

Fucoxanthin Activates Apoptosis via Inhibition of PI3K/Akt/mTOR Pathway and Suppresses Invasion and Migration by Restriction of p38-MMP-2/9 Pathway in Human Glioblastoma Cells

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Abstract Fucoxanthin is rich in seaweed and considered as effective anti-cancer drug because of powerful antioxidant properties. The objective of this study was to investigate the role of fucoxanthin on apoptosis, invasion and migration of glioma cells. Firstly, fucoxanthin showed obvious cytotoxicity against human glioma cancer cell line U87 and U251, however, there was no inhibitory effect on normal neuron. And then, fucoxanthin induced apoptotic cell death showed by the condensation of chromatin material stained with Hoechst 33342, and reduced mitochondrial membrane potential via DIOC6(3) staining, and enhanced apoptosis by annexin V-FITC/SYTOX Green double staining on U87 and U251 cell lines. Transmission electron microscopy and western blotting were used to determine ultrastructure of U87 cell and expression of proteins related to apoptosis. A scratch wound healing assay and the expression of matrix metalloproteinases (MMPs), and a trans-well assay were used to investigate cell migration and invasion, respectively. Additionally, we uncovered upstream signaling Akt/mTOR and p38 pathways induced by incubation U87 and U251 cell lines with fucoxanthin that mediated cell

apoptosis, migration and invasion by using PI3K and p38 inhibitors. Moreover, incubation of fucoxanthin obviously reduced the weight and volume of glioma mass of U87 cells in nude mice. Furthermore, we also examined the glioma mass of U87 cells by hematoxylin-eosin staining, TUNEL assay and western blot, and these outcomes in vivo consistently confirmed that above results in vitro. Taken together, these findings suggest that fucoxanthin augments apoptosis, and reduces cell proliferation, migration and invasion, and reveals a potential mechanism of fucoxanthin-mediated Akt/mTOR and p38 suppression in human glioblastoma cell line.

Keywords Fucoxanthin · U87 and U251 · Apoptosis · Invasion · Migration · PI3K/Akt/mTOR · P38

Introduction

Gliomas comprise primary central nervous (CNS) system tumors, which are derived from progenitor of glial origin and microglia. Glial origin encompasses ependymal cells, oligodendrocytes and astrocytes, which could transform to ependymomas, oligodendroglioma and astrocytomas, respectively [1]. Astrocytomas are the most common type of primary CNS neoplasm which can be classified grade I to IV in term of a histological/prognostic grading system. According to the classification, the human glioma U87 and U251 cell lines are the kind of Grade IV gliomas, which is known as glioblastoma multiforme (GBM) [2]. As most serious malignant astrocytomas, GBM grows uncontrolled, and invades into neighboring brain structures and metastasizes distantly [3]. Although combine surgical resection with radiotherapy or chemotherapy, it difficultly achieves effective at curing primary due to poor prognosis. Therefore, the

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development of novel treatment strategies for induction of glioma cell death may be a new line of research into the treatment of glioma.

Fucoxanthin is a major carotenoid extraction from seaweeds and its molecule formula comprises an unusual allenic bond and conjugated carbonyl group [4–5]. Because of these special functional groups, fucoxanthin represents antioxidant activity [6–8], induction of apoptosis in cancer cells [9]. Additionally, current researches focus on the effect of fucoxanthin on inhibition migration and invasion of metastatic tumors. It has been demonstrated that fucoxanthin reduces the ability of invasion and metastasis of melanoma cells and osteosarcoma cells *in vitro* and *in vivo* [10–11]. Unfortunately, few reports have been claimed the effect of fucoxanthin on glioma cells, particularly in the category of underlying mechanisms of glioma growth inhibition.

Apoptosis is the term of programmed cell death which is the critical action target on cancer treatment induced by drugs or chemicals [12]. Apoptosis is roughly divided into two types according to the different inductive factor: intrinsic and extrinsic apoptosis [13]. The former class of apoptosis is well known as mitochondrial apoptosis which is characterized by depolarization of membrane potential and activation of caspase family [14]. It has been reported that many botanical drugs display the killing of glioma cells by inducing activation of pro-apoptotic proteins and reduction of anti-apoptosis related proteins [15, 16].

Likewise, the invasion and migration are major causes of malignant glioma U87 and U251-associated mortality. Previous studies have proposed that prevention of tumor exacerbation not only inhibits the proliferation and growth but also blocks the metastasis of glioma cells [17]. Various inspections predict that some drugs have the potential capability to prevent invasion and migration of U87 and U251 cells [18]. Fucoxanthin has effects against tumors due to various pharmacological activity, however, the inhibitory effect of fucoxanthin on U87 and U251 cells remains unclear. Hence, we used human glioma U87 and U251 cells as the cell model to examine potential effects of fucoxanthin on apoptosis, invasion and migration of human glioblastoma cell lines, and investigate the possible molecular targets of anti-cancer of fucoxanthin in the present study.

Materials and Methods

Materials

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) bromide (MTT) and fucoxanthin were purchased from Sigma Chemical Company (St. Louis, MO). Hoechst 33342 dye was purchased from Beyotime Biotechnology Inc (Nantong, China). All other chemicals and reagents

were of analytical grade. PI3K inhibitor (LY294002) and p38 inhibitor (SB203580), primary polyclonal antibodies against Bcl-2, Bax, caspase-3, caspase-9, PARP, anti-Akt, anti-phospho-Akt, anti-mTOR, anti-phospho-mTOR and secondary antibodies (goat anti-rabbit and rabbit anti-mouse) were purchased from Cell Signaling Technology (Danvers, MA, USA). Primary rabbit polyclonal anti-p38, anti-phospho-p38, anti-ERK, anti-phospho-ERK, anti-JNK, anti-phospho-JNK, anti-MMP-2, anti-MMP-9 and anti-uPA were purchased from Affinity Biosciences (Affinity, Cincinnati, OH, USA).

Cell Line and Animals

The human glioma U87 and U251 cell lines were purchased from the Chinese Academy of Medical Sciences (Beijing, People's Republic of China). Nude mice (male 5-week-old BALB/c) were obtained from Vitalriver, Beijing, China. The experimental protocol was approved by the Animal Care and Use Committee of General Hospital of Beijing Military Region (approved number: 2015–121), and all the procedures were conducted as per the institutional guidelines. Animals were kept in separate cages at room temperature (25 °C), with free access to food and water under a 12 h light–dark cycle for 1 week before the experiment.

Cell Culture

U87 and U251 Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (DMEM, Invitrogen, Valencia, CA, USA) supplemented with 10% foetal bovine serum, 100 µg/mL streptomycin and penicillin (HyClone, Utah, USA) and maintained at 37 °C in an incubator supplemented with an atmosphere containing humidified 5% CO₂ plus 95% air.

Cell Viability Assay

The effect of fucoxanthin on the viability of glioma U87, U251 cells and normal neurons were determined by using MTT assay, which was based on the reduction of a tetrazolium salt by mitochondrial dehydrogenase in viable cells. Cells of U87 and U251 were seeded on a 96-well plate at a density of 4000 cells/well were treated 16 h later with 6.25, 12.5, 25, 50, 75, 100 µM of fucoxanthin. Normal neurons were planted on a 96-well plate at a density of 1.5×10^4 cells/well were treated 3 days later with 25 and 50 µM of fucoxanthin. After incubation for 12, 24 and 48 h, MTT stock solution (50 µL, 2 mg/mL) was added to each well to yield a total reaction volume of 150 µL. 4 h later, the supernatants were aspirated. The formazan crystals in each well were dissolved in DMSO (150 µL), and the

absorbance at 540 nm was read on a scanning multi-well spectrophotometer [19].

Hoechst 33342 Staining Assay

Glioma U87 and U251 cells were treated with fucoxanthin at a concentration of 25, 50 μM after seeding on the coverslips for 16 h. After additional 24 h incubation at 37 °C, U87 and U251 cells were washed with PBS and then fixed with 4% paraformaldehyde for 20 min at room temperature. The DNA-specific fluorescent dye Hoechst 33342 (1.5 μL of 10 mg/mL stock) was added to coverslips and the fixed cells were incubated for 10 min at 37 °C. The stained cells were visualized under a fluorescence microscope (Leica, Germany). The degree of nuclear condensation was evaluated and the apoptotic cells were quantified.

Cell Apoptosis Assay

Apoptosis was explored by staining U87 and U251 cells with Annexin V-FITC/SYTOX Green according to the manufacturer's recommendations (BD Biosciences, San Diego, CA, USA). Briefly, five hundred thousand cells were seeded into 100 mm dishes incubating for 12 h, and then cells were treated with fucoxanthin (25, 50 μM) for 24 h, and harvested and resuspended at a density of 1×10^5 cells/mL with PBS. After 5 μL of Annexin V-FITC was added and mixed well, 5 μL of SYTOX Green was added and mixed well, and the mixture was incubated for 15 min in the dark at room temperature. The stained cells were analyzed with flow cytometry (BD Biosciences, San Diego, CA, USA). The percentage of early and late apoptotic cells was assessed based on histograms generated using CellQuest and ModFit software (Becton Dickinson).

Mitochondrial Membrane Potential ($\Delta\psi\text{m}$)

The U87 and U251 cells were seeded in a 24-well plate at 2×10^5 cells/well and incubated for 12 h. The U87 cells were treated with fucoxanthin (25, 50 μM) for 24 h at 37 °C. After adding 40 nM (final concentration) of DIOC6(3) solution for 30 min, $\Delta\psi\text{m}$ was analyzed by flow cytometer (BD Biosciences, San Diego, CA, USA).

Transmission Electron Microscopy Analysis

Transmission electron microscopy (TEM) was conducted to observe the ultrastructure of U87 cells after incubation with fucoxanthin. U87 cells were treated with 50 μM fucoxanthin for 24 h, then harvested and fixed for 1 h with 2.5%

glutaraldehyde in a Sørensen 0.1 M phosphate buffer (pH 7.4) at 4 °C. After fixation for 30 min with 1% osmium tetroxide and deshydration, cells were embedded into epon. Ultrathin sections were obtained using a Reichert ultracut S ultramicrotome and mounted on copper grids. Sections were stained with uranyl acetate for 15 min, and lead citrate for 3 min and were examined Jeol 100 CX II transmission electron microscope at 60 kV (Tokyo, Japan).

Scratch Wound Healing Assay

The U87 and U251 cells were seeded at a density of 1×10^5 cells/well in 6-well plates, and incubated for 12 h. The cell monolayer was scraped in a straight line using a 200- μL pipette tip and the cells were washed three times with PBS. The cells were incubated in free media and various concentrations of fucoxanthin (25 and 50 μM) for 18 h. The control group was treated with an equal volume of solvent (DMSO) in culture medium. Images were captured under phase-contrast microscope (Leica, Germany). ImageJ2x software (Rawak Software, Inc. Germany) was used to analyse the relative migration distances.

Trans-Well Invasion Assay

U87 and U251 cells were resuspended at a density of 2×10^4 cells/well with medium containing 1% FBS. Transwell chambers (Corning Life Sciences), which precoated with 100 μL Matrigel (BD Biosciences, Bedford, MA, USA) diluted with culture medium. Medium contained 20% FBS in the lower chambers served as the chemoattractant. Fucoxanthin (25, 50 μM) was added in the lower chambers. Non-invading cells were removed with cotton swabs after 24 h, while invading cells were dyed with 0.1% crystal violet (Amresco, Inc., Solon, OH, USA). Washed the trans-well chambers with the water and captured images by using Image-Pro Plus software under a phase-contrast microscope (magnification, 200 \times). Crystal violet in the invading cells was dissolved with 500 μL of 33% acetic acid. The absorbance of crystal violet dissolved in acetic acid was measured at 570 nm using a microplate reader.

