

Autophagy inhibition contributes to Endostar sensitization in esophageal squamous cell carcinoma

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Abstract. Endostar is a novel artificially-synthesized anti-angiogenesis drug, and has been approved for clinical use. Previous studies have indicated that patients with esophageal cancer could benefit from Endostar combined with chemotherapy or chemoradiotherapy. However, the most advantageous use of this drug remains to be elucidated. The role of autophagy in cancer treatment remains controversial. The results of the present study demonstrated that Endostar promotes autophagy activation, which is regulated via phosphorylation inhibition of the downstream signaling molecules of the vascular endothelial growth factor, AKT serine/threonine kinase and mechanistic target of rapamycin signaling pathways. Furthermore, inhibiting autophagy using the pharmacological inhibitor chloroquine facilitated the antiproliferative effect of Endostar and increased the number of apoptotic cells, compared with Endostar monotherapy. Taken together, the results of the present study suggest that autophagy activation induced by Endostar serves a protective role in human esophageal cancer treatment, and that autophagy inhibition promotes the antiproliferative role of Endostar. Therefore, the combination of Endostar with an autophagy inhibitor may be a novel prospective approach to improving the efficacy of Endostar for the treatment of patients with esophageal cancer.

Introduction

Esophageal cancer is a type of malignant disease with one of the highest causes of cancer-associated mortality in developing countries (1). Chemotherapy and radiotherapy are two indispensable methods for the treatment of esophageal

squamous cell carcinoma (ESCC), particularly for locally advanced or unresectable cases; however, therapeutic toxicity and resistance remain two challenges to overcome. The five-year survival rate for advanced ESCC is ~10% (2). Thus, the development of novel therapeutic agents for the treatment of patients with this disease is warranted.

Advances in understanding of the signaling pathways involved in carcinogenesis, tumor growth and metastasis may aid in identifying potential novel molecular targets for esophageal cancer treatment (3). Angiogenesis serves an essential role in cancer development and progression, and determines the rate of tumor cell growth and invasion (4). Thus, targeting angiogenesis is being considered as a novel therapeutic strategy to overcome tumor growth (4). Endostar[®], a recombinant human-endostatin (rh-endostatin), is a novel artificially-synthesized anti-angiogenesis drug, which was approved by The China Food and Drug Administration (FDA) in 2005 for the treatment of non-small cell lung cancer when combined with cisplatin-based chemotherapy (5). Previous studies have reported that Endostar improves the efficacy of chemotherapy for esophageal cancer with synergistic effects when combined with chemoradiotherapy (CRT), and that it is a good prospect for clinical application (6,7). Furthermore, it has been demonstrated that CRT combined with endostatin markedly improves the complete response rate (endostatin combined with CRT vs. CRT-alone, 44 vs. 30%), and increases the 1-year (72 vs. 50%) and 3-year (32 vs. 22%) overall survival rates in metastatic ESCC when compared with the CRT-alone treatment group (8). However, despite the success of anti-angiogenic therapy regimens for advanced esophageal cancer, the most advantageous use of this drug remains to be identified.

Autophagy is a highly conserved catabolic process that transports cellular macromolecules and organelles to the lysosome for degradation in eukaryotic cells (9). It has been reported that autophagy is upregulated in cancer cells in response to antitumor treatment, including cisplatin, doxorubicin, sorafenib and cetuximab (10-13). Autophagy is generally considered as a means by which carcinoma cells develop resistance to therapy, thus the inhibition of autophagy could represent a promising strategy to improve the efficacy of cancer treatment.

The potential molecular mechanism underlying the anti-angiogenic effect of Endostar involves the vascular

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endothelial growth factor (VEGF)/VEGF receptor (R) signaling pathway (14). The AKT serine/threonine kinase (Akt)/mechanistic target of rapamycin (mTOR) signaling pathway is one of the essential downstream pathways of VEGF (15). As inhibition of the Akt/mTOR signaling pathway is known to activate autophagy, it is hypothesized that there is an association between autophagy and Endostar treatment in ESCC therapy.

