Grifolin induces autophagic cell death by inhibiting the Akt/mTOR/S6K pathway in human ovarian cancer cells

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Abstract. Grifolin, a secondary metabolic product isolated from the mushroom *Albatrellus confluence*, has been reported to possess antitumor activities in various tumors. To date, no report exists on the role of autophagy in grifolin-treated human ovarian cancer cells. In the present study, we investigated the effect and the mechanism of autophagy in ovarian cancer. Ovarian cancer cell lines A2780 and SKOV3 were treated with grifolin. Cell proliferation was assessed by MTT assay and the autophagic effect was determined using flow cytometry, electron microscopy, immunofluorescence staining and GFP-LC3 puncta formation assay. The expression of autophagy markers and the main autophagy-associated Akt/mTOR/S6K pathway proteins were measured by western blot analysis. MTT assay indicated that grifolin inhibits the proliferation of human ovarian cancer cell lines A2780 and SKOV3. Flow cytometry, electron microscopy, immunofluorescence and GFP-LC3 puncta formation assay proved that grifolin induces autophagic cell death in human ovarian cancer. The results of the western blot analysis suggested that grifolin treatment leads to upregulation of autophagy markers LC3B, Atg7, Beclin-1 along with downregulation of P62. In addition, the proteins of the pathways p-Akt, p-mTOR, p-p70S6K and p-4E-BP1 were downregulated while the total of these proteins remained unaffected. The present study indicated that grifolin could induce autophagic cell death in human ovarian cancer by inhibiting the Akt/mTOR/S6K pathway.

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Introduction

Ovarian cancer is regarded as the deadliest gynecological cancer with the highest mortality rate worldwide. In the year 2015, more than 21,290 new cases and 14,180 deaths were reported all over the world (1). Traditional methods with chemotherapy after surgery may cause unwanted side-effects and drug resistance in patients. Therefore, it is necessary to develop more effective drugs against ovarian cancer. In recent studies, natural agents have been widely used for cancer therapy. Grifolin (2-trans, trans-farnesyl-5-methylresorcinol), a secondary metabolite extracted from the mushroom *Albatrellus confluens*, is an antibiotic belonging to basidiomycota (2,3). Several mechanisms have been reported, including inhibition of G1 phase cell cycle (4-6), promotion of apoptosis proteins (7,8) suppression of cell metastasis (9) which have been proposed to explain grifolin's antitumor effects. However, the other mechanisms of the drug's antitumor effects have remained unclear.

In the present study, we investigated the autophagic effect of grifolin on human ovarian cancer cell lines A2780 and SKOV3 *in vitro*. We found that grifolin inhibited cell growth in the two types of cells through inducing autophagic cell death via Akt/mTOR/S6K pathway. These results suggest that grifolin could be a meaningful therapeutic for the treatment of human ovarian cancer.

Materials and methods

Cell lines and culture. The human ovarian cancer cell line A2780 and SKOV3 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS). All cells were maintained at 37˚C in a humidified atmosphere containing 5% CO_2.

Reagents and antibodies. Grifolin was kindly provided by Kunming Institute of Botany, the Chinese Academy of Science and prepared at a concentration of 100 mmol/l stock solution in dimethyl sulfoxide (DMSO). Both chloroquine disphosphate (CQ) and acridine orange were obtained from Abcam (Cambridge, UK). The antibodies to p-Akt, Akt, p-mTOR, mTOR, p-p70S6K, p70S6K, p-S6, S6, p-4E-BP1 and 4E-BP1

were all purchased from Cell Signaling Technology (Danvers, MA, USA), the antibodies to LC3B, P62, Atg7, Beclin-1 and β-actin were obtained from Abcam. The cell culture media and other reagents were obtained from HyClone Laboratories (Logan, UT, USA).

Cell viability assay. Cell viability was measured by MTT assay. The A2780 and SKOV3 cells were seeded at a density of (4-5)x10^3 on 96-well plates and was allowed to adhere overnight. The cells were then treated with various concentration of the drugs (0, 20, 40, 60, 80 and 100 μM) to cells for 24, 48 and 72 h. At indicated time-points, the cells in the 96-well plate were incubated with 20 μl MTT and after 4 h at 37˚C. The formazan product was dissolved in 100 μl DMSO and evaluated at 490 nm with a microplate reader called infinite M200 PRO (Bio-Rad Laboratories, Hercules, CA, USA.)

