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Molecular mechanisms of apoptosis and autophagy elicited by combined treatment with oridonin and cetuximab in laryngeal squamous cell carcinoma

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Abstract

Combined oridonin (ORI), a natural and safe kaurene diterpenoid isolated from *Rabdosia rubescens*, and cetuximab (Cet), an anti-EGFR monoclonal antibody, have been reported to exert synergistic anti-tumor effects against laryngeal squamous cell carcinoma (LSCC) both in vitro and in vivo by our group. In the present study, we further found that ORI/Cet treatment not only resulted in apoptosis but also induced autophagy. AMPK/mTOR signaling pathway was found to be involved in the activation of autophagy in ORI/Cet-treated LSCC cells, which is independent of p53 status. Additionally, chromatin immunoprecipitation (ChIP) assay showed that ORI/Cet significantly increased the binding NF-κB family member p65 with the promotor of *BECN 1*, and p65-mediated up-regulation of *BECN 1* caused by ORI/Cet is coupled to increased autophagy. On the other hand, we demonstrated that either Beclin 1 SiRNA or autophagy inhibitors could increase ORI/Cet induced-apoptosis, indicating that autophagy induced by combination of the two agents plays a cytoprotective role. Interestingly, 48 h after the combined treatment, autophagy began to decrease but apoptosis was significantly elevated. Our findings suggest that autophagy might be strongly associated with the antitumor efficacy of ORI/Cet, which may be beneficial to the clinical application of ORI/Cet in LSCC treatment.

Keywords Cetuximab · Oridonin · Autophagy · Apoptosis · Laryngeal squamous cell carcinoma

Introduction

Autophagy, known to be type II programmed cell death (PCD), maintains cellular biosynthesis through degrading cellular organelles and proteins during nutrient deprivation or metabolic stress [1]. In cancer, autophagy is a vital process to regulate cancer cell growth, development and homeostasis [2]. In addition, autophagy is detected in cancer cells after various types of treatment, such as radio- and chemotherapies [3, 4]. Autophagy induced by different anti-cancer treatments could lead to cancer cell death or survival, which highly depends on the specific context of the cancer cells and treatment types, such as tumor environment and treatment characteristics [5]. Furthermore, autophagy could occur together with apoptosis after treatment with same stimulus [6]. Meanwhile, autophagy can be protective or stimulative when apoptosis occurs [7, 8].

Autophagy is functionally controlled by distinct members of the autophagy-related (Atg) proteins and other upstream signaling pathways. Multiple signaling molecules, such as adenosine monophosphate kinase (AMPK), mammalian

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target of rapamycin (mTOR) or nuclear factor-kappa B (NF-κB), have been shown to regulate autophagy [9, 10]. AMPK is a master regulator of metabolism and stimulates autophagy [11]. AMPK activation leads to autophagy through negative regulation of mTOR [12]. Once mTOR is deactivated, it activates autophagy via dephosphorylating Atg proteins [13]. In addition, NF-κB pathway is also closely associated with regulation of autophagy [14]. NF-κB, a transcription factor, influences autophagic responses through transactivating genes coding for cytokines, for example IL-6, TGF-β [15, 16]. Recent studies have reported that NF-κB could contribute to the stimulation of autophagy by up-regulating Beclin 1 expression [17]. A full understanding of these pathways will be critical for the assessment of anti-cancer strategies.

Some plant-derived natural products such as stellettin B [18] and parthenolide [19] exert significant anti-tumor effects by regulating two critical cellular processes—apoptosis and autophagy. Oridonin (ORI), a diterpenoid isolated from the medicinal herb *Rabdosia rubescens*, has been demonstrated to be effective against various tumors through inducing apoptosis or autophagy [20–22]. Zhang et al. observed that ORI could induce both apoptosis and autophagy in human cervical cancer HeLa cells, and inhibition of autophagy contributes to apoptosis [23]. Our previous reports also proved that the autophagy triggered by ORI participated in upregulation of apoptosis in LSCC cells [22, 24]. Nevertheless, Cui et al. found that ORI could simultaneously induce human breast cancer MCF-7 cells both apoptosis and autophagy, meanwhile inhibition of autophagy significantly reduced the level of apoptosis [25]. Therefore, whether the autophagy induced by ORI treatment promotes or inhibits the apoptosis in tumor cells remains controversial, but it is apparent that the outcome depends highly on tumor cell types.

Cetuximab (Cet) is an IgG1 monoclonal antibody that directly binds to the extracellular domain of EGFR and blocks the activation of EGFR in several human cancers of epithelial origin [26]. We firstly found that in EGFR over expressed laryngeal squamous cell carcinoma (LSCC) cells, combined treatment of ORI with Cet possessed synergistic anti-cancer activities both in vitro and in vivo without severe side effects [27]. However, the underlying molecular mechanisms of the anti-cancer effects of the combination of ORI and Cet are still unknown. In our current work, we established that combination of ORI and Cet induced marked autophagy in two LSCC cell lines. We also explored the role of autophagy in the apoptosis of LSCC cells after treatment with ORI plus Cet. The molecular mechanisms mediating the effect of autophagy on the ORI/Cet-induced apoptosis were further investigated to provide the evidence for improving the antitumor efficacy of combination ORI with Cet via increasing apoptosis by autophagy modulation.

Materials and methods

Reagents

ORI (Fig. 1a) was provided from the Beijing Institute of Biological Products (Beijing, China). Cet (Erbitux, C-225), a humanized IgG1 monoclonal antibody, was obtained from Merck (KGaA, Darmstadt, Germany). The other chemicals including phenylmethanesulfonyl fluoride (PMSF), 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA).

Antibodies

The primary antibodies of p-AMPK (Thr172), AMPK, p-mTOR (Ser2448), mTOR, LC3, Beclin 1, cleaved PARP and β-Actin for western blotting and immunohistochemical studies were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

HEp-2 and Tu212 cell lines (ATCC, Manassas, VA, USA) were maintained at 37 °C and 5% CO_2 in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 100 U/mL penicillin, 10 μg/mL streptomycin and 10% fetal bovine serum (TBD, Tianjin, China).

