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Original Article

Combination effect of curcumin with docetaxel on the PI3K/AKT/mTOR pathway to induce autophagy and apoptosis in esophageal squamous cell carcinoma

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Abstract: Background: Docetaxel (DTX) is widely used to treat many malignant tumors but has many adverse effects. Curcumin (CUR) also has effects on a variety of tumor cells and can reduce the toxicity and side effects of chemotherapy drugs and the occurrence of drug resistance. However, the combination of CUR and DTX for treating esophageal cancer has not been reported. Methods: Human esophageal squamous cell carcinoma (ESCC) KYSE150 and KYSE510 cells were treated with CUR or DTX alone or both drugs and cancer cell viability was detected by CCK8, apoptosis, scratch-healing and migration assays. Electron microscopy and Western blots were used. *In vivo* experiments were used observe anti-tumor effects. Results: CUR combined with DTX significantly inhibited the viability and migration of esophageal cancer cells ($P<0.01$) and further promoted the apoptosis of cancer cells. In addition, CUR induced autophagy in esophageal cancer cells when combined with DTX. DTX combined with CUR may induce apoptosis and autophagy by inhibiting the PI3K/AKT/mTOR signaling pathway. The compound 3-methyladenine (3MA) inhibited the autophagy induced by DTX and CUR (DC), further accelerated apoptosis and inhibited the proliferation of esophageal cancer cells when combined with DC. Conclusion: CUR combined with DTX induced apoptosis and autophagy of ESCC and probably worked through the PI3K/AKT/mTOR signaling pathway. The combination of the autophagy inhibitor, CUR and DTX may become a new treatment strategy for esophageal cancer.

Keywords: Curcumin, esophageal cancer, docetaxel, autophagy, apoptosis

Introduction

The incidence of esophageal cancer ranks seventh in the world, and its death rate ranks sixth among malignancies [1]. It is mainly divided into squamous cell carcinoma and adenocarcinoma. In China, approximately 90% of esophageal carcinomas are ESCCs. Patients with ESCC are mostly diagnosed with late-stage disease without the opportunity for surgery [2]. Platinum-based chemotherapy and chemoradiotherapy are standard regimens for esophageal cancer patients without surgical indications, but the efficacy is not satisfactory [1]. No consistent standard treatment regimen exists for esophageal cancer with first-line chemotherapy failure, progression or metastasis. Chemotherapy has an essential role in the treatment of tumors, but single chemotherapeutic drugs can produce a variety of toxicities and side effects, as well as drug resistance [3, 4]. The combined application of drugs can enhance their antitumor effects and delay the occurrence of drug resistance. In China, Chinese medicine has been used for thousands of years to prevent and cure tumors, because of its wide variety of treatments, availability and low cost. Currently, injections containing traditional Chinese medicine combined with chemotherapeutic drugs are used for cancer treatment and have been found to reduce adverse effects and improve quality of life in patients.

DTX is widely used for treating non-small cell lung cancer, esophageal cancer, breast cancer, gastric cancer, prostate cancer and other tumors [5, 6]. It is one of the most common che-

motherapeutic drugs for patients with recurrent or advanced esophageal cancer after first-line platinum treatment [7]. DTX inhibits cell division by stopping cell proliferation through stabilizing microtubules, and it initiates apoptosis by binding to β-microtubules and thus blocking mitosis [8]. However, because DTX can lead to allergies, water and sodium retention, and toxic effects to the heart, nerves and other organs [9], its clinical application is limited. In addition, cancer cells can develop drug resistance, which is one of the main causes of treatment failure [10].

CUR is a polyphenolic compound extracted from turmeric roots. It is commonly used in Asia, mainly in food dyes, cosmetics and medicine. CUR has been reported to induce tumor cell apoptosis, improve the sensitivity of tumor cells to chemoradiotherapy. It is safe, nontoxic and has anti-tumor effects in the human body [11]. CUR has attracted attention because of its broad biological significance. CUR has been reported to decrease expression of miR-155 by inhibiting the PI3K/AKT signaling pathway and to have anti-inflammatory effects in reducing tissue damage caused by sepsis [12]. Some studies have also found that CUR repairs autophagy damage caused by protein aggregation by inhibiting activation of mTOR, PI3K and AKT, and upregulating LAMP-2, beclin levels [13]. However, the specific mechanism of the anti-tumor effect of CUR has not been clarified. The effect of CUR and the combined effects of CUR and DTX on esophageal cancer have not been reported.

Therefore, this paper studied whether CUR combined with DTX might have antitumor effects in esophageal cancer. We found that the combination of these two drugs induced apoptosis and autophagy of esophageal cancer cells. We further explored related mechanisms, which may provide a new strategy for the treatment of esophageal cancer.

Materials and methods

Cell culture

Human ESCC KYSE150 and KYSE510 cells (Center Laboratory of Southern Hospital) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco, Life Technologies, CA, USA) and 1% penicillin/streptomycin (BI, Beijing, China) at 37°C in a humidified atmosphere containing 5% CO_2.

CCK8 assays

CCK8 assays were conducted to check the viability of cells after drug treatment. KYSE150 and KYSE510 cells were seeded in a 96-well plate (KYSE150 4×10^3/well, KYSE510 3.5×10^3/well) and cultured at 37°C in an atmosphere containing 5% CO_2 for 24 h until cells adhered. The esophageal cancer cells were treated with different concentrations of CUR or DTX (both from Aladdin, Shanghai, China) alone or in combination. Cell viability was detected 48 h after applying drugs by adding 10 μl CCK8 solution and incubating in a dark place for 1-4 h. Absorbance was measured at 450 nm using a microplate reader to determine the half maximal inhibitory concentration (IC50). Cells treated without drugs were used as negative controls and culture medium was used as a control. Concentrations of CUR were 0, 1, 2, 4, and 8 μg/ml. Concentrations of DTX for KYSE150 cells were 0.25, 0.5, 1, 2, 4, 8, 16, and 32 ng/ml and for KYSE510 cells were 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, and 8 ng/ml.

Scratch wound healing assays

Cells in log phase were digested with 0.25% trypsin (Gibco, Life Technologies, CA, USA) and resuspended, seeded (KYSE150 3×10^3/well, KYSE510 4.5×10^3/well) in 6-well plates and incubated at 37°C until they adhered. Cells in the log phase of growth were cultured, and 100 μl cell suspensions (3×10^5 KYSE150 cells, 4.5×10^5 KYSE510 cells) were seeded in 6-well plates for culturing at 37°C until 90% of the cells adhered. KYSE150 cells were treated for 48 h with 4 ng/ml DTX and 4 μg/ml CUR, and KYSE510 cells were treated with 1 ng/ml DTX and 4 μg/ml CUR. Cell monolayers were scraped to create crosses using a 200 μl pipette tip. Cell culture dishes were placed under a phase contrast microscope to acquire images after 0, 24 and 30 h. Images were analyzed quantitatively by ImageJ software. Wound closure % = $[(A_1-A_2)/A_1] \times100\%$ (where A_1 is area of the wound measured immediately after scratching and A_2 is area of the wound measured in h after scratch).

