell clone formation in a dose dependent manner in both cell types. Similarly, cell viability analysis also suggested a dose dependent ICA II effect on both cells (**Figure 1C**, $P<0.05$), with IC_{50} (half maximal inhibitory concentration) of 30 μM in U87 cells and 34.5 μM in A172 cells, which is 4-fold lower than those of temozolomide [18]. Taken together, these data indicated that ICA II could suppress GBM proliferation *in vitro*.

ICA II could induce cell cycle arrest and apoptosis in human glioma cells

To explore the effect of ICA II on human glioma cells, we performed cell cycle and apoptosis assays. Cell cycle analysis demonstrated that ICA II could induce cell cycle arrested at G1 phase, which leads to fewer cells in proliferated G2/M phase in both GBM cells (**Figure 2A**). Apoptosis data indicated that ICA II treatment resulted in a significant increase of early apoptotic cells (Annexin V^+/PI^-) in a dose-dependent manner (**Figure 2B**, $P<0.01$). To further dissect the effect of ICA II treatment, we carried out western blot assay, and showed that a key player for G1/S transition cyclin D was significantly reduced after ICA II treatment. While apoptosis related proteins, including cytochrome c, p53, Poly (ADP-ribose) polymerase (PARP), and cleaved caspase 3, were increased (**Figure 2C**, $P<0.05$). Hence, our data suggested that ICA II could induce apoptosis and cell cycle arrest at G1 phase in GBM cells.

ICA II could inhibit GBM cell migration

To further investigate other potential effect of ICA II effect on GBM cells, wound healing assay and transwell assay were conducted to examine cell migration. We examined three treatment groups: DMSO, 20 μM ICA II, and 40 μM ICA II (dissolved in DMSO). Compared with the control group, we found that ICA II effectively reduced the migration of U87 and A172 cells

