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OP16 induces deadly autophagy and apoptosis of cells by inhibiting Akt in esophageal squamous cell carcinoma

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Abstract

Background OP16, a derivative of the novel ent-kaurene diterpenoid compound separated from *Rabdosia rubescens*, has been confirmed for its efficacy and safety in the treatment of esophageal squamous cell carcinoma (ESCC) in our previous study. However, the precise mechanisms of tumor lethality mediated by OP16 have not yet been fully characterized.

Aims To investigate the effects and molecular mechanism of OP16 on autophagy and apoptosis of ESCC cells.

Methods Effects and mechanism of OP16 on autophagy of ESCC cells were first detected by Western blot, immunofluorescence, mRFP-GFP-LC3 adenovirus infection and transmission electron microscope. Next, function of autophagy and apoptosis induced by OP16 on cell death was investigated by flow cytometry and CCK-8 assay. Finally, molecular mechanism of OP16 affecting autophagy and apoptosis of ESCC cells was explored by Western blot.

Results We demonstrated that OP16 could induce autophagy by promoting the formation of autophagosome and autolysosome, and promote autophagic cell death in ESCC. Furthermore, we also found that OP16 could promote cell apoptosis by activating mitochondria apoptosis pathway in ESCC. Finally, we demonstrated that OP16 affecting autophagy and mitochondria apoptosis pathway was mediated by phosphorylation of Akt.

Conclusion Our data show that OP16 could promote cell death through affecting autophagy and mitochondria apoptosis pathway mediated by Akt in ESCC, which enriches the theoretical mechanism of anti-tumor effects of OP16 and provides a basis for treatment of OP16 on ESCC.

Keywords Oridonin · Autophagy · Akt · Apoptosis · Esophageal squamous cell carcinoma

Introduction

Autophagy, a ubiquitous process induced by stresses such as starvation, is highly conservative in evolution and plays a crucial role in the growth, differentiation and homeostasis of eukaryotic cells. In this process, double-membrane autophagosomes captures intracellular proteins and organelles to provide energy and nutrition for survival of cells at extreme environment [1, 2]. At present, 41 Atgs (autophagy-related genes) have been demonstrated in yeast to participate in the regulation of autophagy, and the homologous genes of them in mammals have also been found, such as microtubule associated protein light chain 3 (LC3), which is a homologous protein of Atg8. LC3 includes three subtypes: LC3A, LC3B and LC3C, in which LC3B is concerned extensively in autophagy-related studies [3]. LC3 has two inter-convertible forms: LC3-I and LC3-II, LC3-I in cell cytoplasm can be sheared and modified by ubiquitination, then combines with PE on the membrane of autophagosomes to form LC3-II [4]. LC3-II distributes widely on both sides of the bilayer membrane of phagocytic vesicle and has key function on bending and stretching of membrane during the formation of autophagosomes [5]. LC3-II, therefore, was recognized as the marker to evaluate autophagy [6]. In addition, SQSTM1/p62 (sequestosome) protein can preferentially recognize and recruit polyubiquitinated proteins into the phagocytic vesicles through interacting with LC3-II, and then is degraded together with of these proteins. SQSTM1/

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p62, therefore, is referred as autophagy substrate protein. The increase of LC3-II companying with the decrease of SQSTM1/p62 was recognized as the mark of autophagy activity [7].

Autophagy has contributions to the pathogenesis of many diseases, including obesity, diabetes, multiple neurodegenerative diseases and malignancies [8, 9]. It is generally believed that autophagy plays a role of "double-edged sword" in the occurrence and development of tumors. At the early stage of tumorigenesis, autophagy can degrade the "cell waste" such as aged organelles and proteins in cells, thereby preventing tumorigenesis caused by cell damage. However, in the formed tumors, autophagy also can provide abundant energy and nutrition for tumor cells for helping them to survive in the stress situation caused by excessive growth and hypoxia [10–12]. Moreover, autophagy in the anti-tumor effects of chemotherapeutic drugs, including the natural drugs, also plays two different roles [13]. Reports have demonstrated autophagy induced by chemotherapeutic drugs, such as fluorouracil (5-FU), sulforaphane, temozolomide [14–17], can repair the cell damage caused by them and help cells resist their lethal effects, that is, chemotherapeutic drugs induce protective autophagy [18–20]. However, some chemotherapeutic drugs, especially some natural compounds such as rottlerin and caffeine [21, 22], could promote the death of tumor cells by inducing autophagy, named as autophagic cell death or type II programmed cell death, which is entirely different with cell apoptosis [13, 23]. However, mechanisms that chemotherapeutic drugs cause two kinds of completely different consequences by inducing autophagy are still obscure now.

Rabdosia rubescens, as a traditional Chinese herbal medicine, has been used to treat esophageal squamous cell carcinoma (ESCC), one of the most frequently diagnosed carcinomas in China for many years [24]. Oridonin, the mainly anti-tumor component of *Rabdosia rubescens*, has also been demonstrated to inhibit many tumors, especially gastrointestinal tumors by inducing autophagy [25, 26]. However, some characteristics of oridonin such as the relatively moderate therapeutic efficacy, limited aqueous solubility and low structural stability have hindered its further clinical applications [27]. OP16 is a derivative of JDA, a novel ent-kaurene diterpenoid compound isolated from *Rabdosia rubescens* by our group, and has the same activity site with oridonin in chemical structure, while has better therapeutic efficacy, aqueous solubility and structural stability than oridonin [28, 29]. We have demonstrated previously that OP16 had obvious anti-tumor effects on ESCC by inhibiting phosphorylation of Akt (Protein kinase B) in vitro and in vivo [30], but effects of OP16 on cell autophagy and the accurate anti-tumor mechanism to ESCC were still obscure.

In this study, we further explored the effect of OP16 on autophagy and the relationship between autophagy and apoptosis as well as the potential molecular mechanism in ESCC.

Materials and methods

Cell lines and cell culture

Two ESCC cell lines EC9706 (poorly differentiated) and KYSE450 (well-differentiated) were obtained from Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (Biological Industries, Israel) containing 10% fetal bovine serum (Biological Industries, Israel) using a CO_2 incubator with saturated humidity, 5% CO_2 at 37 ℃, as described before [31]. Cells were continuously generated no more than three months before used.

Reagents and antibodies

Structure and characters of OP16 have been described in our previous study [30]. Chloroquine diphosphate (CQ, HY-17589), Bafilomycin A1 (HY-100558), Z-VAD-FMK (HY-16658B), SC79 (HY-18749) were obtained from MCE (MedChemExpress, USA). The primary antibodies p-Akt (Ser473) (#4060), Akt (#4691), Cleaved-caspase-9 (#7237), Cleaved-caspase-3 (#9664), Bax (#5023), Bcl-2 (#15071), LC3B (#3868), p62 (#8025), Beclin-1 (#3495), Atg5 (#1299), Atg7 (#8558), Atg12 (#4180), GAPDH (#5174), HRP-linked secondary antibodies, Alexa Flour 488-conjugated (#4416) and Alexa Flour 555 Phalloidin (#8953) were obtained from Cell Signaling Technology (USA).

Western blot analysis

As described before [31, 32]. Equal amounts of proteins (30 μg) separated by SDS-PAGE were transferred onto nitrocellulose membranes and blocked with 5% skimmed milk for 2 h, and then the membranes were incubated with primary antibodies overnight at 4 °C. After the membranes were incubated with corresponding secondary antibodies at room temperature for 2 h, immunoblots were visualized by enhanced chemiluminescence (ECL) reagent (Thermo Scientific, USA). The membranes were rinsed three times with PBST (Phosphate Buffer Solution and Tween20) between the incubations described above. Quantification of protein relative to GAPDH was presented based on ImageJ densitometric analysis.