Transwell migration and invasion assays

Cells were treated with drugs for 24 h as for cell scratch assays. Medium was discarded and cells collected and resuspended. Cell suspensions (200 μl with 5×10^4 cells) were added

to the upper chambers. Medium with 10% fetal bovine serum was added to lower chambers. Cells were incubated at 37°C for 48 h and washed with PBS (Gibco, USA) three times. Cells were fixed with 4% paraformaldehyde (Leagene Biotechnology, Beijing, China) for 30 min, stained with 0.1% Crystal Violet (Leagene Biotechnology, Beijing, China) for 15 min and washed with PBS. Cells that did not migrate across the Transwell membrane were removed by gently wiping with a cotton swab. Migrated cells were viewed with a phase contrast microscope and counted. The invasion assay was the same as the migration assay except for the upper chambers and the embedding in Matrigel (BD Bioscience, USA).

Cell apoptosis assays

Cells were treated as described above. Annexin V, FITC and PI solutions were added in sequence according to instructions for an Annexin V-FITC apoptosis detection kit (Dojindo, Japan). Cells were incubated in the dark for 15 min and apoptosis detected by flow cytometry.

Morphological observation staining with Hoechst 33342

Cells (3×10^3 KYSE150 cells and 5×10^3 KYSE510 cells) were plated in 96-well plates and cultured at 37°C in an atmosphere containing 5% CO_2. Cells were treated with CUR, DTX or combinations for 48 h. Cells were incubated with Hoechst 33342 (Leagene Biotechnology, Beijing, China) in the dark for 30 min. Hoechst 33342 was removed and cells washed with PBS for 3-5 min three times. We viewed cells using a fluorescence microscope.

Transmission electron microscopy

Cells were treated for 48 h as for scratch healing assays. Cells were centrifuged 1,000 rpm for 3 min and fixed with 2.5% glutaraldehyde, preserved at 4°C and placed under an electron microscope to observe the number of autophagosomes and autolysosomes.

Western blots

Cells were treated with drugs as described above for 48 h. RIPA lysis buffer and phosphatase inhibitor (Leagene Biotechnology, Beijing, China) were added. Pyrolysis liquid was placed at 4°C and centrifuged at 13,000 rpm for 20 min. Supernatant was used as protein solution. Protein concentrations of samples were detected according to the instructions of a BCA Protein Assay Kit (Beyotime, Shanghai, China). After calculating concentrations, an appropriate amount of sample loading buffer was added. Solutions were boiled in a water bath at 100°C for 10 min to denature proteins. After preparation of SDS-PAGE gels, protein samples were added for electrophoresis. Gels were cut and transferred to membranes. After membrane transfer, PVDF membranes (Merck Millipore, MA, USA) were placed in 5% BSA and blocked for 1.5 h then washed with TBST three times. Membranes were incubated overnight at 4°C in primary antibodies to the following: PI3K, p-PI3K, AKT, p-AKT, mTOR, p-mTOR, light chain 3 (LC3), P62, Beclin-1, Atg7 and GADPH (Protein Tech, China). Membranes were washed with TBST three times. Membranes were immersed in the secondary antibody goat anti-mouse IgG-HRP or goat anti-rabbit IgG-HRP conjugate (Protein Tech, China) and incubated on a shaking table at room temperature for 90 min. Membranes were washed with TBST and exposed to an ultrasensitive chemiluminescence imager (Bio-Rad, Hercules, CA), and gray values of proteins calculated by ImageJ software.

Experiments *in vivo*

Establishing esophageal cancer xenograft models

Male nude mice (from the animal center, Southern Medical University, Guangzhou, China) aged 4-5 weeks and weighing about 18-20 g were used. KYSE510 cells (1×10^6) were used for subcutaneously inoculations on the backs of nude mice to develop implant tumors. Mice were reared in an SPF laboratory animal environment and lengths and diameters of tumors were measured with vernier calipers every other day. Models were successfully established when tumors grew to 50-100 mm^3.

Antitumor tests in vivo

After establishment of models, drugs were injected into caudal veins once every three days with the concentration of DTX at 8 mg/kg and CUR at 15 mg/kg. Mice were divided into

PBS, DTX, CUR, and DC groups with three animals per group. Mice injected with PBS were used as the control group. The day of the first injection was considered to be day 0, and drugs were injected five times. Tumor sizes and body weights of mice were recorded during medication. Mice were sacrificed for tumor collection at day 15. The calculation formula for tumor volume was: $V = (L \times W^2)/2$ (where L is length of the tumor, and W is width of the tumor).

Immunohistochemistry

Tumor tissue was dissected, fixed, dehydrated, and embedded in paraffin and paraffin blocks were sliced. After dewaxing and hydration, slides were sealed with 3% BSA at room temperature for 30 min. Primary antibody Ki-67 (Servicebio, China) was added and slides were incubated overnight at 4°C in a wet box. Slides were washed with PBS and incubated with the secondary antibody HRP-labeled goat anti-rabbit IgG (Servicebio, China) for 50 min at room temperature. Then the manufacturer's instructions for the immunostaining kit were followed. We observed slides by microscope, with hematoxylin-stained nuclei blue, and positive expression of DAB brown-yellow. ImageJ software was used to analyze the expression of Ki-67 in immunohistochemical sections.

Statistical analysis

The data are presented as mean ± standard deviation (mean ± SD), and the significance between groups was analyzed by one-way analysis of variance (one-way ANOVA) and LSD. The statistical significance was determined with a *P* value threshold of <0.05. All experiments were repeated more than three times. Statistical analysis was performed in SPSS20.0.

Results

CUR enhances the antitumor effect of DTX on esophageal carcinoma cells

CUR inhibited the cell viability of KYSE150 and KYSE510 cells according to CCK8 assays, which were concentration dependent (**Figure 1A**, **1B**). The IC50 for CUR was 5.80 ± 0.98 μg/ml for KYSE150 and 8.91 ± 0.88 μg/ml for KYSE510 cells. We found that the inhibition of proliferation of esophageal cancer cells was stronger for the combined drug group was stronger than for the DTX monotherapy group. The higher the concentration of CUR, the lower the cell activity (**Figure 1C**, **1D**). The IC50 value was significantly lower (P<0.05) at a CUR concentration of 4 μg/ml (KYSE150 4.13 ± 1.51 vs. 13.65 ± 2.42 ng/ml, KYSE510 0.70 ± 0.65 vs. 1.90 ± 0.49 ng/ml) (**Tables 1**, **2**).

The inhibition effect on tumor cells was not significant at a concentration of CUR of 4 μg/ml. The antitumor effect of DTX was significantly enhanced when combined with CUR at a concentration of 4 μg/ml. According to the cell activity curve of DTX and CUR, the concentration when the cell activity was above 60% and lower than the IC50 value of single drug was selected. Thus, DTX 4 ng/ml + CUR 4 μg/ml for KYSE150 cells and DTX 1 ng/ml + CUR 4 μg/ml for KYSE510 cells were used for follow-up studies.

CUR combined with DTX reduced migration and invasion of ESCC

Cell healing was observed after scratch-healing tests. CUR and DTX weakened the healing capability of esophageal cancer cells after sufficient treatment time with certain concentrations, whereas CUR combined with DTX further weakened the healing ability (**Figure 2**). Transwell migration assays showed that DTX combined with CUR further inhibited the migration and invasion of esophageal cancer cells (**Figure 3**).