Flow cytometric analysis of acidic vesicular organelles (AVOs). The ovarian cancer cells were treated with grofolin with different concentrations (0, 25, 50 and 75 μM) for 24 h and then stained with acridine orange (1 μg/ml) in PBS at 37˚C for 15 min in dark. Then the cells were washed with cold PBS twice and re-suspended in PBS for analysis in 1 h. The data were analyzed by CellQuest software.

Immunofluorescence staining. Analysis of LC3B protein localization: the cells were plated in 24-well plates and treated with 50 μM grifolin for 24 h. The cells were then fixed with 4% paraformaldehyde for 15 min at room temperature and were permeabilized with 0.2% Triton 150-200 μl in PBS for 10 min. After that, the cells were blocked with indicated anti-LC3B antibody (1:200) overnight and related secondary antibody (1:250) for 1 h in dark and then stained with 4,6-diamidino-2-pheny-lindole (DAPI) for 5 min in dark and at room temperature. At last, the fluorescence images were observed using a DP71 fluorescence microscope (Olympus, Tokyo, Japan).

Electron microscopy. The grifolin-treated cells were used to detect the induction of autophagy in ovarian cancer. The cells were treated with 50 μM grifolin for 48 h and harvested by trypsinization and then fixed with cold fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer. Then the samples were post-fixed in 1% osmium tetroxide buffer (OsO_4). We observed the representative areas (90 nm thin sections were cut) with a JEM-1010 transmission electron microscope (JEOL USA, Inc., Peabody, MA, USA) at 80 kV.

Western blot analysis. Vehicle- or drug-treated cells were lysed in a mixed buffer which contained RIPA, NaF, PMSF, and the supernatants were collected and the protein levels were measured. Protein (20 μg) were resolved by 12 or 10% SDS-PAGE and transferred to PVDF membranes (Immobilon P; Millipore, Bedford, MA, USA). After blocking for 2 h using 5% non-fat milk, the stripes were incubated with the indicated primary antibodies overnight at 4˚C. This was followed by incubation with secondary antibodies at room temperature for 1-2 h. The protein signals were then detected by ImageQuent LAS 4000.

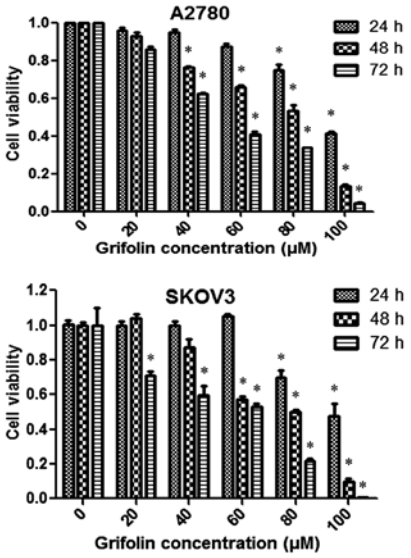

Figure 1. Anti-proliferative effects of grifolin on human ovarian cancer cell lines A2780 and SKOV3. Cells were seeded in 96-well with varying concentrations of grifolin (0, 20, 40, 60, 80 and 100 μM) for 24, 48 and 72 h. Then cell proliferation rate was assessed by MTT assay. Results are representative of three independent experiments. Compared with control group (0 μM) *P<0.05.

GFP-LC3 puncta formation assay. Cells were plated in 6-well cell culture plates at the confluence of 1x10^5 cells/well and incubated at 37˚C temperature with 5% CO_2 overnight. Then the cells were transfected with GFP-LC3 plasmid using Olifectamine and Opti-MEM medium according to the manufacturer's protocol.

shRNA transfection. ShRNA was used to silence LC3B protein expression in A2780 and SKOV3 cells. Establishment of A2780-plko.1-shLC3B, A2780-plko.1-NC, SKOV3-plko.1-shLC3B and SKOV3-plko.1-NC cell lines (shRNA-Forward primer CCGGCGCTTACAGCTCAATGCTAATCTCGAGATTAGCATTGAGCTGTAAGCGTTTTTG and shRNA-Reverse primer AATTCAAAAACGCTTACAGCTCAATGCTAATCTCGAGATTAGCATTGAGCTGTAAGCG).