Immunofluorescence assay

Immunofluorescence was used to analyze the subcellular distribution of LC3. In brief, EC9706 cells were seeded in a six-well plate with coverslips placed in advance at 3×10^6 cells/well and treated with 10 μM of OP16 and 100 nM of Bafilomycin, respectively, for 12 h. After coverslips were taken out and cells on them were fixed with 4% formaldehyde for 15 min, 3% bovine serum albumin (BSA) and 0.3% Triton X-100 were dropped on the cells for 60 min. Cells were followed by being incubated with LC3B primary antibody (Rabbit mAb) diluted with buffer (0.3% Triton X-100 and 1% BSA, 1:200) overnight at 4 °C, then incubated with Alexa Flour 488-conjugated secondary antibody (1:500) in the dark for 1 h. Cells were stained with Alexa Flour 555 Phalloidin for 45 min and DAPI for 10 min in the dark, followed by being observed and photographed under a confocal microscope. Cells were rinsed 3 times with phosphate-buffered saline (PBS) between the incubations described above.

mRFP-GFP-LC3 adenovirus infection assay

Double labeled adenovirus vector mRFP-GFP-LC3 was used to label LC3 for observing autophagosome and autolysosome. In brief, EC9706 cells were seeded on U-Slide plates to 70% confluency and transfected with mRFP-GFP-LC3 adenovirus (Hanbio Biotechnology Co., Ltd., Shanghai, China) at 800 of multiplicity of infection (MOI = 800) using polybrene for 48 h. Then cells were treated with OP16 (10 μM) and Bifilomycin A1 (100 nM), respectively, for 6 h, the fluorescence of cells was detected using a confocal microscope.

Transmission electron microscope assay

EC9706 cells were plated in 6-well plates and treated with OP16 (10 μM) or rapamycin (50 nM) (as positive control) for 6 h. After cells were collected and fixed with 2.5% glutaraldehyde at 4 °C for 2 h, the ultrastructure of autophagy and autolysosome in cells were observed using a transmission electron microscope (TEM).

Cell apoptosis assay

Cell apoptosis was measured by Annexin V-FITC/PI Apoptosis Detection Kit (KeyGEN, China). ESCC Cells treated with different measures were collected, washed twice with cold PBS and re-suspended using binding buffer. Cell was stained with 5 μL Annexin V-FITC and 5 μL PI, respectively, for 15 min in the dark, and then analyzed by flow cytometer (Becton Dickinson, USA).

Mitochondrial membrane potential (ΔΨm) assay

The fluorescent dye JC-1 (Beyotime Biotech, Shanghai, China) was used to detect the alteration of ΔΨm. In brief, EC9706 and KYSE450 cells disposed with the disparate dosages of OP16 (0, 10, 20, and 30 μM) for 24 h were harvested and co-fostered with 500 μl JC-1 working solution at 37 °C for 20 min in the dark. After cells were rinsed twice and re-suspended with JC-1 buffer, the alteration of ΔΨm in both EC9706 and KYSE450 cells was monitored by flow cytometry.

shRNA plasmid transfection

EC9706 cells were cultured overnight with antibiotic-free medium and transfected with Beclin-1 shRNA vector (5′-GGA CAA CAA GTT TGA CCA TGC-3′) and control shRNA vector (5′-GTT CTC CGA ACG TGT CAC GT-3′) (Gene Pharma Co., Ltd, Shanghai, China), respectively, using Lipofectamine® 3000 (Invitrogen) as described previously [32]. Cells with stable expression of Beclin-1 shRNA were screened using culture medium with 2.5 μg/mL puromycin.

Cell viability assay

After ESCC cells seeded in 96-well plate at 6×10^3 cells/well were incubated overnight and treated with different measures, 5 μL Cell Counting Kit-8 reagent (CCK-8, Beyotime Biotech, China) was added into every well. Cells were incubated for another 2 h, and the absorbance (optical density) of cells at 450 nm was measured by BioTEK system (PerkinElmer Enspire).

Protein–protein interaction (PPI) analysis

The STRING database (Search Tool for the Retrieval of Interacting Genes/Proteins) was used to analyze the functional associations between Akt, autophagy-related proteins and apoptosis-related proteins in tumors of a global scale (STRING, https://string-db.org) [33, 34]. Confidence score ≥ 0.4 was considered as significant.

Statistical analysis

All experiments were triply repeated independently and the results were analyzed statistically by one-way analysis of variance (ANOVA) using SPSS21.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 8.0 (San Diego, CA, USA). The data were expressed as

mean ± standard deviation and $P < 0.05$ was considered statistically significant.

Results

OP16 induced autophagy through promoting the formation of autophagosome and autolysosome

To explore whether OP16 (Fig. 1a), the new compound with the same activity site with oridonin, can affect cell autophagy in ESCC as oridonin [35, 36], expressions of autophagy key proteins LC3B and SQSTM1/p62 were firstly investigated by Western blot after EC9706 and KYSE450 cells, respectively, were treated with OP16 at different concentration (0, 1, 5, 10 μM) for 12 h. Compared with the corresponding untreated cells, the expression of LC3B-II increased ($P < 0.01$ or $P < 0.001$) and SQSTM1/p62 decreased ($P < 0.05$ or $P < 0.001$) in EC9706 and KYSE450 cells along with the concentration increase of OP16, indicating that OP16 could affect expression of autophagy proteins in a concentration-dependent manner in ESCC cells (Fig. 1b).

Next, Effects of OP16 on the subcellular distribution of LC3B protein were assayed by immunofluorescence. As shown in Fig. 1c, LC3B with green fluorescence was observed in the cytoplasm of EC9706 cells treated with Bafilomycin A1, which can induce the accumulation of LC3B by inhibiting the function of lysosome. The similar phenomenon was observed after cells were treated with OP16, indicating that OP16 could promote accumulation of LC3B in cytoplasm. In addition, we detected the expression change of LC3B after EC9706 cells were treated with bafilomycin A1 (100 nM) and chloroquine (CQ, 20 μM), alone or combined with OP16 (10 μM), respectively, for 6 h. The results showed that the expression of LC3B in cells treated with bafilomycin A1, CQ or OP16 alone significantly increased compared to the control cells ($P < 0.01$ or $P < 0.001$), while there was highest expression in cells treated with OP16 combined with bafilomycin A1 or CQ, respectively ($P < 0.001$) (Fig. 1d), which suggested that OP16 might further increase the expression of LC3-II through promoting the formation of more autophagosome to activate autophagy flux after Bafilomycin A1 and CQ inhibited the combination of autophagosome and lysosome.