CUR combined with DTX intensified apoptosis of ESCC

Flow cytometry was used to detect cell apoptosis. In KYSE150 and KYSE510 cells, early apoptosis of quadrant 2 and late apoptosis of quadrant 4 in the DTX and DC combined groups were significantly higher than those in the control group (**Figure 4A**). The apoptosis rate of the CUR group was slightly higher than that in the control group for KYSE150 cells (P>0.05) and was significantly higher than that in the control group (P<0.01) for KYSE510 cells. The apoptosis rate of the DC group was 23.7%, which was higher than that in the DTX (15.4%) and CUR groups (10.1%) for KYSE150 cells, and the apoptosis rate of the DC group of KYSE510 cells was 18.8% (**Figure 4B**, **4C**).

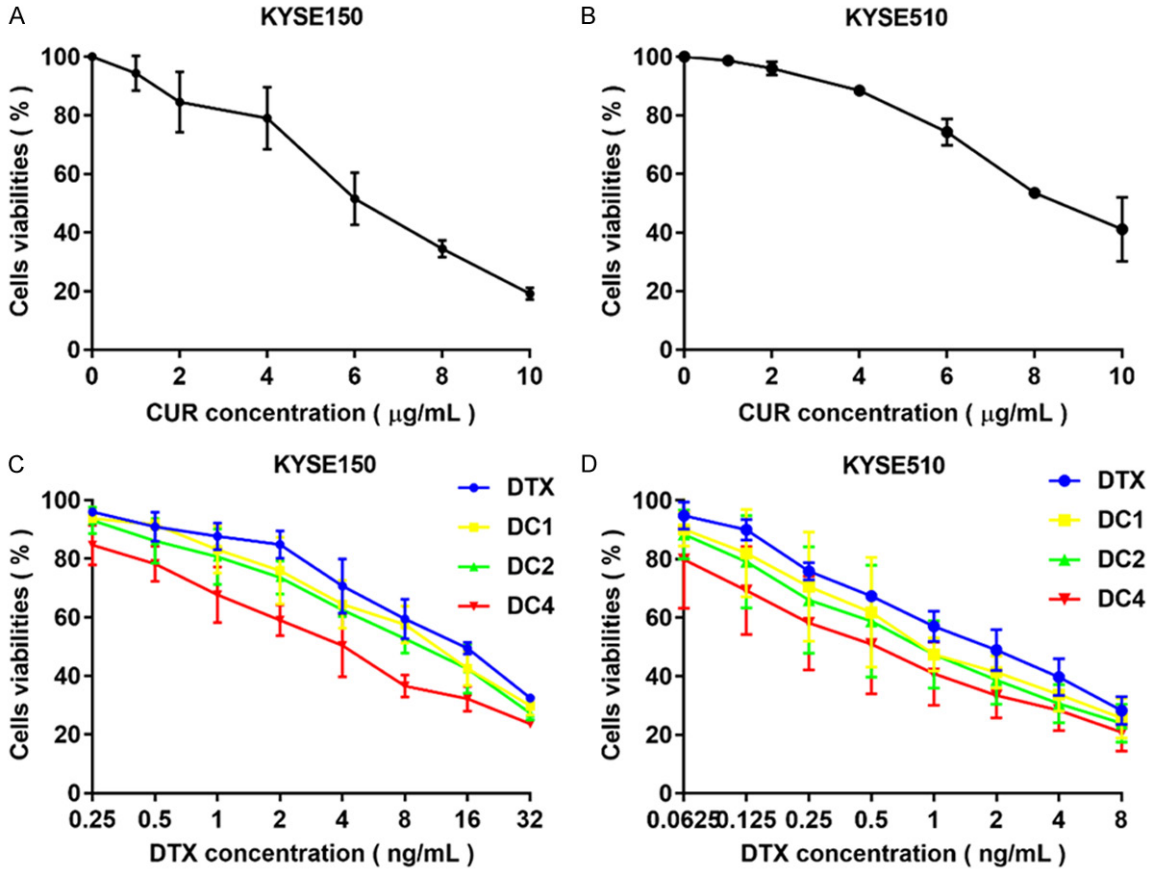

Figure 1. CCK8 assay showing that curcumin inhibits the proliferation of both KYSE150 and KYSE510. Cell viability declined as the concentration increased (A, B). Docetaxel combined with curcumin suppress the proliferation of esophageal squamous carcinoma cells, as compared with that in the single docetaxel group. As the concentration of curcumin increased, cell proliferation inhibition was enhanced (C, D). The IC50 of docetaxel decreased with increasing curcumin concentrations and significantly decreased when the curcumin concentration reached 4 μg/ml ($P<0.05$).

Table 1. IC50 for the same DTX concentration combined with different CUR concentrations for proliferation inhibition of KYSE150 cells

Group	DTX IC50 values (ng/ml)
DTX	13.65 ± 2.42
DTX+CUR 1 μg/ml	10.42 ± 2.52
DTX+CUR 2 μg/ml	9.09 ± 2.19*
DTX+CUR 4 μg/ml	4.13 ± 1.51**

*P<0.05, **P<0.01.

Table 2. IC50 for the same DTX concentration combined with different CUR concentrations for proliferation inhibition of KYSE510 cells

Group	DTX IC50 values (ng/ml)
DTX	1.90 ± 0.49
DTX+CUR 1 μg/ml	1.30 ± 0.83
DTX+CUR 2 μg/ml	1.13 ± 0.79
DTX+CUR 4 μg/ml	0.70 ± 0.65*

*P<0.05.

Both results were higher than for monotherapy groups, and differences were significant ($P<0.01$).

We viewed changes in cell morphology after drug treatment using Hoechst 33342 staining. Cell nuclei in the control group were regularly and evenly stained. Some pyknosis and karyolysis were observed in the monotherapy groups. In the DC group, nuclei became pyknotic and hyperchromatic (**Figure 4D**). The experiments demonstrated that DTX further induced apoptosis when combined with CUR.