In the current study, the pro-survival role of autophagy for ESCC cells was studied. Furthermore, the inhibiting effects of autophagy on ESCC cells were discussed. The study evaluated a novel and promising approach for enhancing the clinical benefits of Endostar for the treatment of ESCC.

Materials and methods

Reagents and antibodies. Endostar was kindly donated by Xiansheng Pharmaceutical Co., Ltd. (Nanjing China; <http://www.simcere.com/>). The autophagy inhibitor chloroquine (CQ) was purchased from J&K Chemical Ltd. (Beijing, China). The primary antibodies directed against microtubule-associated protein 1 light chain 3- α (LC3), nucleoporin p62 (p62), Beclin-1, Akt, phosphorylated Akt, mTOR and phosphorylated mTOR were obtained from Cell Signaling Technology, Inc., (Danvers, MA, USA, cat. nos. 4455, 4691, 13038, 2972 and 2971, respectively). Horseradish peroxidase-conjugated anti-rabbit (cat. no. 2357) and anti-mouse (cat. no. 516190) secondary antibodies were obtained from Santa Cruz Biotechnology, Inc., (Dallas, TX, USA), and a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG was purchased from Beyotime Institute of Biotechnology (Nanjing, China).

Cell cultures. Eca-109 and TE-1 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and were incubated at 37°C in a humidified incubator with 5% CO₂. Endostar was aliquoted to achieve desired working volumes of 20 μ l and stored at 4°C. The drug was further diluted with DMEM to 25, 50, 100 and 200 μ g/ml prior to use.

Cell viability assays. The viability of Eca-109 and TE-1 cells was determined using an MTS assay kit (Jiangsu Kaiji Bio-Technology Co., Nanjing, China). Cells were seeded into 96-well plates at a density of 3x10³ cells/well in 100 μ l DMEM (n=6) and treated with Endostar (25, 50, 100 and 200 μ g/ml), CQ (5 mM), combination therapy or vehicle control (DMSO, 0.1%) for 24, 48, or 72 h when cells were in the logarithmic phase. After 24, 48 or 72 h incubation at 37°C, 10 μ l MTS was added into each well and incubated for 4 h at 37°C. Subsequently, the absorbance was measured using an ELX800 microplate reader (Omega Bio-Tek, Inc., Norcross, GA, USA) at a wave length of 490 nm and used to calculate the cell viability rates. The half maximal inhibitory concentration (IC₅₀) for Endostar was calculated for esophageal cancer cell lines using standard algorithms (16).

Transmission electron microscopy (TEM). For TEM, treated Eca-109 cells were washed with PBS and fixed at

room temperature for 30 min in 2.5% glutaraldehyde. Then, the samples were treated with 1.5% osmium tetroxide at room temperature for 30 min, dehydrated with acetone and embedded in Durcupan resin. The sections were stained at room temperature for 2 h with 0.2% lead citrate and 1% uranyl acetate, and examined using a Tecnai 10 electron microscope (FEI; Thermo Fisher Scientific, Inc.; x4,000-50,000) at 60 kV in five fields of view. For quantification of autophagic vesicles, cells with >3-4 double-membrane vesicles were scored as positive for autophagosomes.

Immunofluorescence. Cells seeded at a density of 3x10⁵ cells/well into six-wells plates were treated with a specific dose (25, 50, 100 and 200 μ g/ml) of Endostar for 24 h at 37°C, then incubated with LysoTracker (Invitrogen; Thermo Fisher Scientific, Inc.) for 90 min at 37°C. Subsequently, cells were washed twice with PBS, fixed with 4% paraformaldehyde at 37°C for 2 h and permeabilized with 1% CHAPS buffer (150 mM NaCl, 10 mM HEPES, 1.0% CHAPS) at room temperature for 15 min. Then, cells were incubated with an anti-LC3 antibody (dilution, 1:1,000; cat. no. 4455) for 2 h at 37°C, washed with PBS three times and incubated with FITC-conjugated goat anti-rabbit IgG (dilution, 1:200; cat. no. A0562) for 1 h at 37°C. Next, the cell nuclei were stained using DAPI (Invitrogen; Thermo Fisher Scientific, Inc.) for 15 min at 37°C. Samples were examined under a Zeiss LSM 710 fluorescence microscope system (Carl Zeiss AG, Oberkochen, Germany; magnification, x1,000). Images were processed using ZEN LE software (version 2.0; Carl Zeiss AG).