Trans-Well Migration Assay

U87 and U251 cells were resuspended at a density of 2×10^4 cells/well with medium containing 1% FBS. Medium contained 20% FBS in the lower chambers served as the chemoattractant. Fucoxanthin (25, 50 μM) was added in the lower chambers. Non-invading cells were removed with cotton swabs after 24 h, while

Fig. 1 Fucoxanthin attenuates glioblastoma cells generation. Fucoxanthin was added to **a** U87 and **b** U251 cells at a concentration of 6.25, 12.5, 25, 50, 75 and 100 μ M, and after 12, 24 and 48 h cell viability was determined by the MTT assay. **c** The normal neurons were exposed to culture medium containing various concentrations of fucoxanthin for 72 h. *Significant difference from control ($p < 0.05$). Each bar represents the mean \pm SEM in triplicate experiments

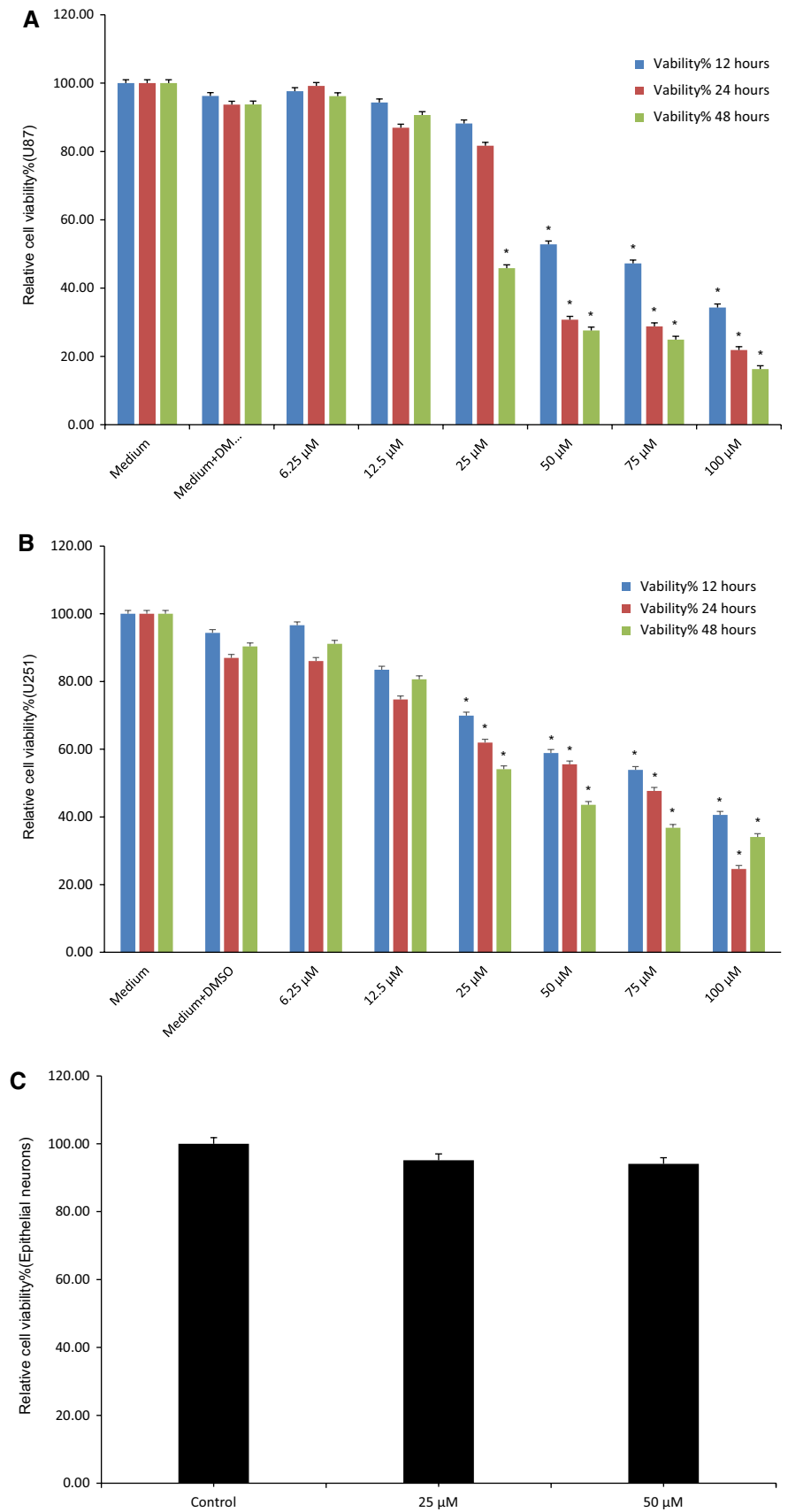
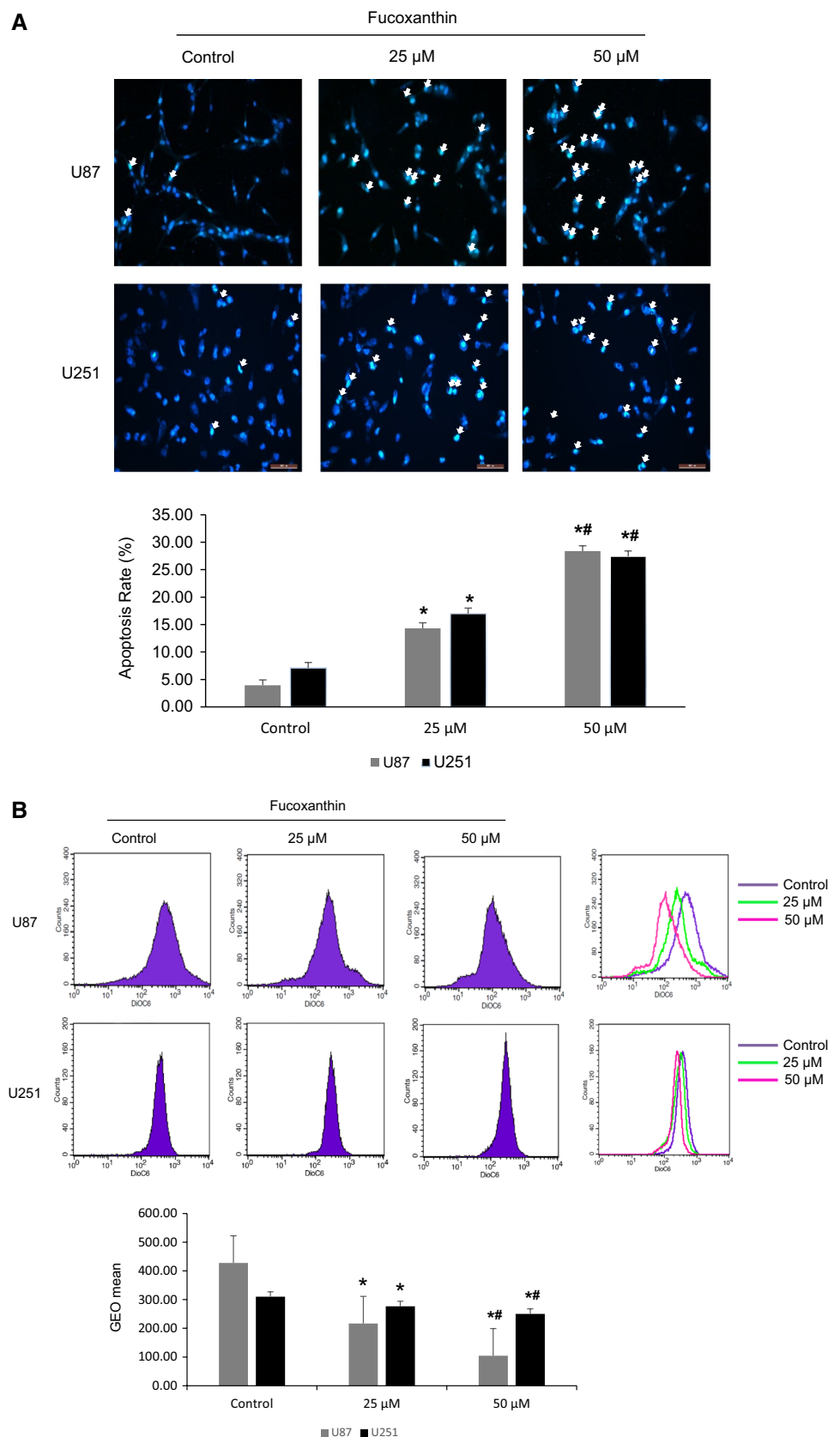


Fig. 2 Fucoxanthin induces the activation of glioblastoma cells apoptosis progression. **a** Apoptotic bodies were observed by fluorescence microscopy in cells stained with Hoechst 33342 dye and quantified. *Significant difference from control ($p < 0.05$), and #significant difference from lower concentration-treated cells ($p < 0.05$). **b** $\Delta\psi_m$ was analyzed by flow cytometer after staining cells with DIOC6(3) (GEO, geometric). *Significant difference from control ($p < 0.05$), and #significant difference from lower concentration-treated cells ($p < 0.05$). **c** Different stages of apoptosis were performed by flow cytometry. *Significant difference from control ($p < 0.05$), and #significant difference from lower concentration-treated cells ($p < 0.05$). **d, e** Cell lysates were electrophoresed and apoptotic proteins were detected by their respective specific antibodies in indicated concentrations. Each *bar* represents the mean \pm SEM in triplicate experiments. **f** Observation of the microstructure of U87 cells treated with fucoxanthin. *Left image* untreated cells had a normal nucleus, cytoplasm and organelle morphology. *Middle image* the early stage of apoptosis had chromosome condensation after treatment U87 cells with 50 μM for 12 h. *Right image* the late stage of apoptosis moved enriched chromatin to area around nuclear membrane after treatment U87 cells with 50 μM for 24 h



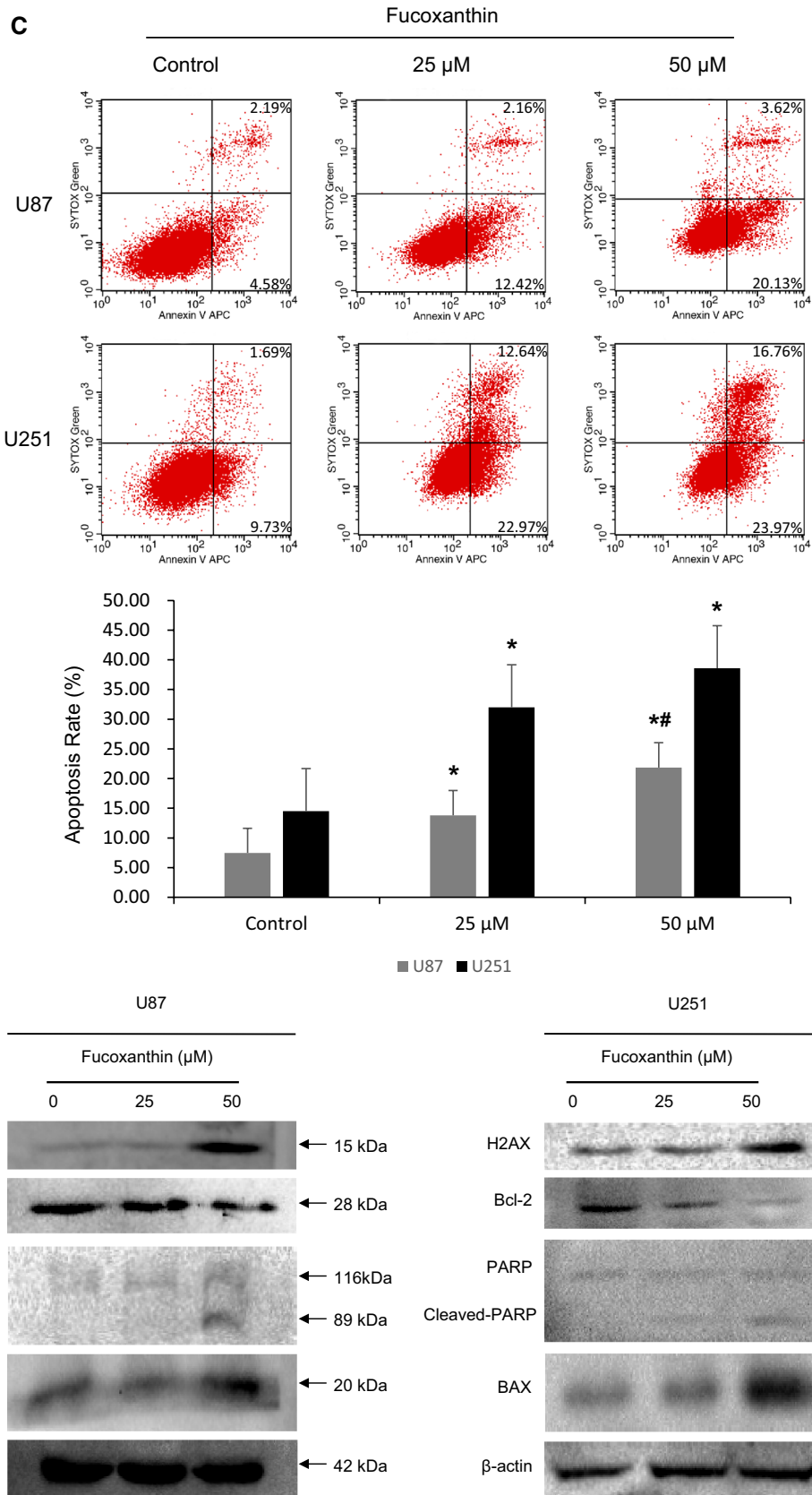
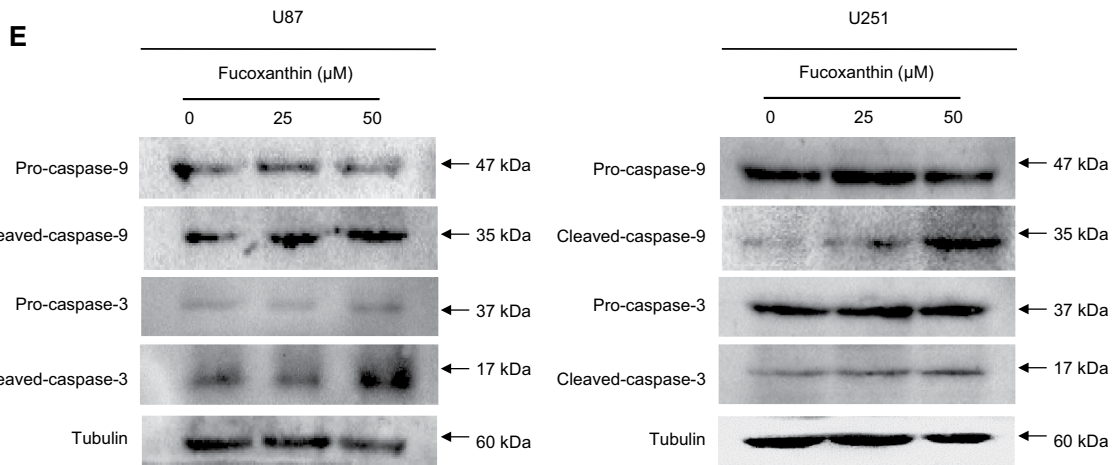
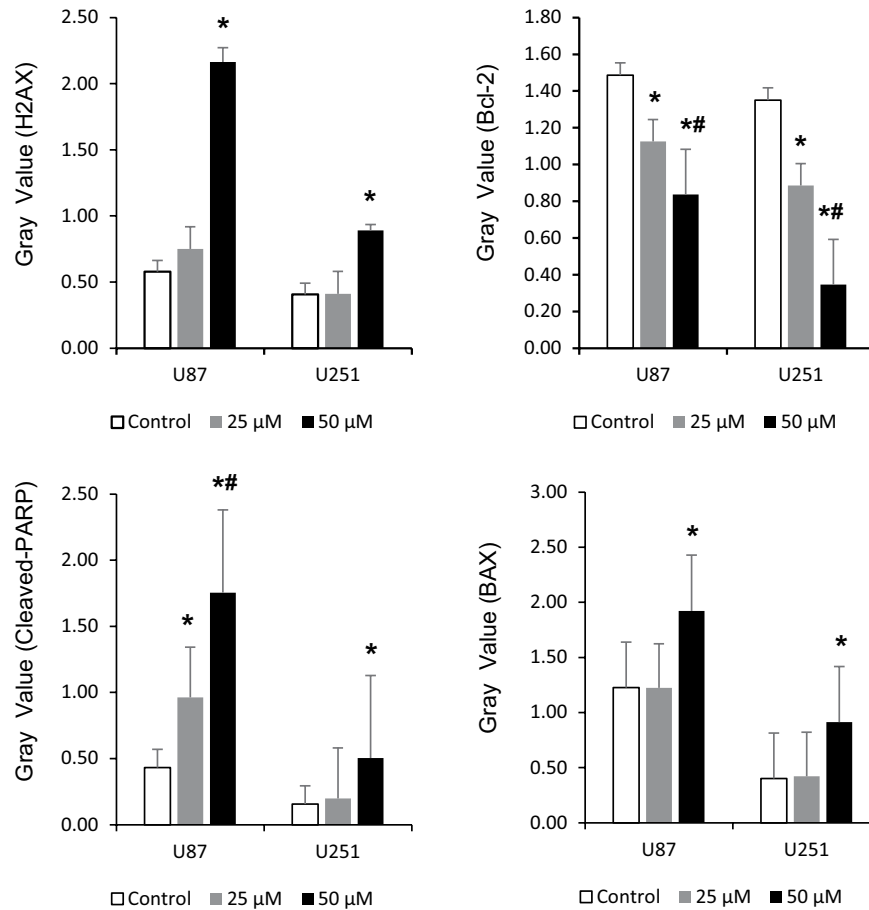