The expression of apoptosis-related proteins was detected by western blots. The anti-apoptotic protein Bcl-2 was lower in the DC group in KYSE150 and KYSE510 cells than the control and monotherapy groups. The pro-apoptotic proteins BAX and cleaved-caspase3/9 incre-

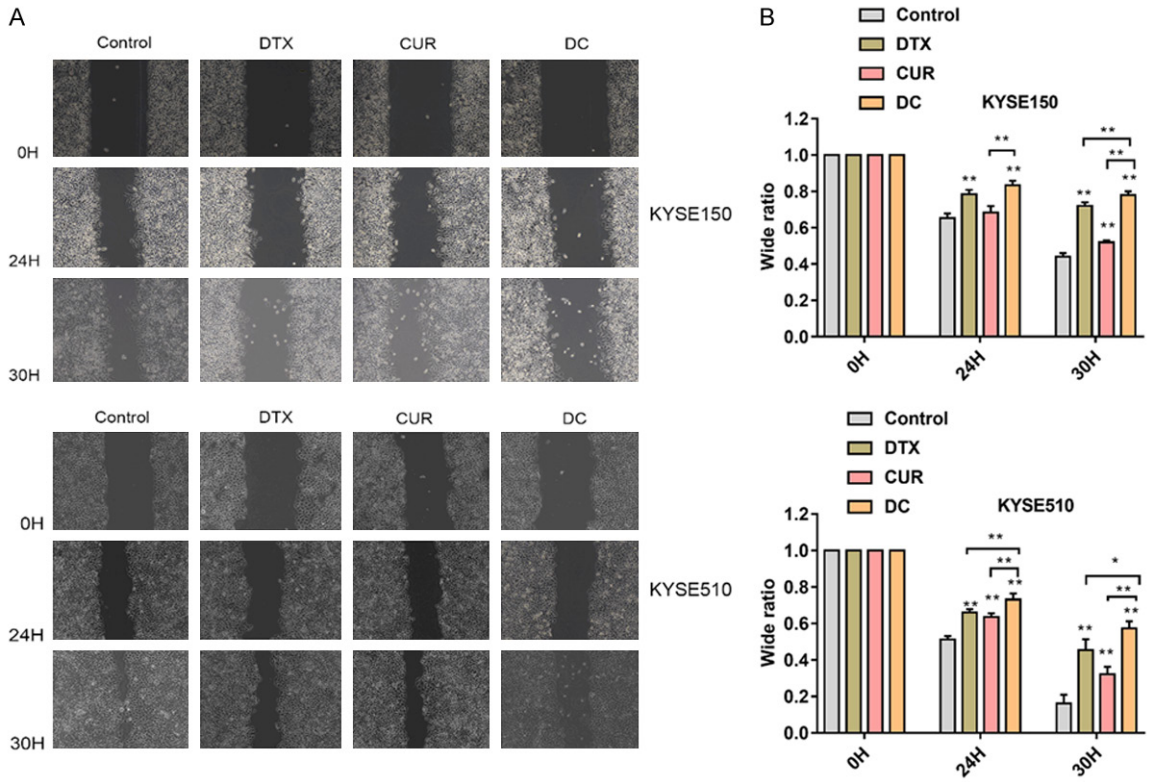

Figure 2. Effects of combined administration on the healing ability of KYSE150 and KYSE510. For KYSE150, the healing rate of the DTX and DC group was significantly lower than that of the control group after 24 h (P<0.01). After 30 h, the healing rates of the DTX, CUR and DC groups were lower than that of the control group, and the DC group had the lowest rate. The healing rate of the DC group was significantly lower than that of the DTX and CUR groups (P<0.01). After drug treatment for 24 h in KYSE510, the healing rates of the other three groups were lower than that of control group. The healing rate of the DC group was significantly lower than those of the DTX and CUR monotherapy groups (A and B). *P<0.05, **P<0.01.

ased (**Figure 4E**). Compared with the monotherapy groups, the combination of CUR and DTX induced apoptosis, consistent with the results of flow cytometry assays.

CUR induces autophagy in ESCC with further promotion when combined with DTX

Transmission electron microscopy was used to observe autophagy of esophageal cancer cells. Autophagic vacuoles and autolysosomes increased in the monotherapy groups compared to the control group, with significant increases in the combined group (**Figure 5A**, **5B**).

LC3 is an important autophagy marker. When autophagy is activated, LC3I in the cytoplasm is transformed into LC3II and aggregates on the membrane surface of autophagosomes. Western blots were used to detect expression of autophagy-related proteins. In the DTX group, LC3II expression was slightly higher than in the control group with no difference in KYSE150 cells (P>0.05). However, LC3II expression in the DTX group was significantly different from that in the control group in KYSE510 cells (P<0.05). The expression of LC3II was raised in the CUR group and with more increases in the combined group (**Figure 5C**). In contrast to LC3II, P62 expression was lower in the CUR and combination groups than in the control and DTX groups. The expression of autophagy-related proteins Beclin-1 and Atg7 were significantly higher in the DC group than the CUR group (P<0.05). From these results, we hypothesized that CUR induced autophagy in esophageal cancer cells, with further promotion of autophagy when combined with DTX.

CUR and DTX induce apoptosis and autophagy through the PI3K/AKT/mTOR pathway

To investigate the mechanism of how CUR synergizes with DTX in inducing apoptosis and