Statistical analysis. T-test and one-way ANOVA were performed to determine significance by using the software SPSS 18.0. Statistical significance was determined at P<0.05. All the experiments were performed in triplicate.

Results

Grifolin induces cell proliferation in human ovarian cancer cell lines. We examined the effect of grifolin on cell viability of A2780 and SKOV3 cells which did decrease cell viability in the two cell lines in a dose- and time-dependent manner, as shown in Fig. 1.

Grifolin induces autophagy in ovarian cancer cell lines. The induction of autophagy by grifolin was confirmed by flow cytometry using acridine orange staining. As shown in Fig. 2, grifolin-treated cells increased formation of AVOs in a dose-dependent manner.

In the grifolin-treated cells, autophagy could cause ultrastructural changes. In Fig. 3, we observed autophagy in

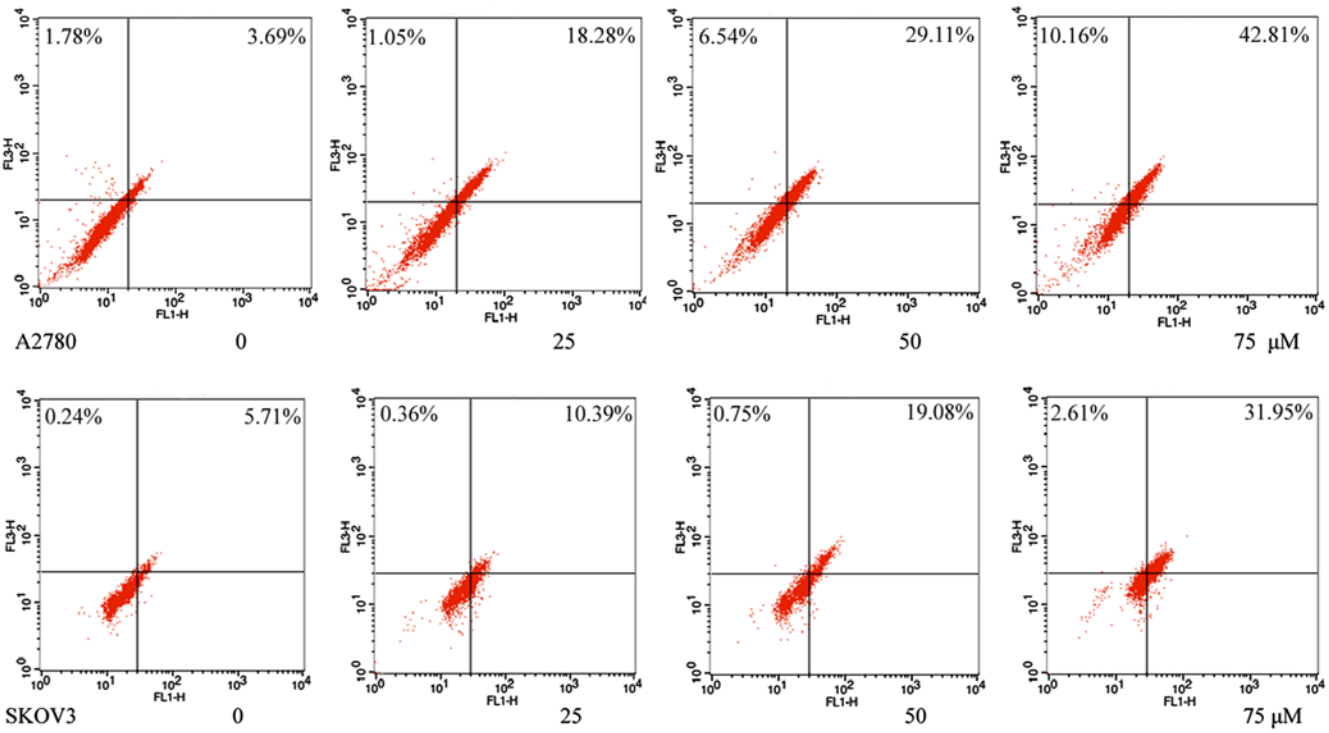

Figure 2. A2780 and SKOV3 cells were treated with different concentrations of grifolin (0, 25, 50 and 75 μM) for 24 h and then harvested. The suspended cells were marked with acridine orange and quantified using flow cytometry. FL1-H shows green color and Fl1-H indicates red color (AVO). Cells in upper and left quadrants were considered AVO-positive. Results are the mean ± SD of duplicate samples and repeated three times with similar results experiments. Compared with control group (0 μM) *P<0.05.

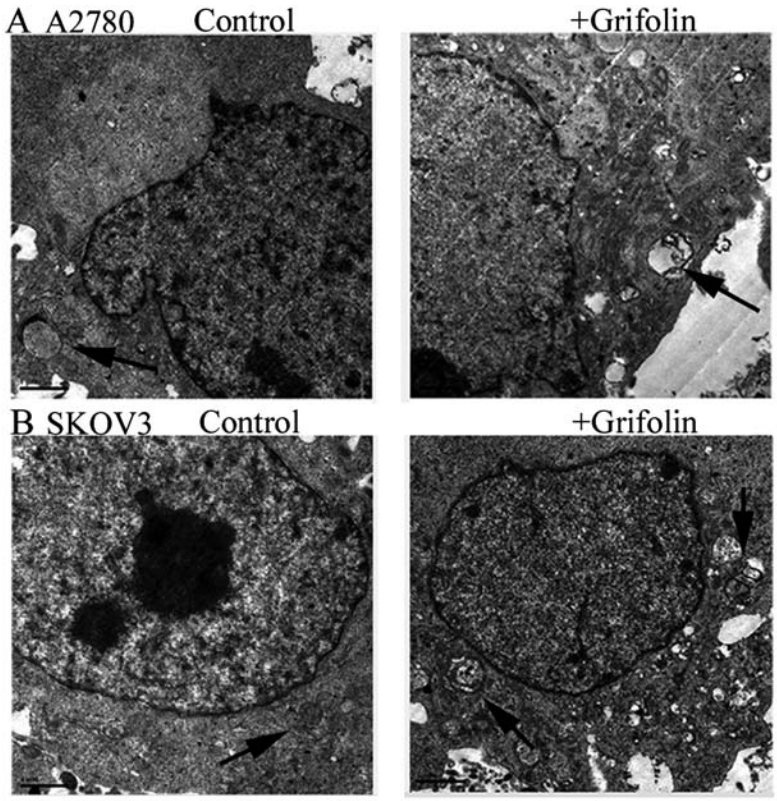

Figure 3. A2780 and SKOV3 cells were treated with or without 50 μM grifolin for 48 h using representative electron micrographs.

grifolin-treated cells by transmission electron microscopy. The cells were treated with 50 μM grifolin for 48 h, after which, we were able to observe the characteristics of cells undergoing autophagy.