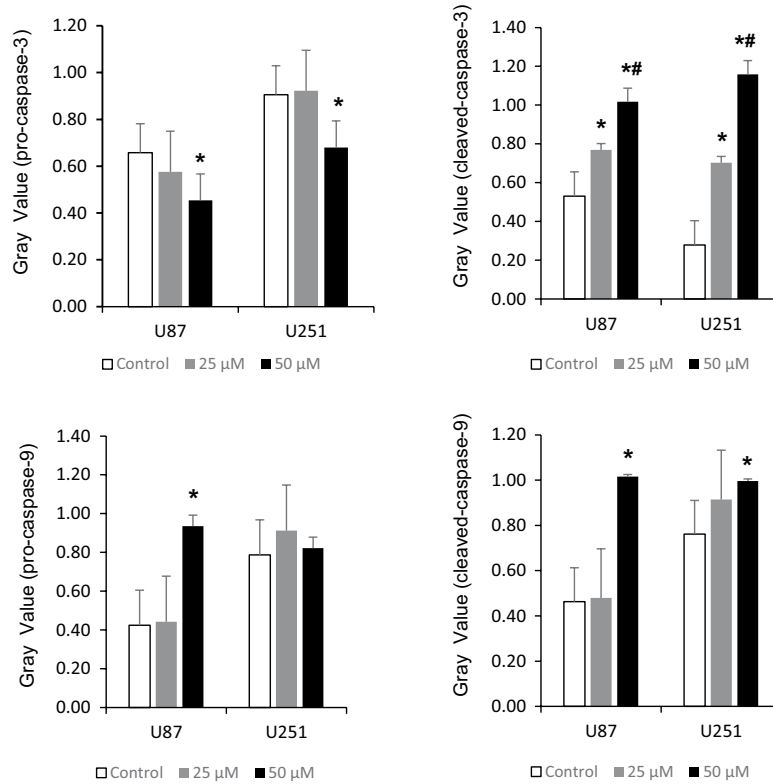
Fig. 2 continued

D Continuing**Fig. 2** continued

invading cells were dyed with 0.1% crystal violet. Washed the trans-well chambers with the water and captured images by using Image-Pro Plus software under a phase-contrast microscope (magnification, 200 \times).

Crystal violet in the invading cells was dissolved with 500 μL 33% acetic acid. The absorbance of crystal violet dissolved in acetic acid was measured at 570 nm using a microplate reader.

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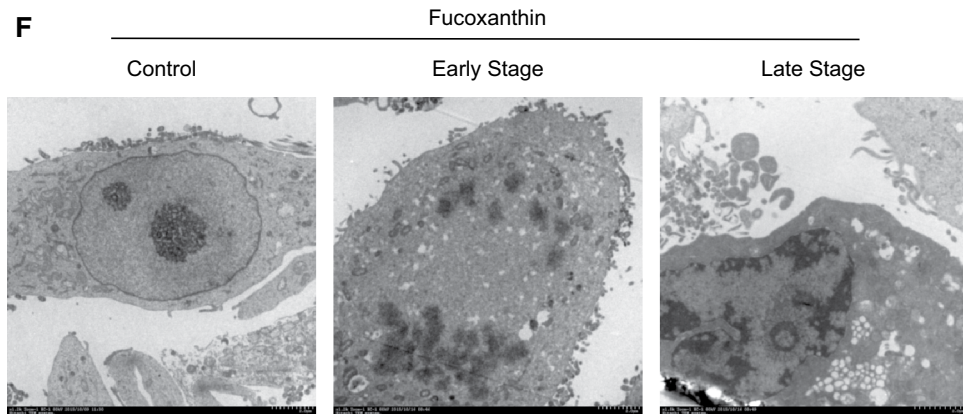


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Western Blot Analysis

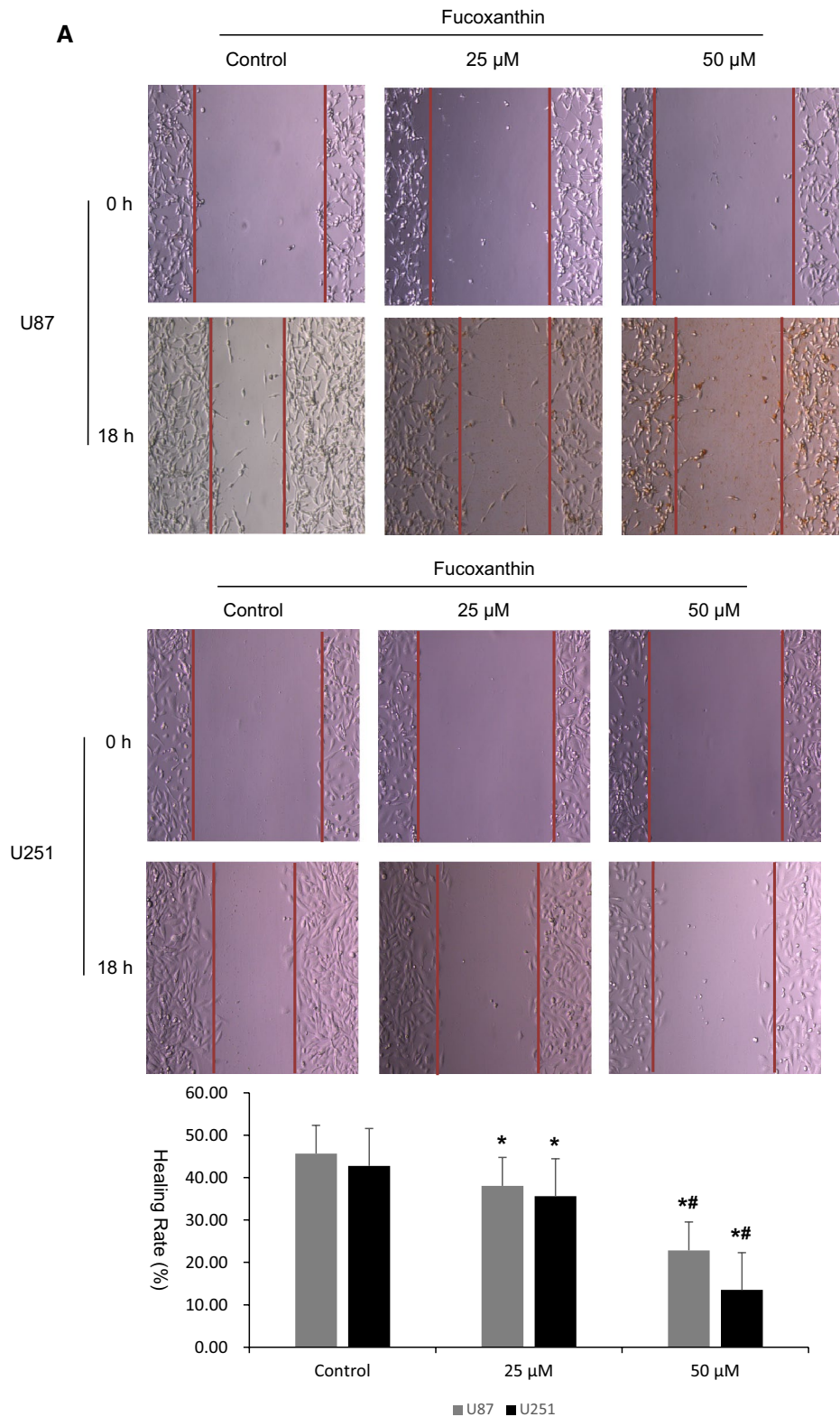
Cells were lysed on ice for 30 min in 100 μL lysis buffer (120 mM NaCl, 40 mM Tris (pH 8), and 0.1% NP-40) and centrifuged at 13,000×g for 15 min. Supernatants were collected and the protein concentrations were determined. Aliquots containing 40 μg of protein were boiled for 5 min and electrophoresed on 10% SDS–polyacrylamide gels. Proteins were transferred to nitrocellulose membranes, which were subsequently incubated with a primary antibody (dilution of 1:1000) overnight at 4°C. The membranes were

further incubated with horseradish peroxidase-conjugated anti-immunoglobulin-G (Pierce, Rockford, IL, USA). Protein bands were detected using an enhanced chemiluminescence western blotting detection kit (Amersham, Little Chalfont, Buckinghamshire, UK).

In Vivo Tumour Xenograft Study

Five-week-old female nude mice, 20–25 g body weight (BALB/c-nude; Vitalriver, Beijing, Beijing, People’s Republic of China) were housed into individually ventilated

Fig. 3 Fucoxanthin suppresses the invasion and migration of glioblastoma cells. **a** Images of the scratchwound healing assay were captured at 0 and 18 h, and the wound gaps were analyzed by measuring the distance of migrating cells for three regions of each wound. *Significant difference from control ($p < 0.05$), and #significant difference from lower concentration-treated cells ($p < 0.05$). **b** Cells were incubated with indicated concentrations of fucoxanthin for 24 h. Photographs are of the cell invasion through the matrigel membrane stained by crystal violet. **c** Cells were treated with indicated concentrations of fucoxanthin for 24 h. The representative images of migrate cells at the bottom of the membrane stained with crystal violet were visualized. *Significant difference from control ($p < 0.05$), and #significant difference from lower concentration-treated cells ($p < 0.05$). **d** Cell lysates were electrophoresed and MMP-2/9 and uPA proteins were detected by their respective specific antibodies in indicated concentrations. *Significant difference from control ($p < 0.05$), and #significant difference from lower concentration-treated cells ($p < 0.05$)



cages, and free accessed to food and drinking water. U87 cell (5×10^6 cells) dissolved in 0.1 ml PBS was injected subcutaneously into the abdominal. Tumor sizes were measured with a caliper and calculated as tumor size (mm^3) = $1/2 \times \text{length} \times \text{width}^2$.