Cell viability assay

MTT assay was used to determine the inhibitory effects of ORI/Cet on LSCC cells, as described in our previous work [27]. HEp-2 and Tu212 cells were seeded into 96-well culture plates, and incubated with ORI plus Cet for 48 h. Then, the medium was removed, 100 μL MTT (0.5 μg/mL) was added, and the cells were incubated for 2.5 h at 37 °C. The formazan crystals were dissolved in 150 μL DMSO for 15 min on an oscillator. The optical density (OD) was measured at 490 nm using a Microplate Reader (BioTek, MV, USA). The cell inhibitory rate (%) was calculated as follows:

$$\text{Cell Inhibitory rate}(\%) = 100 - \left[\left(\text{OD}_{490,Sample} - \text{OD}_{490,\text{Blank}}\right)/\left(\text{OD}_{490,\text{Control}} - \text{OD}_{490,\text{Blank}}\right)\right] \times 100.$$

Autophagy and apoptosis analysis by flow cytometry

After incubation with ORI plus Cet for the different times, HEp-2 and Tu212 cells were cultured with MDC (0.05 mM in PBS) at 37 °C for 30 min. The fluorescence microscope (Olympus, Tokyo, Japan) was used to observe the cellular

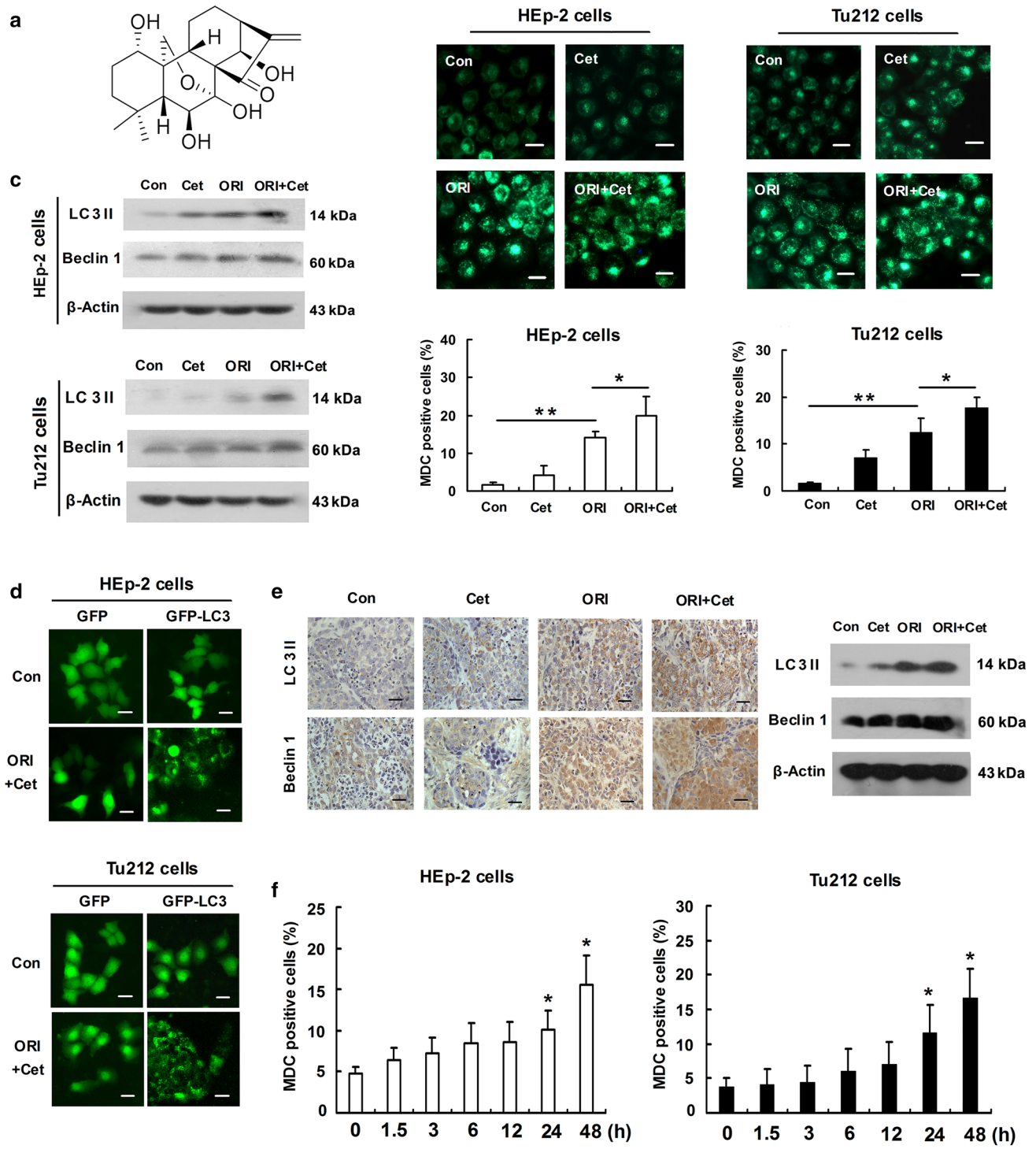

Fig. 1 Induction of autophagy by combination treatment with oridonin (ORI) and cetuximab (Cet) in LSCC in vitro and in vivo. **a** The chemical structure of ORI. **b** HEp-2 and Tu212 cells were treated with ORI (24 μM for HEp-2 cells and 36 μM for Tu212 cells, respectively) and Cet (10 μg/mL) for 48 h and stained with monodansylcadaverine (MDC) for detection of autophagic vacuoles. Bar represents 10 μm. The MDC fluorescent intensity was analyzed by flow cytometry. n = 3, Mean ± S.D. *P < 0.05; **P < 0.01. **c** Western blotting was used to detect the levels of LC3II and Beclin 1. **d** Formation of LC3-positive vesicles in ORI/Cet-treated GFP-LC3 transduced cells. **e** The expression of LC3II and Beclin 1 in the HEp-2 xenografts were examined by immunohistochemistry and western blotting. Bar represent 100 μm. **f** ORI/Cet time-dependently induces autophagy. HEp-2 and Tu212 cells were exposed to ORI (24 μM for HEp-2 cells and 36 μM for Tu212 cells, respectively) and Cet (10 μg/mL) for different hours. The cells were stained with MDC and analyzed by flow cytometry. n = 3, Mean ± S.D. *P < 0.05

fluorescent changes. Furthermore, the fluorescence intensity of cells was analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

Cell apoptosis was analyzed using a commercial Annexin V-PE/7-AAD apoptosis kit (BD Pharmingen Biosciences, San Diego, CA, USA). After the LSCC cells treated with ORI and Cet for different hours, the apoptotic cells were measured using the flow cytometry.

