Vitexin induces G2/M-phase arrest and apoptosis via Akt/mTOR signaling pathway in human glioblastoma cells

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Abstract. Glioblastoma is a common primary brain tumor with aggressive malignancy, which results in poor outcomes, short survival time and high mortality. Vitexin, an active ingredient from natural products, has been reported to inhibit cell growth and induce cell apoptosis in various cancer cell lines including hepatocellular carcinoma, oral and esophageal cancer. To the best of the authors knowledge, the present study was the first to investigate anticancer effects of vitexin on human glioblastoma cells and potential underlying mechanisms. The present study demonstrated that vitexin inhibited cell viability in a dose- and time-dependent manner. In the present study, vitexin induced G2/M cell cycle arrest, as demonstrated by flow cytometry. Induction of cell apoptosis following vitexin treatment, was further indicated by observation of morphological alterations, flow cytometry analysis and detection of cleaved-poly (ADP-ribose) polymerase. The present study also demonstrated that vitexin inhibited RAC-alpha serine/threonine-protein kinase (Akt)/mechanistic target of rapamycin kinase (mTOR) signaling in human glioblastoma cells. Collectively, the results of the present study demonstrated that vitexin induced G2/M cell cycle arrest and apoptosis by inhibiting Akt/mTOR signaling in human glioblastoma cells. Vitexin may in the future be used as a therapeutic agent for treatment of malignant glioblastoma.

Introduction

Glioblastoma (GBM), a common primary malignant brain tumor, accounts for 70% of total brain tumors and demonstrates aggressive proliferation, metastasis and recurrence (1-3). These biological behaviors contribute to its poor prognosis and high mortality with 15-20% patients surviving >3 months post-diagnosis (4,5). Due to simultaneous occurrence of multiple lesions, it is challenging to treat patients with GBM and obtain a desirable clinical outcome (6). At present, a clinical therapeutic regimen for patients with GBM is to coordinate several methods including neurosurgery, radiotherapy and chemotherapy (7). Despite the progress made in these treatment methods, the outcome for patients with GBM remains poor due to disease recurrence, with 12-15 months median survival time (8). Therefore, there is a need to investigate more effective therapies targeting aggressive biological processes associated with GMB to improve prognosis.

Vitexin, an apigenin-8-C-D-glucopyranoside, is a flavonoid compound derived from natural products, serving the role of active ingredient in a number of traditional Chinese medicines (9,10). Numerous studies have demonstrated that vitexin has anti-oxidative, anti-inflammatory, anti-hyperalgesic and neuroprotective effects (11-14). It has also been reported that vitexin may suppress cell growth and induce cell apoptosis in a number of cancer cell lines including hepatocellular carcinoma, oral and esophageal cancer (15-17). However, to the best of the authors knowledge, whether vitexin exhibits an effect on GBM remains to be elucidated.

Cell proliferation requires completion of a cell cycle, and cell apoptosis results from cell cycle arrest (18). A complete cell cycle is composed of G1, S, G2 and M phases, in which a successful G2/M transition is important. It has been previously demonstrated that uncontrolled cell proliferation is a consequence of imbalanced cell cycle regulation, which is a characteristic of cancer cells (19,20). Numerous anticancer drugs have been reported to induce G2/M cell cycle arrest which may effectively repress proliferation of cancer cells (21-23).

Apoptosis, also known as type I programmed cell death, is a regulated process triggered in response to cell damage (24). It is primarily triggered by caspase-dependent intrinsic or extrinsic pathways and may be predictive factor for the cytotoxicity of anti-cancer drugs (25). During apoptosis, a characteristic morphological alteration in cells is observed, including cell shrinkage, chromatin condensation and nuclear fragmentation (26). Additionally, poly(ADP-ribose) polymerase (PARP), a component of the DNA damage response mechanism elicited by cytotoxic agents, is cleaved by functional caspases activated in the apoptotic process (27). Phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K)/RAC-α serine/threonine-protein kinase (Akt)/mechanistic target of rapamycin kinase (mTOR) signaling pathway

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is a common cancer-associated pathway and previous studies have reported that it is negatively associated with cell apoptosis in various cancers (28-30).

To the best of the authors knowledge, the present study was the first to investigate the anti-cancer effect of vitexin on human GBM cells and to attempt to elucidate the underlying mechanisms. The present study demonstrated that vitexin induced G2/M cell cycle arrest and cell apoptosis by inhibiting Akt/mTOR signaling in human GBM cells.