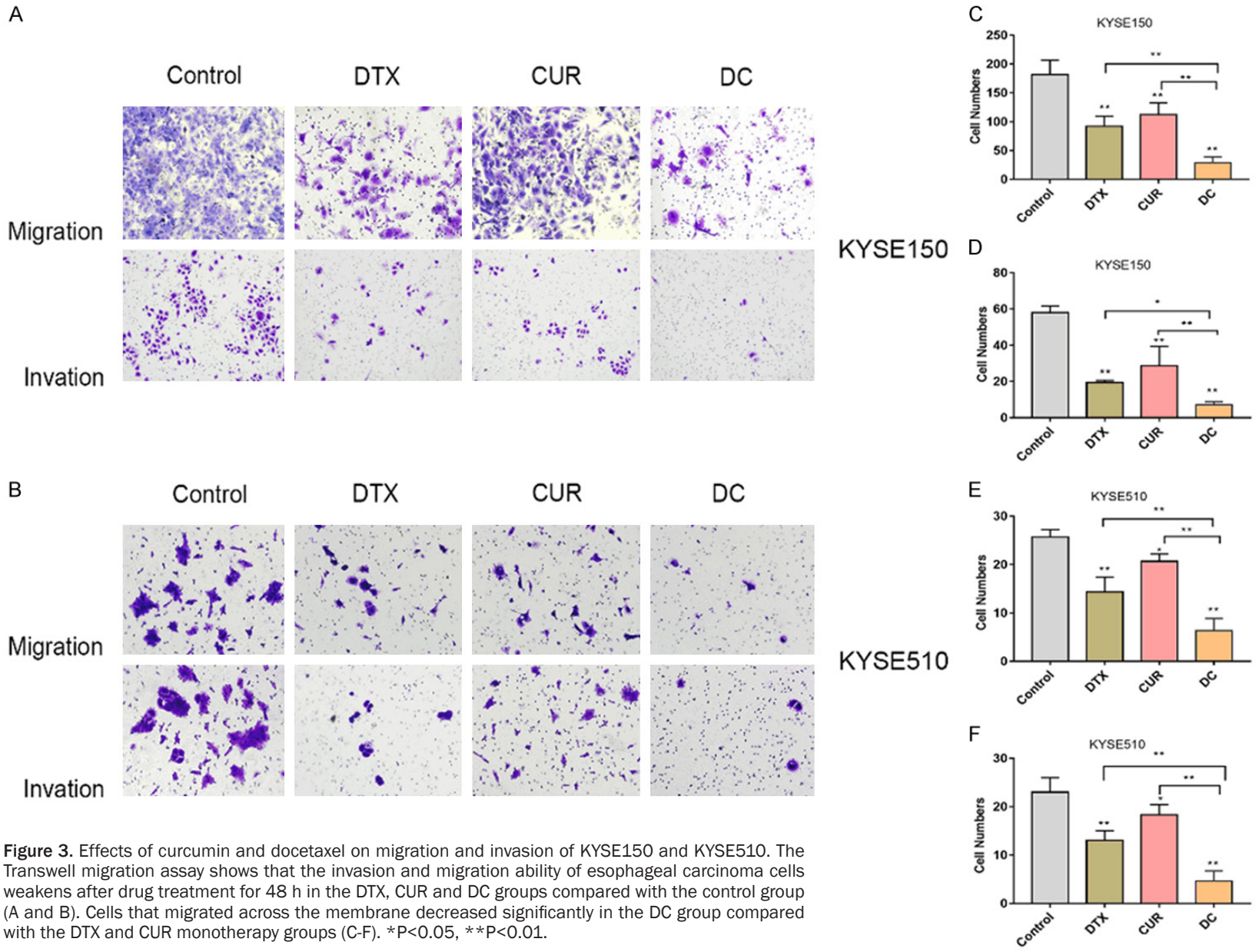

Figure 3. Effects of curcumin and docetaxel on migration and invasion of KYSE150 and KYSE510. The Transwell migration assay shows that the invasion and migration ability of esophageal carcinoma cells weakens after drug treatment for 48 h in the DTX, CUR and DC groups compared with the control group (A and B). Cells that migrated across the membrane decreased significantly in the DC group compared with the DTX and CUR monotherapy groups (C-F). *P<0.05, **P<0.01.

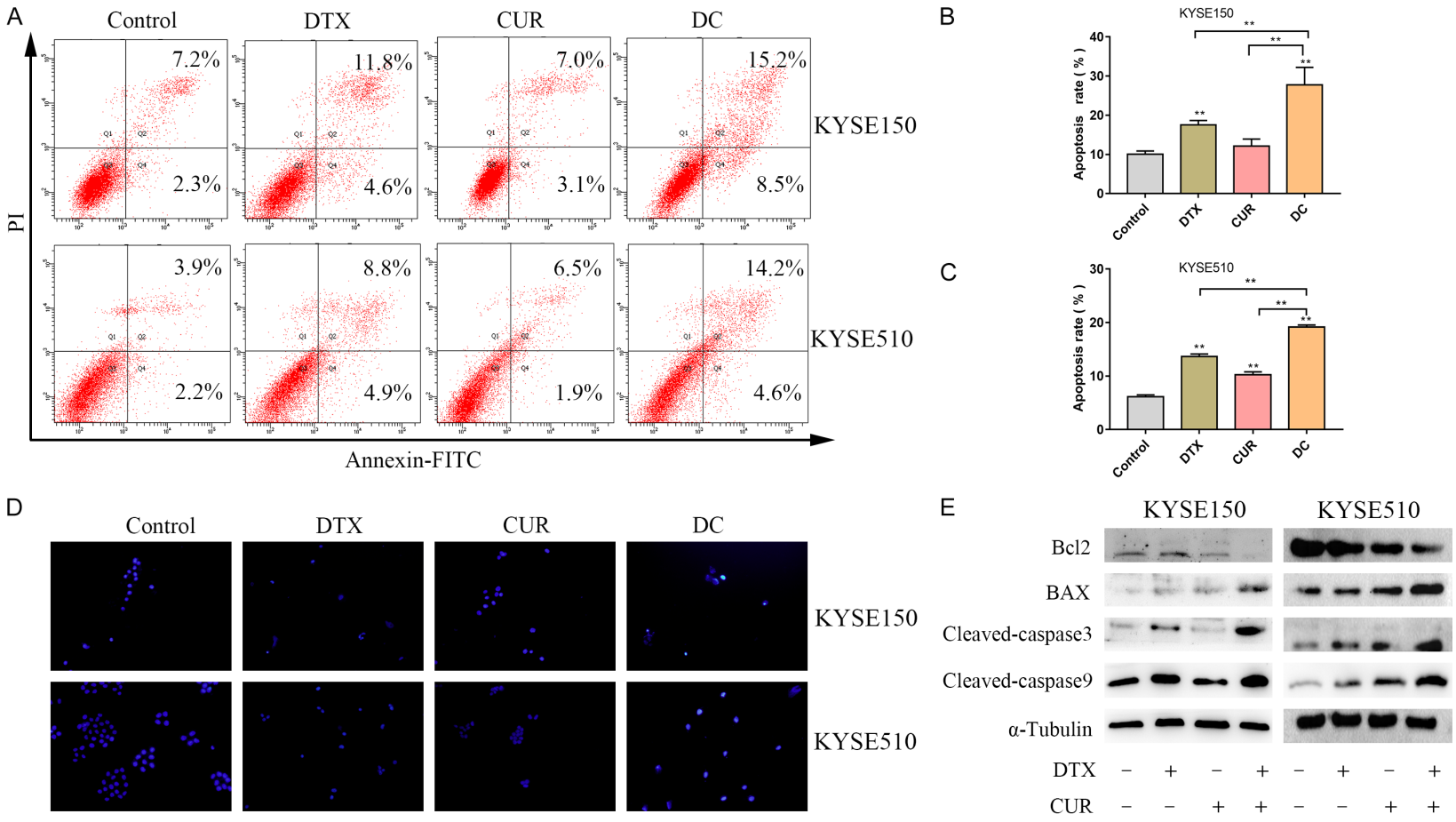

Figure 4. Flow cytometry indicated that apoptosis in the DTX monotherapy group and DC group was greater than that in the control group (A). The apoptosis rate of the CUR monotherapy group was slightly higher than that of the control group in KYSE150 but higher than that in the control group ($P<0.01$) in KYSE510. The apoptosis rate of the DC group was significantly higher than that of the monotherapy groups ($P<0.01$) (B and C). The nuclei appeared normal in the control group but were pyknotic and hyperchromatic in the DC group. Only a few instances of pyknosis and karyolysis were seen in the monotherapy groups (D). The antiapoptotic protein Bcl-2 was lower in the DC group, but pro-apoptotic proteins, such as BAX and Cleaved-caspase3/9, were visibly increased (E).