GFP-LC3 plasmid

LSCC cells were transfected with GFP-LC3 plasmid using the lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). LSCC cells stable expressing GFP-LC3 were treated with ORI/Cet and then fixed as previously described [28]. The fluorescence of GFP-LC3 was observed under a fluorescence microscope.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay with LSCC cells was carried out as previously described [29]. The standard PCR and real-time PCR were used to analyze the bound DNA fragments. The specific primers for the κB site in the *BECN 1* promoter (forward 5′-CCCGTATCATACCATTCCTAG-3′; reverse 5′-GAA ACTCG TGTCCAGTTTCAG-3′) were synthesized from GenePharma Biotech (Shanghai, China).

siRNA transfection

LSCC cells were transfected with 10 nM Beclin 1 siRNA and Con SiRNA (GenePharma Biotech, Shanghai, China) utilizing lipofectamine 2000. After 24 h, the transfected cells were ready for experimental use.

Mouse xenograft and immunohistochemistry assay

Mouse xenograft model was performed as previously described [27]. Tumor tissues used for immunohistochemistry analysis were obtained from the previous experiment. Immunohistochemistry analysis was performed following the standard protocol as described previously [27].

Western blotting assays

LSCC cells and the tumor tissues of HEp-2 xenograft nude mouse model treated with ORI and Cet were lysed in cell lysis buffer, and the protein concentrations were determined using Bradford assay. Expression of related proteins of apoptosis, autophagy and AMPK-mTOR pathway were analyzed by western blotting as previously described [24].

Data analysis

All data were expressed as the mean ± standard deviation (S.D) and analyzed using SPSS 17.0 software. The differences between sets of data were analyzed by One-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

Cet enhances ORI-induced autophagy in LSCC in vitro and in vivo

To establish whether combined treatment with ORI and Cet induced autophagy in LSCC cells, several assays to monitor autophagy were conducted. First, monodansylcadaverine (MDC) staining was used to detect autophagic vacuoles. In Fig. 1b, the amount of MDC-positive structures in LSCC cells increased after ORI treatment, as reported in our previous study [24], while compared with control group Cet did not significantly change MDC-positive vesicles. However, combination of these two agents markedly increased the abundance of MDC-positive cells in two LSCC cell lines. Meanwhile, quantification of MDC staining proved that combined treatment induced more autophagy than the ORI alone group. Further the expression levels of LC3II and Beclin 1, two major molecular markers of autophagy, were examined by western blotting (Fig. 1c). The LSCC cells in the combination group revealed significantly higher levels of LC3II when compared to all other treatment groups. In addition, ORI increased the expression of Beclin 1 in LSCC cells, and their combination showed an even stronger effect on the up-regulation of Beclin 1 protein levels. Moreover, the LSCC cells in the control group exhibited diffused distribution of GFP-LC3 in the cytoplasm, whereas combined ORI and Cet induced a punctuated fluorescent pattern of LC3 (Fig. 1d). These results suggest that combined ORI and Cet indeed induced significant autophagy in the LSCC cells.

Next, we performed immunohistochemistry and western blotting to detect the levels of LC3II and Beclin 1 in HEp-2 xenografts. As shown in Fig. 1e, LC3II staining was diffusely distributed in the cytoplasm, and little LC3II puncta staining was observed in control and Cet alone treated groups. However, LC3II puncta indicative of autophagosomes could clearly be detected after staining of tumor tissue for LC3II from ORI-treated nude mice. ORI plus Cet

further increased the expression of LC3II. Moreover, a large number of cells in ORI alone and ORI/Cet treated groups exhibited significant staining of Beclin 1, compared with control and Cet group. Similarly, the expression of Beclin 1 in the combined group was higher than ORI alone treated group. The result of western blotting also showed that ORI alone could upregulate the levels of LC3II and Beclin 1, while Cet further enhanced ORI-induced upregulation of LC3II and Beclin 1 on HEp-2 xenograft tumor in nude mouse model.

Finally, time-course MDC staining demonstrated that combined treatment with ORI and Cet increased autophagy in a time-dependent manner in both HEp-2 and Tu212 cells, especially at 24 and 48 h (Fig. 1f). Taken together, these results demonstrate that combined treatment with ORI and Cet significantly induces autophagy both in vitro and in vivo.

Inhibition of autophagy enhances ORI/Cet-triggered apoptosis in LSCC cells

Autophagy caused by different treatments in cancer cells might show different roles: promoting cell survival or cell death [30]. We used two autophagy inhibitors, 3-methyladenine (3-MA) and chloroquine (CQ) to assess the role of autophagy in LSCC cells death caused by ORI/Cet. Herein, LSCC cells were pretreated with 3-MA or CQ for 1 h, after which the cells were exposed to ORI/Cet for 48 h. As shown in Fig. 2a, a significant increase in ORI/Cet-induced cell death was observed in LSCC cells after autophagy was inhibited with CQ or 3-MA. Next, we investigated the effect of autophagy on the introduction of apoptosis by ORI/Cet in LSCC cells. Apoptosis and its related protein were analyzed after co-exposure to ORI/Cet in the presence or absence of