Materials and methods

Cell line and culture. The human GBM cell line LN-18 was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin (100 U/ml) and 1% streptomycin (100 μg/ml). The culture was maintained at 37˚C, in a humid atmosphere containing 5% CO_2.

Reagents. DMEM and FBS were acquired from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Vitexin was purchased from Selleck Chemicals (Houston, TX, USA) and stored at -20˚C following preparation of the stock solution at 300 mM vitexin dissolved in dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Antibodies against cleaved-PARP (cat. no. 5625), Akt (cat. no. 4685), mTOR (cat. no. 2983), phosphor (p)-Akt (cat. no. 4060), p-mTOR (cat. no. 5536), GAPDH (cat. no. 5174) and anti-rabbit immunoglobulin G, horseradish peroxidase-linked antibody (cat. no. 14708) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell viability assay. Viability of GBM cells following treatment with vitexin was assessed using Cell Counting Kit-8 (CCK8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Cells were plated in 96-well plates at a density of $5x10^5$ cells/ml for 24 h and subsequently treated with different concentrations of vitexin (0, 10, 20, 40, 80 and 160 μM). Following incubation for another 24 or 48 h at 37˚C, cells were washed with PBS twice and cultured with CCK8 solution for 2 h according to the manufacturer's instructions. The absorbance was measured at a wavelength of 450 nm using a microplate reader iMark (Molecular Devices, LLC, Sunnyvale, CA, USA) and IC_{50} values were calculated using SPSS software 19.0 statistical software package (IBM Corp., Armonk, NY, USA).

Cell cycle analysis by flow cytometry. The influence of vitexin on the proportion of cells at differing stages of the cell cycle was examined using flow cytometry. Cells were plated in a 6-well plate at a density of $5x10^6$ cells/ml and treated with different concentrations of vitexin (0, 20, 40 and 80 μM) for 24 h at 37˚C. Cells were digested using trypsin at 37˚C for 1 min and centrifuged at 600 x g for 5 min at room temperature following washing with PBS, fixed in 75% ethanol at 4˚C overnight and subsequently stained with propidium iodide assay kit (BD Biosciences, Franklin Lakes, NJ, USA) for 15 min in the dark. The cell cycle distribution was analyzed using an Accuri C6 flow cytometer (BD Biosciences) and the resulting data were presented following analysis with ModFit LT software v3.1 (FACSCalibur; BD Biosciences).

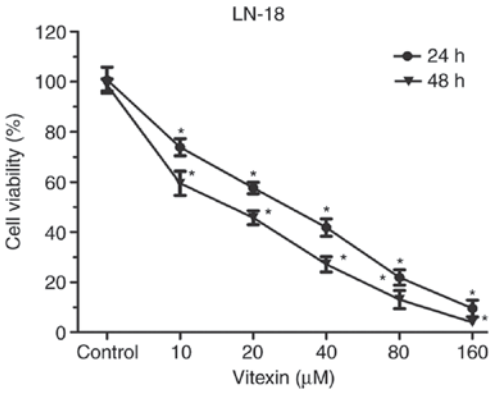

Figure 1. Vitexin inhibits cell viability of human GBM cells. Human GBM cells were treated with vitexin (0, 10, 20, 40, 80 and 160 μM) for 24 or 48 h. The effect of vitexin on viability of GBM cells was determined by using Cell Counting Kit-8. Data are presented as the mean ± standard deviation. *P<0.05 vs. the untreated control group. GBM, glioblastoma.

Morphological apoptosis. Morphological characteristics of apoptotic cells were observed using Hoechst 33342 staining. Cells were incubated in 6-well plates with a coverslip at a density of $5x10^6$ cells/ml and treated with 40 μM vitexin for 24 h at 37˚C. Following incubation, cells on the coverslip were washed with PBS three times and fixed in 4% paraformaldehyde for 20 min at room temperature and washed again with PBS. Subsequently, 0.1% Triton X-100 was used to permeabilize the cells and Hoechst 33342 solution (5 μg/ml) was added to stain the nucleus of apoptotic cells, for 10 min at room temperature without light. A fluorescence microscope (Olympus Corporation, Tokyo, Japan) was used to observe morphological alterations in the stained cells.