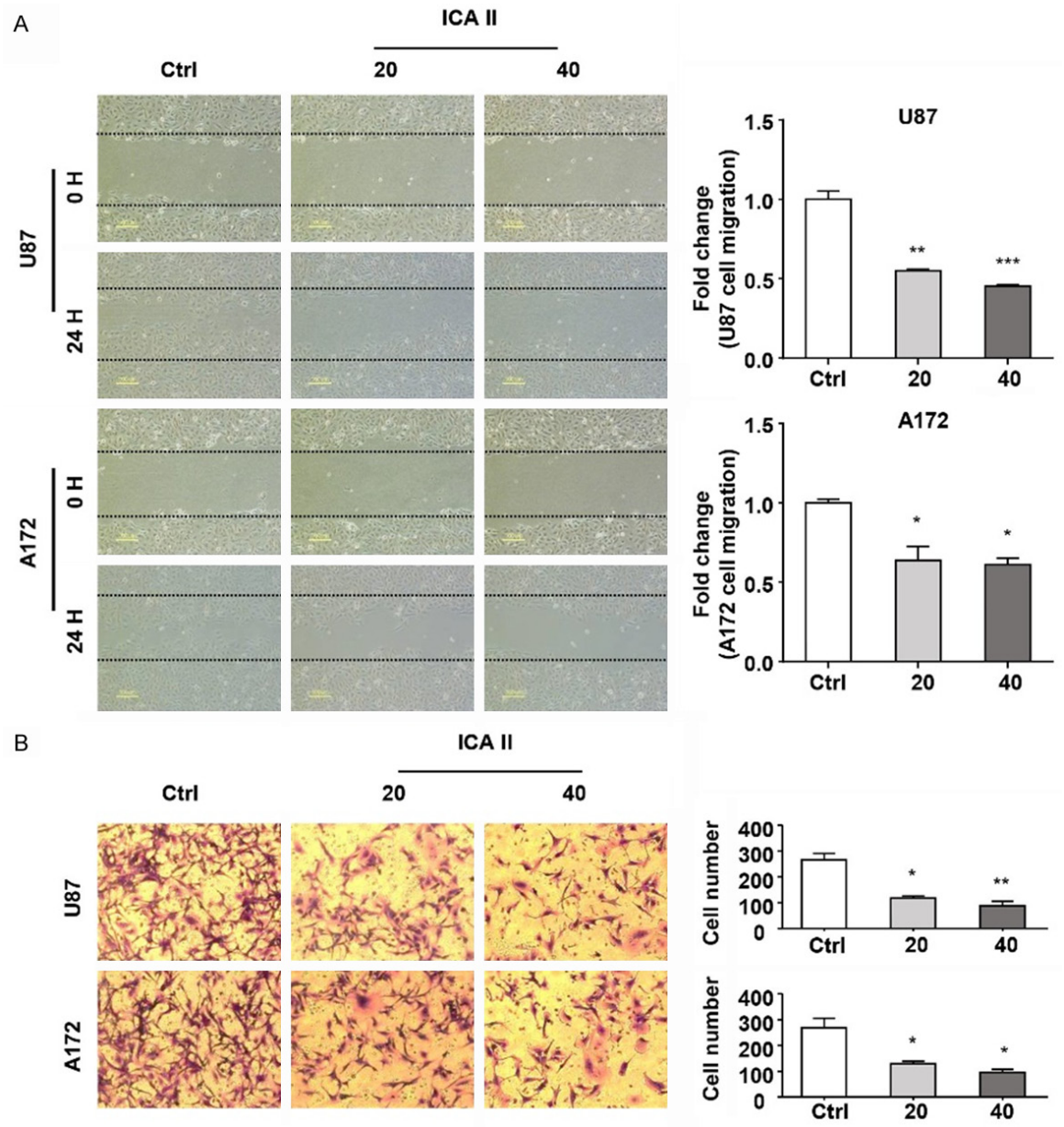


Figure 3. ICA II inhibits the cell motility in U87 and A172 glioblastoma cell lines. A: U87 and A172 cells were first treated with ICA II for 24 h, and cells were scraped with pipette tips for the wound healing assay. After cells were further incubated for another 24 h, wound closure was detected by light microscopy. B: Transwell assays were performed to determine the invasiveness of the cells after 24 h of treatment.

after 24 hours treatment (**Figure 3A**, **3B**, $P<0.05$). These findings indicated that ICA II also could suppress GBM cell migration.

ICA II inhibits glioma cell proliferation via Akt/FOXO3a signaling pathway

Akt activates the transcription of several critical regulators involved in glioma proliferation and survival [19], including FOXO3a, a downstream phosphorylation target of the PI3K/Akt pathway and a member in a family of transcription factors that are characterized by a distinct forkhead DNA-binding domain [8]. Therefore, we also examined whether Akt/FOXO3a signaling plays a role in effect of ICA II on GBM cells. Compared with a control group, p-Ser473-Akt and p-Ser318/321-FOXO3a levels were significantly reduced in glioma cells after ICA II treatment (**Figure 4**, $P<0.05$). To assess how ICA II regulates FOXO3a distribution in glioma cells,

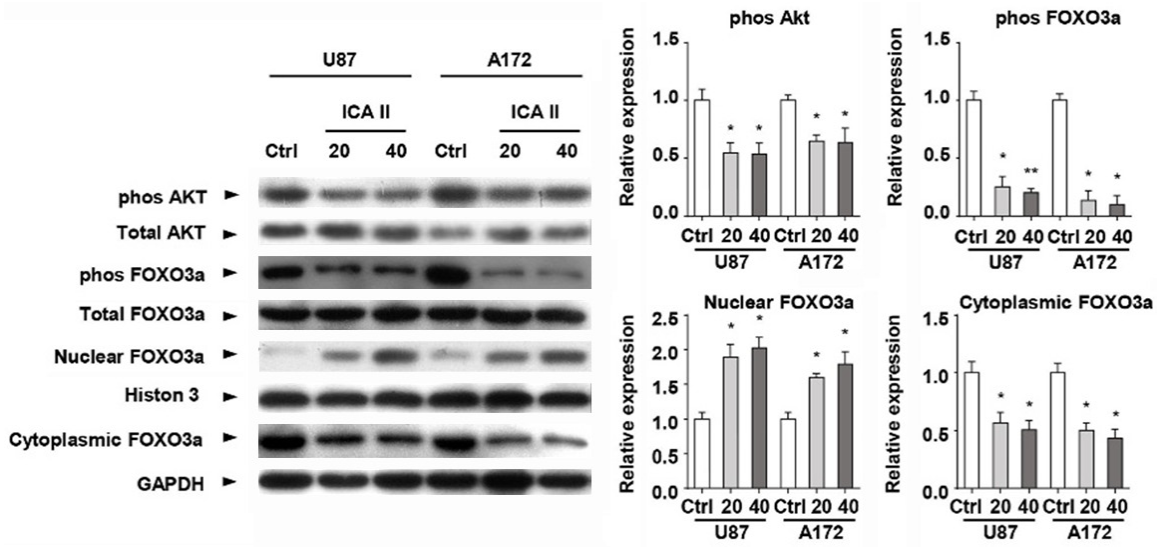


Figure 4. Akt/FOXO3a signaling pathway is involved in the effect of ICA II on cell proliferation. After U87 and A172 cells were treated with ICA II (20 and 40 μM) for 48 h, western blot assay was performed to measure the protein levels of Akt, p-Ser473-Akt, FOXO3a, p-Ser318/321-FOXO3a in cytoplasmic and nuclear fractions (left). The corresponding quantified data were shown in the right panel.

the levels of nuclear and cytoplasmic fractions of FOXO3a were determined by western blot, and no change of total FOXO3a level was observed. However, ICA II treatment promoted the translocation of FOXO3a from the cytoplasm to the nucleus, which led to accumulation of FOXO3a in the nucleus (**Figure 4**, $P<0.05$). These findings indicated the ICA II could inhibit cell proliferation by blocking Akt-dependent FOXO3a phosphorylation and promoting FOXO3a transportation into the nucleus.