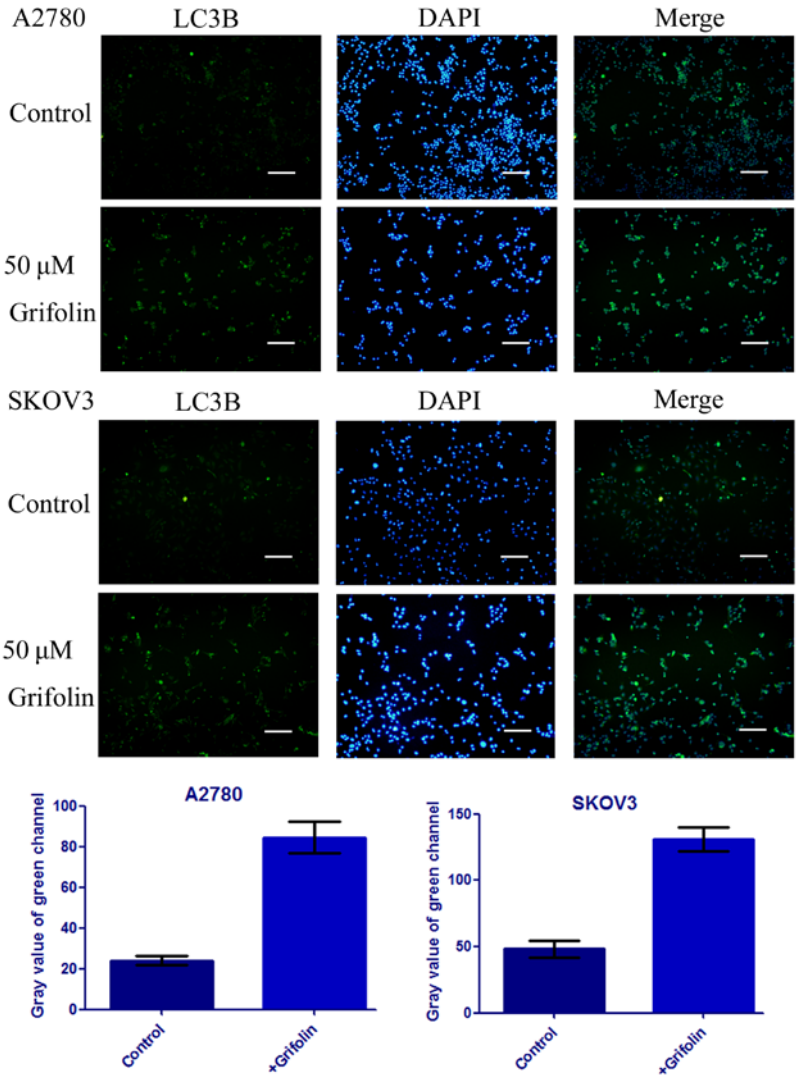

Figure 4. A2780 and SKOV3 cells were treated with or without 50 μM grifolin for 24 h and stained with the LC3B antibody by immunofluorescence staining. Bars, 100 μm.

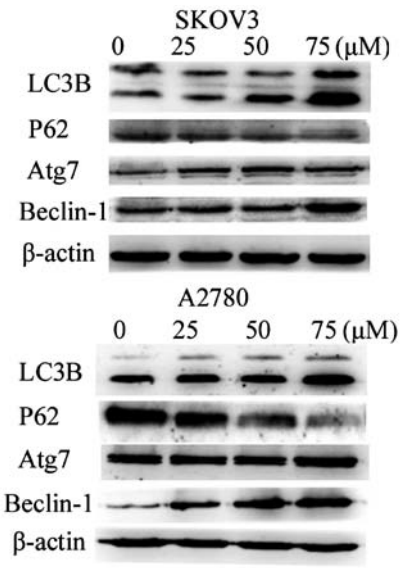

Figure 5. Western blot analysis was used to detect well-known autophagy markers. Cells were harvested after being exposed to various concentrations of grifolin (0, 25, 50 and 75 μM) 24 h. Data were obtained from three independent experiments. β-actin was used as a loading control.

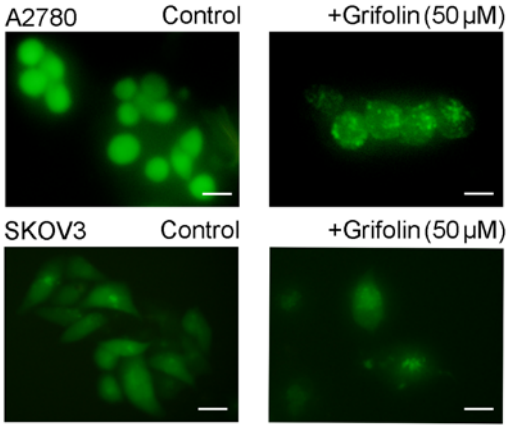

Figure 6. A2780 and SKOV3 cells were transfected with a GFP-LC3 plasmid and then treated with or without 50 μM grifolin for 24 h. The cells were observed under a fluorescence microscope. Bars, 25 μm.