Flow cytometric analysis of apoptosis. Apoptosis detection was identified using a Annexin V-fluorescein isothiocyanate (FITC)/PI assay kit (Beyotime Institute of Biotechnology, Beijing, China) according to the manufacturer's instructions. In brief, the GBM cells were incubated in 6-well plates at a density of $5x10^6$ cells/ml and treated with 0, 20, 40 and 80 μM vitexin. Following incubation for 24 h at 37˚C and two washes in ice-cold PBS, cells were collected and re-suspended in annexin-binding buffer. Subsequently, cells were stained by adding Annexin V-FITC and PI at room temperature for 15 min without light. Apoptosis analysis of each sample was performed using an flow cytometer with Accuri C6 software (BD Biosciences).

Western blotting. Protein samples were extracted by adding radioimmunoprecipitation assay buffer (Sigma-Aldrich; Merck KGaA) to lyse GBM cells. Protein concentrations were quantified using a Bicinchoninic Acid Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Proteins, 30 μg/lane, were added and separated on 8-12% SDS-PAGE prior to being transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Subsequently, membranes were blocked

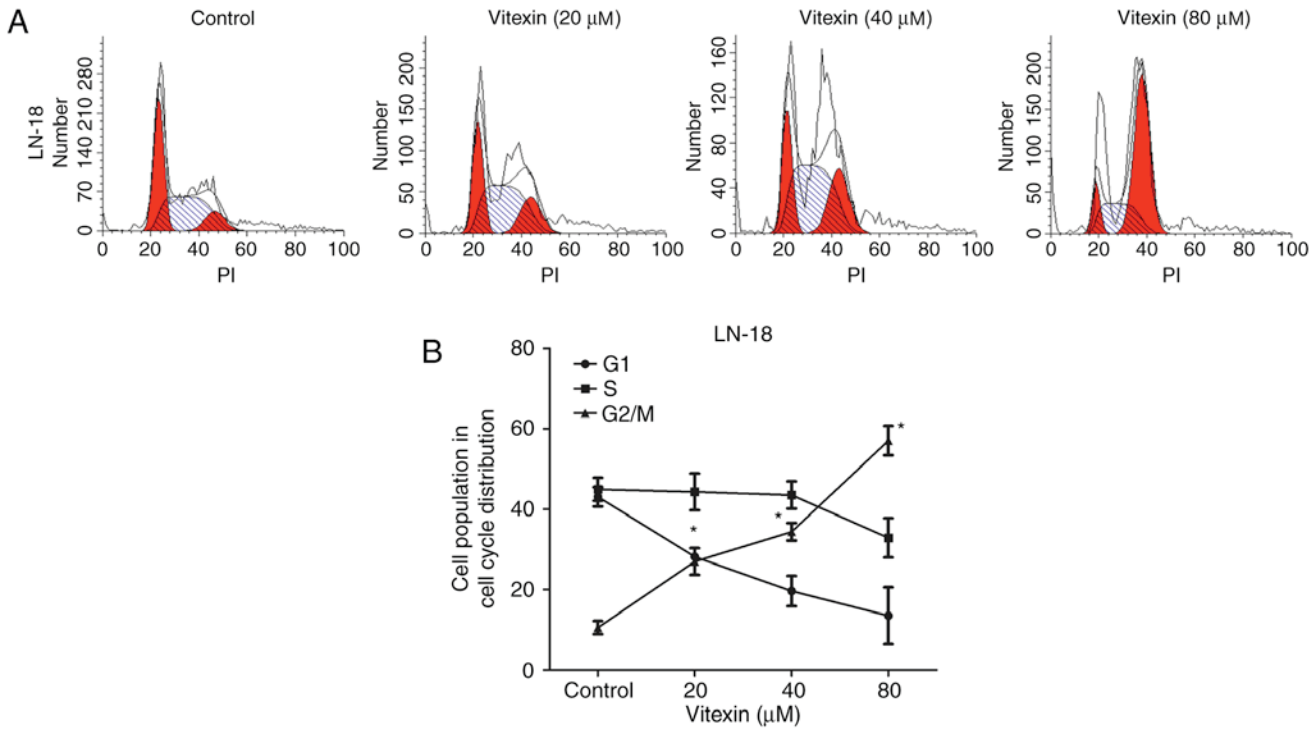

Figure 2. Vitexin induces G2/M cell cycle arrest in human GBM cells. Human GBM cells were treated with vitexin at 0, 20, 40 and 80 μM for 24 h. Following incubation, cells were harvested and cell cycle distribution was analyzed by flow cytometry. (A) Vitexin led to the accumulation of cells in G2/M phase (first red peaks) and a corresponding decrease in G0/G1 (the second red peaks). The number of cells in the S phase (blue stripes) did not significantly change; however, a marked increase was observed in response to 40 μM. (B) A line chart presenting the significant increase of the cell population in the G2/M phase (from 40.3 to 17.7%), and the decrease in the G1 phase (from 14.0 to 52.5%). Data are presented as the mean ± standard deviation. *P<0.05 vs. the untreated control group. GBM, glioblastoma; PI, propidium iodide.

using 5% skimmed milk at room temperature for 1 h. Primary antibodies (1:1,000; cleaved-PARP, Akt, phospho-Akt, mTOR, phospho-mTOR and GAPDH) were added to the membranes and incubated at 4˚C overnight. Subsequently, horseradish peroxidase-conjugated secondary antibody (1:5,000) was added for 1 h at room temperature. Detection of proteins was performed using an Enhanced Chemiluminescence kit (EMD Millipore).