ICA II could regulate the cyclin-dependent kinase inhibitors p21 and p27 dependent on Akt-FOXO3a pathway

We further investigated downstream effectors of FOXO3a which participate in the anti-GBM effect of ICA II. Previous studies have suggested that p21 and p27, cyclin-dependent kinase inhibitors and downstream effectors of FOXO3a, are important anti-tumor targets [20-22]. Interestingly, the mRNA and protein levels of p21 and p27 were upregulated in ICA II-treated cells (**Figure 5A**). To further test this hypothesis, we performed the reporter assay to check whether ICA II influences p21 and p27 activity in glioma cells. Indeed, ICA II potentiated the transcriptional activity of p21 and p27 in GBM cells (**Figure 5E**). To examine the role of FOXO3a in this process, FOXO3a siRNA or scramble control siRNA were transfected into U87 and A172 cells, and significant reduction of FOXO3a protein level were observed in GBM cells (Figure S1). Notably, silencing FOXO3a expression abolished the increase of p21 and p27 expression levels and transcriptional activities induced by ICA II (**Figure 5A**, **5B** and **5E**, $P<0.05$). Consistently, ICA II mediated cell growth suppression and cell cycle arrest were reversed after FOXO3a siRNA transfection (**Figure 5C**, **5D**). In summary, the upregulation of p21 and p27 are essential for ICA II-mediated growth inhibition in GBM cells, and FOXO3a plays a critical role in this event. The working model of the mechanism was shown in **Figure 6**.

Discussion

Malignant glioma is among the deadliest types of brain cancer, and currently there is no satisfactory treatment. Many scholars pay much attention to explore new medicines. However, most drugs would induce neurotoxicity, which will unfortunately limit their clinical application. Growing evidence indicates that flavone compounds have anti-cancer activities, which has been applied in traditional Chinese medicine for thousands of years. Several clinical studies suggested that dietary intake of plant flavonoids could reduce the risk of various cancers [23-25]. ICA II, an active flavonoid compound derived from *Epimedium koreanum*, is a metabolite and pharmacological active form of Icariin [26, 27]. With the effective concentration rang-