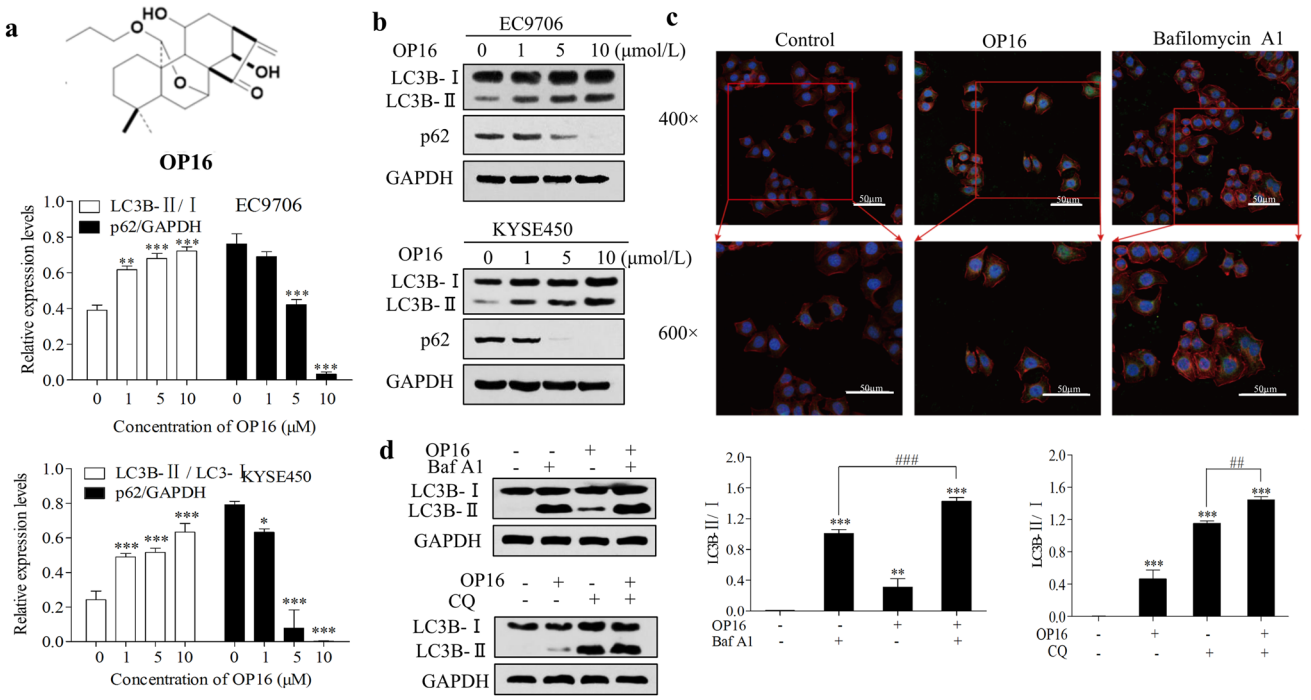

Fig. 1 OP16 induced autophagy of ESCC cells through promoting the formation of autophagosome and autolysosome. **a** Chemical structure of OP16. **b** Expression levels of LC3B and SQSTM1/p62 in EC9706 and KYSE450 cells treated with various doses of OP16 (0, 1, 5 and 10 μM) for 12 h. **c** LC3B distribution in EC9706 cells treated with bafilomycin A1 (100 nM) or OP16 (10 μM) for 12 h. **d** Expression of LC3B-II in EC9706 cells treated with bafilomycin A1 (100 nM) and chloroquine (20 μM) alone or combined with OP16 (10 μM) for 6 h. **e** Representative images of autophagosomes in EC9706 cells infected with mRFP-GFP-LC3 adenovirus treated with OP16 (10 μM) or bafilomycin A1 (100 nM) for 6 h. **f** Electron microscopy showed the increased autophagosomes and autolysosomes in EC9706 cells treated with OP16 (10 μM) or rapamycin (50 nM) for 6 h. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, versus control group; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, versus single-drug treatment group

To further demonstrate the promoting effects of OP16 on autophagy flux, EC9706 cells were transfected with mRFP-GFP-LC3B adenovirus vector, which could mark autophagosome (yellow dot) and autolysosome (red dot), and observed under the confocal microscope. The results are shown as Fig. 1e, the fluorescence was distributed dispersively and had no aggregate spot in control cells, while in cells treated with OP16, the yellow and red fluorescent spots were observed obviously and green fluorescence partly quenched compared to cells treated with bafilomycin A1, indicating that OP16 could activate autophagy flux by promoting the formation of autophagosome and autolysosome.

Further, we observed the ultrastructure of autophagosome and autolysosome in cells using transmission electron microscope after EC9706 cells was treated with OP16 and rapamycin, a typical autophagy inducer [36, 37]. Compared with the control cells, a large number of bilayer-membrane autophagosome and monolayer-membrane autolysosome (red arrows) were observed in cells treated with OP16, as shown in cells treated with rapamycin (Fig. 1f). Moreover, since cell autophagy is an energy-consuming process, a large number of mitochondria (yellow arrows) could be observed around autophagosome and autolysosome in cells treated with OP16 or rapamycin (Fig. 1f).

These results above demonstrated that OP16 could induce autophagy by promoting the formation of autophagosome and activating autophagy flux in ESCC cells.

OP16 can induce partly apoptosis of ESCC cells through activating mitochondrial apoptosis pathway

To explore the effects of OP16 on cell apoptosis, cell apoptosis and JC-1 mitochondrial membrane potential (ΔΨm) were detected by a flow cytometer, and expression of the related proteins (Bcl-2, Bax, Cleaved-caspase-9 and Cleaved-caspase-3) in the mitochondrial apoptosis pathway was detected by Western blot after EC9706 and KYSE450 cells, respectively, were treated with OP16 at different concentrations (0, 10, 20 and 30 μM) for 24 h. As shown in Fig. 2a, the apoptosis rate of EC9706 or KYSE450 cells increased along with the increasing of OP16 concentration compared to control cells ($P < 0.001$), indicating that OP16 can induce apoptosis of ESCC cells in a concentration-dependent manner. The results of ΔΨm assay showed that the JC-1 monomers (%) increased gradually along with the increase of OP16 concentration compared to control cells ($P < 0.01$ or $P < 0.001$), indicating that OP16 could promote apoptosis by decreased the mitochondrial membrane potential (Fig. 2b). Moreover, the expression of Bcl-2 decreased, while Bax, Cleaved-caspase-9 and Cleaved-caspase-3 increased in cells treated with OP16 compared to untreated cells ($P < 0.05$ or $P < 0.001$, Fig. 2c). The above results indicated that OP16 could induce cell apoptosis through activating mitochondrial apoptosis pathway.

Another, to further demonstrate whether OP16 induced cell apoptosis completely through activating mitochondrial apoptosis pathway, apoptosis of EC9706 and KYSE450 cells treated with OP16 (20 μM) and caspase inhibitor Z-VAD-FMK (20 μM) alone or combined for 24 h was detected by flow cytometry. As shown in Fig. 2d, compared to control group, Z-VAD-FMK alone had no obvious inducing apoptosis effects on EC9706 and KYSE450 cells, OP16 alone or combined with Z-VAD-FMK sharply induced cell apoptosis ($P < 0.001$), while the apoptosis rate in OP16 + Z-VAD-FMK group was obviously lower than that in OP16 group ($P < 0.01$ or $P < 0.05$), indicating that Z-VAD-FMK partly, but not completely, offset the inducing effects of OP16 on cell apoptosis. In other words, OP16 might induce apoptosis through other mechanism except for its activation to mitochondrial apoptosis pathway.

OP16 inducing autophagy promotes apoptosis of ESCC cells

To explore whether OP16 activating autophagy could induce cell apoptosis in ESCC, EC9706 and KYSE450 cells were treated with chloroquine (20 μM) or free-serum starvation alone or combined with OP16 (20 μM) for 24 h, cell apoptosis were detected by flow cytometry. As shown in Fig. 3a, compared to control cells, both CQ and free-serum starvation had no obvious apoptosis-inducing effects on ESCC cells, but inhibiting autophagy by CQ impaired the apoptosis-inducing effects of OP16 on cells and the apoptosis rate decreased 40.7% and 23.4%, respectively, in EC9706 and KYSE450 cells treated with OP16 + CQ compared to OP16 group ($P < 0.001$ or $P < 0.01$), while inducing autophagy by free-serum starvation enhanced the apoptosis-inducing effects of OP16 on cells and the increasing rate was 84.8% and 24.5%, respectively, in EC9706 and KYSE450 cells treated with OP16 + starvation compared to cells treated with OP16 ($P < 0.001$ or $P < 0.01$).

Moreover, we investigated effects of OP16 (20 μM for 24 h) on apoptosis and proliferation of EC9706 cells by flow cytometry after the expression of Beclin-1, an important protein for regulating the early stage of autophagy, was downregulated with Beclin-1 shRNA. As shown in Fig. 3b, Beclin-1 shRNA significantly downregulated the expression of Beclin-1 in EC9706 cells and the decrease rate reached to 73.6%. Beclin-1 shRNA had no obvious effects on cell apoptosis and proliferation, while when cells transfected with Beclin-1 shRNA treated with OP16, apoptosis rate was decreased 37.9% and cell viability rate was increased 18.0% compared with that in OP16 group ($P < 0.001$), indicating that inhibiting the early stage of autophagy could also