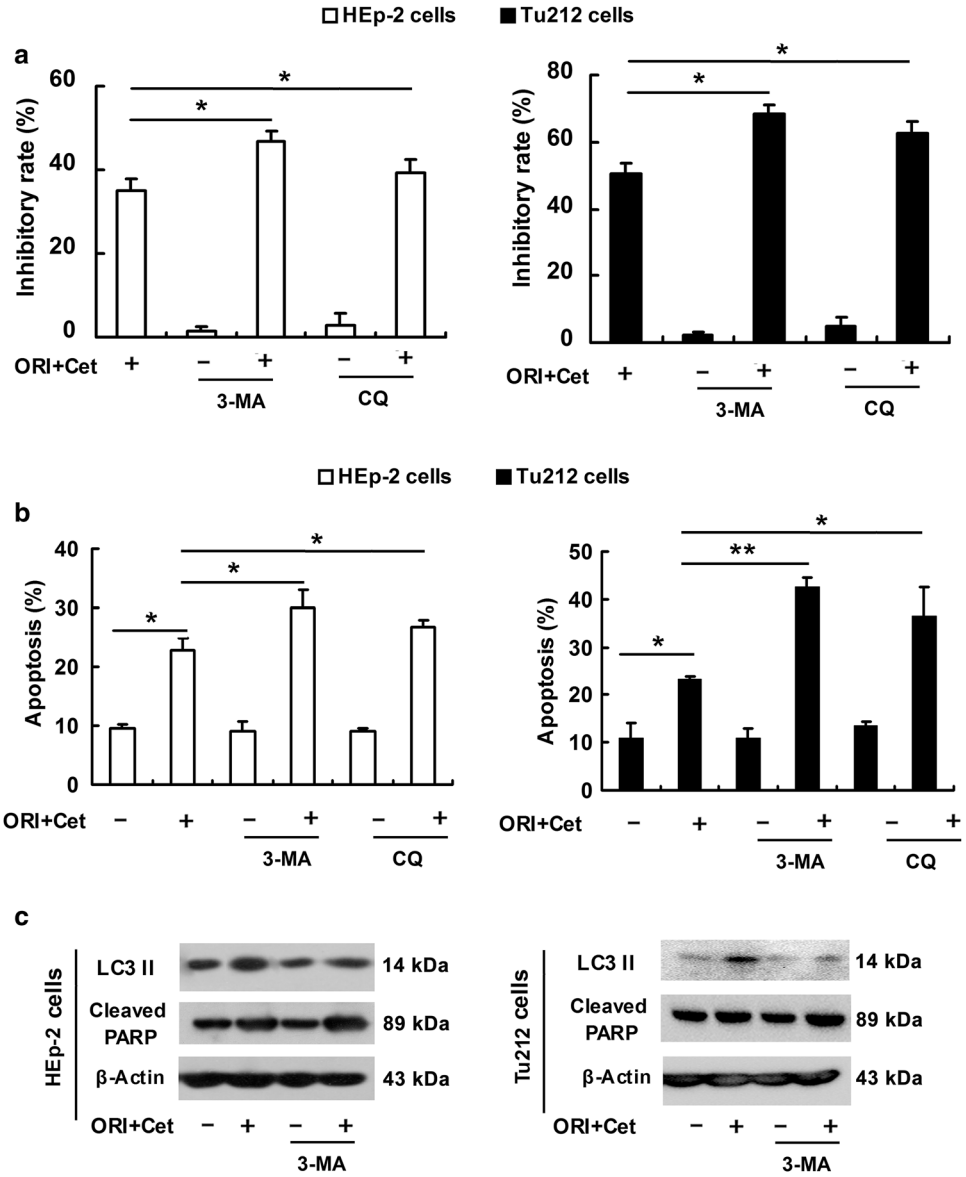

Fig. 2 Inhibition of autophagy increases ORI/Cet-induced apoptosis. LSCC cells were treated with autophagy inhibitors 3-methyladenine (3-MA) or chloroquine (CQ) for 1 h before the addition of ORI/Cet. **a** MTT assay was used to determined cell proliferation. **b** The percentage of apoptosis cells was evaluated by Annexin V-PE/7-AAD staining. **c** The levels of LC3II and cleaved PARP were measured by western blot analysis. β-Actin was used as a standard to ensure equivalent loading of cell extracts. n = 3, Mean ± S.D. $^{*}P < 0.05$; $^{**}P < 0.01$

two autophagy inhibitors. Annexin V-PE/7-AAD staining revealed a significant increase in apoptotic cells after ORI/Cet plus 3-MA or CQ treatment (Fig. 2b). We discovered that, ORI/Cet plus 3-MA treatment significantly decreased the protein expression of LC3II in two LSCC cells, compared with ORI/Cet. At the same time, in HEp-2 cells, ORI/Cet plus 3-MA further enhanced cleaved PARP expression, while the upregulation of cleaved PARP by ORI/Cet plus 3-MA treatment is not significant in the Tu212 cells (Fig. 2c).

To further confirm whether autophagy is associated with the apoptosis induced by ORI/Cet, small interfering RNA (SiRNA) was used to knock down the mRNA expression of Beclin 1, a critical regulator of autophagy, and then checked the changes of autophagy and apoptosis. Unsurprisingly, transfection with Beclin 1 siRNA resulted in a remarkable decrease of Beclin 1 expression in HEp-2 and Tu212 cells (Fig. 3d, first panel). Furthermore, the inhibition of Beclin 1 by using SiRNA significantly decreased the percentage of MDC positive cells of the combination group (Fig. 3a) and at the same time inhibited ORI/Cet-induced up-regulation of Beclin 1 and LC3II (Fig. 3d). Next, MTT assay result showed that knockdown of Beclin 1 markedly enhanced ORI/Cet-induced proliferation inhibitory effects in two LSCC cell lines (Fig. 3b). In addition, Beclin 1 SiRNA significantly increased the effect of ORI/Cet on the apoptosis in LSCC cells (Fig. 3c). Consistent with the results using 3-MA and CQ, the protein levels of cleaved-PARP were also markedly increased by repression of autophagy through knockdown of Beclin 1 gene in HEp-2 cells, whereas Beclin 1 SiRNA only showed slightly effect on the upregulation of cleaved-PARP in the Tu212 cell (Fig. 3d). Taken together, these results demonstrate that the inhibition of autophagy by 3-MA, CQ or Beclin-1 SiRNA promoted ORI/Cet-induced apoptosis in LSCC cells.

Cet enhances ORI-induced autophagy through the AMPK-mTOR pathway

Previous reports proved that AMPK-mTOR pathway mediated autophagy in cancer cells [13]. We evaluated whether AMPK and mTOR were involved in this autophagy caused by combination of ORI and Cet in LSCC cells. As shown in Fig. 4a, ORI induced AMPK phosphorylation in LSCC cells. In addition, ORI inhibited the activation of mTOR by decreasing phosphorylation, especially in HEp-2 cells. Cet did not affect the levels of p-AMPK and p-mTOR, but significantly enhanced ORI-induced activation of AMPK and inactivation of mTOR (Fig. 4a). Next, the inhibitors of AMPK and mTOR were used to test whether AMPK-mTOR pathway is associated with the cytoprotective autophagy by ORI/Cet. Pretreatment with AMPK inhibitor Compound C (Comp C) inhibited ORI/Cet-induced down-regulation of p-mTOR (Fig. 4b). In addition, pretreatment with Comp C decreased the MDC positive cells of combined treatment with ORI and Cet, whereas mTOR inhibitor rapamycin (Rap) promoted the percentage of MDC positive cells (Tu212 cells) (Fig. 4c), suggesting that the AMPK-mTOR signaling pathway may contribute to the activation of autophagy by ORI/Cet in LSCC cells. Furthermore, Comp C markedly increased ORI/Cet-induced cytotoxicity in the LSCC cells, while pretreatment with Rap demonstrated significant inhibitory effect on cytotoxicity of the combination treatment (Fig. 4d). Together, these results clearly prove that Cet enhanced ORI-induced cytoprotective autophagy through AMPK-mTOR signaling in LSCC cells.