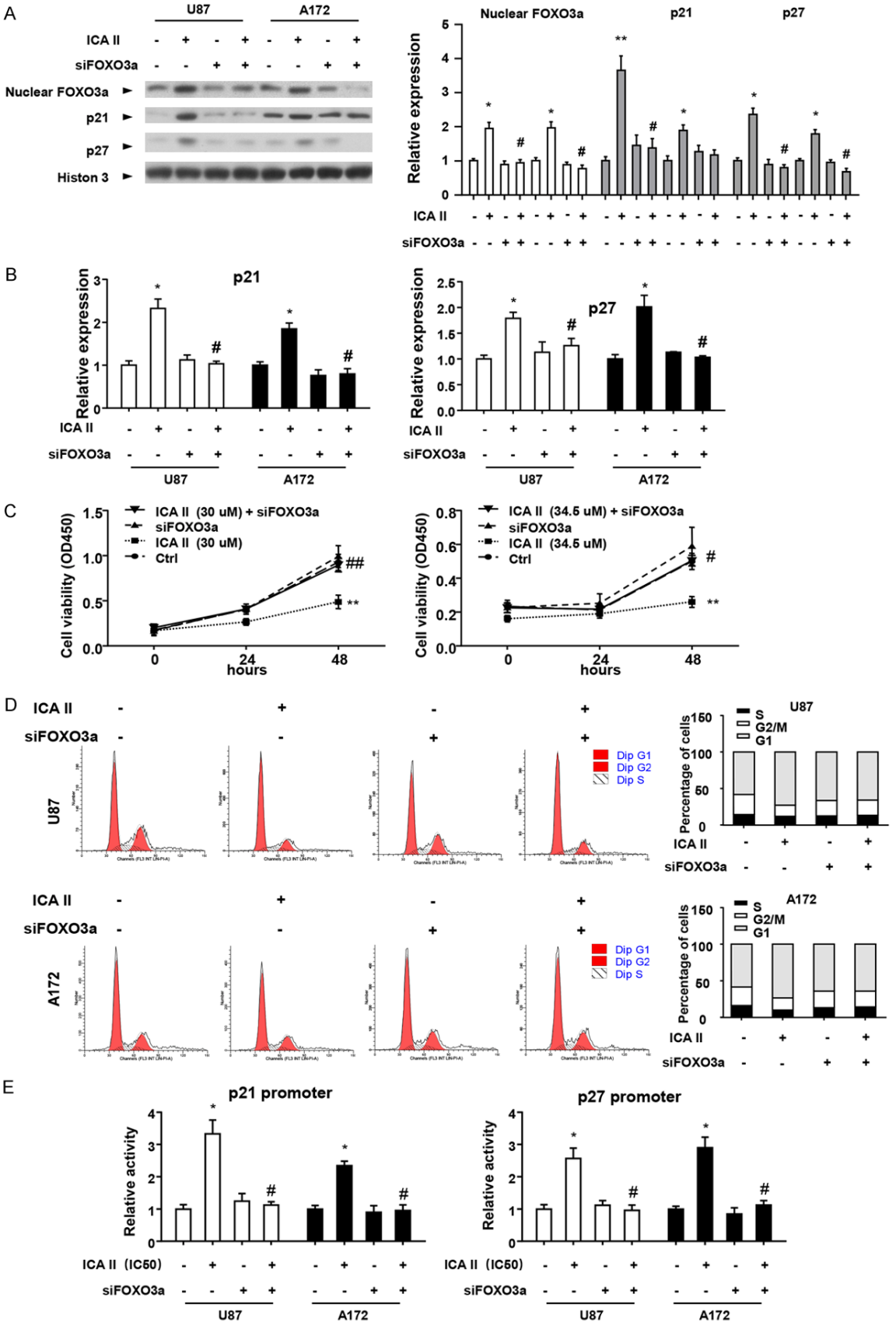

A
U87
A172
ICA II
siFOXO3a
Nuclear FOXO3a
p21
p27
Histon 3
Nuclear FOXO3a
p21
p27
Relative expression
B
p21
p27
Relative expression
ICA II
siFOXO3a
U87
A172
C
ICA II (30 uM) + siFOXO3a
siFOXO3a
ICA II (30 uM)
Ctrl
ICA II (34.5 uM) + siFOXO3a
ICA II (34.5 uM)
Cell viability (OD450)
hours
D
ICA II
siFOXO3a
U87
A172
Dip G1
Dip G2
Dip S
S
G2/M
G1
Percentage of cells
E
p21 promoter
p27 promoter
Relative activity
ICA II (IC50)
siFOXO3a
U87
A172

Figure 5. ICA II regulates the expression of p21 and p27 through the Akt-FOXO3a signaling pathway. A: After U87 and A172 cells were treated with ICA II at IC_{50} concentration for 24 h, increased expression levels of nuclear FOXO3a , p21, and p27 were detected, and this effect was abolished after FOXO3a knockdown. B: After U87 and A172 cells were treated with ICA II at IC_{50} concentration for 24 h, upregulated mRNA levels of p21 and p27 were detected, and this effect was reversed after FOXO3a knockdown. C: Cell viability was analyzed using CCK-8 assay in control and siFOXO3a transfected cell treated with ICA II (IC_{50} concentration) or vehicle control. D: siFOXO3a transfected glioma cells were treated with ICA II for 24 h prior to cell cycle analysis by flow cytometry. The percentage of cells in each phase of the cell cycle (sub G1, G0/G1, G2/-M and S) was shown. E: After cells were co-transfected with the pCMV-p21/p27 construct or with an equimolar amount of the pCMV empty vector and the pGL4-p21/p27 construct, cells were treated with ICA II at IC_{50} concentration for another 24 h before the luciferase signal was measured to determine the transcriptional activity of p21 and p27. **P*<0.05, ***P*<0.01 *vs*. control group, #*P*<0.05 *vs*. scramble group.