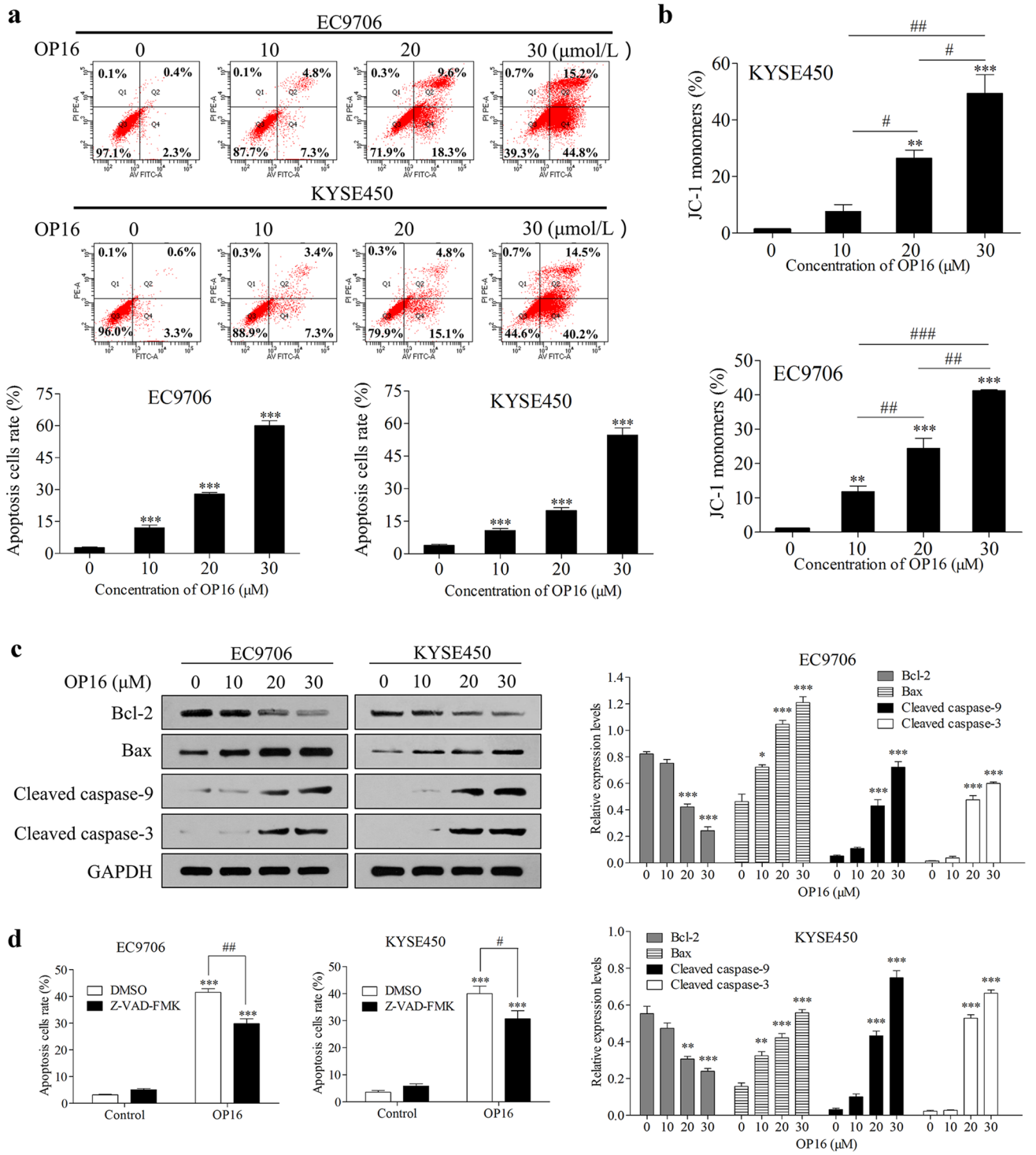

Fig. 2 OP16 induces apoptosis of ESCC cells partly through activating mitochondrial apoptosis pathway. **a**, **b** Cell apoptosis and mitochondrial membrane potential (ΔΨm), respectively, of EC9706 and KYSE450 cells treated with OP16 at different concentrations (0, 10, 20 and 30 μM) for 24 h. **c** Expression levels of Bcl-2, Bax, Cleaved-caspase-9 and Cleaved-caspase-3 in EC9706 and KYSE450 cells treated with OP16 (0, 10, 20 and 30 μM) for 12 h. **d** Cell apoptosis rate of EC9706 and KYSE450 cells treated with OP16 (10 μM) alone and combined with Z-VAD-FMK (20 μM) for 24 h. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, versus control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, versus single-drug treatment group

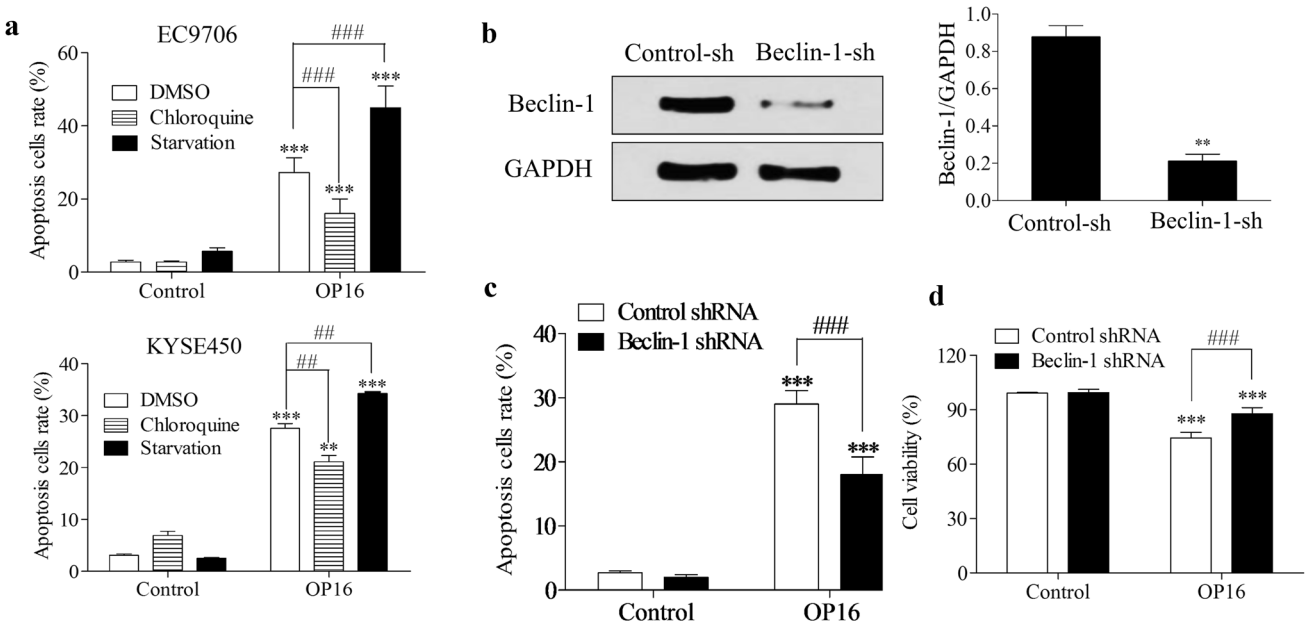

Fig. 3 OP16 promotes apoptosis by inducing autophagic cell death in ESCC. **a** Apoptosis rate of EC9706 and KYSE450 cells treated with OP16 (20 μM) alone or combined with chloroquine (20 μM) and serum-free starvation, respectively, for 24 h. **b** Expression of Beclin-1 in EC9706 cells with Beclin-1 shRNA. **c** and **d** Apoptosis rate and cell viability, respectively, of EC9706 cells with Beclin-1 shRNA treated with OP16 (20 μM) for 24 h. $^{**}P<0.01$, $^{***}P<0.001$, versus control group, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, versus single-drug treatment group

weaken the apoptosis-inducing and proliferation-inhibiting effects of OP16 on ESCC cells (Fig. 3c, d).

These results above showed that inhibiting autophagy could weaken while inducing autophagy could enhance the apoptosis-inducing effects of OP16 on ESCC cells, suggesting that OP16 could induce deadly autophagy of cells in ESCC.

OP16 induces autophagy and activates mitochondria apoptosis pathway by inhibiting Akt phosphorylation in ESCC cells

Akt has been demonstrated to be a key factor for the antitumor efficiency of OP16 to ESCC in our previous study [30]. To explore whether OP16 regulating autophagy and apoptosis had correlation with Akt in ESCC, we analyzed the interaction relationship of Akt, autophagy and apoptosis in tumors using STRING database. PPI network is shown as Fig. 4a, there were 54 nodes and 757 edges between Akt, autophagy- and apoptosis-related proteins, indicating that there was regulating relationship between Akt and autophagy- or apoptosis-related proteins. Moreover, the potential biological processes were further analyze as shown in Fig. 4b, there were 12 nodes and 56 edges, indicating that Akt was involved in the regulation of autophagy, apoptosis and mitochondrial membrane potential but not in the primary process of autophagy in tumorigenesis.