Autophagy-induced by ORI/Cet is associated with NF-κB-mediated up-regulation of Beclin 1

Since NF-κB pathway stands at a cross-road of various signaling pathways relating to apoptosis and autophagy in cancer cells, we decided to investigate whether the contribution of NF-κB pathway in the response of LSCC cells to ORI/Cet. As shown in Fig. 5a, we observed that ORI inhibited the expression of nucleus NF-κB, and Cet further enhanced the downregulation of NF-κB. Pretreating cells with NF-κB inhibitor PDTC or MG132 before adding ORI/Cet strongly promoted LSCC cells death (Fig. 5b) and apoptosis (Shown in Supplementary Data, Fig. 1R), especially PDTC. In addition, MDC positive cells in the presence of PDTC or MG132 were significantly decreased compared with that in the absence of PDTC or MG132 (Fig. 5c). Further, the results of western blotting shown in Fig. 5d indicated that PDTC treatment significantly inhibited ORI/Cet-induced up-regulation of Beclin 1 and LC3II, confirming that NF-κB are both associated with ORI/Cet-triggered cytoprotective autophagy and apoptosis in LSCC cells.

Next, ChIP assay was conducted to determine whether the interaction of NF-κB with the putative κB site in the promoter of *BECN 1*. Figure 5e shows that, following combined treatment with ORI and Cet, the amount of the amplified product of *BECN 1* promoter significantly increased in both HEp-2 cells and Tu212 cells, suggesting that ORI/Cet promotes the binding of NF-κB to the *BECN 1* promoter.

ORI plus Cet induces both apoptosis and autophagy at early stages but autophagy reduces and apoptosis increases at late stages

To delineate the relationship between autophagy and apoptosis activated by combined ORI and Cet, we extended the observation of these two cellular processes to 96 h. As

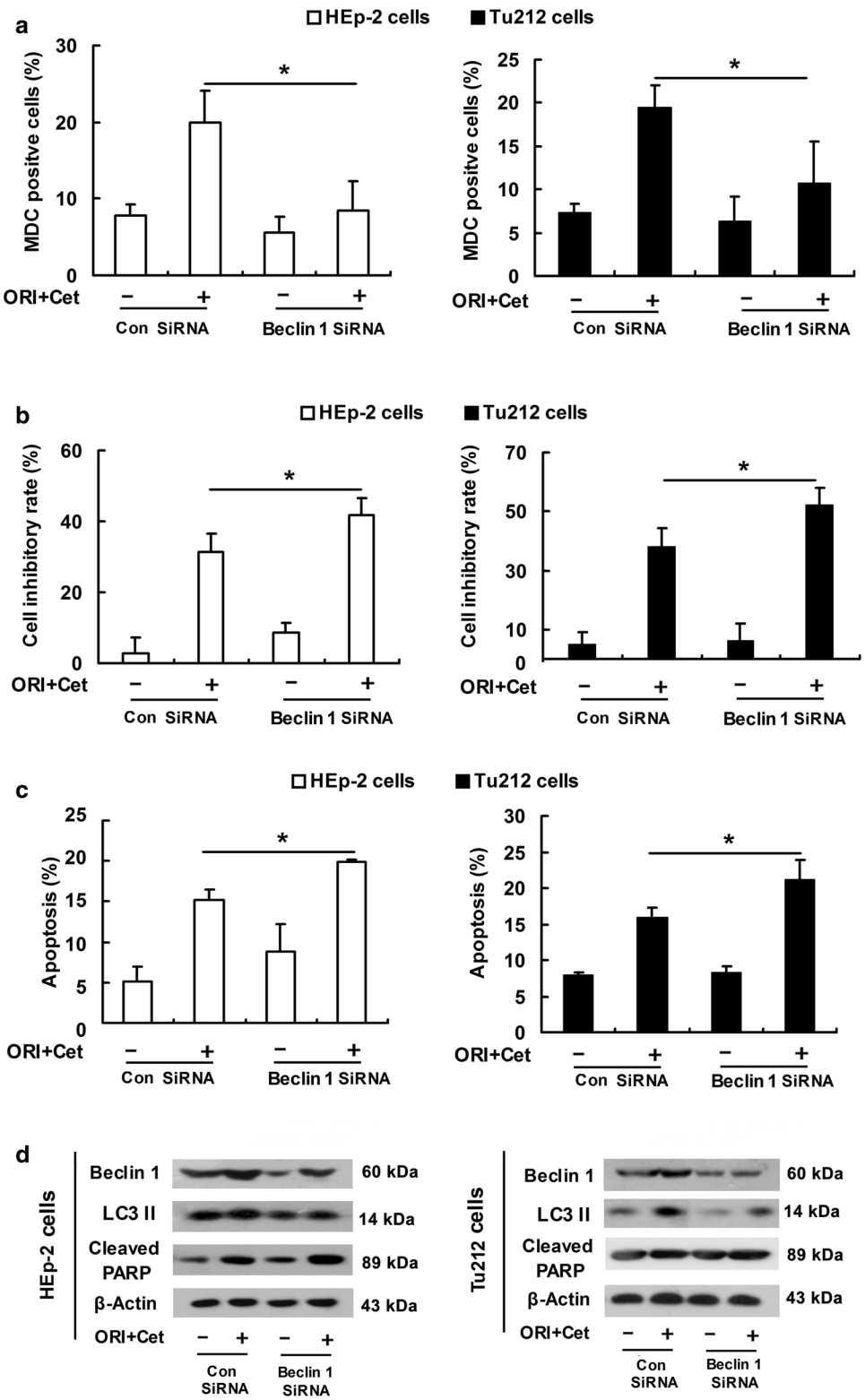

Fig. 3 Knockdown of Beclin 1 enhanced ORI/Cet-induced LSCC cell apoptosis. LSCC cells were transfected with Beclin 1 SiRNA and Con SiRNA for 1 h before the addition of ORI/Cet. **a** Autophagy was determined by flow cytometry after MDC staining. **b** MTT assay was used to detect cell inhibition. **c** Apoptosis was monitored by flow cytometry using Annexin V-PE/7-AAD staining assay. **d** The expression of Beclin 1, LC3II and cleaved PARP were detected by western blotting. β-Actin was used as a standard to ensure equivalent loading of cell extracts. All blots are representative of at least 3 repeats. n = 3, Mean ± SD. $^*P < 0.05$

shown in Fig. 6a, c, MDC staining fluorescent intensity and the levels of LC3II demonstrated that autophagy increased time-dependently until 48 h then declined at 72 and 96 h. Annexin V-PE/7-AAD dual staining demonstrated that apoptosis increased time-dependently at 24, 48, 72 and 96 h, bursting at 96 h when autophagy significantly declined in both HEp-2 and Tu212 cells (Fig. 6b). Notably, with the decrease of LC3II after 48 h, the levels of cleaved PARP