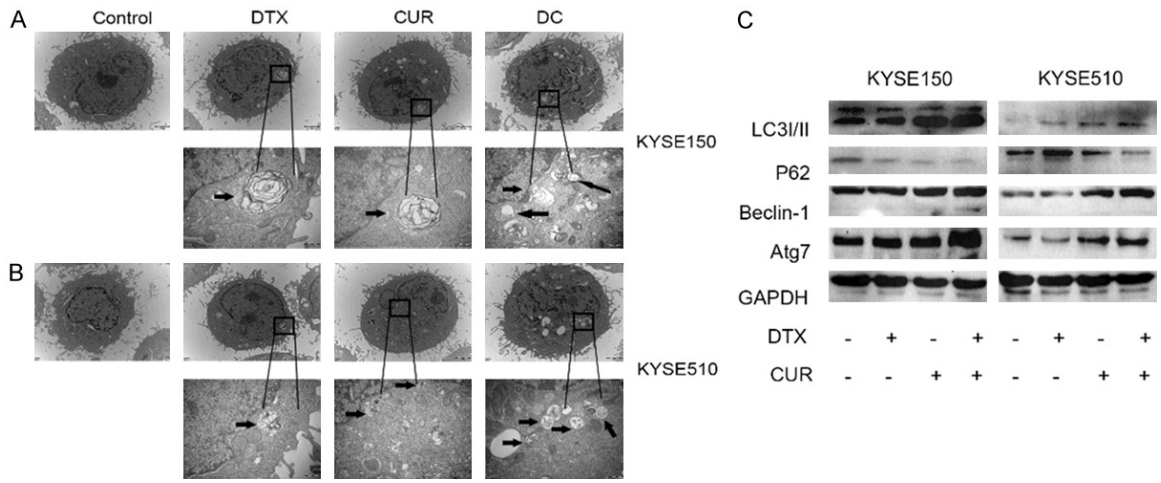

Figure 5. Electron microscopy was used to observe the effects of drugs on autophagy in KYSE150 and KYSE510. Autophagic vacuoles and autolysosomes (arrow) were seen in the monotherapy groups and were more significant in the combined group (A and B). LC3II in the DTX group was slightly higher in KYSE150 but increased in KYSE510. LC3II increased in the CUR group and did so to a greater extent in the combined group. P62 had lower expression in the CUR group and combination group than the control group and DTX group. The expression of Beclin-1 and Atg7 was also up-regulated in the CUR group and the DC group, and was more significant in the combination group (C).

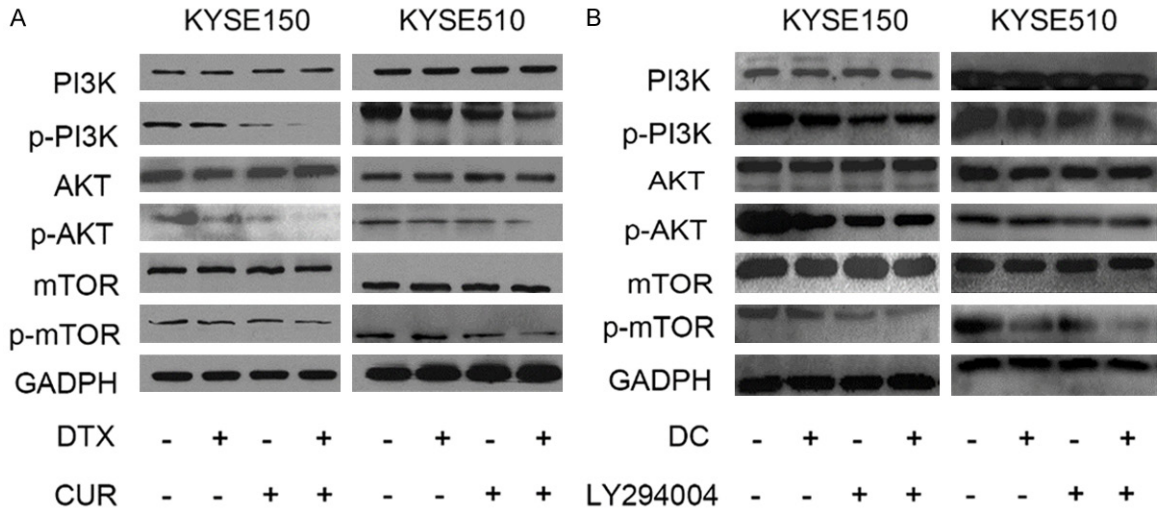

Figure 6. WB assays were used to detect the expression of PI3K/AKT/mTOR protein after drug treatment. The DTX group showed a slight decrease in p-PI3K/PI3K in KYSE150 but a significant decrease in KYSE510. The combined drug group showed a substantially lower p-PI3K/PI3K than the single drug group ($P<0.05$). The expression of p-AKT/AKT in the monotherapy groups was lower than that in the control group ($P<0.05$), whereas p-AKT/AKT in the combined treatment group was clearly downregulated ($P<0.01$). The expression of p-mTOR in the combined group was also significantly lower than that in the DTX and CUR monotherapy groups ($P<0.05$) (A). The ratios of p-PI3K/PI3K, p-AKT/AKT and p-mTOR/mTOR in KYSE150 and KYSE510 treated with LY294004 and DC were significantly lower than those in the DC group and LY294004 group ($P<0.05$) (B).

autophagy, Western blots were used to detect the expression of apoptosis-related and autophagy-related proteins in esophageal cancer cells. Earlier studies found that the PI3K/AKT/mTOR pathway is important in apoptosis and autophagy [14]. Our results showed that expression of PI3K, AKT and mTOR was not significantly different in the experimental groups than the control group. In the DTX group, p-PI3K/PI3K was not significantly different from the blank group in KYSE150 cells ($P>0.05$), but was decreased significantly in KYSE510 cells ($P<0.05$) (**Figure 6A**). In both kinds of cells, the CUR and DC groups had

decreases in p-PI3K/PI3K compared with the control group, and the combined group had significant decreases in p-PI3K/PI3K compared with the single-drug groups (P<0.05). Expression of p-AKT/AKT in the DTX and CUR groups was downregulated compared with the control group (P<0.05), while expression of p-AKT/AKT in the combined group was downregulated compared with the single drug groups (P<0.01). The expression of p-mTOR in the combined group was also significantly lower than in the DTX and CUR groups (P<0.05). Esophageal cancer cells were pretreated with the PI3K pathway inhibitor LY294004, then with DC. The ratios of p-PI3K/PI3K, p-AKT/AKT, and p-mTOR/mTOR were significantly lower in both types of cancer cells treated with LY294004 than in the control group (P<0.05). The ratios in the group with DC combined with LY294004 were further decreased compared with the DC and LY294004 groups, and the difference was significant (P<0.05) (**Figure 6B**). These results suggested that CUR and DTX further inhibited the PI3K/AKT/mTOR signaling pathway after use of a pathway inhibitor. We hypothesized that CUR induced apoptosis and autophagy with DTX by inhibiting activation of the PI3K/AKT/mTOR signaling pathway.

Inhibition of autophagy enhances the apoptotic effect of CUR and DTX

A commonly used inhibitor of early autophagy is 3MA. Cells were treated with 3MA for 30 min, then treated with DTX and CUR for 48 h. CCK8 assays, flow cytometry and western blots were used to determine the effect of autophagy induced by DC on cell apoptosis. CCK8 assays showed that the cell activity in the 3MA group was not significantly different from the control group (P>0.05). The cell activity of the DC group was lower than that in the control group, the difference was significant (P<0.01). The cell activity of the 3MA+DC group was reduced compared to the DC group and was a significant difference (P<0.01) (**Figure 7A**). These findings suggested that 3MA combined with DC further enhanced the inhibitory effect of DC on cell proliferation.