Statistical analysis. Data are presented as the mean ± standard deviation of three independent experiments and analyzed by one-way analysis of variance and Dunnett's post hoc test were used in order to compare differences among groups. P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS software (version 18.0; SPSS, Inc., Chicago, IL, USA).

Results

Vitexin inhibits cell viability in human GBM cells. The influence of vitexin on proliferation of human GBM cells was assessed by CCK-8 assay (Fig. 1). Cells were cultured with different concentrations of vitexin (0, 10, 20, 40, 80 and 160 μM) for 24 or 48 h. Following incubation, cell viability significantly decreased in a dose and time dependent manner. IC_{50} values for GBM cells were 32.32 and 25.32 μM following 24 and 48 h of incubation, respectively. The aforementioned results indicated that vitexin suppressed cell proliferation in human GBM cells in a dose- and time-dependent manner.

Vitexin induces G2/M cell cycle arrest in human GBM cells. To investigate whether vitexin inhibited GBM cell proliferation by inducing cell cycle arrest, cell cycle progression was assessed by flow cytometry. Following treatment with vitexin at 20-80 μM for 24 h, cells were collected to perform cell cycle analysis (Fig. 2A and B). Compared with the control group, a significant increase in the percentage of the cell population in the G2/M phase, and a decrease in the number of cells in G1 phase, were observed in cells treated with vitexin. The aforementioned data indicated that vitexin exhibited an effect on cell cycle progression by inducing cell cycle arrest at G2/M phase.

Vitexin induces apoptosis in human GBM cells. Cell apoptosis is a consequence of cell cycle arrest. Therefore, the present study further investigated the influence of vitexin on cell apoptosis of human GBM cells. Hoechst 33258 staining was performed to observe nuclear morphological alterations characteristic of apoptotic cells, following treatment of GBM cells with 40 μM vitexin for 24 h. GBM cells treated with vitexin exhibited features including cell shrinkage, chromatin condensation and nuclear fragmentation, compared with the control group (Fig. 3A). Subsequently, Annexin V/PI double staining was used to assess the proportion of apoptotic cells. The results indicated a dose-dependent increase in the number of early and late apoptotic cells (Fig. 3B and C). Western blotting was performed to determine the expression level of the intracellular apoptosis-associated protein, cleaved-PARP. PARP recruits DNA repair proteins by binding to DNA