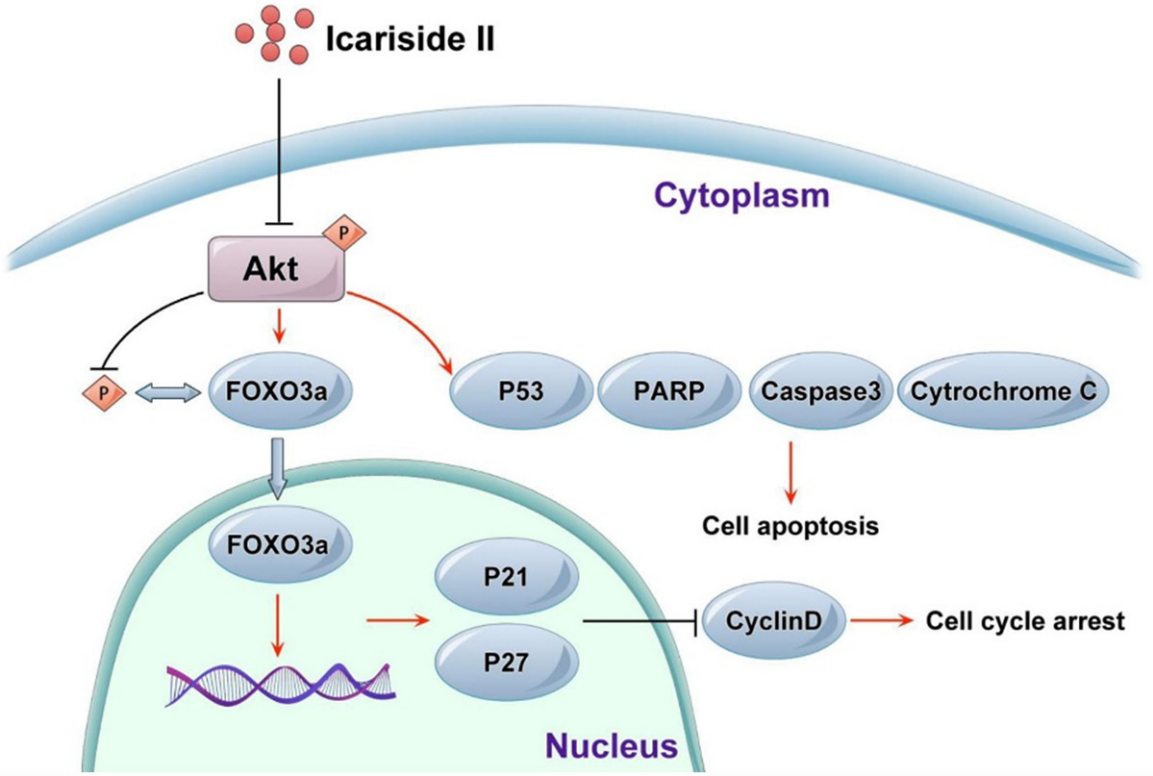


Figure 6. A schematic model to describe the mechanism of ICA II's anti-GBM effect. ICA II inhibits the phosphorylation and activation of Akt, induces the expression of apoptotic related proteins (cytochrome c, p53, PARP, and cleaved caspase 3), and promotes FOXO3a to transport into nucleus and drives the transcription of p21 and p27, which eventually leads to glioma cell apoptosis and cell cycle arrest.

ing from 5 to 100 μM, ICA II was demonstrated to be efficacious against lung cancer, prostate cancer, breast cancer and leukemia [28-31]. In present study, we first demonstrated that ICA II could suppress cell proliferation and cell cycle progression in human glioblastoma cells, and IC_{50} of ICA II in GBM cells (about 30 μM) was 4-fold lower than that of temozolomide (IC_{50} 134.97 μM), a drug widely used in current clinical practice, suggesting that ICA II may have broader application prospects.

Our data indicated that ICA II could induce apoptosis-related proteins expression and suppress Akt phosphorylation in GBM cells, as phosphorylated-Akt might help GBM cells grow without control of apoptosis and also enhance tumor invasion. ICA II, icariin, and icaritin, all have ability against tumors with different mechanisms [13]. FOXO3a, a tumor suppressor that is downregulated in glioma cells [32], is the substrate of Akt and plays a vital role in the initiation of cell cycle arrest. Activated Akt could subsequently phosphorylate FOXO3a and inhibit its activity by translocating it into the cytoplasma, where it is degraded by proteasomal machinery [33]. In line with this, recent reports

suggested that the cytoplasmic FOXO3a is correlated with Akt phosphorylation and poor prognosis in gliomas [34]. Our data demonstrated that ICA II treatment could inhibit Akt-dependent FOXO3a phosphorylation and induce FOXO3a accumulation in the nucleus. Furthermore, ICA II did not alter the total expression of FOXO3a in U87 and A172 cells, indicating the specificity of ICA II in FOXO3a nuclear translocation in GBM cells. Nuclear FOXO3a could target multiple genes such as *p21*, *p27*, and *cyclin D*, which will result in the arrest of cell cycle in G0/G1 phases and cell growth inhibition [21, 35]. Consistent with these reports, our data indicated that the anti-GBM effect by ICA II was dependent on the activation of p21 and p27 and the reduction of cyclin D, leading to growth inhibition and cell cycle arrest. Moreover, the cytotoxic effect of ICA II could be partially rescued when FOXO3a was knocked down. Thus, we proposed that the anti-cancer activities of ICA II can be exerted dependent on Akt-FOXO3a-p21/p27 pathway in GBM cells.

Since the mechanism of cytotoxicity induced by ICA II seems different from TMZ, ICA II might be an alternative drug and a candidate of combination therapy for recurrent gliomas. It was reported that combo of ICA II and paclitaxel could suppress tumor growth in melanoma and minimize paclitaxel resistance [36]. Icariin has been tried to synergize the anti-glioma effect with temozolomide, the additive anti-tumor activities was associated with suppression of NF-κB activity [37]. ICA II has also been shown to improve cerebral microcirculatory disturbance by decreasing IL-1β and TGF-β1 levels via PPARs signaling in cerebral ischemia-reperfusion injury rat models, and is considered to be a neuroprotective drug in cerebral ischemic stroke [14, 38]. In addition, ICA II can readily penetrate the blood brain barrier [39], implicating that ICA II could be a potential drug to prevent both brain tissue damage and enhance the effectiveness of chemotherapy in GBM patients.