For the quantification of LC3-positive cells, 150-200 cells were randomly selected from the acquired image and counted. Cells with >5 dots of specific red signals were considered to be autophagic (LC3-positive).

Western blot analysis. Cells were lysed by cell lysis buffer (Beyotime Institute of Biotechnology, Nanjing, China; cat. no. P0013) and immune blotted as previously described (15). Briefly, proteins from a total cell extract (20 μ g/lane) were separated using 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA) and blocked with non fat milk powder (5%) for 2 h at room temperature, then detected using the aforementioned primary antibodies (dilution, 1:200, 4°C, overnight) and secondary antibodies (dilution, 1:1,000, room temperature, 2 h), prior to visualization using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology). Visualization was performed using the ImageQuant LAS-4000 imager with Multi Gauge software (version 1.03; both from Fujifilm, Tokyo, Japan).

Cell apoptosis assay. Cells were treated with Endostar (25 μ g/ml), CQ (5 mM) or vehicle control (DMSO, 0.1%) for 24 h when cells were in the logarithmic phase. After 24 h, cells were collected by trypsinization, washed twice with cold PBS and resuspended in 1X binding buffer. The final concentration of cells was 1x10⁶/ml. Then, 100 μ l cells were mixed with 5 μ l Annexin V/FITC and 5 μ l propidium iodide, then gently vortexed and incubated for 15 min at room temperature in the dark according to the manufacturer's protocol (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Cell apoptosis was determined using flow cytometric analysis

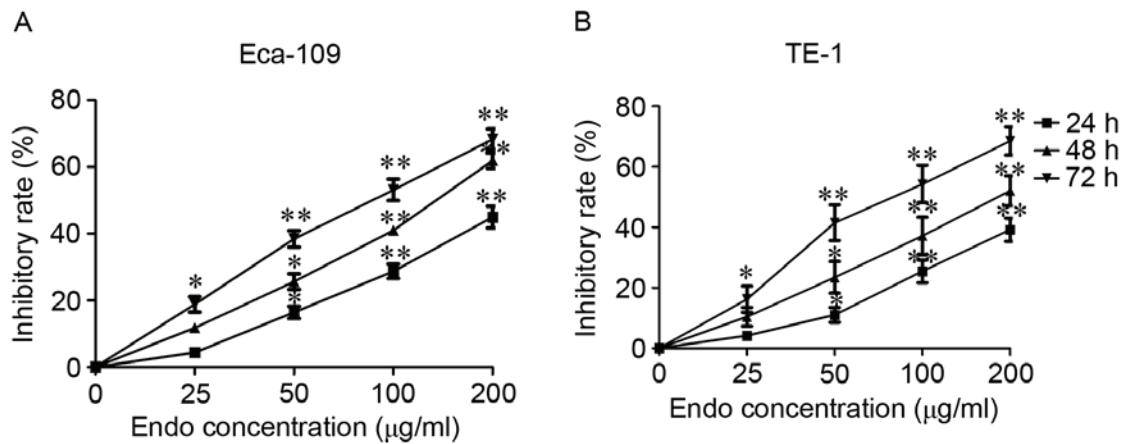


Figure 1. Treatment with Endostar inhibits human esophageal cancer cell proliferation *in vitro*. Eca-109 and TE-1 cells were treated with Endostar (0-200 µg/ml) or the vehicle control (DMSO 0.1%) for 24, 48, or 72 h. Significant reductions in the growth rate of esophageal cancer cells were detected following treatment with Endostar in a dose- and time-dependent manner, as compared with in the control group. Results are presented as the mean ± standard deviation following ≥3 independent experiments. *P<0.05; **P<0.01, vs. vehicle-treated cells. Endo, Endostar; DMSO, dimethyl sulfoxide.

with the Annexin V/FITC Apoptosis Detection kit (Nanjing KeyGen Biotech Co., Ltd.). The samples were then detected by flow cytometric analysis within 1 h. The results were analyzed using the BD FACSCalibur™ system and FACStation™ software version 6.1 (BD Biosciences, Franklin Lakes, NJ, USA), as suggested by the manufacturer.