According to the results of biological information analysis, we explored the molecular mechanism of OP16 inducing autophagy in ESCC, we firstly detected the effects of OP16 on the expression of Atg protein in start stage of autophagy by Western blot after EC9706 or KYSE450 cells were treated with OP16 (0, 1, 5 and 10 μM) for 12 h. The results are shown as Fig. 4c, expression of Atg5, Atg7 and Atg12 in EC9706 or KYSE450 cells had no significant change compared with the control cells ($P>0.05$), indicating that OP16 had no effects on autophagy-primary stage, which was in accordance with the results of PPI analysis. Next, we explored the effects of Akt phosphorylation on cell autophagy induced by OP16. EC9706 and KYSE450 cells were treated with OP16 (10 μM) and SC79 (40 μM), an activator of Akt, alone or combined for 12 h, expression of LC3B and p-Akt (Ser473) were detected. As shown in Fig. 4d, compared with the untreated group, SC79 promoted expression of p-Akt (Ser473), OP16 alone could significantly decrease the expression of p-Akt (Ser473) and increase the expression of LC3B ($P<0.001$), while the expression of p-Akt (Ser473) was significantly higher and LC3B was lower in cells treated with OP16 combined with SC79 than that in cells treated with OP16 alone ($P<0.001$), suggesting that SC79 activating Akt could impair the inhibiting effects of OP16 on p-Akt (Ser473) and the inducing effects of OP16 on LC3B. The results indicated that OP16 induces autophagy by inhibiting Akt phosphorylation in

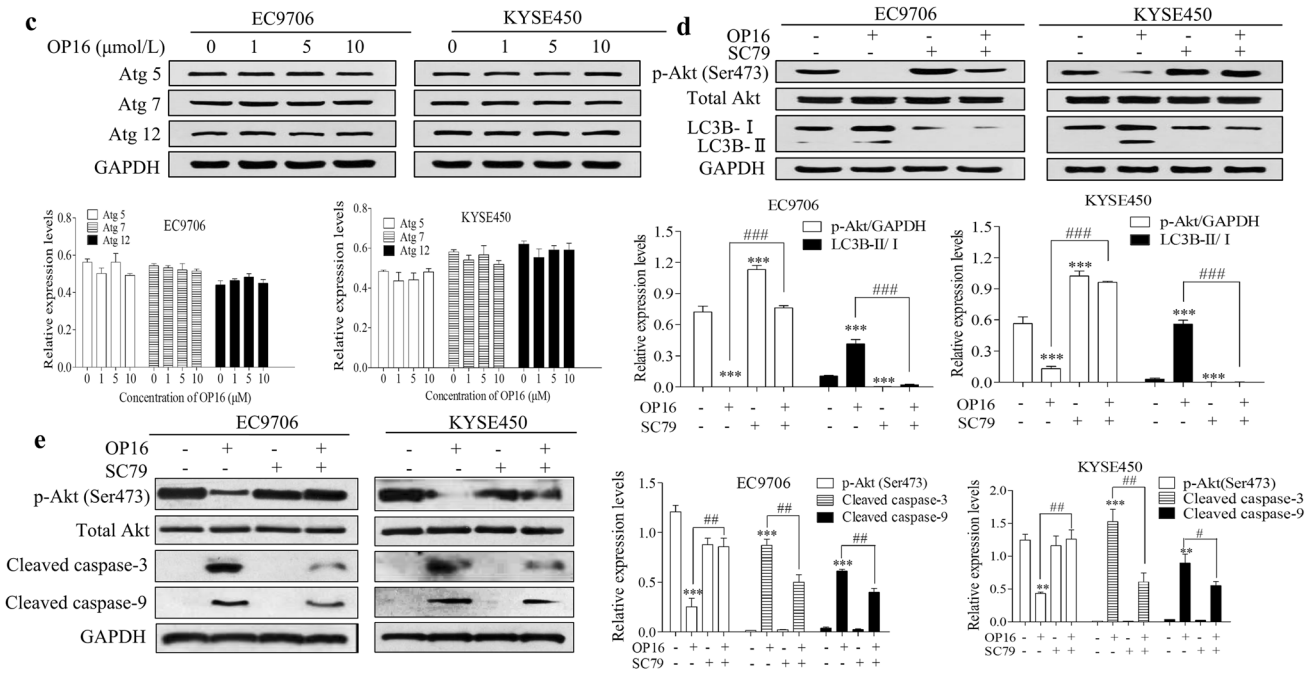

Fig. 4 OP16 induces autophagy and apoptosis by inhibiting Akt in ESCC. **a** Information of PPI network. PPI network consisted of 54 nodes and 757 edges were constructed by overlapping Akt, and yellow nodes represent proteins associated with Akt. **b** Multiple Proteins PPI network included 12 nodes and 56 edges, Each node represents all the proteins produced by a single, protein-coding gene locus, and color of blue, red, green and yellow were represent regulation of apoptotic process, regulation of mitochondrial membrane potential, regulation of autophagy and autophagy, respectively. Filled nodes indicated some 3D structure known or predicted. Edges: Edges represent specific and meaningful. Line thickness indicates the strength of data support. MAP1LC3A: LC3A, MAP1LC3B: LC3B, SQSTM1: SQSTM1/p62, BECN1: Beclin-1, CASP3: Caspase-3, CASP9: Caspase-9. **c** Expression of Atg5, Atg7 and Atg12 in EC9706 and KYSE450 cells treated with different concentrations of OP16 (0, 1, 5 and 10 μM) for 12 h. **d**, **e** Expression of LC3B, p-Akt (Ser473), Cleaved-caspase-9 and Cleaved-caspase-3 in EC9706 and KYSE450 cells treated with OP16 (10 μM) and SC79 (40 μM) alone or combination for 12 h. $^{**}P<0.01$, $^{***}P<0.001$, versus control group, $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, versus single-drug treatment group

ESCC. In addition, we investigated the expression of proteins in mitochondrial apoptosis pathway after cells were treated as described above. The results demonstrated that SC79 also impaired the promoting effects of OP16 on Cleaved-caspase-9 and Cleaved-caspase-3 ($P<0.05$ or $P<0.01$) (Fig. 4e), indicating that OP16 activated mitochondrial apoptosis pathway through inhibiting phosphorylation of Akt in ESCC. The above results indicated that the OP16 induces both cell autophagy and activates mitochondrial apoptosis pathway apoptosis in ESCC through inhibiting phosphorylation of Akt.

Discussion

ESCC is an aggressive human malignancy, which has a high morbidity and mortality rate in North China, Japan, Northern Iran and South Africa [38], while the number of ESCC patients in China is more than 50% [39]. Autophagy, as an evolutionarily conserved catabolic process, has been extensively studied, the alterations in autophagy have been linked to human pathologies, including neurodegeneration, inflammatory bowel disease, and cancers [40, 41]. We have shown the anti-tumor effects of OP16 on ESCC by inhibiting p-Akt in our previous study [30], in this study, we further explore the mechanism of OP16 inhibiting ESCC and demonstrated that the apoptosis-promoting effects of OP16 on ESCC have related to its inducing effects on cell autophagy and activating effects on mitochondria apoptosis pathway, and both of which were mediated by the inhibition effects of OP16 on phosphorylation of Akt (Fig. 5).