The tumor-bearing mice were randomly divided into two groups ($n = 6/\text{group}$) and treatment of mouse with fucoxanthin was initiated on the day after cell injection. Fucoxanthin was dissolved in soybean oil and 1 g/kg or soybean oil by oral gavage every day for 28 days. Tumor sizes and

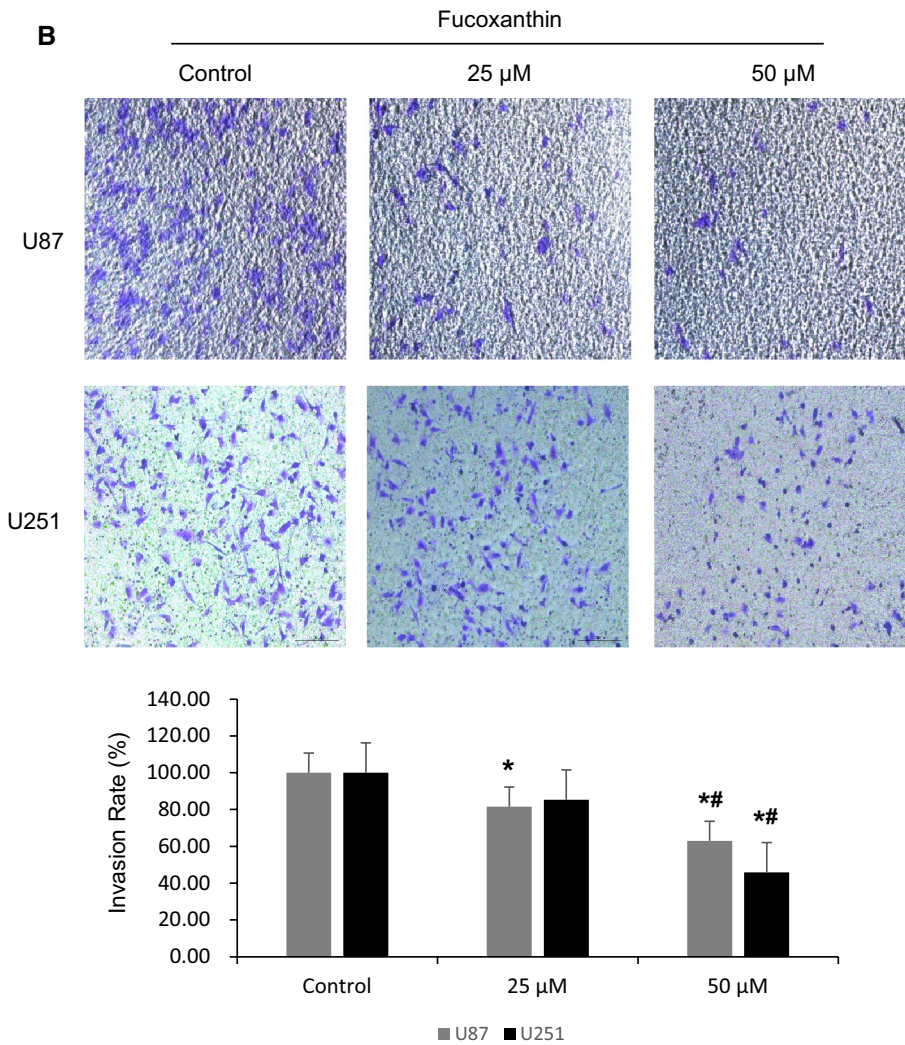


Fig. 3 continued

body weights were measured two or three times weekly. On day 28, all mice were euthanized. Primary tumors were dissected out for measurement of weight and volume, and related proteins with western blot, and staining with hematoxylin and eosin (H&E) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) using a commercial kit (Beyotime Biotechnology Inc, Nantong, China).

Statistical Analysis

All measurements were performed in triplicate and all values are expressed as the mean±the standard error. The results were subjected to an analysis of variance (ANOVA) using Tukey’s test to analyze differences between means. In each case, a P value <0.05 was considered statistically significant.

Results

Fucoxanthin Inhibites Cell Proliferation of Human Glioblastoma Cells

The effect of fucoxanthin on inhibition of U87 and U251 cells growth were detected by MTT assay, which was conducted in a concentration- and time-dependent manner. The MTT data revealed that treatment U87 and U251 cells with 25, 50, 75 and 100 μ M of fucoxanthin significantly decreased the level of cell viability incubation for 12, 24 and 48 h, compared with blank control and control groups (Fig. 1a, b). It is noteworthy that fucoxanthin had no suppression on the cell viability of normal neurons which indicated that the drug could be developed into the clinical chemicals because of avirulence for human normal neurons. Additionally, the

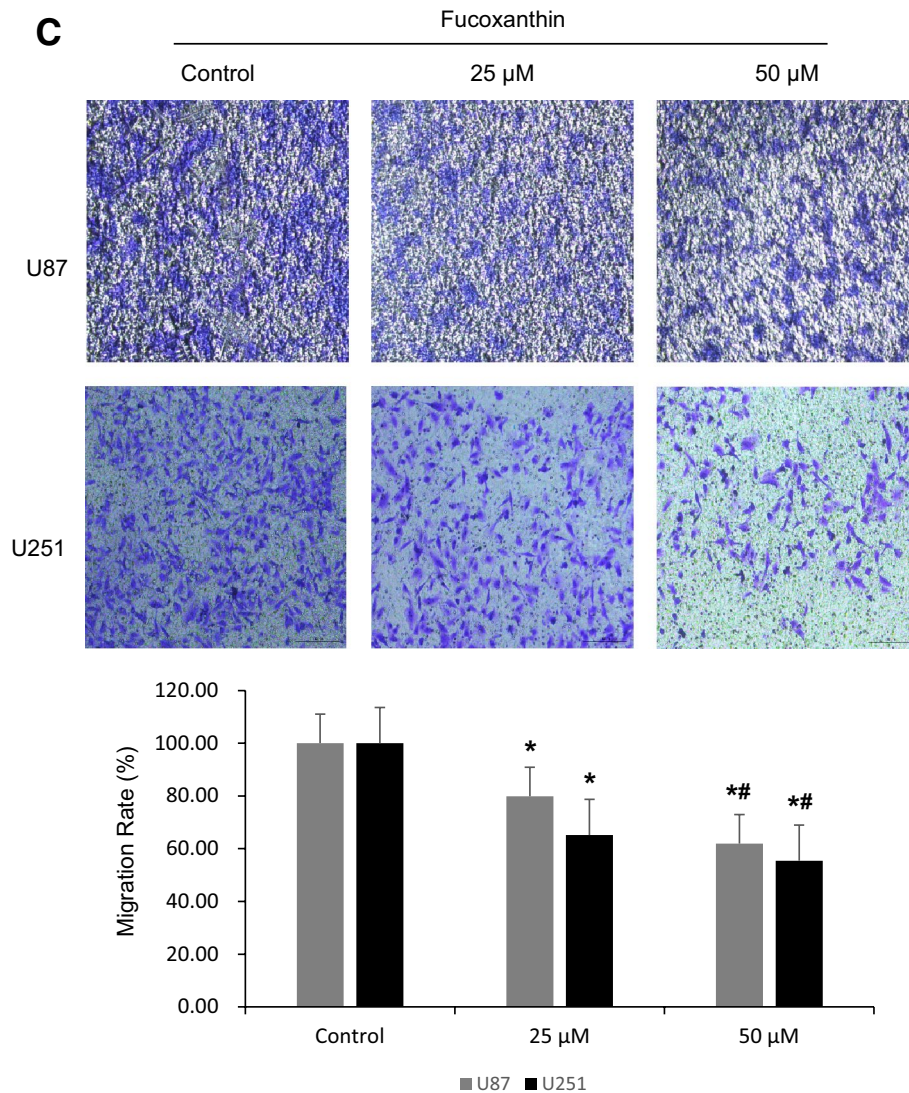


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lower concentration of fucoxanthin (6.25 and 12.5 μM) was not cytotoxic towards glioma cells and 25 μM is the middle concentration to initiate the anti-proliferation of fucoxanthin. Based on these results, 25 and 50 μM were chosen as the optimal concentrations of fucoxanthin for further investigation.

Fucoxanthin Induces Apoptotic Progress in Human Glioblastoma Cells

To evaluate the toxic effect of U87 and U251 cells on apoptosis induced by fucoxanthin, the cellular nuclei were stained with hoechst 33342 and then assessed by microscopy. Observation with microscopy, fractured nuclei were both viewed in fucoxanthin-treated U87 and U251 cells at 25 and 50 μM compared to intact nuclei observed in control cells (Fig. 2a) and the apoptotic rates were 14.32 and

17.00 at 25 μM , and 28.36 and 27.31 at 50 μM , respectively. Similarly, the expression of H2AX, a hallmark of chromosome damage, was increased which indicated that fucoxanthin impaired U87 and U251 cells via apoptosis initiation (Fig. 2d). In addition, depolarization of mitochondrial potential ($\Delta\psi\text{m}$) is indispensable part involved in apoptotic program and we examined whether fucoxanthin caused apoptosis via disruption of mitochondria normal function. DiOC6(3) is a cyanine dye that suggests $\Delta\psi\text{m}$ change by shifting its fluorescence emission from blue to yellow [20]. As shown in Fig. 2b, the maximum absorption wavelength was blue shift after treatment U87 and U251 cells with fucoxanthin by showing reduced GEO means suggesting the disruption of $\Delta\psi\text{m}$ and destroyed the mitochondrial function, and effect of 50 μM was better than that of 25 μM concentration. Impaired mitochondrial membrane releases the cytochrome *c* into cytoplasm which activates

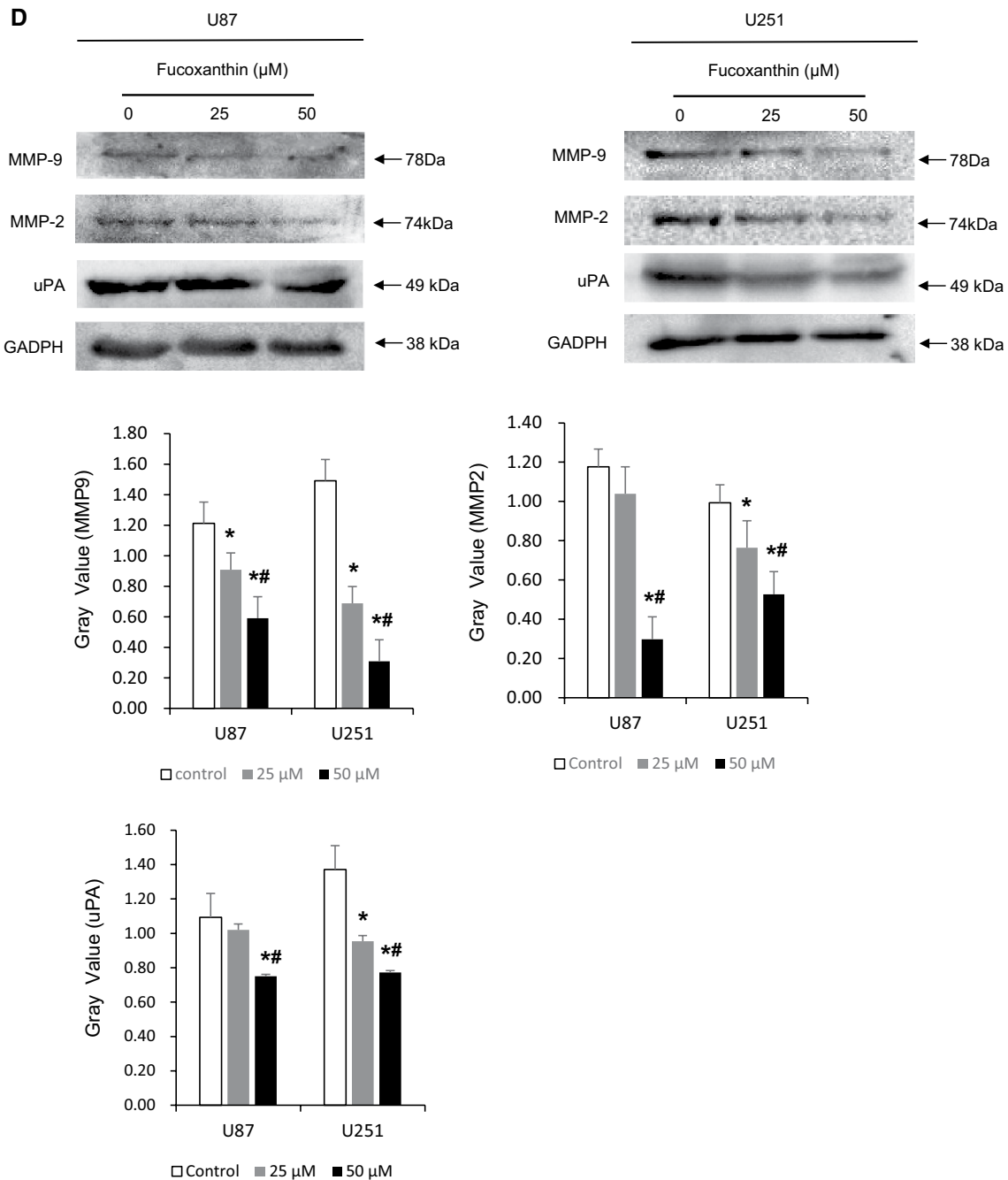


Fig. 3 continued

the apoptosis progression [21]. As shown in the Fig. 2c, apoptosis was detected in the fucoxanthin-incubated cells by performing Annexin V-FITC/SYTOX Green double staining which could distinguish different stage of apoptotic cell. Positive staining of annexin means the early apoptosis by binding of phosphatidylserine which changes the location from the inner to outer of cell membrane at the beginning of the apoptosis [22]. SYTOX Green then binds to DNA when membrane permeability is increased caused by further development of apoptosis [23]. The degree of early

and late apoptosis of U87 and U251 were 2.16 and 12.42%, and, 12.64 and 22.97% at the lower concentration of fucoxanthin, respectively, and it increased to 3.62 and 20.13%, and, 16.76 and 23.97% at the higher concentration which were significant difference compared with that of control groups (Fig. 2c). To further understand the mechanism of fucoxanthin-induced apoptotic process, we explored the expressions of proteins related to mitochondrial apoptosis. Firstly, we examine the changes of protein expression between Bcl-2 and Bax, which are anti-apoptotic protein