According to flow cytometry, no significant difference occurred in cell apoptosis between the 3MA and control groups (P>0.05) (**Figure 7B**). The apoptosis rate of the DC and 3MA+DC groups was higher than those in the control and 3MA groups. The apoptosis rate of the 3MA+DC group was even higher than that in the DC group with a significance difference (P<0.05) (**Figure 7C**). Western blots showed no significant difference in apoptosis-related proteins Bcl-2, BAX, cleaved caspase3 and cleaved caspase9 between the 3MA and control groups. The expression of the anti-apoptotic protein Bcl-2 was downregulated in the DC group compared with the control group with a significant difference (P<0.05). Pro-apoptotic proteins BAX and activated cleaved caspase-3 and cleaved caspase-9 were upregulated with significant differences (P<0.05). Compared with the DC group, Bcl-2 was significantly downregulated in the 3MA+DC group (P<0.05) while BAX, cleaved caspase-3 and cleaved caspase-9 were significantly upregulated (P< 0.05). Compared with the control group, the ratio of LC3II/LC3I increased in the DC group and decreased in the 3MA group (P<0.05) (**Figure 7D**). After treatment with 3MA and DC, the ratio of LC3II/LC3I was significantly lower than that in the DC group in the two types of cancer cells.

These results suggested that autophagy inhibitors inhibited autophagy induced by DC and further increased antiproliferative and apoptosis-inducing effects of DC. We hypothesized that autophagy induced by CUR and DTX had protective effects on the growth of esophageal cancer cells.

CUR enhances the antitumor effect of DTX in vivo

Animal *in vivo* experiments illustrated that tumor volume growth in the CUR group was slightly slower than that in the control group and significantly slower in the DTX compared to the control group (P<0.01). The tumor volume growth rate in the combined group was significantly slower than in the control, DTX and CUR groups, and tumor volume was significantly reduced. DC appeared to inhibit tumor growth (**Figure 8A**, **8B**). Changes in the body weight of nude mice decreased at a later stage in each group. The weight loss showed no significant difference between the CUR group and PBS group. The weight loss in the DTX group was significant compared to that in the PBS

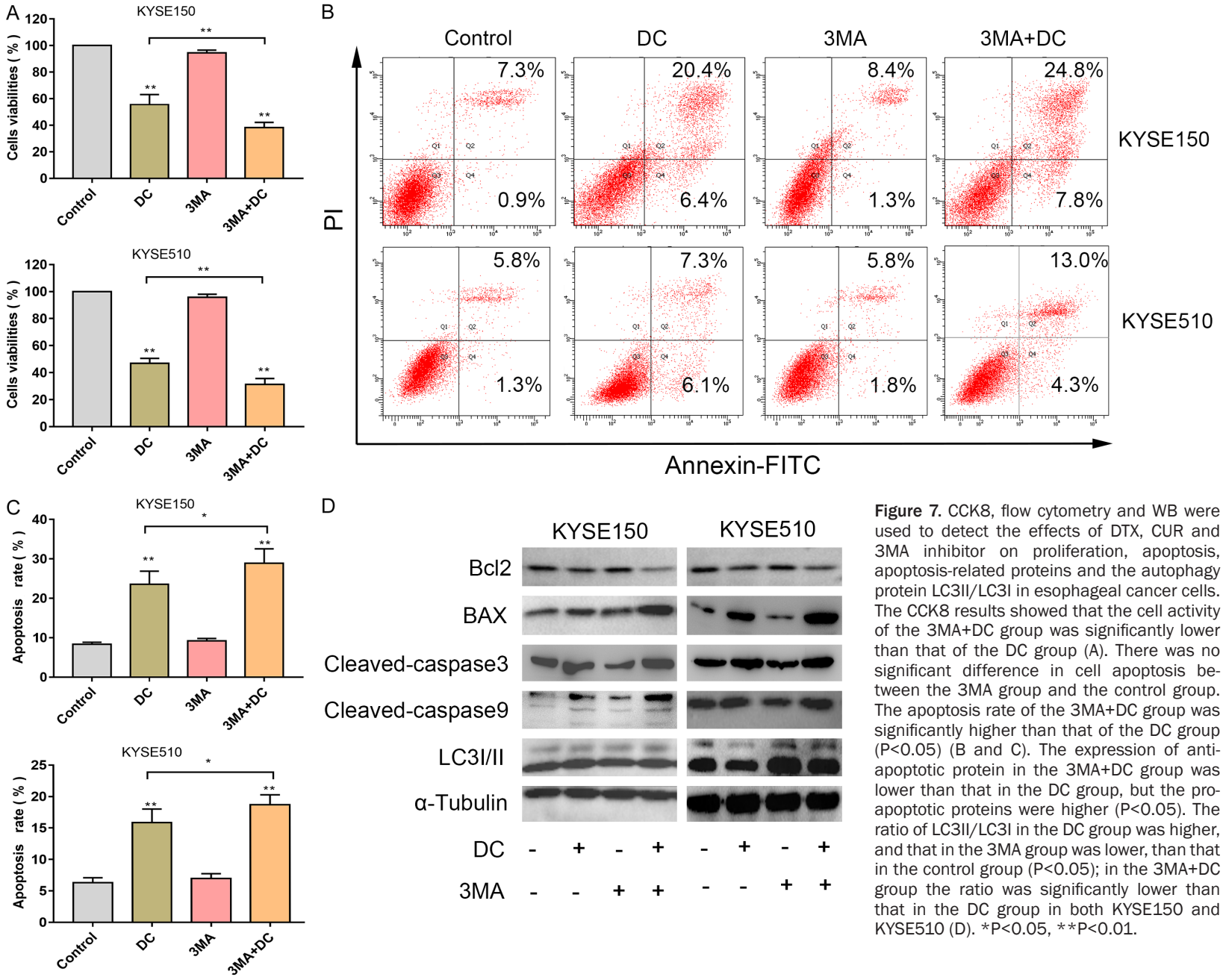

Figure 7. CCK8, flow cytometry and WB were used to detect the effects of DTX, CUR and 3MA inhibitor on proliferation, apoptosis, apoptosis-related proteins and the autophagy protein LC3II/LC3I in esophageal cancer cells. The CCK8 results showed that the cell activity of the 3MA+DC group was significantly lower than that of the DC group (A). There was no significant difference in cell apoptosis between the 3MA group and the control group. The apoptosis rate of the 3MA+DC group was significantly higher than that of the DC group ($P<0.05$) (B and C). The expression of anti-apoptotic protein in the 3MA+DC group was lower than that in the DC group, but the pro-apoptotic proteins were higher ($P<0.05$). The ratio of LC3II/LC3I in the DC group was higher, and that in the 3MA group was lower, than that in the control group ($P<0.05$); in the 3MA+DC group the ratio was significantly lower than that in the DC group in both KYSE150 and KYSE510 (D). *$P<0.05$, **$P<0.01$.