It has been well-established that the microtubule-associated protein 1 light chain 3 (LC3) is a protein associated with the autophagosomal membrane (10). After being treated with

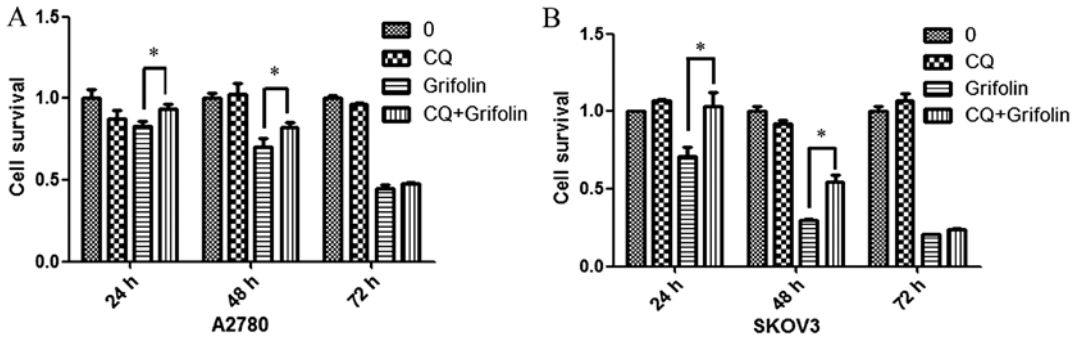

Figure 7. A2780 and SKOV3 cells were incubate in a 96-well plate with 10 μM CQ before being treated with grifolin. The cell survival rate was measured using MTT assay. Representative data from three independent experiments are shown. *P<0.05.