Researches have demonstrated that there are high autophagy activity in subsets of melanoma, colorectal cancer, myeloma and renal cell carcinoma patients [42–44], including ESCC [45], and tumors are more sensitive to autophagy inhibition than normal tissues [46]. However, it has been demonstrated that oridonin, the key anti-tumor component of *Rabdosia rubescens* widely grown in China and Japan, could promote apoptosis of tumor cells by inducing autophagy. We, therefore, explored the autophagy-inducing effects of OP16, which has the same active site with oridonin. Bafilomycin A1, CQ and rapamycin, here, were used as the control drugs in the experiments. Bafilomycin A1 and CQ, the regular autophagy inhibitors, can inhibit fusion

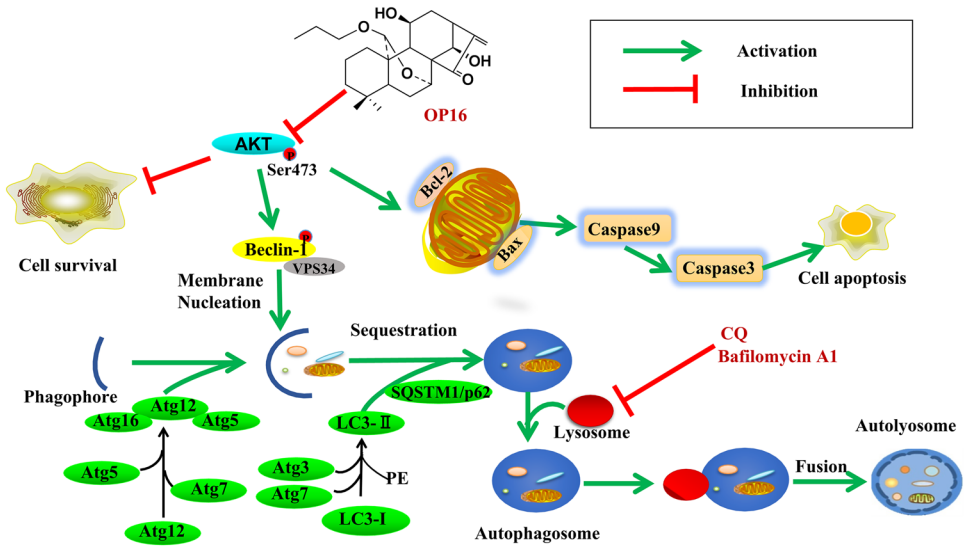

Fig. 5 OP16 induces deadly autophagy and apoptosis of ESCC cells by inhibiting Akt

of autophagosome and lysosome by inhibiting the function of lysosome, and cause the accumulation of autophagy in the cells by increasing expression of LC3B protein located on the membrane of autophagy [47, 48], while rapamycin is not only the inhibitor of mTOR pathway [49], but also a typical autophagy inducer [37]. We firstly showed that OP16 could promote LC3B and decrease SQSTM1/p62 expression in ESCC cells by Western blot. Next, we indicated that OP16 could promote accumulation of LC3B in cytoplasm by immunofluorescence assay. Moreover, when ESCC cells were treated with OP16 combined with bafilomycin A1 or CQ, respectively, expression of LC3B-II was obviously higher than cells treated with OP16, bafilomycin A1 or chloroquine alone. In other words, Bafilomycin A1 and CQ promoted the expression of LC3B-II through inhibition the fusion of autophagosome and lysosome, OP16 might further increase of LC3B-II expression through promoting the formation of more autophagosomes to activate autophagy flux. However, the results above were inadequate to judge the inducing effects of OP16 on autophagy, because both promoting autophagy flux and retarding the information of autolysosome could cause the aggregation of LC3B. We, therefore, further detected the effects of OP16 on the autophagic flux activity using double-marked adenovirus vector mRFP-GFP-LC3B, which is regularly used to mark autophagosome and autolysosome. Yellow fluorescence represents autophagosome, which produced by the co-localization of green and red fluorescence, while when autophagosome combines with lysosomes into autolysosome, the acidity in cells will increase and green fluorescence will quench, red fluorescence therefore represents autolysosome [50, 51]. Bafilomycin A1 can gather autophagosome in cells by blocking the combination of autophagosome and lysosomes, so in cells treated with bafilomycin A1, strong yellow fluorescence could be observed because of no quench of green fluorescence, while in cells treated with OP16, strong yellow and red fluorescence as well as the partly quenched green fluorescence could be observed (Fig. 1e), which suggested that OP16 promoted the formation of autophagy flux. Finally, we showed morphologically OP16 promoted the formation of autophagosomes and autolysosomes of ESCC cells by transmission electron microscopy. The results above demonstrated sufficiently that OP16 could induce cell autophagy in ESCC.

Researches confirmed that oridonin can induce apoptosis of tumor cells by activating caspase apoptosis signal cascade in many cancers such as cervical cancer and osteosarcoma [52, 53]. We also demonstrated the promoting-apoptosis effects of OP16 on ESCC through activating mitochondria apoptosis pathway in our previous study [30] and in this study (Fig. 2a-c). However, when we treated ESCC cells with the combination of OP16 and caspase inhibitor Z-VAD-FMK, we found Z-VAD-FMK could not neutralize completely the inhibition of OP16 on cell apoptosis (Fig. 2d), which indicated that there might be other mechanism in OP16-induced apoptosis. To explore whether OP16-induced apoptosis had relationship with OP16-induced autophagy, we treated ESCC cells with OP16 combined with autophagy inhibitor CQ or serum-free starvation as well as Beclin-1 shRNA, and found that CQ and Beclin-1 shRNA impaired the promoting effects of OP16 on cell apoptosis, while serum-free starvation promoted the apoptosis-inducing effects of OP16, indicated that OP16 inducing autophagy could promote apoptosis of ESCC cells. Therefore, OP16 induced apoptosis of ESCC cell not only because of its activation to mitochondria apoptosis pathway, but also its induction to cell autophagy.

To explore the mechanism of OP16 inducing cell autophagy in ESCC, we detected the effect of OP16 on the key initiative proteins in autophagy Atg5, Atg7 and Atg12. However,

we found OP16 had no obvious effects on these proteins (Fig. 4c), indicating that OP16 inducing autophagy by promoting autophagosome and autolysosome formation has no relationship with the initiative stage of autophagy. Researches demonstrated that autophagy can be regulated by many signal pathways, such as Akt [54, 55]. Akt is a negative regulatory factor of autophagy and plays a key role in autophagy regulation [56], and the results of biological information also primarily demonstrated the regulation relationship of Akt to autophagy- and apoptosis-related proteins (Fig. 4a, b). Because we have demonstrated the inhibiting effects of OP16 on the phosphorylation of Akt [30], to explore whether autophagy induced by OP16 was regulated by Akt, an Akt-activating agent SC79 was used to treat ESCC cells to promote phosphorylation of Akt, and then the expression of LC3B was detected. We found SC79 inhibited expression of LC3B and neutralized the promoting effects of OP16 on LC3B (Fig. 4d), indicating that OP16 induced autophagy by inhibiting phosphorylation of Akt. A large number of studies have shown that Akt is an important pro-proliferation and pro-survival factor, which can control the proliferation and apoptosis of cells by regulating the downstream substrates as well as the mitochondrial apoptosis pathway [57, 58]. Oridonin could induce apoptosis of tumor cells by activating caspase signal cascade mediated by inhibiting PI3K/Akt signal pathway [25]. In this study, we demonstrated activating Akt by SC79 impaired the activating effects of OP16 on cleaved caspase3/9 (Fig. 4e), suggesting that OP16 activated mitochondrial apoptosis pathway by inhibiting phosphorylation of Akt.

In conclusion, our data disclosed that OP16 could promote cell apoptosis by activating mitochondria apoptosis pathway and inducing cell autophagy through inhibiting phosphorylation of Akt (Fig. 5). These findings would enrich the theoretical mechanism of anti-tumor effects of OP16 and provide a basis for future clinical trials of OP16 treating ESCC.

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Compliance with ethical standards

Conflict of interest No potential conflicts of interest are disclosed.