Statistical analysis. SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Data are presented as the mean ± standard deviation. Comparisons between two groups were performed by unpaired t-test. Multiple comparisons were performed by one-way analysis of variance with Scheffe's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Endostar reduces the viability of Eca-109 and TE-1 cells. Human ESCC Eca-109 and TE-1 cells were selected to evaluate the cytotoxic activity of Endostar. Cell viability was measured using an MTS assay. Time- and dose-dependent inhibitory effects on cell viability were observed following 72 h of Endostar treatment in Eca-109 and TE-1 cells, and the IC₅₀ was calculated as being 67.23±8.42, and 75.39±11.56 µg/ml, respectively (Fig. 1). These results suggest that Endostar can significantly inhibit ESCC cell viability (Fig. 1).

Endostar induces autophagy in Eca-109 and TE-1 cells. To evaluate the significance of autophagy in esophageal cancer therapy, the occurrence of autophagy in Endostar-treated human ESCC Eca-109 and TE-1 cells was detected. Autophagosomes were observed using TEM, a standard approach for detecting autophagy. As presented in Fig. 2A and B, typical double-membrane autophagosomes emerged following exposure to Endostar for 24 h in Eca-109 and in TE-1 cells. The control cells exhibited normal nuclei with a significantly lower number of autophagosomes compared with in the Endostar-treated group (Fig. 2A).

These results were further confirmed by analyzing the endogenous punctae fluorescence of LC3 in cells following

Endostar treatment using immunofluorescence staining. Eca-109 and TE-1 cells demonstrated overlapping of LC3 punctae, and lysosomes in the Endostar-treated group, indicating the formation of autolysosomes (Fig. 2C). Autophagic punctae were scarce in the untreated cell group compared with Endostar-treated group (Fig. 2C).

Although increased autophagosome numbers and autophagic punctae were observed following Endostar incubation, it remains unclear whether these changes are the result of autophagy activation or inhibition of the process at the late degradation stage. Thus, the expression of autophagy-associated proteins LC3, Beclin-1 and p62 were assessed using western blotting. LC3 is used as a marker of autophagosomes and expressed by two forms: LC3-I and LC3-II. The ratio of LC-II to LC3-I is increased when autophagy activated. Beclin-1 performed an important role in the regulation of autophagy activation. P62 was a selective substrate of autophagy. P62 facilitated binding of LC3 to ubiquitinated proteins and degraded ubiquitinated proteins in autolysosomes (9). The transition of LC3-I to LC3-II was induced after 24 h of Endostar treatment in a dose-dependent manner (Fig. 2D). Similarly, under various doses of Endostar treatment (0-200 µg/ml), the expression of Beclin-1 increased in Eca-109 and TE-1 cell lines in a dose-dependent manner. In addition, when ESCC cells were treated with Endostar the p62 protein expression was markedly inhibited, which indicates that autophagy was activated by Endostar (Fig. 2D). These findings provide evidence that Endostar favors autophagy activation in ESCC cells.

Endostar-induced autophagy is regulated through suppression of the Akt/mTOR signaling pathway. The Akt/mTOR signaling pathway serves an essential role in the regulation of autophagy. As a multi target inhibitor of VEGF and platelet derived growth factor receptor family members, linifanib-induced autophagy has been reported to be associated with the inhibition of Akt/mTOR phosphorylation in hepatocellular carcinoma (17). It has been demonstrated that Endostar suppresses VEGF and its receptor, thus inhibiting the downstream Akt/mTOR signaling pathway (18). However, the precise effect of Endostar