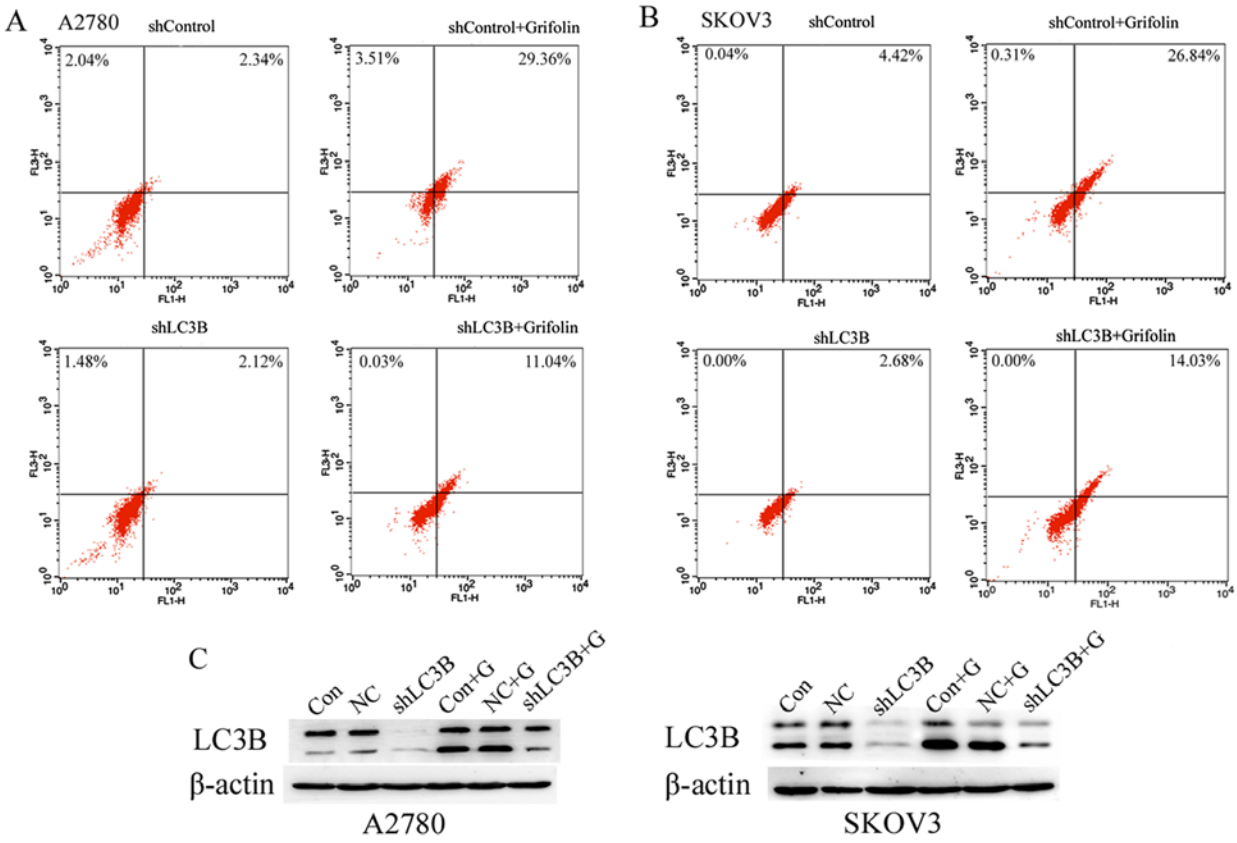

Figure 8. (A and B) LC3B was knocked down by shRNA. A2780 and SKOV3 cells were transfected with shLC3B. shControl was used as the negative control (NC). The cells were treated with or without grifolin for 24 h and then harvested. Then the suspended cells were marked with acridine orange and quantified via flow cytometry. (C) Con, normal cells, and NC was used as the negative control, G, grifolin. Cells were harvested after being exposed to grifolin (50 μM) for 24 h. Data were obtained from three independent experiments. β-actin was used as a loading control.

50 μM grifolin for 24 h, the number and intensity of punctuate LC3B fluorescence increased as shown in Fig. 4. Next, we investigated the expression of autophagy-related genes by western blot analysis. We found a significant increase in LC3B, Atg7, Beclin-1 and a decrease in P62 in a dose-dependent manner as shown in Fig. 5.

For further studying the role of grifolin in inducing autophagy in human ovarian cancer cell lines, the effect of grifolin on autophagy was confirmed by a GFP-LC3 puncta formation assay. As shown in Fig. 6, we find increased number of GFP-LC3 puncta in the grifolin-treated A2780 and SKOV3 cells.