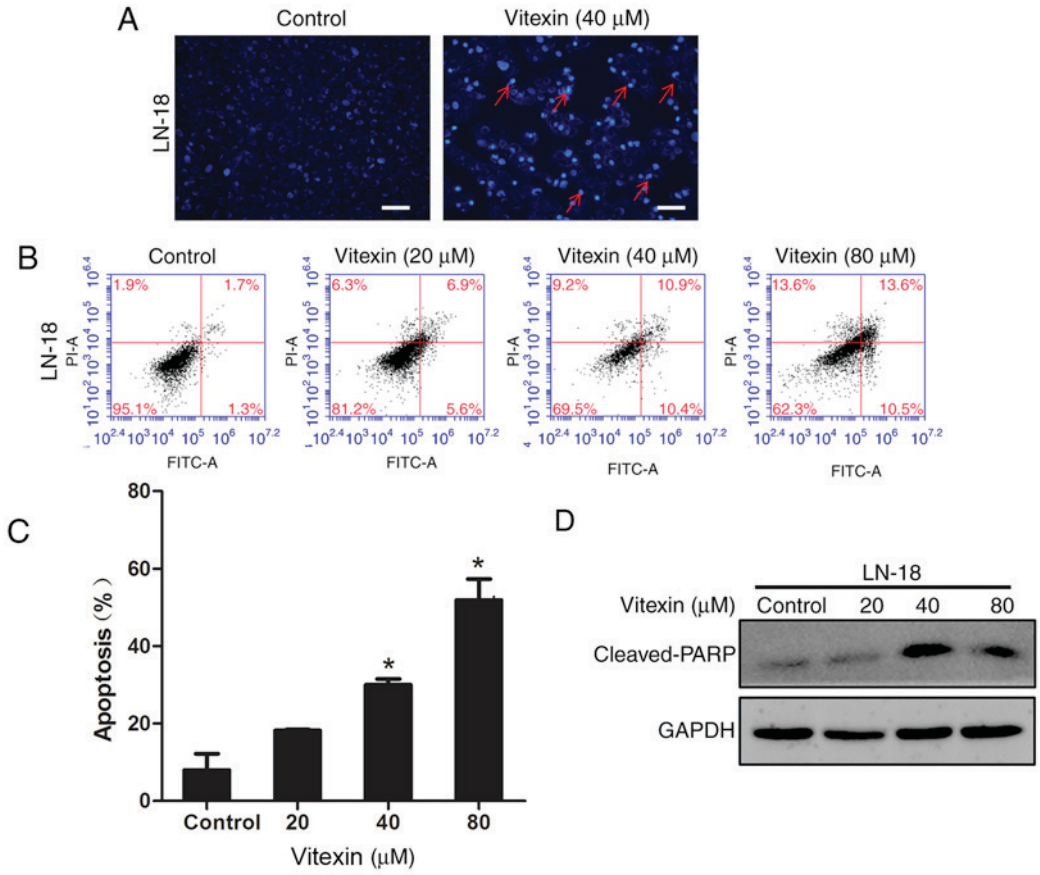

Figure 3. Vitexin induces apoptosis of human GBM cells. GBM cells were treated with vitexin for 24 h. (A) Nuclear morphological alterations characteristic of apoptotic cells (indicated by red arrows), including chromatin condensation and nuclear fragmentation, were observed under the fluorescence microscope through Hoechst 33258 staining. Scale bar, 50 μm. (B) Cells were harvested, stained by Annexin V and PI, and subsequently analyzed by flow cytometry. (C) Apoptotic cell proportion was presented in the histogram. Data are presented as the mean ± standard deviation. *P<0.05 vs. the untreated control group. (D) Following incubation, expression level of cleaved-PARP, an apoptosis-associated protein, was assessed by western blotting. PARP, poly(ADP-ribose) polymerase; GBM, glioblastoma; PI, propidium iodide.

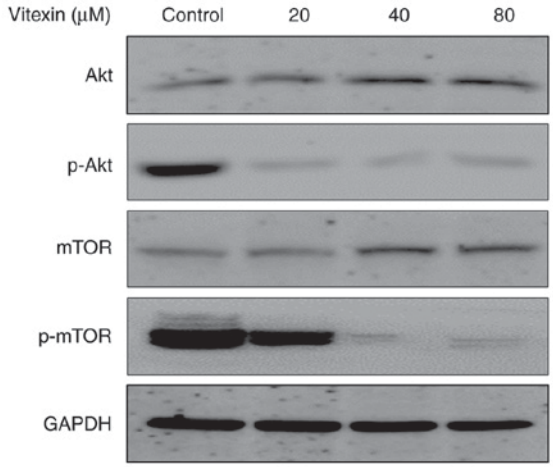

Figure 4. Vitexin inhibits the Akt/mTOR signaling pathway in GBM cells. Human GBM cells were treated with 0, 20, 40 and 80 μM vitexin for 24 h. Expression levels of Akt, p-Akt, mTOR, p-mTOR were analyzed by western blotting. GBM, glioblastoma; Akt, RAC-α serine/threonine-protein kinase; p, phosphorylated; mTOR, mechanistic target of rapamycin kinase.

breaks. Levels of cleaved-PARP markedly increased following vitexin treatment. The aforementioned results demonstrated that vitexin induced cell apoptosis to inhibit cell proliferation.