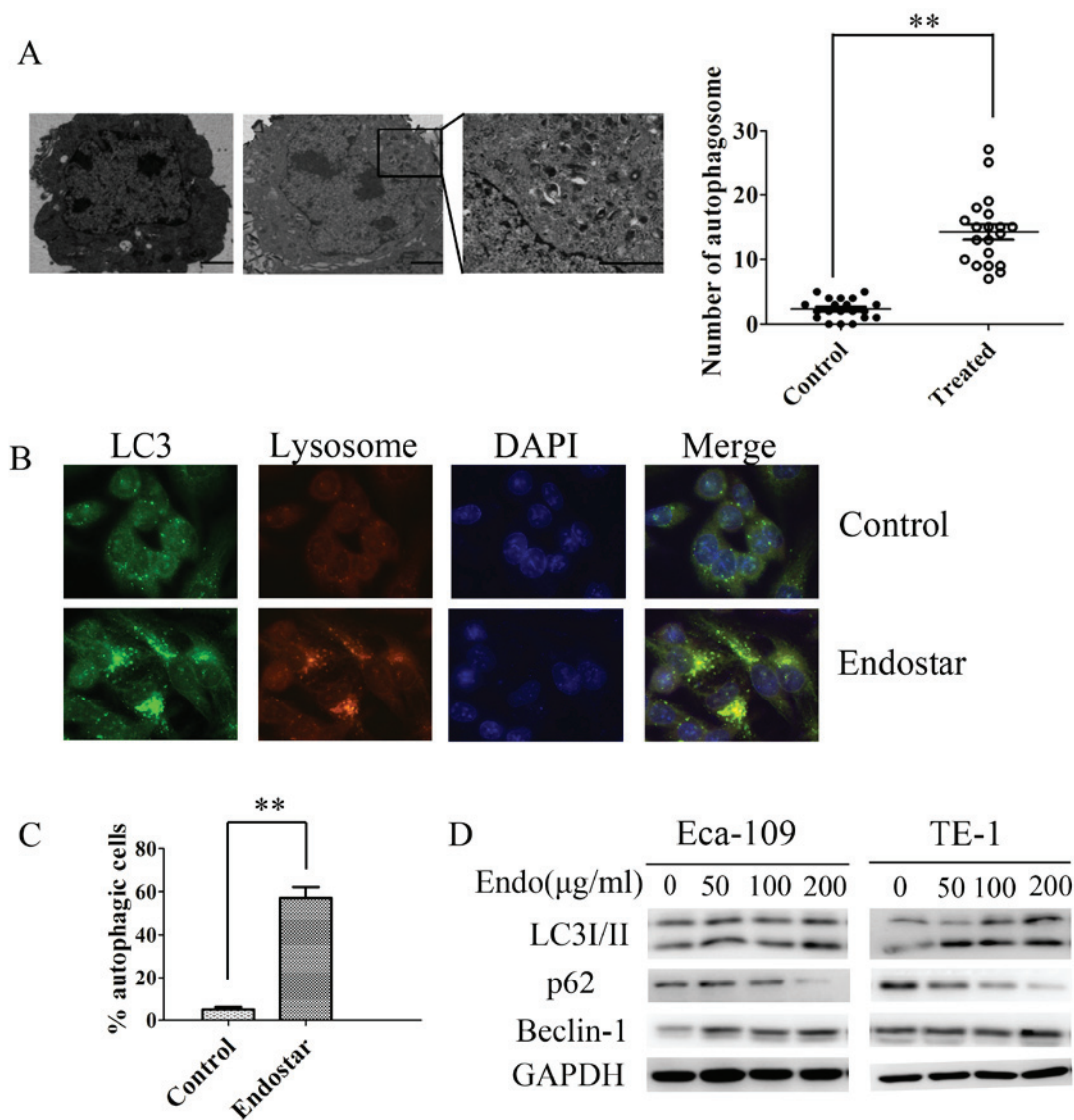


Figure 2. Endostar promotes autophagy activation in human esophageal cancer cells. (A) Representative transmission electron microscopy images demonstrating autophagic vacuoles, including autophagosomes, in Eca-109 cells treated with Endostar or 0.1% DMSO. Magnification, $\times 4,000$ ($\times 50,000$ in the highlighted area). (B) Representative immunofluorescence microscopy images of endogenous punctae fluorescence of LC3 in Eca-109 cells after 24 h treatment with Endostar. Magnification, $\times 1,000$. Green, FITC-labeled LC3; Red, lyso-tracker-labeled lysosome; Blue, DAPI-labeled nucleus. (C) The percentage of green or yellow punctae-positive cells was quantified and statistically analyzed using a threshold of >5 dots/cell. Results are presented as the mean \pm standard deviation. (D) Western blot analysis indicating the transition of LC3-I to LC3-II, p62 and Beclin-1 protein expression in Eca-109 and TE-1 cells treated with Endostar (0-200 $\mu\text{g/ml}$). ** $P < 0.01$. LC3, microtubule-associated protein 1 light chain 3 α ; p62, nucleoporin p62; Endo, Endostar; DMSO, dimethyl sulfoxide.