Many other effects could be also induced by icariin against tumors, such as angiogenesis inhibition, immunomodulation, and hormone dependent cytotoxicity. So, a limitation of this study is that ICA II is an anti-tumor drug with multiple targets, thus our results might only elaborate a partial mechanism. Other pathways like JAK2/STAT3 and PI3K pathway might also be involved [28, 40]. More precise treatment by ICA II needs lots of delicate work. More importantly, the anti-GBM effect of ICA II *in vivo* still needs further investigation, which will be an objective of our further studies.

Conclusion

In summary, our data provided critical evidence of anti-tumor effect induced by ICA II against GBM cells *in vitro*. We also verified that ICA II treatment could suppress the axis of Akt-FOXO3a-p21/p27 and lead to cell cycle arrest and apoptosis in GBM cells. These findings suggested that ICA II could inhibit GBM cells via inactivation of Akt-related signaling pathway, as a potential therapeutic candidate.

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Disclosure of conflict of interest

None.

Authors' contribution

WH and WZ mainly conceived the project and designed the study. KQ and XZ performed cell culture experiments and participated in the experimental design. They also drafted the manuscript. FK, QY and BL analyzed and interpreted part of data. JFW, BJL and YX mainly contributed to the conception and revised the manuscript. All authors read and approved the final manuscript.

Abbreviations

ICA, Icariside II; GBM, Glioblastoma multiforme; FOXO3a, Forkhead box O3a; PARP, Poly (ADP-ribose) polymerase; MGMT, O(6)-methylguanine-DNA methyltransferase; TMZ, Temozolomide.

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References

[1] Gallego O. Nonsurgical treatment of recurrent glioblastoma. Curr Oncol 2015; 22: e273-281.

[2] Wick W, Weller M, van den Bent M, Sanson M, Weiler M, von Deimling A, Plass C, Hegi M, Platten M and Reifenberger G. MGMT testing--the challenges for biomarker-based glioma treatment. Nat Rev Neurol 2014; 10: 372-385.

[3] Chinot OL, Wick W, Mason W, Henriksson R, Saran F, Nishikawa R, Carpentier AF, Hoang-Xuan K, Kavan P, Cernea D, Brandes AA, Hilton M, Abrey L and Cloughesy T. Bevacizumab plus radiotherapy-temozolomide for newly diagnosed glioblastoma. N Engl J Med 2014; 370: 709-722.

[4] Herrlinger U, Schafer N, Steinbach JP, Weyerbrock A, Hau P, Goldbrunner R, Friedrich F, Rohde V, Ringel F, Schlegel U, Sabel M, Ronellenfitsch MW, Uhl M, Maciaczyk J, Grau S, Schnell O, Hanel M, Krex D, Vajkoczy P, Gerlach R, Kortmann RD, Mehdorn M, Tuttenberg J, Mayer-Steinacker R, Fietkau R, Brehmer S, Mack F, Stuplich M, Kebir S, Kohnen R, Dunkl E, Leutgeb B, Proescholdt M, Pietsch T, Urbach H, Belka C, Stummer W and Glas M. Bevacizumab plus irinotecan versus temozolomide in newly diagnosed O6-methylguanine-DNA methyltransferase non-methylated glioblastoma: the randomized GLARIUS trial. J Clin Oncol 2016; 34: 1611-1619.

[5] Gilbert MR, Dignam JJ, Armstrong TS, Wefel JS, Blumenthal DT, Vogelbaum MA, Colman H, Chakravarti A, Pugh S, Won M, Jeraj R, Brown PD, Jaeckle KA, Schiff D, Stieber VW, Brachman DG, Werner-Wasik M, Tremont-Lukats IW, Sulman EP, Aldape KD, Curran WJ Jr and Mehta MP. A randomized trial of bevacizumab for newly diagnosed glioblastoma. N Engl J Med 2014; 370: 699-708.

[6] Taylor JW, Dietrich J, Gerstner ER, Norden AD, Rinne ML, Cahill DP, Stemmer-Rachamimov A, Wen PY, Betensky RA, Giorgio DH, Snodgrass K, Randall AE, Batchelor TT and Chi AS. Phase 2 study of bosutinib, a Src inhibitor, in adults with recurrent glioblastoma. J Neurooncol 2015; 121: 557-563.

[7] Gatson NT, Weathers SP and de Groot JF. ReACT Phase II trial: a critical evaluation of the use of rindopepimut plus bevacizumab to treat EGFRvIII-positive recurrent glioblastoma. CNS Oncol 2016; 5: 11-26.

[8] Schuster J, Lai RK, Recht LD, Reardon DA, Paleologos NA, Groves MD, Mrugala MM, Jensen R, Baehring JM, Sloan A, Archer GE, Bigner DD, Cruickshank S, Green JA, Keler T, Davis TA, Heimberger AB and Sampson JH. A phase II, multicenter trial of rindopepimut (CDX-110) in newly diagnosed glioblastoma: the ACT III study. Neuro Oncol 2015; 17: 854-861.