Next, we used the autophagy inhibitor chloroquine (CQ) to investigate whether grifolin induced ovarian cancer cell death through the induction of autophagy. CQ is a well-known inhibitor of autophagy and inhibits lysosome acidification and degradation (11). We used MTT assay to detect cell viability (as shown in Fig. 7) using 10 μM of CQ which was able to significantly reduce the cell death in A2780 and SKOV3 cell lines. To prove the role of LC3B on autophagy in the grifolin-treated human ovarian cancer cells, we silenced the expression of LC3B and measured the autophagy level by flow cytometry-AVO analysis. As demonstrated in Fig. 8A and B, we found that transfection with shLC3B blocked the autophagic effect of

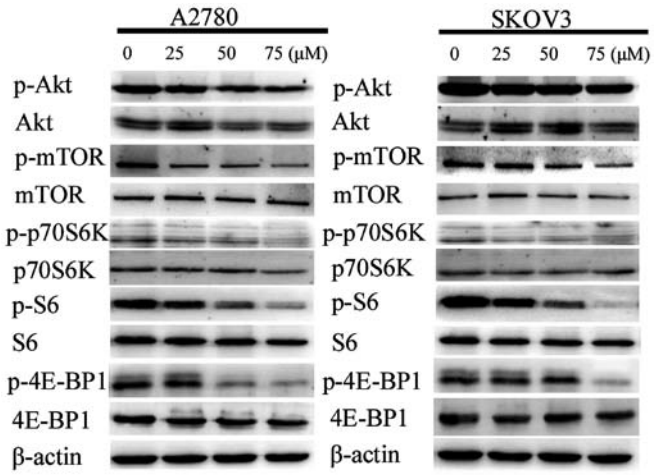

Figure 9. Grifolin inhibits the activity of Akt/mTOR/S6K pathway as detected by western blot analysis. Cells were exposed to grifolin with different concentrations (0, 25, 50 and 75 μM) for 24 h and were harvested. Results are representative of three independent experiments. β-actin was used as a loading control.

grifolin-treated A2780 and SKOV3 cells. Then we measured the level of LC3B by western blot analysis. Fig. 8C demonstrates that knockdown of LC3B in A2780 and SKOV3 cells significantly decreased its expression level, and the expression level of LC3B protein in grifolin-treated shLC3B cells expressed was higher than the negative control group. These results suggested that grifolin could reduce autophagic cell death in human ovarian cancer cell lines.