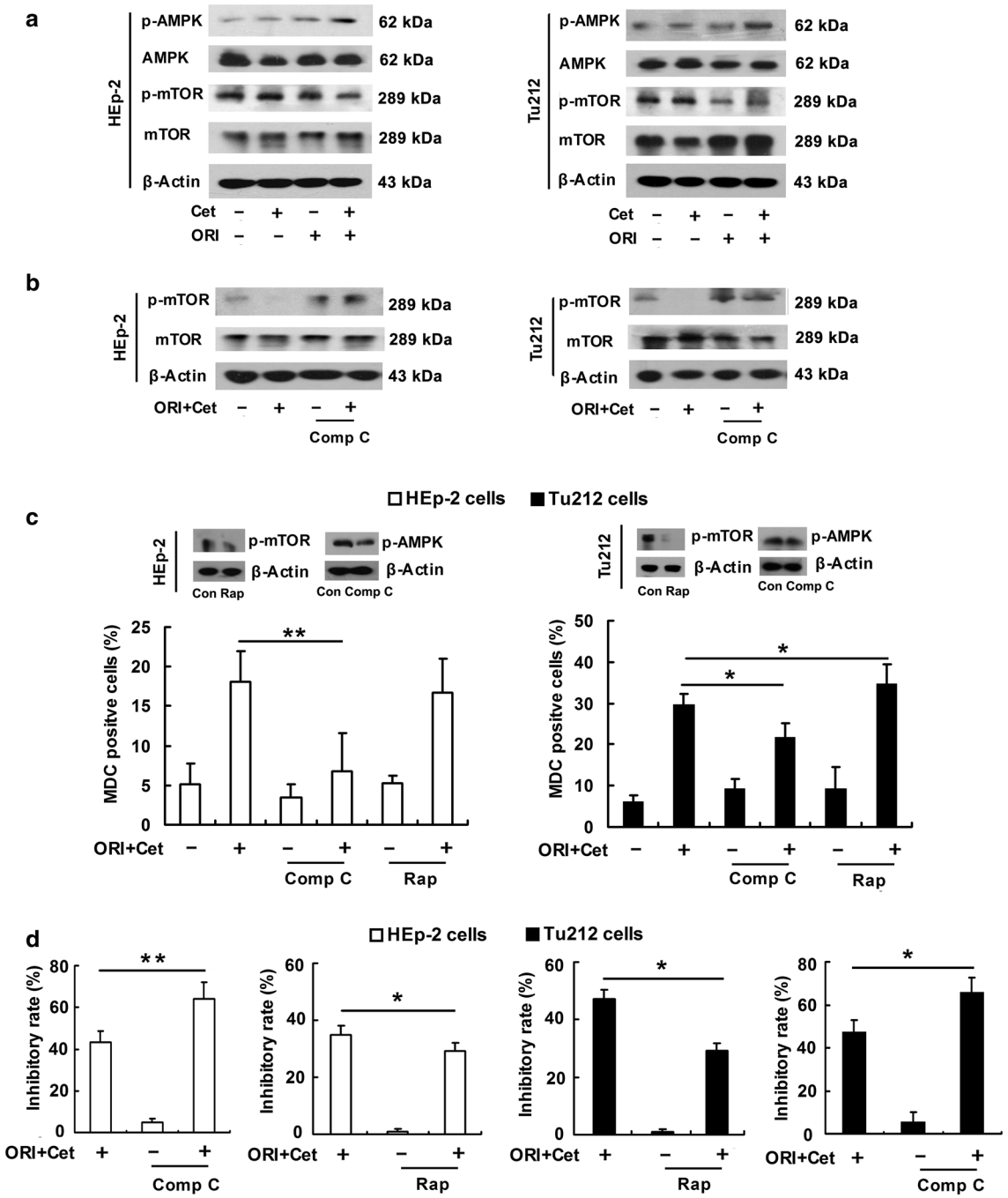

Fig. 4 ORI/Cet induces autophagy by activating AMPK-mTOR pathway. **a** HEp-2 and Tu212 cells were treated with ORI (24 μM for HEp-2 cells and 36 μM for Tu212 cells, respectively) and Cet (10 μg/mL) for 48 h and western blot analysis was used to detect the levels of p-AMPK, AMPK, p-mTOR and mTOR. β-Actin worked as the loading control. **b–d** LSCC cells were pre-treated with AMPK inhibitor Compound C (Comp C) or mTOR inhibitor Rapamycin (Rap) for 1 h, then treated with ORI/Cet for 48 h. Levels of protein expression were analyzed by western blotting (**b**). MDC staining analysis was used to determine to autophagy and the inhibitory effect of Comp C or Rap on AMPK and mTOR were tested by western blotting, respectively (**c**). Cell inhibitory rates were analyzed by MTT assay (**d**). n = 3, Mean ± SD. *P < 0.05; **P < 0.01

was further increased (Fig. 6c). These results suggest that reduced autophagy may contribute to the induction of apoptosis by ORI/Cet in the long term.

Discussion

Recently, anti-tumor agents from natural plants have shown significant effect against LSCC, such as