[9] Wheeler LA, Manzanera AG, Bell SD, Cavaliere R, McGregor JM, Grecula JC, Newton HB, Lo SS, Badie B, Portnow J, Teh BS, Trask TW, Baskin DS, New PZ, Aguilar LK, Aguilar-Cordova E and Chiocca EA. Phase II multicenter study of gene-mediated cytotoxic immunotherapy as adjuvant to surgical resection for newly diagnosed malignant glioma. Neuro Oncol 2016; 18: 1137-1145.

[10] van Gool S. Immunotherapy for high-grade glioma: how to go beyond Phase I/II clinical trials. Immunotherapy 2013; 5: 1043-1046.

[11] Kim SH, Ahn KS, Jeong SJ, Kwon TR, Jung JH, Yun SM, Han I, Lee SG, Kim DK, Kang M, Chen CY, Lee JW and Kim SH. Janus activated kinase 2/signal transducer and activator of transcription 3 pathway mediates icariside II-induced apoptosis in U266 multiple myeloma cells. Eur J Pharmacol 2011; 654: 10-16.

[12] Geng YD, Yang L, Zhang C and Kong LY. Blockade of epidermal growth factor receptor/mammalian target of rapamycin pathway by Icariside II results in reduced cell proliferation of osteosarcoma cells. Food Chem Toxicol 2014; 73: 7-16.

[13] Chen M, Wu J, Luo Q, Mo S, Lyu Y, Wei Y and Dong J. The Anticancer properties of Herba Epimedii and its main bioactive componentsicariin and icariside II. Nutrients 2016; 8.

[14] Deng Y, Xiong D, Yin C, Liu B, Shi J and Gong Q. Icariside II protects against cerebral ischemia-reperfusion injury in rats via nuclear factor-kappaB inhibition and peroxisome proliferator-activated receptor up-regulation. Neurochem Int 2016; 96: 56-61.

[15] Yin C, Deng Y, Gao J, Li X, Liu Y and Gong Q. Icariside II, a novel phosphodiesterase-5 inhibitor, attenuates streptozotocin-induced cognitive deficits in rats. Neuroscience 2016; 328: 69-79.

[16] Chao Y, Wang Y, Liu X, Ma P, Shi Y, Gao J, Shi Q, Hu J, Yu R and Zhou X. Mst1 regulates glioma cell proliferation via the AKT/mTOR signaling pathway. J Neurooncol 2015; 121: 279-288.

[17] Lan F, Yue X, Han L, Yuan X, Shi Z, Huang K, Yang Y, Zou J, Zhang J, Jiang T, Pu P and Kang C. Antitumor effect of aspirin in glioblastoma cells by modulation of beta-catenin/T-cell factor-mediated transcriptional activity. J Neurosurg 2011; 115: 780-788.