Grifolin inhibits the activity of the Akt/mTOR/S6K pathway. Researchers have found that the inhibition of Akt/mTOR/S6K pathway is associated with autophagy in various cancer cells (12-14). Thus, we examined whether the pathway is associated with grifolin-treated ovarian cancer cell autophagic death using western blot analysis (Fig. 9). The results demonstrated that grifolin caused a decrease in levels of the phosphorylated form of Akt, mTOR, p70S6K, S6 and 4E-BP1 while the total of these proteins remained unaffected by the treatment. Those results suggested that grifolin could induce autophagic cell death by inhibiting the activity of Akt/mTOR/S6K pathway.

Discussion

In the present study, we investigated the role of autophagy in human ovarian cancer cell lines A2780 and SKOV3 which were treated with grifolin by inhibiting the Akt/mTOR/S6K pathway. This is the first study on the role of grifolin in inducing autophagy in ovarian cancer. A previous study showed that grifolin induced cell cycle arrest in G1 phase via the ERK1/2 pathway (6), and also induced apoptosis through the Bax/Bcl-2 and caspase-3/-9 families via inhibition of PI3K/Akt signalling pathway in human osteosarcoma cells (8). In addition, it has been reported that grifolin upregulated death-associated protein kinase 1 DAPK1 via p53 and mediated G1 phase arrest in nasopharyngeal carcinoma cells (4,7). According to the latest reports, grifolin suppressed cancer cell metastasis by inhibiting ERK1/2 pathway (9).

The Akt/mTOR/S6K pathway plays a significant role in biological functions on various human cancers. A body of evidence proved that Akt is a major component of the Akt/mTOR/S6K pathway and its inactivation promotes cell proliferation and reduces cell death (15). mTOR, a major down-stream target of the pathway, is essential to regulate tumor growth (16). In addition, S6K possesses a key role in cell proliferation and survival (17). The Akt/mTOR/S6K pathway is becoming an attractive therapeutic target for cancer therapy and its inactivation is reported to occur in various tumors (18,19). Kondo *et al* (20) found that autophagy is induced mainly through the PI3K/Akt/mTOR pathway. In recent years, many studies demonstrated that the pathway regulates autophagy in various cancers such as breast cancer (21), human hepatocellular carcinoma (22), ovarian cancer (12), melanoma (13), non-small cell lung (23), and nasopharyngeal cancer (14) and the mechanisms of autophagy are well-known. The main mechanism is led by mTOR kinase that acts as an upstream factor of all autophagy-associated genes and regulates transcription and translation (24). The other important mechanism involves the effect of mTOR on two main scaffold proteins the Atg11 and Atg 17, the changes in which lead to activation of Atg1, a key autophagy kinase and then the activated Atg1 is able to affect the output of autophagsome formation (25). In the present study, we investigated that the main proteins of Akt/mTOR/S6K pathway in grifolin-treated ovarian cancer cells. Our results show that grifolin treatment downregulates phosphorylation of Akt and mTOR and also their downstream targets, p70S6K, S6 and 4E-BP1 while the total proteins have no obvious changes. Those findings prove that grifolin could inhibit autophagy via inhibiting Akt/mTOR/S6K pathway on human ovarian cancer cells.

In summary, we have shown for the first time that grifolin induces autophagic cell death in human ovarian cancer cells by inhibiting the Akt/mTOR/S6K pathway. We provide evidence to prove grifolin could play a role as a novel antitumor agent for human ovarian cancer through the induction of autophagy. However, it is still crucial to explore the responsible molecular mechanisms of grifolin inducing human ovarian cancer cell death.

Acknowledgements

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