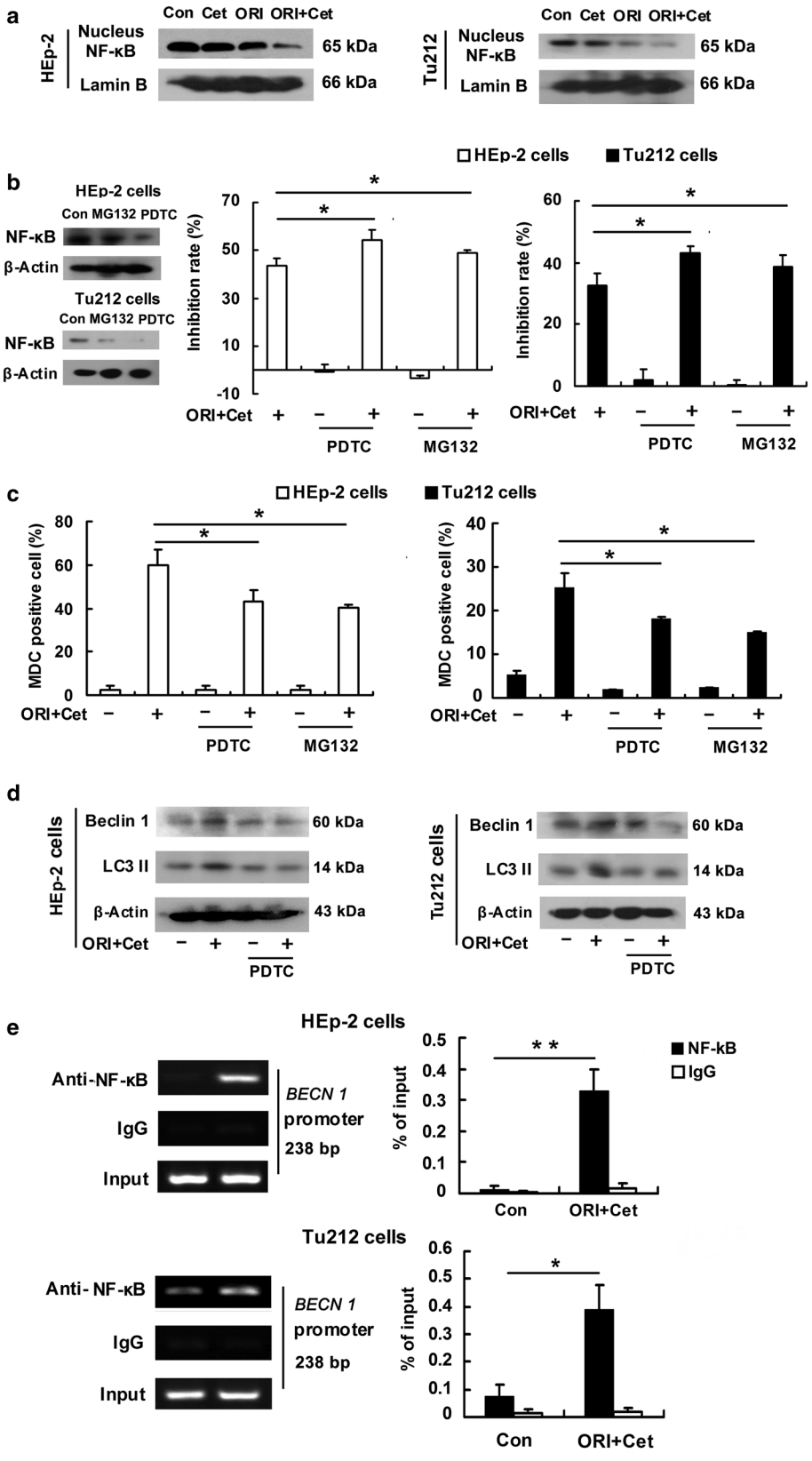

Fig. 5 NF-κB mediated-Beclin 1 upregulation is associated with ORI/Cet- induced LSCC cell autophagy. **a** HEp-2 and Tu212 cells were treated with ORI and Cet for 48 h and western blotting was conducted to detect the levels of nucleus NF-κB. Lamin B was used as a loading control. **b** LSCC cells were incubated with ORI/Cet with or without NF-κB inhibitors PDTC or MG132. MTT assay was used to detect cell proliferation. The inhibition of NF-κB of PDTC or MG132 was detected by western blotting. **c** MDC fluorescent intensity was analyzed by flow cytometry. **d** Western blotting was performed to detect the levels of Beclin 1 and LC3II. β-Actin was used as a loading control. **e** The ChIP assay reveals the binding of endogenous NF-κB to the BECN 1 promoter by addition of ORI/Cet. n = 3, Mean ± S.D. *P < 0.05; **P < 0.01

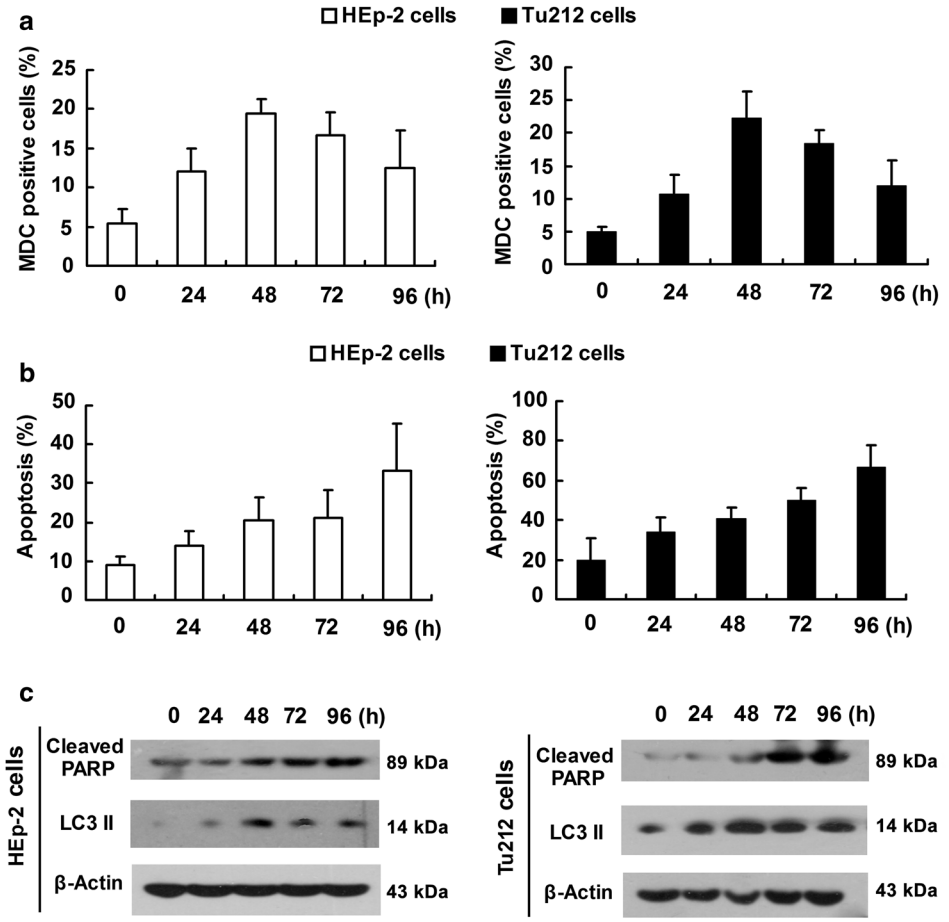

Fig. 6 Quantitative analysis of autophagy and apoptosis in ORI/Cet-treated LSCC cells. The cells were treated with ORI/Cet for 0, 24, 48, 72 and 96 h, and then MDC fluorescent intensity (**a**) and apoptotic cell proportion (**b**) were analyzed by flow cytometry. **c** Cleaved PARP and LC3II protein levels were detected by western blotting. β-Actin worked as the loading control. n = 3, Mean ± SD