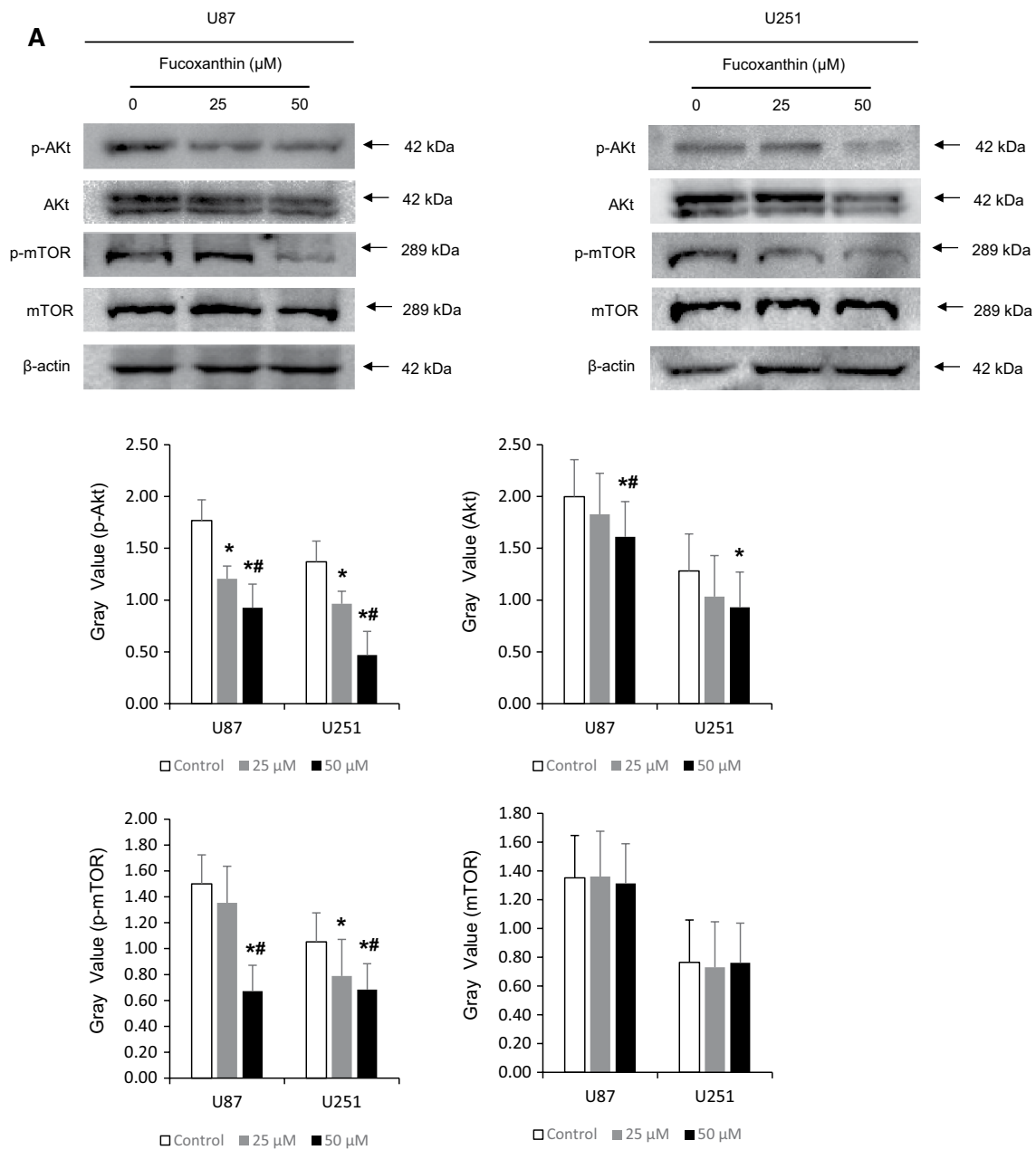


Fig. 4 Fucoxanthin promotes apoptosis via inhibition of PI3K/Akt/mTOR signaling pathway in glioblastoma cells. **a** Cell lysates were electrophoresed and Akt, p-Akt, mTOR and p-mTOR proteins were detected by their respective specific antibodies in indicated concentrations. *Significant difference from control ($p < 0.05$), and #significant difference from lower concentration-treated cells ($p < 0.05$). After

treatment with LY294002 for 1.5 h, and with fucoxanthin for 24 h, **(b)** apoptotic related proteins were detected using their respective specific antibodies in indicated times. *Significant difference from control ($p < 0.05$), #significant difference from fucoxanthin-treated cells ($p < 0.05$), and ##significant difference from LY294002-treated cells ($p < 0.05$)

and pro-apoptotic protein. As shown in Fig. 2d, fucoxanthin showed an increment in Bax expression and a decrement in Bcl-2 expression compared with the control cells both in U87 and U251 cells. The balance of Bcl-2/BAX dominates the switch which turns on or off the apoptosis [24]. Notably, fucoxanthin initiated the activation of apoptotic pathway via Bax folded expression and it transduces the signal to the downstream-caspase system [25]. As expected, the

enhanced activation of cleaved-PARP, caspase-9 and -3 was showed in fucoxanthin-treated groups (Fig. 2e). Likewise, similar result was also obtained by electron microscopy. Transmission electron microscope (TEM) could find some changes of ultrastructure, including organelle and chromosome [26]. We visualized the phenomenon of early and late apoptosis after treatment of U87 cells with fucoxanthin by means of electron microscope. The middle picture displayed

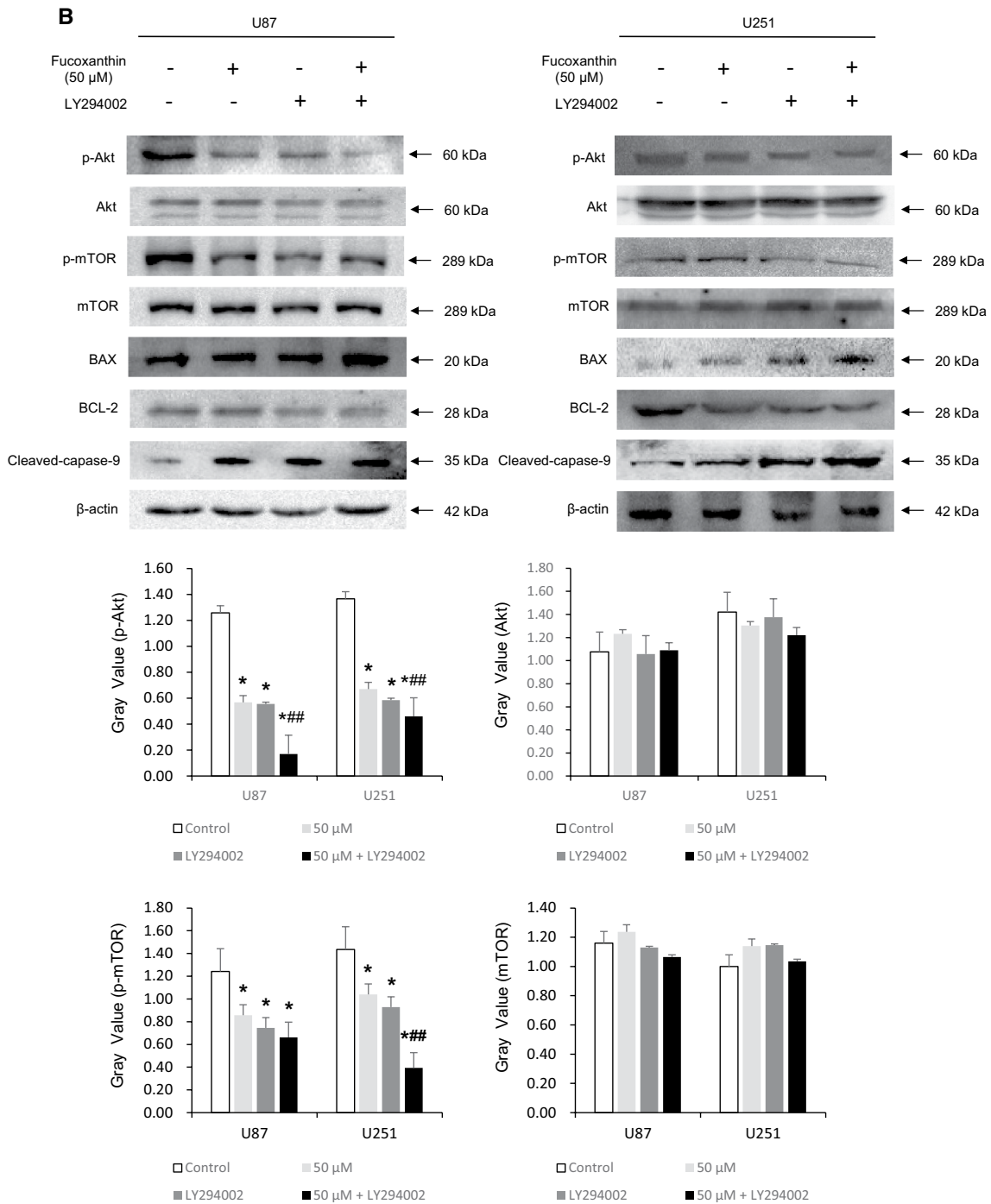


Fig. 4 continued

the chromosome condensation which suggested that fucoxanthin initiated the apoptotic process as well as the early stage of apoptosis after incubation for short period (Fig. 2f). And then, the enriched chromatin was moved to the edge of nuclear membrane shown in right one, which was the indicator of advanced period of apoptosis. These results suggest that fucoxanthin promotes cell death by induction apoptosis via mitochondrial pathway.