References

1. Choi AM, Ryter SW, Levine B (2013) Autophagy in human health and disease. N Engl J Med 368(7):651–662. https://doi.org/10.1056/NEJMra1205406
2. He C, Klionsky DJ (2009) Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet 43:67–93. https://doi.org/10.1146/annurev-genet-102808-114910
3. Nguyen TN, Padman BS, Usher J, Oorschot V, Ramm G, Lazarou M (2016) Atg8 family LC3/GABARAP proteins are crucial for autophagosome-lysosome fusion but not autophagosome formation during PINK1/Parkin mitophagy and starvation. J Cell Biol 215(6):857–874. https://doi.org/10.1083/jcb.201607039
4. Huang Y, Hou JK, Chen TT, Zhao XY, Yan ZW, Zhang J, Yang J, Kogan SC, Chen GQ (2011) PML-RARalpha enhances constitutive autophagic activity through inhibiting the Akt/mTOR pathway. Autophagy 7(10):1132–1144. https://doi.org/10.4161/auto.7.10.16636
5. Reggiori F, Ungermann C (2017) Autophagosome Maturation and Fusion. J Mol Biol 429(4):486–496. https://doi.org/10.1016/j.jmb.2017.01.002
6. Ge L, Baskaran S, Schekman R, Hurley JH (2014) The protein-vesicle network of autophagy. Curr Opin Cell Biol 29:18–24. https://doi.org/10.1016/j.ceb.2014.02.005
7. Bjorkoy G, Lamark T, Brech A, Outzen H, Perander M, Overvatn A, Stenmark H, Johansen T (2005) p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. J Cell Biol 171(4):603–614. https://doi.org/10.1083/jcb.200507002
8. Amaravadi R, Kimmelman AC, White E (2016) Recent insights into the function of autophagy in cancer. Genes Dev 30(17):1913–1930. https://doi.org/10.1101/gad.287524.116
9. Mizushima N, Levine B, Cuervo AM, Klionsky DJ (2008) Autophagy fights disease through cellular self-digestion. Nature 451(7182):1069–1075. https://doi.org/10.1038/nature06639
10. White E (2015) The role for autophagy in cancer. J Clin Invest 125(1):42–46. https://doi.org/10.1172/JCI73941
11. White E, DiPaola RS (2009) The double-edged sword of autophagy modulation in cancer. Clin Cancer Res 15(17):5308–5316. https://doi.org/10.1158/1078-0432.CCR-07-5023
12. Hippert MM, O'Toole PS, Thorburn A (2006) Autophagy in cancer: good, bad, or both? Cancer Res 66(19):9349–9351. https://doi.org/10.1158/0008-5472.CAN-06-1597
13. Fulda S, Kogel D (2015) Cell death by autophagy: emerging molecular mechanisms and implications for cancer therapy. Oncogene 34(40):5105–5113. https://doi.org/10.1038/onc.2014.458
14. Rangwala R, Chang YC, Hu J, Algazy KM, Evans TL, Fecher LA, Schuchter LM, Torigian DA, Panosian JT, Troxel AB, Tan KS, Heitjan DF, DeMichele AM, Vaughn DJ, Redlinger M, Alavi A, Kaiser J, Pontiggia L, Davis LE, O'Dwyer PJ, Amaravadi RK (2014) Combined MTOR and autophagy inhibition: phase I trial of hydroxychloroquine and temsirolimus in patients with advanced solid tumors and melanoma. Autophagy 10(8):1391–1402. https://doi.org/10.4161/auto.29119
15. Li J, Hou N, Faried A, Tsutsumi S, Kuwano H (2010) Inhibition of autophagy augments 5-fluorouracil chemotherapy in human colon cancer in vitro and in vivo model. Eur J Cancer 46(10):1900–1909. https://doi.org/10.1016/j.ejca.2010.02.021
16. Vyas AR, Hahm ER, Arlotti JA, Watkins S, Stolz DB, Desai D, Amin S, Singh SV (2013) Chemoprevention of prostate cancer by d, l-sulforaphane is augmented by pharmacological inhibition of autophagy. Cancer Res 73(19):5985–5995. https://doi.org/10.1158/0008-5472.CAN-13-0755
17. Herman-Antosiewicz A, Johnson DE, Singh SV (2006) Sulforaphane causes autophagy to inhibit release of cytochrome C and apoptosis in human prostate cancer cells. Cancer Res 66(11):5828–5835. https://doi.org/10.1158/0008-5472.CAN-06-0139
18. Gewirtz DA (2014) The four faces of autophagy: implications for cancer therapy. Cancer Res 74(3):647–651. https://doi.org/10.1158/0008-5472.CAN-13-2966

19. Sui X, Chen R, Wang Z, Huang Z, Kong N, Zhang M, Han W, Lou F, Yang J, Zhang Q, Wang X, He C, Pan H (2013) Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. Cell Death Dis 4:e838. https://doi.org/10.1038/cddis.2013.350
20. Yang ZJ, Chee CE, Huang S, Sinicrope FA (2011) The role of autophagy in cancer: therapeutic implications. Mol Cancer Ther 10(9):1533–1541. https://doi.org/10.1158/1535-7163.mct-11-0047
21. Kumar D, Shankar S, Srivastava RK (2014) Rottlerin induces autophagy and apoptosis in prostate cancer stem cells via PI3K/Akt/mTOR signaling pathway. Cancer Lett 343(2):179–189. https://doi.org/10.1016/j.canlet.2013.10.003
22. Saiki S, Sasazawa Y, Imamichi Y, Kawajiri S, Fujimaki T, Tanida I, Kobayashi H, Sato F, Sato S, Ishikawa K, Imoto M, Hattori N (2011) Caffeine induces apoptosis by enhancement of autophagy via PI3K/Akt/mTOR/p70S6K inhibition. Autophagy 7(2):176–187. https://doi.org/10.4161/auto.7.2.14074
23. Farrow JM, Yang JC, Evans CP (2014) Autophagy as a modulator and target in prostate cancer. Nat Rev Urol 11(9):508–516. https://doi.org/10.1038/nrurol.2014.196
24. Tan W, Lu J, Huang M, Li Y, Chen M, Wu G, Gong J, Zhong Z, Xu Z, Dang Y, Guo J, Chen X, Wang Y (2011) Anti-cancer natural products isolated from chinese medicinal herbs. Chin Med 6(1):27. https://doi.org/10.1186/1749-8546-6-27
25. Song M, Liu X, Liu K, Zhao R, Huang H, Shi Y, Zhang M, Zhou S, Xie H, Chen H, Li Y, Zheng Y, Wu Q, Liu F, Li E, Bode AM, Dong Z, Lee MH (2018) Targeting AKT with oridonin inhibits growth of esophageal squamous cell carcinoma in vitro and patient-derived xenografts in vivo. Mol Cancer Ther 17(7):1540–1553. https://doi.org/10.1158/1535-7163.mct-17-0823
26. Li Y, Wang Y, Wang S, Gao Y, Zhang X, Lu C (2015) Oridonin phosphate-induced autophagy effectively enhances cell apoptosis of human breast cancer cells. Med Oncol 32(1):365. https://doi.org/10.1007/s12032-014-0365-1
27. Ding Y, Ding C, Ye N, Liu Z, Wold EA, Chen H, Wild C, Shen Q, Zhou J (2016) Discovery and development of natural product oridonin-inspired anticancer agents. Eur J Med Chem 122:102–117. https://doi.org/10.1016/j.ejmech.2016.06.015
28. Liu H, Zhu W, Zhu C, Wang Q, Ke Y, Liu Z, Yan X, Zhang J, Qu H Novel ent-kaurene diterpene compound and its derivatives, their preparation and their use. Patent No. 8084430 (in the United States).
29. Liu H, Zhu W, Zhu C, Wang Q, Ke Y, Liu Z, Yan X, Zhang J, H Q Novel ent-kaurene intervene compound and its derivatives, their preparation and their use. Patent No. AZ (in China).
30. Peng KZ, Ke Y, Zhao Q, Tian F, Liu HM, Hou G, Lu Z (2017) OP16, a novel ent-kaurene diterpenoid, potentiates the antitumor effect of rapamycin by inhibiting rapamycin-induced feedback activation of Akt signaling in esophageal squamous cell carcinoma. Biochem Pharmacol 140:16–27. https://doi.org/10.1016/j.bcp.2017.05.013
31. Hou G, Zhang Q, Wang L, Liu M, Wang J, Xue L (2010) mTOR inhibitor rapamycin alone or combined with cisplatin inhibits growth of esophageal squamous cell carcinoma in nude mice. Cancer Lett 290(2):248–254. https://doi.org/10.1016/j.canlet.2009.09.015
32. Hou G, Zhao Q, Zhang M, Fan T, Liu M, Shi X, Ren Y, Wang Y, Zhou J, Lu Z (2018) Down-regulation of Rictor enhances cell sensitivity to PI3K inhibitor LY294002 by blocking mTORC2-medicated phosphorylation of Akt/PRAS40 in esophageal squamous cell carcinoma. Biomed Pharmacother 106:1348–1356. https://doi.org/10.1016/j.biopha.2018.07.075
33. Chen L, Lu D, Sun K, Xu Y, Hu P, Li X, Xu F (2019) Identification of biomarkers associated with diagnosis and prognosis of colorectal cancer patients based on integrated bioinformatics analysis. Gene 692:119–125. https://doi.org/10.1016/j.gene.2019.01.001
34. von Mering C, Huynen M, Jaeggi D, Schmidt S, Bork P, Snel B (2003) STRING: a database of predicted functional associations between proteins. Nucleic Acids Res 31(1):258–261. https://doi.org/10.1093/nar/gkg034
35. Liu Z, Ouyang L, Peng H, Zhang WZ (2012) Oridonin: targeting programmed cell death pathways as an anti-tumour agent. Cell Prolif 45(6):499–507. https://doi.org/10.1111/j.1365-2184.2012.00849.x
36. Kim J, Kundu M, Viollet B, Guan KL (2011) AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat Cell Biol 13(2):132–141. https://doi.org/10.1038/ncb2152
37. Sotthibundhu A, McDonagh K, von Kriegsheim A, Garcia-Munoz A, Klawiter A, Thompson K, Chauhan KD, Krawczyk J, McInerney V, Dockery P, Devine MJ, Kunath T, Barry F, O'Brien T, Shen S (2016) Rapamycin regulates autophagy and cell adhesion in induced pluripotent stem cells. Stem Cell Res Ther 7(1):166. https://doi.org/10.1186/s13287-016-0425-x
38. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. CA Cancer J Clin 61(2):69–90. https://doi.org/10.3322/caac.20107
39. Chen J, Pan J, Zheng X, Zhu K, Li J, Chen M, Wang J, Liao Z (2012) Number and location of positive nodes, postoperative radiotherapy, and survival after esophagectomy with three-field lymph node dissection for thoracic esophageal squamous cell carcinoma. Int J Radiat Oncol Biol Phys 82(1):475–482. https://doi.org/10.1016/j.ijrobp.2010.08.037
40. Racanelli AC, Kikkers SA, Choi AMK, Cloonan SM (2018) Autophagy and inflammation in chronic respiratory disease. Autophagy 14(2):221–232. https://doi.org/10.1080/15548627.2017.1389823
41. Karabiyik C, Lee MJ, Rubinsztein DC (2017) Autophagy impairment in Parkinson's disease. Essays Biochem 61(6):711–720. https://doi.org/10.1042/ebc20170023
42. Vogl DT, Stadtmauer EA, Tan KS, Heitjan DF, Davis LE, Pontiggia L, Rangwala R, Piao S, Chang YC, Scott EC, Paul TM, Nichols CW, Porter DL, Kaplan J, Mallon G, Bradner JE, Amaravadi RK (2014) Combined autophagy and proteasome inhibition: a phase 1 trial of hydroxychloroquine and bortezomib in patients with relapsed/refractory myeloma. Autophagy 10(8):1380–1390. https://doi.org/10.4161/auto.29264
43. Rangwala R, Leone R, Chang YC, Fecher LA, Schuchter LM, Kramer A, Tan KS, Heitjan DF, Rodgers G, Gallagher M, Piao S, Troxel AB, Evans TL, DeMichele AM, Nathanson KL, O'Dwyer PJ, Kaiser J, Pontiggia L, Davis LE, Amaravadi RK (2014) Phase I trial of hydroxychloroquine with dose-intense temozolomide in patients with advanced solid tumors and melanoma. Autophagy 10(8):1369–1379. https://doi.org/10.4161/auto.29118
44. Mahalingam D, Mita M, Sarantopoulos J, Wood L, Amaravadi RK, Davis LE, Mita AC, Curiel TJ, Espitia CM, Nawrocki ST, Giles FJ, Carew JS (2014) Combined autophagy and HDAC inhibition: a phase I safety, tolerability, pharmacokinetic, and pharmacodynamic analysis of hydroxychloroquine in combination with the HDAC inhibitor vorinostat in patients with advanced solid tumors. Autophagy 10(8):1403–1414. https://doi.org/10.4161/auto.29231
45. Hall TM, Tetreault MP, Hamilton KE, Whelan KA (2018) Autophagy as a cytoprotective mechanism in esophageal squamous cell carcinoma. Curr Opin Pharmacol 41:12–19. https://doi.org/10.1016/j.coph.2018.04.003
46. Karsli-Uzunbas G, Guo JY, Price S, Teng X, Laddha SV, Khor S, Kalaany NY, Jacks T, Chan CS, Rabinowitz JD, White E (2014) Autophagy is required for glucose homeostasis and lung