on the Akt/mTOR signaling pathway in ESCC cell lines has not been clearly established. To investigate the effect on Akt/mTOR signaling, the activity of Akt/mTOR was detected in ESCCs following treatment with Endostar using western blot analysis. The phosphorylation of Akt/mTOR in ESCC cell lines was markedly reduced following Endostar treatment in a dose-dependent manner, whereas the total levels were unaffected (Fig. 3). This suggests that the activation of autophagy following Endostar treatment is regulated through inhibition of the Akt/mTOR signaling pathway.

Inhibition autophagy sensitizes esophageal cancer to Endostar. To investigate the biological effect of autophagy activation following Endostar treatment, ESCC cells were incubated with Endostar combined with the autophagy inhibitor CQ for 24 h to detect cell viability. The conversion of LC3-I to LC3-II was

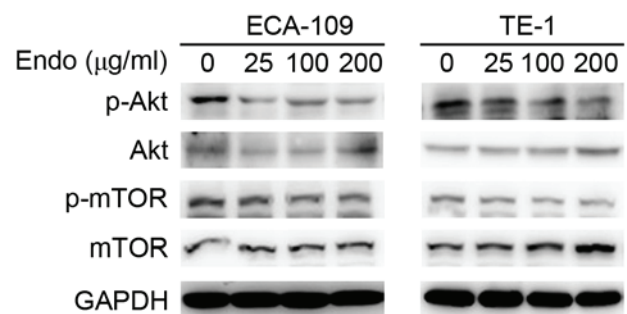


Figure 3. The effects of Endostar on Akt and mTOR in Eca-109 and TE-1 human esophageal cancer cells. Western blot analysis demonstrated markedly increased levels of phosphorylated-Akt and -mTOR, and the stable expression of total Akt, and mTOR protein expression in esophageal cancer cells following Endostar treatment (0-200 $\mu\text{g/ml}$). Endo, Endostar; p, phosphorylated; Akt, AKT serine/threonine kinase; mTOR, mechanistic target of rapamycin.