Vitexin inhibits Akt/mTOR signaling pathway in human GBM cells. The aforementioned *in vitro* results indicated that vitexin treatment resulted in cell cycle arrest and cell apoptosis to inhibit cell viability in human GBM cells. To further investigate the underlying molecular mechanism of the anti-tumor effect of vitexin, the present study investigated the alterations in the Akt/mTOR signaling pathway. Western blotting was performed to measure the expression levels of Akt, mTOR, p-Akt, and p-mTOR proteins. The results demonstrated a dose-dependent decrease in phosphorylation of Akt and mTOR following treatment with vitexin (Fig. 4). These results indicated that vitexin inhibited activation of the Akt/mTOR signaling pathway, which may contribute to vitexin-induced cell cycle arrest and apoptosis.

Discussion

Natural products may serve as a source of flavonoids used for cancer prevention and treatment (31,32). The flavonoid vitexin has previously gained attention due to its multiple pharmacological effects, including neuroprotective and anti-cancer properties. Vitexin was reported to markedly inhibit cell growth and induce cell apoptosis in a number of cancer cell lines, including hepatocellular carcinoma, oral and esophageal cancer (33,34).

However, whether it exhibits an effect on malignant GBM remains to be elucidated. The present study demonstrated that vitexin induced G2/M cell cycle arrest and cell apoptosis by inhibiting Akt/mTOR signaling in human GBM cells.

The cell cycle is a precisely regulated process composed of G1, S, G2 and M phases. A successful transition between two phases serves a role in cell proliferation, particularly the G2/M transition, which promotes the symmetric division of a cell (35). Disruption of the cell cycle is characteristic of cancer cells and results in uncontrolled cell proliferation. Therefore, the therapeutic aim of anti-cancer agents is to suppress cell proliferation, via an induction of G2/M cell cycle arrest. The present study demonstrated that vitexin exhibited an effect on cell cycle progression by inducing cell cycle arrest at G2/M transition phase. Compared with the control group, the percentage of cells at G2/M phase increased markedly, whereas the percentage of cells at G1 phase decreased.

Apoptosis is a result of cell cycle arrest and its rate may be used to predict the cytotoxicity of anti-cancer agents (36,37). In the presence of pro-death stimuli, including cytotoxic agents, cells respond by initiating programmed death, characterized by certain detectable morphological alterations, including nuclear fragmentation, chromatin condensation, cell shrinkage, membrane blebbing and formation of apoptotic bodies (26,38). The aforementioned morphological alterations serve as indicators of cell apoptosis. In the present study, morphological alterations indicated cell apoptosis following treatment with vitexin. A number of studies have demonstrated that cell apoptosis is triggered by caspase-dependent or caspase-independent pathways and the former is more common (39-42). In the caspase-dependent apoptotic process, caspases-3 and -8, and other downstream caspases are activated and subsequently cleave their common substrate, PARP, inducing apoptosis. Therefore, an increased expression level of cleaved-PARP is an indicator of cell apoptosis. Western blotting results demonstrated an increase in the expression level of cleaved-PARP in human GBM cells.

It has previously been demonstrated that certain signaling pathways are involved in the cell apoptosis process, including the PI3K/Akt/mTOR and mitogen-activated protein kinase pathways (29,43,44). The present study investigated Akt/mTOR signaling due to its association with cell apoptosis in various cancer diseases (45). In the present study, phosphorylation of Akt and mTOR molecules was suppressed compared with the control group, which indicated negative regulation of Akt/mTOR signaling during vitexin-induced cell apoptosis.

In conclusion, the present study demonstrated that vitexin induced G2/M cell cycle arrest and cell apoptosis by inhibiting the Akt/mTOR signaling pathway in human GBM cells. To the best of the authors knowledge, the present study is the first to investigate anticancer effects of vitexin on human GBM cells and the underlying molecular mechanisms. Since flavonoids have been extensively used for cancer prevention and treatment, vitexin may in the future be used for GBM chemotherapeutic treatment, which requires further investigation.

Acknowledgements

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