Fucoxanthin Inhibits the Invasion and Migration of Human Glioblastoma Cells

Invasiveness and migration are the pathological signatures of human malignant gliomas, the effects of fucoxanthin on the invasiveness and migration of U87 cells were checked by scratch wound healing assay and trans-well assays [27]. The scratch wound healing assay revealed that both lower

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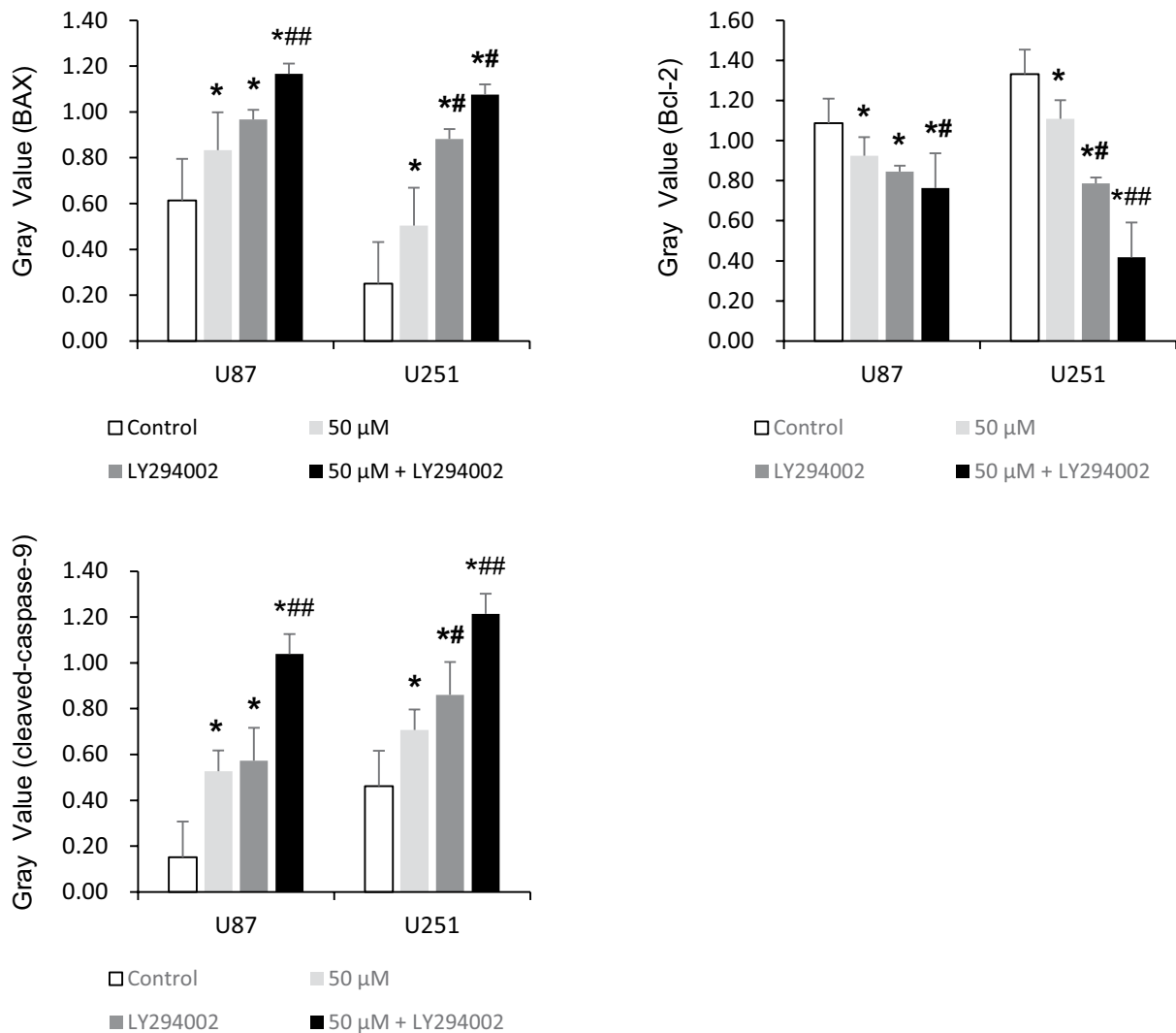


Fig. 4 continued

and higher concentration of fucoxanthin exposure remarkably restrained the invasion of U87 and U251 cells showing by the reduced healing rate compared to that of untreated-cells (Fig. 3a). Similarly, another outcome of the in vitro, trans-well assay, certified that treatment of U87 and U251 cells with fucoxanthin obviously reduced cells to infiltrate the membranes with (Fig. 3b) or without matrigel (Fig. 3c) compared with untreated groups which indicated that fucoxanthin indeed had ability to control the translocation of U87 cells to adjacent tissue or other parts of body. Besides, we further investigated the mechanisms underlying fucoxanthin-inhibited properties of migration and invasion of glioblastoma cells. It has been proven that the increased degree of matrix metalloproteases (MMPs) expression is closely associated with invasiveness and migration of human gliomas [28]. MMPs promote tumor growth, angiogenesis,

metastasis and invasion by degradation of extracellular matrix to break down structure of tissue and cell or provide various growth factors [29]. Therefore, we selected two kind of gelatinase, MMP-2 and MMP-9, as the objective to explore the potential mechanism of fucoxanthin on human glioblastoma cells. As expected, the protein levels of MMP-2 and MMP-9 were undoubtedly suppressed by incubation glioma cells with fucoxanthin at the concentration of 25 and 50 μ M (Fig. 3d). The serine protease urokinase-type plasminogen activator (uPA) is upstream signaling and regulates the expression of MMPs to influence the characteristics of malignant glioma cells [30]. As shown in Fig. 3d, we observed that the expression of uPA was reduced by exposure of glioma cells with fucoxanthin suggesting the effect of drug on U87 and U251 cells via inhibition of uPA-MMPs systems. These findings indicate that the application

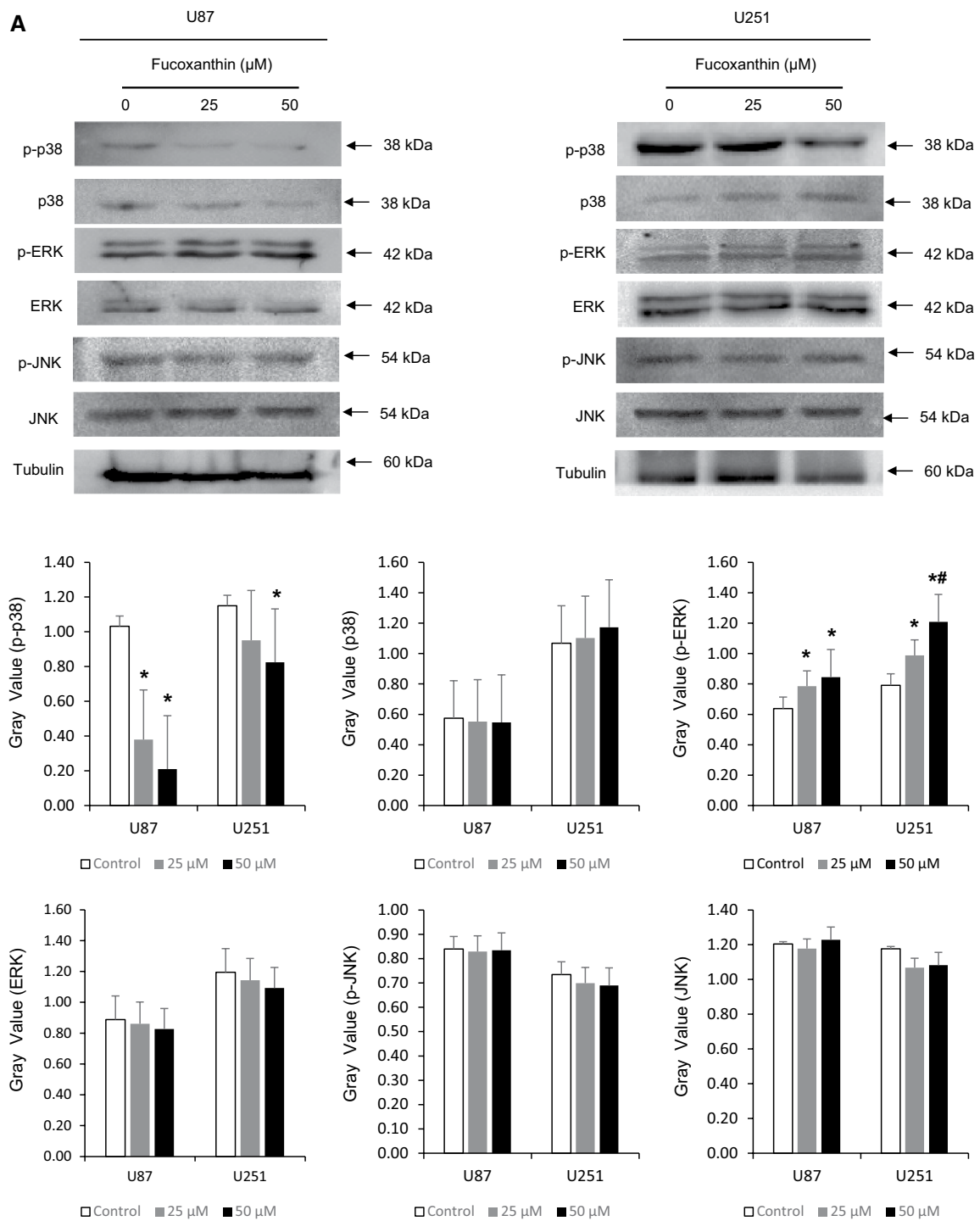


Fig. 5 p38 kinase involves the fucoxanthin-mediated reduction of invasion and migration of U87 cells. **a** Cell lysates were electrophoresed and ERK, p-ERK, p38 and p-p38 proteins were detected by their respective specific antibodies in indicated concentrations. *Significant difference from control ($p < 0.05$), and #significant difference from lower concentration-treated cells ($p < 0.05$). After treatment with

SB203580 for 1.5 h, and with fucoxanthin for 24 h, **(b)** metastasis related proteins were detected using their respective specific antibodies in indicated times. *Significant difference from control ($p < 0.05$), #significant difference from fucoxanthin-treated cells ($p < 0.05$), and ##significant difference from SB203580-treated cells ($p < 0.05$)

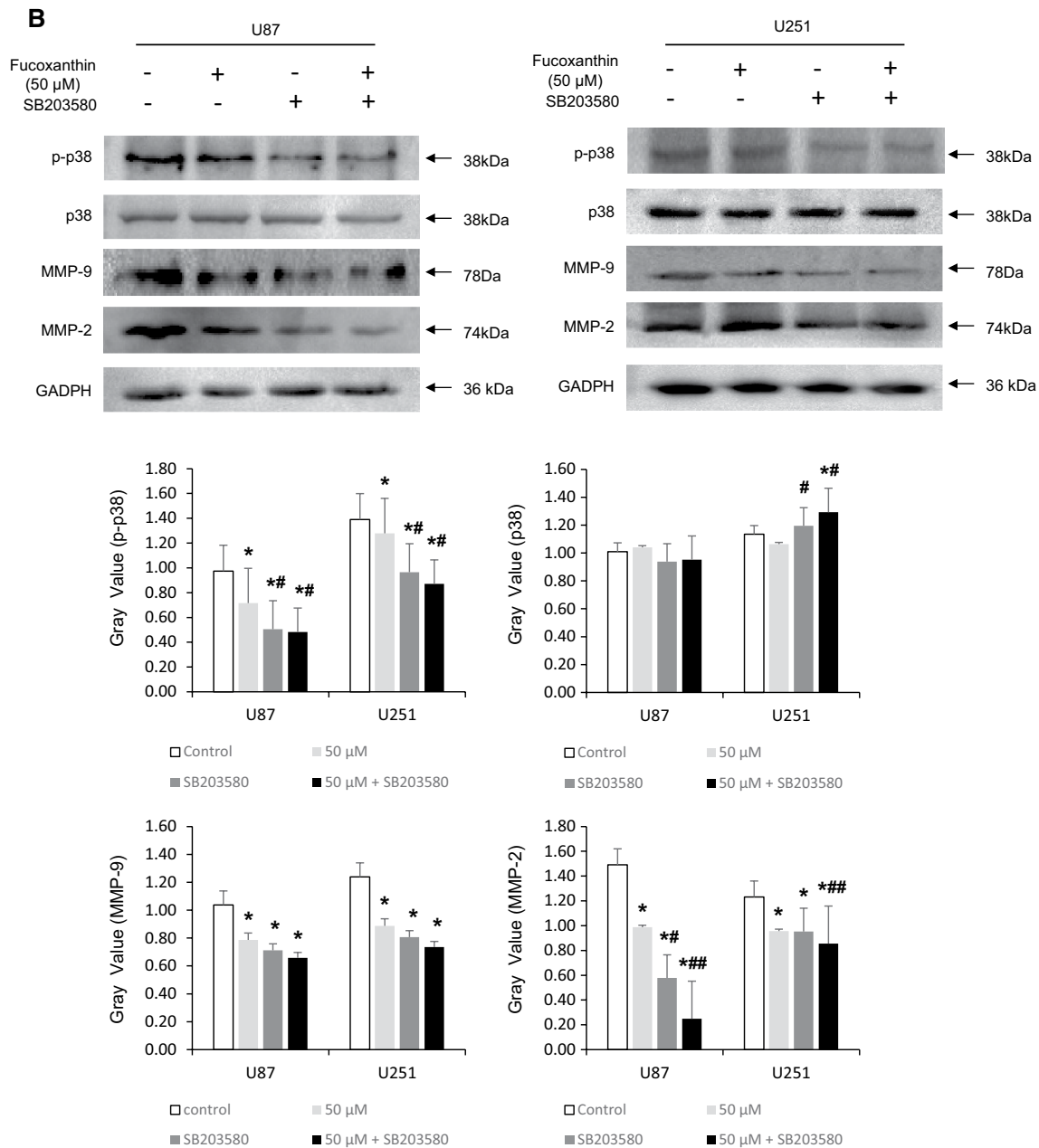


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of fucoxanthin inhibits the invasive and migrative ability of glioma cells in vitro.

Fucoxanthin Induces Apoptosis by Inhibition of PI3K/Akt/mTOR Signaling Pathway

It's well known that apoptosis is closely related to many molecular signaling pathways which are involved in the process of initiation, activation or deactivation. It has been reported that the apoptosis is down-regulated by the PI3K/Akt/mTOR signaling pathway [31]. Moreover, the induction

of the programmed cell death, as termed apoptosis, is a promising therapeutic approach and target when the PI3K/Akt/mTOR system is inhibited by drugs treatment [32]. Therefore, we confirmed that whether the effect of fucoxanthin on apoptosis of glioma cells was caused by negative regulation of PI3K/Akt/mTOR pathway. As shown in Fig. 4a, we observed that the expression of phosphorylation of Akt and mTOR was dramatically decreased by treatment of glioma cells with fucoxanthin at the concentration of 25 and 50 μ M, and the level of phosphorylated mTOR of glioma cells was notably reduced only at higher concentration of fucoxanthin.