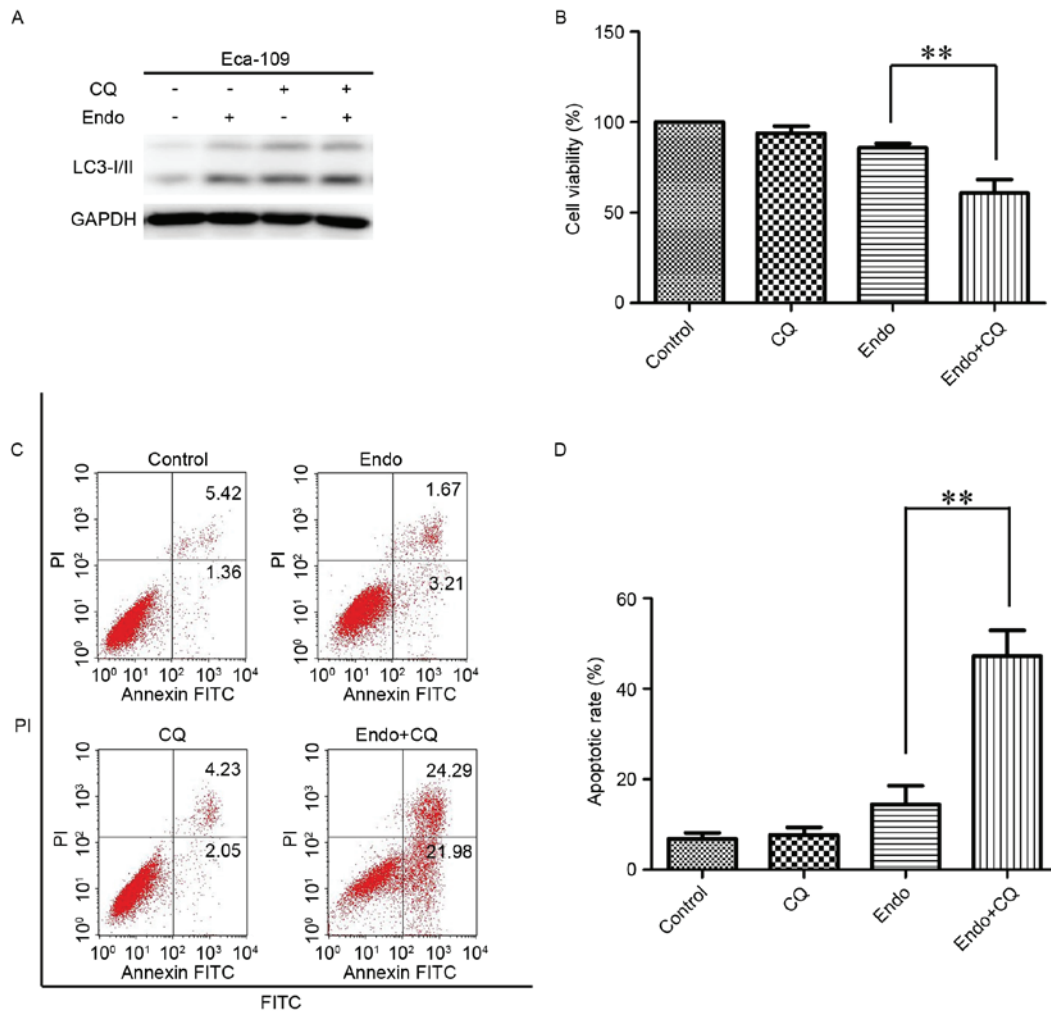


Figure 4. Inhibition of autophagy increases the sensitivity of human esophageal cancer cells to Endostar *in vitro*. (A) Western blot analysis demonstrated LC3 activation in Eca-109 cells treated with or without Endostar, in the presence or absence of the lysosomal inhibitor CQ. (B) Cell viability was measured using an MTS assay. Eca-109 cells were treated with Endostar or DMSO in the presence or absence of CQ for 24 h. (C) Cell apoptosis was measured using a flow cytometric assay. Cells were treated with Endostar alone or in combination with CQ for 24 h, then stained with Annexin V and PI. (D) Compared with the Endostar-alone treated group, the combination of Endostar with CQ resulted in a significant increase in the apoptotic rate of Eca-109 cells. Data are presented as the mean ± standard deviation. **P<0.01. CQ, chloroquine; PI, propidium iodide; FITC, fluorescein isothiocyanate; Endo, Endostar; LC3, microtubule-associated protein 1 light chain 3 α ; DMSO, dimethyl sulfoxide.

markedly increased in the combined group compared with the Endostar-alone treatment group (Fig. 4A). Next, the viability of ESCC cells was detected. In combination with 5 mM CQ, which inhibits the fusion of autophagosomes and lysosomes, Endostar treatment significantly suppressed the viability of ESCC cells compared with in the Endostar-alone treatment group (Fig. 4B). As presented in Fig. 4B, the cell viability of the untreated, CQ-alone, Endostar-alone and combined groups was 99.7 ± 0.75 , 93.8 ± 3.21 , 85.8 ± 1.21 and $62.6 \pm 8.42\%$, respectively. Furthermore, a significant increase in the number of apoptotic cells was observed in the Endostar combined with CQ group, as compared with in the Endostar-alone treatment group (46.27 ± 5.61 vs. $14.78 \pm 3.42\%$; Fig. 4C and D). Together, this data indicates that autophagy activation promotes cell growth and survival in response to Endostar treatment.