tumor maintenance. Cancer Discov 4(8):914–927. https://doi.org/10.1158/2159-8290.cd-14-0363
47. Mauvezin C, Neufeld TP (2015) Bafilomycin A1 disrupts autophagic flux by inhibiting both V-ATPase-dependent acidification and Ca-P60A/SERCA-dependent autophagosome-lysosome fusion. Autophagy 11(8):1437–1438. https://doi.org/10.1080/15548627.2015.1066957
48. Redmann M, Benavides GA, Berryhill TF, Wani WY, Ouyang X, Johnson MS, Ravi S, Barnes S, Darley-Usmar VM, Zhang J (2017) Inhibition of autophagy with bafilomycin and chloroquine decreases mitochondrial quality and bioenergetic function in primary neurons. Redox Biol 11:73–81. https://doi.org/10.1016/j.redox.2016.11.004
49. Schreiber KH, Arriola Apelo SI, Yu D, Brinkman JA, Velarde MC, Syed FA, Liao CY, Baar EL, Carbajal KA, Sherman DS, Ortiz D, Brunauer R, Yang SE, Tzannis ST, Kennedy BK, Lamming DW (2019) A novel rapamycin analog is highly selective for mTORC1 in vivo. Nat Commun 10(1):3194. https://doi.org/10.1038/s41467-019-11174-0
50. Luo T, Fu J, Xu A, Su B, Ren Y, Li N, Zhu J, Zhao X, Dai R, Cao J, Wang B, Qin W, Jiang J, Li J, Wu M, Feng G, Chen Y, Wang H (2016) PSMD10/gankyrin induces autophagy to promote tumor progression through cytoplasmic interaction with ATG7 and nuclear transactivation of ATG7 expression. Autophagy 12(8):1355–1371. https://doi.org/10.1080/15548627.2015.1034405
51. Liu F, Chen J, Wang P, Li H, Zhou Y, Liu H, Liu Z, Zheng R, Wang L, Yang H, Cui Z, Wang F, Huang X, Wang J, Sha W, Xiao H, Ge B (2018) MicroRNA-27a controls the intracellular survival of Mycobacterium tuberculosis by regulating calcium-associated autophagy. Nat Commun 9(1):4295. https://doi.org/10.1038/s41467-018-06836-4
52. Jin S, Shen JN, Wang J, Huang G, Zhou JG (2007) Oridonin induced apoptosis through Akt and MAPKs signaling pathways in human osteosarcoma cells. Cancer Biol Ther 6(2):261–268. https://doi.org/10.4161/cbt.6.2.3621
53. Hu HZ, Yang YB, Xu XD, Shen HW, Shu YM, Ren Z, Li XM, Shen HM, Zeng HT (2007) Oridonin induces apoptosis via PI3K/Akt pathway in cervical carcinoma HeLa cell line. Acta Pharmacol Sin 28(11):1819–1826. https://doi.org/10.1111/j.1745-7254.2007.00667.x
54. Munson MJ, Ganley IG (2015) MTOR, PIK3C3, and autophagy: Signaling the beginning from the end. Autophagy 11(12):2375–2376. https://doi.org/10.1080/15548627.2015.1106668
55. Gao L, Wang Z, Lu D, Huang J, Liu J, Hong L (2019) Paeonol induces cytoprotective autophagy via blocking the Akt/mTOR pathway in ovarian cancer cells. Cell Death Dis 10(8):609. https://doi.org/10.1038/s41419-019-1849-x
56. Sun Y, Huang YH, Huang FY, Mei WL, Liu Q, Wang CC, Lin YY, Huang C, Li YN, Dai HF, Tan GH (2018) 3'-epi-12beta-hydroxyfroside, a new cardenolide, induces cytoprotective autophagy via blocking the Hsp90/Akt/mTOR axis in lung cancer cells. Theranostics 8(7):2044–2060. https://doi.org/10.7150/thno.23304
57. Brenner D, Mak TW (2009) Mitochondrial cell death effectors. Curr Opin Cell Biol 21(6):871–877. https://doi.org/10.1016/j.ceb.2009.09.004
58. Mundi PS, Sachdev J, McCourt C, Kalinsky K (2016) AKT in cancer: new molecular insights and advances in drug development. Br J Clin Pharmacol 82(4):943–956. https://doi.org/10.1111/bcp.13021