These results suggest that fucoxanthin indeed inhibits the PI3K/Akt/mTOR pathway by reduction of their phosphorylated forms. To further prove the positive effect of fucoxanthin on apoptosis via suppression of PI3K/Akt/mTOR system, we incubated glioblastoma cells with PI3K inhibitor prior to fucoxanthin treatment (50 μ M). Undoubtedly, the expressions of active form of Akt/mTOR were lessened by exposure of glioma cells with fucoxanthin or PI3K inhibitor, individually (Fig. 4b). Ulteriorly, the application of LY294002 had the semblable bands with fucoxanthin of western blotting on the expression of Bax, Bcl-2, cleaved-caspase-9, which indicated that the inhibition of PI3K/Akt/mTOR by fucoxanthin exactly induced the activation of apoptosis (Fig. 4b). Eventually, the combination of LY294002 and fucoxanthin had more effect on apoptosis related proteins, compared to that of fucoxanthin or PI3K inhibitor. These results demonstrate that the inductive effect of fucoxanthin on apoptosis is performed via suppression of PI3K/Akt/mTOR system.

Fucoxanthin Suppresses the Capability of Invasion and Migration by Inhibition of p38 Signaling Pathway

Previous reports have demonstrated that MMPs-caused invasion and migration of tumor cells are mediated by the family of mitogen-activated protein kinases (MAPKs) [33]. There three classes of MAPK, including extracellular signal-regulated kinases (ERK), stress-activated JUN N-terminal kinases (JNKs) and p38 [34]. Next, we investigated that the whether the diminishment of invasion and migration was facilitated by MAPKs after treatment of cells with fucoxanthin. As illustrated in Fig. 5a, the degree of phosphorylated-p38 expression was markedly decreased in a concentration-dependent manner. Conversely, fucoxanthin abnormally increased the protein levels of phosphorylated-ERK which should promote the cancerous cells to invade or infiltrate into other parts. However, fucoxanthin could not influence the expression of p-JNK/JNK. Therefore, we verified that if the p38 was the upstream target of MMPs which was inhibited by chemical in our article. As expected, the MMPs was significantly reduced by the p38 inhibitor (SB203580) treatment which indicated that the p38-MMPs signaling pathway involved the fucoxanthin-mediated the reduction of disruption of other normal tissue of glioma cells (Fig. 5b). Hence, fucoxanthin negatively regulated the activity of glioma cells on invasion and migration mediated by suppression of activation of p38.

Effects of Fucoxanthin on Glioblastoma Xenografts of U87 Cells in BALB/c-Nude Mice

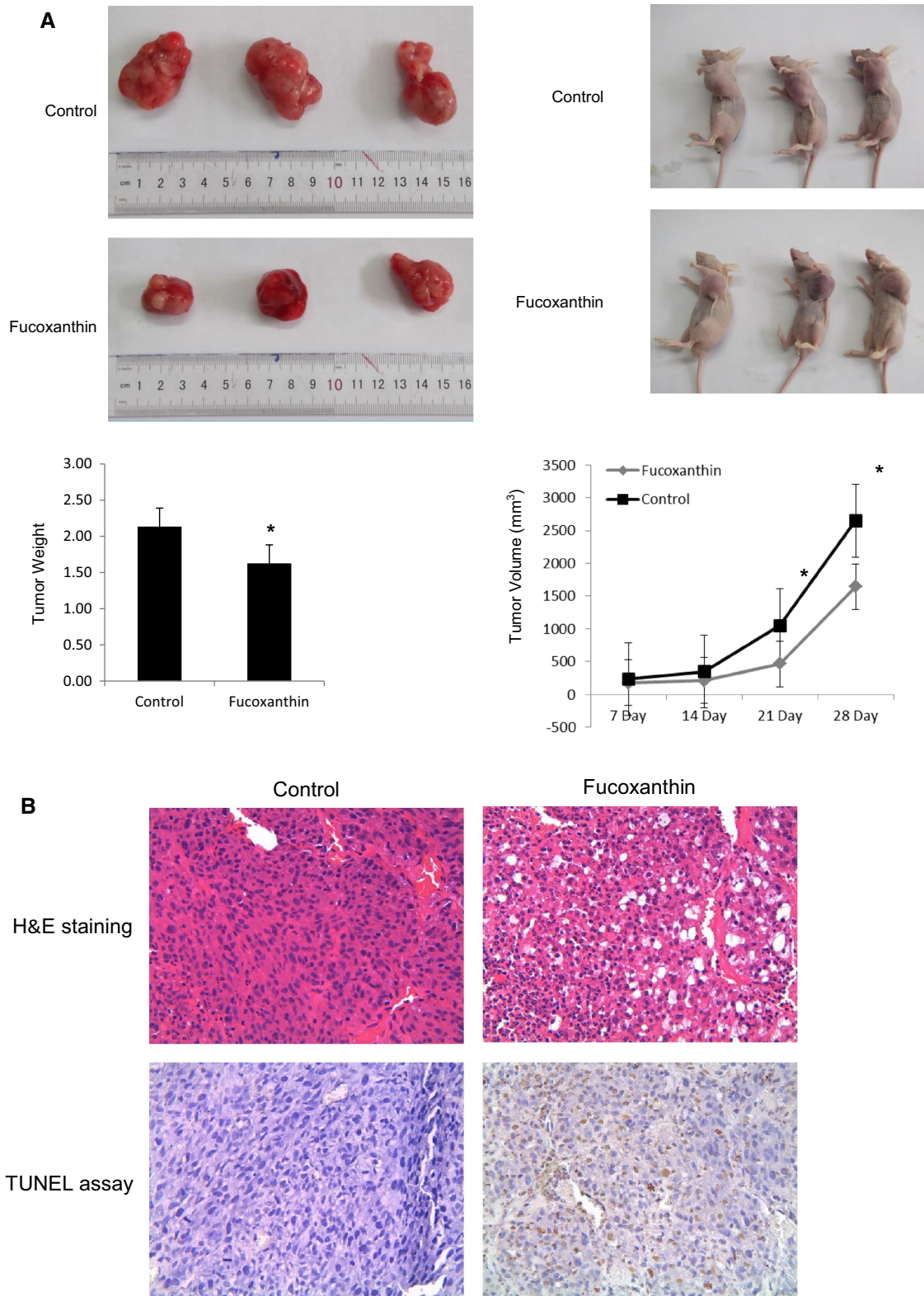
We had demonstrated that fucoxanthin was capable of inhibition of proliferation, invasion and migration, and induction of apoptosis on U87 cells in vitro. Next, we inspected

Fig. 6 Fucoxanthin inhibited U87 xenograft growth in vivo. **a** U87 cells were injected into the nude mice and tumors were allowed to grow. Subsequently, fucoxanthin (dissolved in soybean oil, 1 g/kg/day body weight) was given by oral gavage daily i.p. for 28 day (n=6). The control group was given a gavage of soybean (1 g/kg body weight) every day i.p. (n=6). The changes in tumor weight and growth were shown. *Significant difference from control ($p < 0.05$), and #significant difference from lower concentration-treated cells ($p < 0.05$). **b** Pathological sections derived from tumor mass were examined by HE staining and TUNEL assay. *Significant difference from control ($p < 0.05$), and #significant difference from lower concentration-treated cells ($p < 0.05$). **c** At the end of the experiment, tumor tissues were excised from mice, and the protein lysates extracted from the tumor tissues were assessed by immunoblotting for p-Akt, p-mTOR, p-p38, MMP-2/9, BCL-2, BAX and cleaved-caspases-9, respectively. *Significant difference from control 1 ($p < 0.05$), #significant difference from control 2 ($p < 0.05$), and &significant difference from control 3 ($p < 0.05$)

that whether fucoxanthin had the similar results in vivo. We assessed the anti-cancer capability of fucoxanthin using immune deficient nude mice (BALB/c-nude) as the testing model. Subcutaneous injection of U87 cells significantly increased from 234 mm³ to 2647 mm³ in size within 3 weeks, respectively, exhibiting rapid growth of glioma cells in the host mice (Fig. 6a). However, the rates of tumor growth in fucoxanthin-treated group was conspicuously reduced showing the average tumor volume were 1644 mm³. Additionally, we examined the function of fucoxanthin on U87 cells in vivo by histochemistry and immunohistochemistry methods. H&E staining illustrated that the cell density was declined and the morphology was changed with irregular cell shape, and scattered nuclei showing by light and uneven purple color (Fig. 6b). TUNEL assay certified that apoptosis was appeared after treatment U87 cell with fucoxanthin showing by staining aberrated chromosome with brown color (Fig. 6b). And then, we explored the underlying signaling pathway mediated by fucoxanthin in vivo via extraction proteins from tumor mass of U87 cells. As illustrated in Fig. 6c, fucoxanthin enhanced the expression level of apoptotic-related proteins and inhibited the activation of PI3K/Akt/mTOR and p38 system to accomplish reduction of invasion and migration of U87 tumor. These results denote that administration of these mice with 200 mg/kg/day of fucoxanthin after inoculation of U87 cells significantly reduce of the tumor volume and weight due to the emerging apoptotic cells and decline of invasion and migration in the U87 cells.

Discussion

In our study, we applied glioma U87 and U251 cells as a testing model to explore the role of the fucoxanthin on the induction of apoptosis, and suppression of invasion and migration. The main question addressed by this study was whether fucoxanthin could exhibit anti-cancer property on



glioma cells and excavated the feasible mechanism that played a pivotal role in the functions of fucoxanthin on U87 and U251 cells. Herein, we proven that fucoxanthin promoted apoptosis through activation of PI3K/Akt/mTOR, and suppressed invasion and migration via involvement of

p38/MMPs signaling pathway, leading to the novel chemical strategy of defense of malignant glioma cells.

Gliomas are the most common malignant primary brain tumors with enhanced signal of computed tomography and magnetic resonance image [1], and U87 and U251 cell lines