[18] Liu Q, Xu X, Zhao M, Wei Z, Li X, Zhang X, Liu Z, Gong Y and Shao C. Berberine induces senescence of human glioblastoma cells by downregulating the EGFR-MEK-ERK signaling pathway. Mol Cancer Ther 2015; 14: 355-363.
[19] Guan H, Song L, Cai J, Huang Y, Wu J, Yuan J, Li J and Li M. Sphingosine kinase 1 regulates the Akt/FOXO3a/Bim pathway and contributes to apoptosis resistance in glioma cells. PLoS One 2011; 6: e19946.
[20] Miyauchi H, Minamino T, Tateno K, Kunieda T, Toko H and Komuro I. Akt negatively regulates the in vitro lifespan of human endothelial cells via a p53/p21-dependent pathway. EMBO J 2004; 23: 212-220.
[21] Lin CH, Chang CY, Lee KR, Lin HJ, Chen TH and Wan L. Flavones inhibit breast cancer proliferation through the Akt/FOXO3a signaling pathway. BMC Cancer 2015; 15: 958.
[22] Wagenknecht B, Hermisson M, Eitel K and Weller M. Proteasome inhibitors induce p53/p21-independent apoptosis in human glioma cells. Cell Physiol Biochem 1999; 9: 117-125.
[23] Vidak M, Rozman D and Komel R. Effects of flavonoids from food and dietary supplements on glial and glioblastoma multiforme cells. Molecules 2015; 20: 19406-19432.
[24] Romagnolo DF and Selmin OI. Flavonoids and cancer prevention: a review of the evidence. J Nutr Gerontol Geriatr 2012; 31: 206-238.
[25] Geybels MS, Verhage BA, Arts IC, van Schooten FJ, Goldbohm RA and van den Brandt PA. Dietary flavonoid intake, black tea consumption, and risk of overall and advanced stage prostate cancer. Am J Epidemiol 2013; 177: 1388-1398.
[26] Khan M, Maryam A, Qazi JI and Ma T. Targeting apoptosis and multiple signaling pathways with icariside II in cancer cells. Int J Biol Sci 2015; 11: 1100-1112.
[27] Cai WJ, Huang JH, Zhang SQ, Wu B, Kapahi P, Zhang XM and Shen ZY. Icariin and its derivative icariside II extend healthspan via insulin/IGF-1 pathway in C. elegans. PLoS One 2011; 6: e28835.
[28] Kang SH, Jeong SJ, Kim SH, Kim JH, Jung JH, Koh W, Kim JH, Kim DK, Chen CY and Kim SH. Icariside II induces apoptosis in U937 acute myeloid leukemia cells: role of inactivation of STAT3-related signaling. PLoS One 2012; 7: e28706.
[29] Huang C, Chen X, Guo B, Huang W, Shen T, Sun X, Xiao P and Zhou Q. Induction of apoptosis by Icariside II through extrinsic and intrinsic signaling pathways in human breast cancer MCF7 cells. Biosci Biotechnol Biochem 2012; 76: 1322-1328.
[30] Lee KS, Lee HJ, Ahn KS, Kim SH, Nam D, Kim DK, Choi DY, Ahn KS, Lu J and Kim SH. Cyclooxygenase-2/prostaglandin E2 pathway mediates icariside II induced apoptosis in human PC-3 prostate cancer cells. Cancer Lett 2009; 280: 93-100.
[31] Song J, Shu L, Zhang Z, Tan X, Sun E, Jin X, Chen Y and Jia X. Reactive oxygen species-mediated mitochondrial pathway is involved in Baohuoside I-induced apoptosis in human non-small cell lung cancer. Chem Biol Interact 2012; 199: 9-17.
[32] Gopinath S, Malla RR, Gondi CS, Alapati K, Fassett D, Klopfenstein JD, Dinh DH, Gujrati M and Rao JS. Co-depletion of cathepsin B and uPAR induces G0/G1 arrest in glioma via FOXO3a mediated p27 upregulation. PLoS One 2010; 5: e11668.
[33] Plas DR and Thompson CB. Akt activation promotes degradation of tuberin and FOXO3a via the proteasome. J Biol Chem 2003; 278: 12361-12366.
[34] Dai B, Pieper RO, Li D, Wei P, Liu M, Woo SY, Aldape KD, Sawaya R, Xie K and Huang S. FoxM1B regulates NEDD4-1 expression, leading to cellular transformation and full malignant phenotype in immortalized human astrocytes. Cancer Res 2010; 70: 2951-2961.
[35] Park SY, Piao Y, Thomas C, Fuller GN and de Groot JF. Cdc2-like kinase 2 is a key regulator of the cell cycle via FOXO3a/p27 in glioblastoma. Oncotarget 2016; 7: 26793-26805.
[36] Wu J, Guan M, Wong PF, Yu H, Dong J and Xu J. Icariside II potentiates paclitaxel-induced apoptosis in human melanoma A375 cells by inhibiting TLR4 signaling pathway. Food Chem Toxicol 2012; 50: 3019-3024.
[37] Yang L, Wang Y, Guo H and Guo M. Synergistic anti-cancer effects of icariin and temozolomide in glioblastoma. Cell Biochem Biophys 2015; 71: 1379-1385.
[38] Yan BY, Pan CS, Mao XW, Yang L, Liu YY, Yan L, Mu HN, Wang CS, Sun K, Liao FL, Fan JY, Wang XM and Han JY. Icariside II improves cerebral microcirculatory disturbance and alleviates hippocampal injury in gerbils after ischemia-reperfusion. Brain Res 2014; 1573: 63-73.
[39] Liu B, Xu C, Wu X, Liu F, Du Y, Sun J, Tao J and Dong J. Icariin exerts an antidepressant effect in an unpredictable chronic mild stress model of depression in rats and is associated with the regulation of hippocampal neuroinflammation. Neuroscience 2015; 294: 193-205.
[40] Wu J, Zuo F, Du J, Wong PF, Qin H and Xu J. Icariside II induces apoptosis via inhibition of the EGFR pathways in A431 human epidermoid carcinoma cells. Mol Med Rep 2013; 8: 597-602.

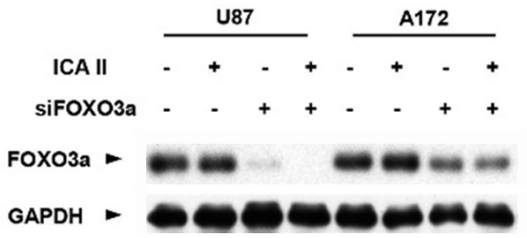

Figure S1. After U87MG and A172 cells were transfected with FOXO3a siRNA or scramble control siRNA and stimulated with ICA II at IC50 concentration or DMSO control for 24 h, the expression levels of total FOXO3a was examined.