epigallocatechin-3-gallate and quercetin [31, 32]. We previously reported that ORI causes LSCC cells death through different mechanism, including cell cycle arrest and apoptosis [22, 33]. Moreover, autophagy, the typeII programmed cell death (PCD), is another process associated with ORI-induced tumor cell death [34]. ORI treatment could induce autophagy in HEp-2 cells [24], human histocytic lymphoma U937 cells [35] and human multiple myeloma RPMI8266 cells [36]. In line with these studies, we also proved that ORI treatment promoted autophagy evidenced by elevated levels of LC3II and Beclin 1. Furthermore, our recent studies showed that combination of Cet and ORI confers synergistic anti-tumor effects against high expression of EGFR LSCC cells in vitro and in vivo [27]. In the current work, combination of Cet and ORI induced more autophagic vacuoles and higher expression of LC3II and Beclin 1 compared with ORI alone group in vitro and in vivo, suggesting that Cet could further enhance ORI-induced autophagy.

Apoptosis and autophagy are the two cellular processes known to affect anti-cancer effect of numerous agents [37, 38]. Recently, an abundance of evidences points to the fact that autophagy for therapeutic purposes in cancer shows a dual role with response either protecting cell survival or contributing to cell death [8, 39]. Similar with other anti-cancer agents, the role of ORI/Cet-caused autophagy in LSCC cells is complex and controversial. Here, we observe that ORI induced both apoptosis and autophagy, and that in the presence of Cet, both the percentage of apoptosis and autophagy in LSCC cells were further increased for 48 h. Since autophagy is either a pro-death or pro-survival response, we then examined the role of autophagy in the anti-tumor effects of ORI/Cet. We found that the inhibition of autophagy by 3-MA, CQ or Beclin 1 SiRNA significantly increased the cytotoxicity of ORI/Cet. In addition, the suppression of autophagy increased the percentage of ORI/Cet-induced apoptosis in LSCC cells and the levels of cleaved PARP in HEp-2 cells, indicating that ORI/Cet-induced autophagy is a pro-survival mechanism in LSCC cells. After 48 h co-treatment with ORI and Cet, the percentage of autophagy decreased while apoptosis significantly increased. Moreover, combined treatment with ORI and Cet after 48 h also caused a reduction of LC3II, but led to an elevation of cleaved PARP up to 96 h, suggesting that reduced autophagy may contribute to the induction of apoptosis by ORI/Cet. Our results are consistent with Cheng et al. [40] and Zeng et al. [41] who reported that the swift from autophagy to

apoptosis was found in MK2206-treated human glioma cell lines and nutrient depletion induced-multiple myeloma cells, respectively. The mechanisms behind this phenomenon are still unknown and need to be investigated in future.

Multiple signaling pathways including MAPK [42] and AMPK-mTOR signaling [43] are involved with the induction of autophagy. Among these signaling pathways, AMPK-mTOR pathway plays an important role in the modulation of autophagy by integrating and coordinating different sensory from other upstream factors [9]. Herein, we observed that ORI increased the levels of p-AMPK and decreased p-mTOR. Inhibiting AMPK using Compound C enhanced ORI/Cet-induced cell death and decreased autophagy. Additionally, when mTOR was inhibited by rapamycin, the aforementioned changes were reversed, indicating that AMPK and mTOR are involved with the effect of ORI/Cet on autophagy. Moreover, we also found that inhibiting AMPK with Compound C increased mTOR activity compared with ORI/Cet group, indicating that AMPK-mTOR pathway partially promotes ORI/Cet-induced autophagy. To the best of our knowledge, this study direct evidence for the first time that ORI/Cet induces protective autophagy through the AMPK-mTOR pathway in LSCC cells. Meanwhile, p53, an upstream kinase associated with cancer cell death, mediates autophagy through an AMPK-mTOR-dependent pathway [44]. Several plant-derived natural products induce autophagy through AMPK-mTOR signaling, which is dependent on p53 activation [13, 45]. However, we found that, in both HEp-2 (deficient p53) [33] and Tu212 (muted p53) [46] cells, ORI/Cet could induce autophagy through activation of AMPK-mTOR pathway, indicating that p53 may not involve with the activation of AMPK/mTOR pathway induced by combined ORI and Cet in the LSCC cells.

It is well-known that NF-κB shows complex role in various cancer or different conditions. It was reported that NF-κB could promote cell survival and inhibit apoptosis in cancer [47]. Whereas, Li et al. found that fenofibrate induces apoptosis in the triple-negative breast cancer cells via activation of NF-κB [48]. In addition, the NF-κB pathway is also closely associated with regulation of autophagy [49]. Here, we also observed that NF-κB has complex function on the cell death by combination ORI with Cet in LSCC cells. ORI plus Cet could significantly downregulate the expression of nucleus NF-κB, and inhibition of NF-κB using PDTC or MG132 increased ORI/Cet-induced cell proliferation inhibition, apoptosis, but reduced the MDC positive cells. Furthermore, PDTC pretreatment partly reversed ORI/Cet-induced up-regulation of LC3II and Beclin 1, indicating that NF-κB was involved with ORI/Cet-induced protective autophagy. A recent work reported by Copetti et al. suggested that three NF-κB binding sites were on the first intron of the promoter of the essential autophagic gene *BECN 1* [50]. NF-κB-mediated up-regulation of *BECN 1* induced by several drugs is coupled to increased autophagy [51]. Our data also clearly indicated that ORI/Cet treatment triggered NF-κB to bind to the *BECN 1* promoter and up-regulated its expression, which further caused autophagy in LSCC cells.

In conclusion, we have demonstrated for the first time that combination of Cet and ORI significantly induces autophagy in LSCC cells by activation of AMPK-mTOR pathway and NF-κB-mediated up-regulation of Beclin 1. In addition, autophagy after the long-term treatment with ORI/Cet contributes to apoptotic cell death. Our findings demonstrate the role of autophagy and its regulation in ORI/Cet-induced apoptosis, which may have important implications in developing future strategies to use ORI/Cet in LSCC prevention and therapy.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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