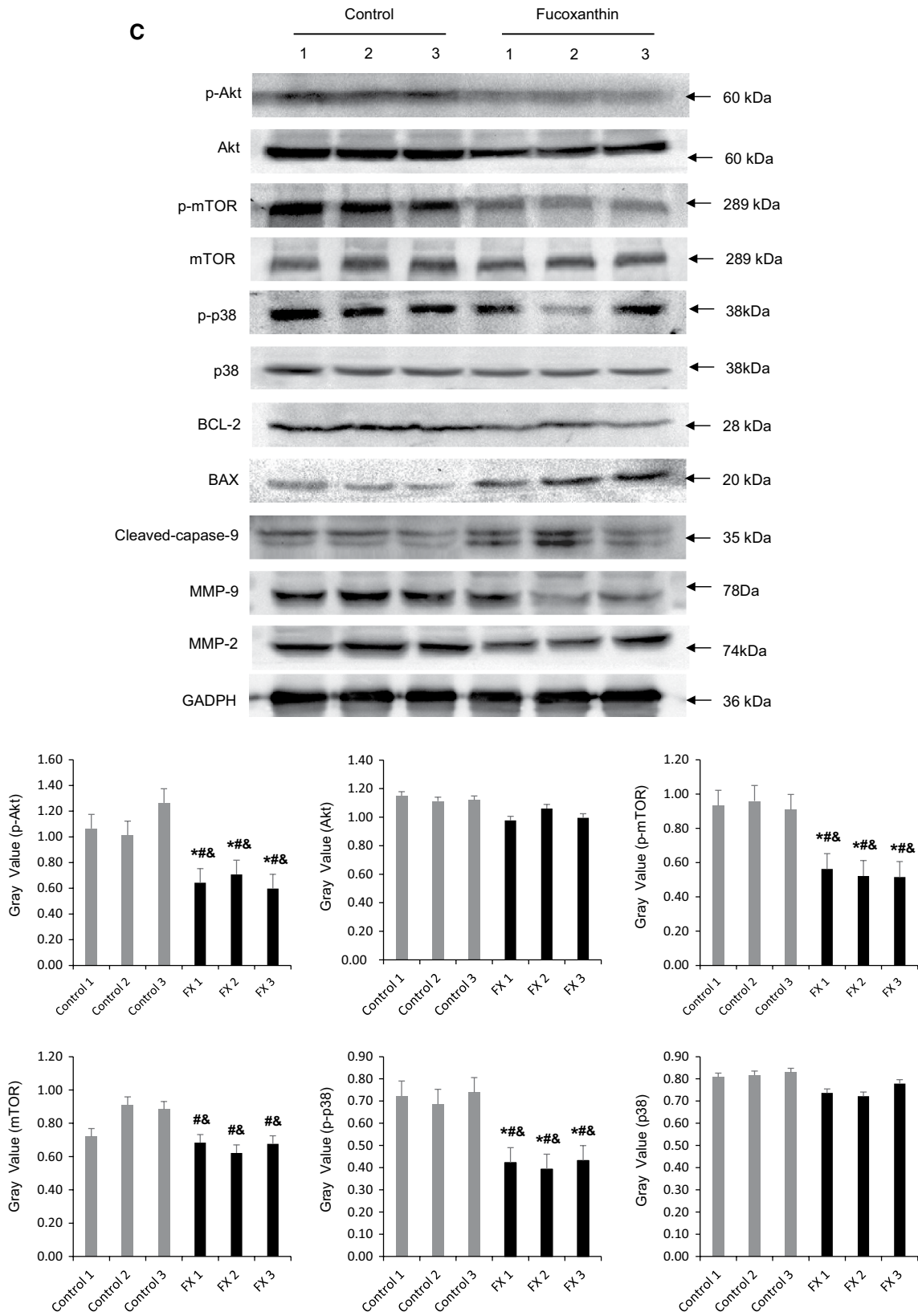


Fig. 6 continued

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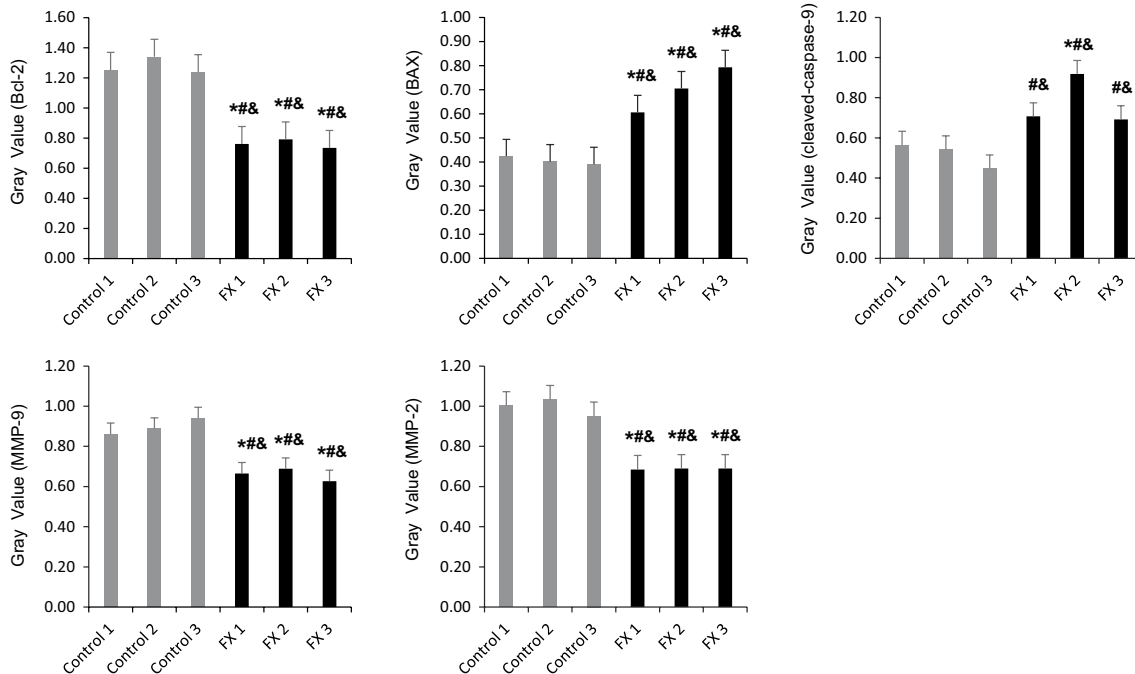


Fig. 6 continued

in this study are belonging to GBM which presents irregular growth pattern of cell proliferation and angiogenesis, and robustly invade or infiltrate into the surrounding normal brain tissue or migrate to distant regions of body [35–37]. Although employing resection surgery, radiation or chemotherapy, it ultimately difficult to achieve complete killing the cancer cells, therefore, the new treatment method and therapeutic targets are indeed required [38]. Previous studies have certified that vitamin A or its metabolic intermediates retinoic acid possess pretty effect on treatment of glioma cells in the aspects of inhibition of growth, and induction of apoptosis, cell cycle arrest, and prevention of invasion and migration [39–43]. As carotenoid, fucoxanthin has the similar chemical structure with retinoic acid, sharing the conjugated groups and active hydroxyl groups, and may display the anticancer functions. However, there is rare report about fucoxanthin's effect on glioma cells, hence, we investigated the foreseeable effect of fucoxanthin on the objective U87 and U251 cells.

Firstly, we explored that fucoxanthin reduced cell viability in a dose-dependent manner via MTT assay, suggesting the inhibitory efficacy of fucoxanthin on glioblastoma cells (Fig. 1a–b). As an added bonus, fucoxanthin did not impair the cell growth of normal neurons. Then, we investigated that whether fucoxanthin reduced cell proliferation via activation of programmed cell death apoptosis. Hoechst 33342 is an indicator of apoptosis which detects chromatin condensations and binds to them [44]. Observation with

microscopy, the binding between Hoechst 33342 and apoptotic cells was apparently increased after incubation of U87 and U251 cells with fucoxanthin in concentration-dependent manner (Fig. 2a). In addition, the double-strand breaks of DNA caused by external stress rapidly recruits abundant γ -H2AX which could be visualized by western blotting [45]. As shown in Fig. 2d, the expression of γ -H2AX was obvious accumulation after treatment with higher concentration of fucoxanthin. As a dynamic process, initiation, activation and execution comprise the entire system of apoptosis, and Hoechst 33342 and γ -H2AX are on behalf of primary stage of apoptosis. Next, the activation of apoptosis is characterized by mitochondrial membrane potential ($\Delta\psi_m$) alteration and abnormal expression of apoptotic related proteins [46, 47]. The staining result of DiOC6(3) revealed that the depolarization of $\Delta\psi_m$ of glioma cells were evidently exacerbated by exposure to fucoxanthin which not only displaced the maximum absorption wavelength but also influenced the intensity of fluorescence (Fig. 2b). Later, we analyzed the percentage of different stage of apoptotic cells through flow cytometry, and both early and late apoptosis cells were markedly augmented by fucoxanthin confirmed by SYTOX Green and FITC-Annexin staining, respectively (Fig. 2c). Moreover, the repression of $\Delta\psi_m$ changes the level of apoptotic proteins in mitochondrial and amplifies the permeability of mitochondrial membrane to release of cytochrome *c* into cytoplasm, and activates the induction of Bax/Bcl-2 ratio and execution of caspase cascade [48]. The expression

of Bcl-2 and Bax was significantly declined and added by 50 μ M fucoxanthin, individually (Fig. 2d). On the other hand, the pro-apoptotic proteins were remarkably increased by the drug including cleaved-PARP, -caspase-3 and -caspase-9 (Fig. 2e). Furthermore, we visualized the phenomenon of early and late apoptosis after treatment of U87 and U251 cells with fucoxanthin by means of electron microscope. In conclusion, fucoxanthin evoked the mitochondrial apoptosis to implement the inhibitory effect of fucoxanthin on glioblastoma cells.

Next, we uncovered the upstream signaling pathway which took participated in the fucoxanthin-mediated apoptosis. It has been reported that PI3K/Akt/mTOR system negatively regulates the programmed cell death in the gliomas, resulting in deterioration of normally pathophysiological functions. Phosphatidylinositol 3 kinase (PI3K) promotes accumulation of phosphatidylinositol (3–5) triphosphate [PtdIns (3,4,5)P3] by phosphorylation, which is an anchor for the Akt/protein kinase B (PKB/Akt) and 3-phosphoinositide-dependent protein kinase 1 (PDK1) [49–51]. Then, Akt translocates to inner side of the cytoplasmic membrane and becomes the active form by PDK1 phosphorylation [52]. Ultimately, Akt dislodges the inhibitor of mammalian target of rapamycin (mTOR), tuberous sclerosis complex (TSC1/2), leading the activation of mTOR and whole PI3K/Akt/mTOR system is finally activated [53]. More reports have been claimed that the activation of Akt directly or indirectly suppresses the apoptosis by influence the apoptosis related proteins, such as phosphorylation of Bax and caspase-9 [54, 55]. Compared to control group, the accumulation of phosphorylated-Akt and -mTOR was significantly decreased (Fig. 4a), and the degree of apoptosis was enhanced (Fig. 2) by application of fucoxanthin. To further investigate the possible relationship between programmed cell death and PI3K/Akt/mTOR signaling pathway, we utilized the PI3K inhibitor on western blotting. The handle of PI3K inhibitor certified that the activation of apoptosis indeed was caused by the inhibition of PI3K/Akt/mTOR, and the similar results were observed in fucoxanthin-treated cells (Fig. 4b). Of particular importance is the combination of PI3K inhibitor and fucoxanthin induced more level of apoptosis which indicated that simultaneously treatment incorporates or catalyze the capability of LY294002 or fucoxanthin for induction of apoptosis, or fucoxanthin maybe activates programmed cell death not only via restriction of PI3K/Akt/mTOR system but also other potential signaling pathway which could be a more interesting point in future.

Invasion and migration are thorny barriers of treatment of glioma with various methods for therapy. Malignant glioma cells invade ambient area to destroy normal functions of tissue, cells and organelle, or migrate to far away regions to form tissue mass, resulting in disfunction of body's organs.

The matrix metalloproteinases (MMPs) play an important role in tissue remodeling associated with various physiological or pathological processes such as morphogenesis, angiogenesis, tissue repair, invasion and metastasis which is activated by cytokines, growth factors, tumor promoters, physical stress, oncogenic transformation, and cell-matrix and cell–cell interactions [56, 57]. The amplifying impact of MMPs derived from cancer is the beginners of cancer pathogenesis and promotes cell proliferation, angiogenesis, invasion and metastasis by the degradation of the extracellular matrix [58]. Therefore, reduction of activity of MMPs is thought to be important target which prevents the tumor spread and migration. More researchers have demonstrated that MMP-2 and MMP-9 modulate the migration and invasion in glioma cells [59, 60]. In our data, fucoxanthin inhibited the invasion and migration via evidenced by scratch wound healing assay and trans-well with or without matrigel (Fig. 3a–c). In addition, the bands of western blotting supported the relationship between fucoxanthin-prohibitive metastasis and the reduced expressions of MMP-2 and MMP-9 (Fig. 3d). As expected, the serine protease urokinase-type plasminogen activator (uPA), upstream signal of MMPs, was down-regulated by fucoxanthin. Currently, many proofs sustain that reductive cell motility and migration is via suppression of p38-MMP-2/9 signaling pathway, suggesting a potential mechanism existing in fucoxanthin-treated glioblastoma cells [61, 62]. The western blots of three isoforms of MAPKs revealed that inducer of fucoxanthin-regulated invasion or spared in glioblastoma is p38 rather than ERK or JNK kinase (Fig. 5a). Then, fucoxanthin exerted approximate outcomes that induced by incubation of glioblastoma cells with p38 inhibitor (Fig. 5b). Analogously with data of PI3K inhibitor, the effect of co-treatment of U87 and U251 cells with fucoxanthin and p38 inhibitor on the p38-MMP-2/9 core proteins had the significant differences with that of only fucoxanthin or SB203580, respectively, suggesting underlying mechanism existing apart from p38-MMP-2/9 central pivot (Fig. 5b). Therefore, prospective studies are exploring deep into the involvement signaling pathway of fucoxanthin independent of p38-MMP-2/9 operating system. In a word, fucoxanthin depresses the activity of p38-MMP-2/9 signal reflection and then lowers the extent of invasion and migration.

Moreover, the tumor xenograft studies shown that the tumor volume and weight were obviously declined reflecting the inhibitory effect of fucoxanthin on the tumor mass of U87 cells (Fig. 6a). Besides, staining histologic sections with H&E dye indicated that inflammation and low cell density were appeared by fucoxanthin compare to that of control cells (Fig. 6b). Moreover, we observed the presence of apoptosis conducted by TUNEL assay (Fig. 6b). Furthermore, we inspected that the molecules such as p-Akt, p-mTOR, p-p38, MMP-2/9 and BCL-2 were all down-regulated, and

BAX and cleaved-caspases-9 were both up-regulated in tumor tissues after fucoxanthin treatment which was consistent with *in vitro* findings.

In summary, we present the first report of anticancer activity of fucoxanthin in glioblastoma cells, and this is also the first evidence that fucoxanthin undertakes a suppresser of cell migration and invasion *in vitro* and *in vivo*. In the present study, fucoxanthin significantly promotes apoptosis via inhibition of PI3K/Akt/mTOR signaling pathway, leading to the inhibition of cell proliferation of glioblastoma cells. In addition, p38-MMP-2/9 signaling pathway involves the fucoxanthin-mediated inhibition of invasion and migration of glioma cells. Therefore, as an active ingredient of marine algae with considerable toxicity, fucoxanthin may represent a new anticancer drug that can impair glioma cells and prevent metastasis, and will become an emerging therapeutic agent in the future.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

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