Discussion

Endostatin is a 22 kD a polypeptide and was obtained from the C-terminal fragment of collagen type XVIII. Endostatin

has an anti angiogenic effect on cancer cells (19). Endostar is a novel rh-endostatin that has an additional nine-amino acid sequence and an attached six-histidine tag at the N-terminal end of the protein (20). It has been reported that Endostar suppresses the proliferation and migration of endothelial cells by inhibiting VEGFR signaling (14). In tumor cells, this results in anti-proliferative and -invasive effects. Treatment with Endostar has been demonstrated to suppress the growth of lung cancer cells (21,22). Furthermore, Endostar significantly enhanced the inhibitory effect of chemotherapy on an ESCC Eca-109 xenograft model (23). In the present study, it was demonstrated that Endostar significantly reduced the viability of Eca-109 and TE-1 ESCC cells in a dose- and time-dependent manner *in vitro*.

Autophagy is considered to be a pro-survival mechanism in cancer therapy (17). Prior studies have revealed that angiogenesis inhibitor-induced autophagy is initiated in the presence of VEGF (24). Furthermore, Selvakumaran *et al* (25) reported that bevacizumab, the first anti-angiogenic medicine approved by the FDA, induces autophagy in colon cancer cells,

and effect that was also observed in hepatocellular carcinoma cells (26). Thus, it was hypothesized that Endostar may induce autophagy in the treatment of esophageal cancer. In the present study, it was demonstrated that the number of autophagosomes significantly increased following Endostar exposure, compared with that in the vehicle-treated cells. In addition, the punctae fluorescence of LC3 and the LC3-I to LC3-II transition were enhanced in the Endostar-treated group, compared with in the vehicle-treated control group. To elucidate whether the changes observed were due to increases in autophagy activation or to inhibition of the late stage of degradation, the expression patterns of Beclin-1 and p62 were determined. Following exposure to Endostar, the expression of Beclin-1 markedly increased and p62 decreased in a dose-dependent manner, suggesting that Endostar initiates autophagy in ESCC cells.

The precise biological significance of autophagy activation in cancer treatment is controversial and context-dependent. It has been reported that endostatin causes autophagic cell death in human EAhy927 endothelial cells (27). However, in the present study, it was demonstrated that autophagy activation promotes the survival of ESCC cells, contributing to the occurrence of innate or acquired resistance. CQ, an inhibitor of autophagy, sensitized the ESCC cells to Endostar and significantly enhanced the growth inhibitory effect of Endostar. Thus, autophagy may serve a protective role in ESCC treated with Endostar, and inhibiting autophagy provides a potential approach for improving the clinical efficacy of Endostar for ESCC therapy.

The underlying mechanisms by which Endostar activates autophagy remain to be elucidated. Previous studies have revealed that the Akt/mTOR signaling pathway serves a significant role in cell survival and migration (28). It is well known that the Akt/mTOR signaling pathway is significantly involved in autophagy (29). In endothelial and gastric cancer, Endostar has been demonstrated to inhibit the interaction between VEGF, and its receptor, which consequently inhibits downstream biological activity, including Akt/mTOR signaling (30,31). In the current study, the expression of phosphorylated Akt and mTOR were markedly decreased, suggesting their involvement in Endostar-mediated autophagy.

In conclusion, it was demonstrated that Endostar induces autophagy activation and significantly reduces the viability of ESCC cells. A potential reason for the autophagy initiation was identified to be the inhibition of Akt/mTOR signaling, as suggested by the decrease in phosphorylated Akt and mTOR protein levels. Furthermore, autophagy initiation was revealed to serve a pro-survival role in cancer cells; thus, autophagy inhibition may confer clinical benefits in the treatment of patients with ESCC.

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