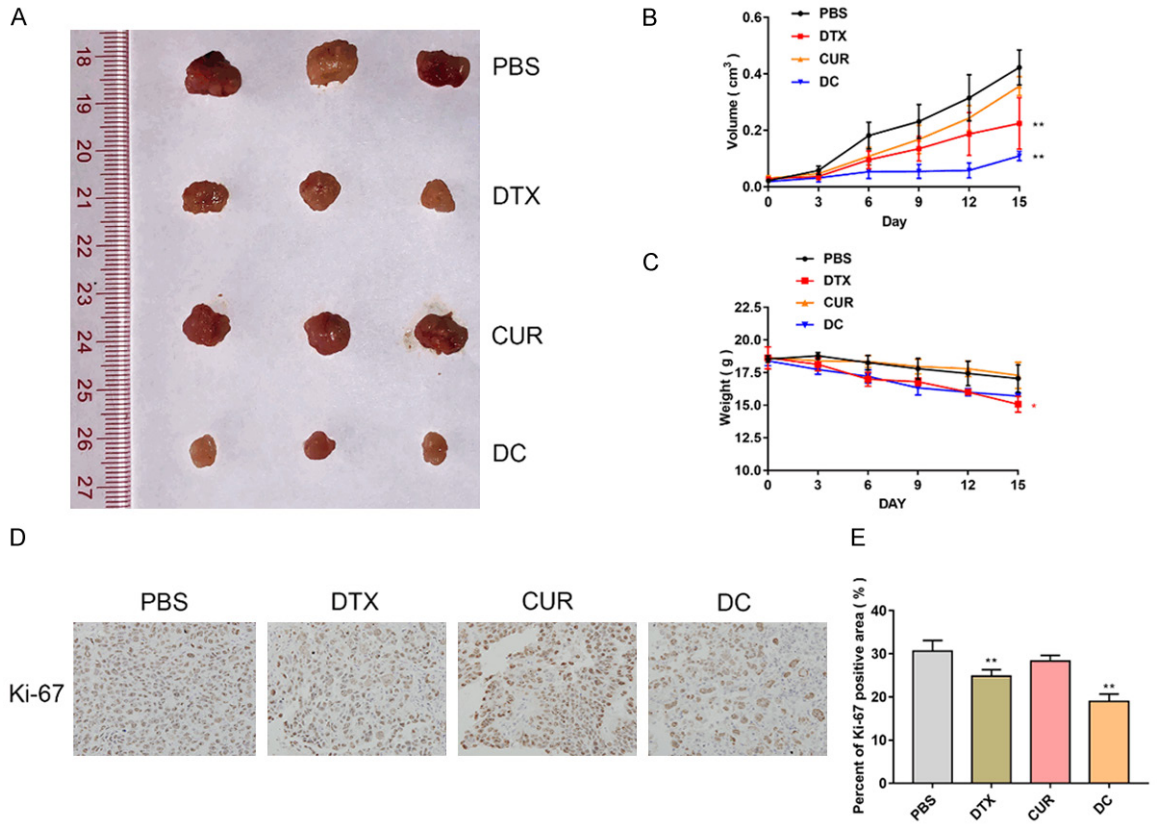

Figure 8. The tumor volumes and body weights of nude mice and Ki-67 in different groups. The volumes of tumors in the DTX and DC groups were lower than those in the control group, and the latter were more apparent (A). The growth of tumor volume was slightly slower in the CUR group but was significantly slower in the DTX group than in the control group (P<0.01); the slowest growth was observed in the combined group (B). Nude mice in each group showed weight loss. There was no significant difference between the CUR group and the control group, but the weight changes in the DTX group and the DC group were statistically significant, as compared with the control group (P<0.05) (C). The expression of Ki-67 was highest in the PBS group, followed by the CUR and DTX groups, and lowest in the DC group, according to immunohistochemical methods (D and E).

group (P<0.05) (**Figure 8C**). Immunohistochemistry demonstrated that expression of Ki-67 was higher in the PBS and the CUR groups than the DTX and DC groups, and significantly lower in the DC group than in the PBS, DTX and CUR groups (P<0.01) (**Figure 8D**, **8E**). The results indicated that tumor proliferation was significantly decreased after combined DTX and CUR therapy.

Discussion

Our experiments showed that CUR inhibited proliferation, weakened the healing ability, decreased the invasion and migration capability, and induced apoptosis of esophageal cancer cells *in vitro*, in a concentration dependent manner. CUR has been reported to inhibit the proliferation and induce the apoptosis of tumor cells, such as those from nasopharyngeal [15], gastric [16], endometrial [17], breast [18], thyroid, cervical [20] and other cancers [21], through multiple signaling pathways. CUR is safe and nontoxic, and it has the potential to become a new generation antitumor drug. Our results also showed that, compared with a single drug, CUR combined with DTX synergistically enhanced the proliferation inhibition effect of DTX and required less drug to achieve comparable anti-tumor effects, thus reducing the toxicity and adverse effects of DTX. Experiments *in vivo* showed that the tumor volumes in the combined group of nude mice were smaller than those in the monotherapy and PBS group. The proliferation index Ki-67 also decreased, thus suggesting that the proliferation of esophageal cancer cells significantly decreased after treatment with DC. The

experiments also showed that the weight loss in nude mice was more noticeable in the DTX group than the PBS and CUR groups. The decreases in the weights of nude mice in the combined group were significantly different from those in the PBS and CUR groups, thus suggesting that CUR decreased the toxic and side effects of DTX.

The findings of scratch healing experiments and Transwell assays showed that DC significantly impaired the scratch-healing ability of esophageal cancer cells and enhanced the ability of DTX to inhibit invasion and metastasis of esophageal cancer cells. CUR may inhibit the migration of endometrial cancer cells by downregulating slit-2-mediated CXCR4, SDF-1 and MMP2/MMP9 [17], in agreement with our findings. Banerjee's group has suggested that CUR greatly improves the efficacy of DTX in the treatment of androgen-independent prostate cancer by inhibiting proliferation and inducing apoptosis, and decreasing the concentration of DTX in PCA cells [22]. Likewise, CUR greatly improves the antitumor effects of DTX in lung cancer *in vitro* and *in vivo* [23].

Apoptosis is also known as programmed cell death. The initiation of this process depends on the activation of a series of caspases [24]. The combination of CUR and DTX promoted the apoptosis of esophageal cancer cells. In addition, expression of the anti-apoptotic protein Bcl-2 decreased and the pro-apoptotic protein Bax was significantly upregulated in the combined group. The expression of caspase-3 and caspase-9, which are cleaved by apoptosis-related protein also increased [24-26]. Most apoptosis in vertebrates occurs through a mitochondria-dependent pathway that is mainly mediated by pro-apoptotic proteins Bax and Bak from the Bcl-2 family. This pathway leads to a mitochondrial permeability transition by inhibiting Bcl-2. After that, promoter procaspase-9 is recruited to the caspase recruitment domain (CARD) of the apoptotic body to carry out self-activated and proteolytic, which activates the downstream proteinases caspases-3, caspases-6, and caspases-7. This process leads to cell lysis and apoptotic cell death. Therefore, we hypothesized that the synergistic effect of CUR and DTX on promoting apoptosis may be from inhibiting the anti-apoptotic protein Bcl-2 and promoting the mitochondrial pathway mediated by the pro-apoptotic protein Bax. The activity of caspase-3 determines the characteristics of apoptotic cells such as capsular vesicle formation, DNA degradation, and nuclear fragmentation [27]. Through Hoechst 33342 staining assays, we observed a series of typical apoptotic morphological changes in the nuclei of esophageal cancer cells in the DC group, including nuclear condensation and partial nuclear lysis, thus further confirming the conclusion that DC may release cytochrome C through an intracellular mitochondrial pathway mediated by the Bcl-2 family, stimulate caspase-9 to self-activate, and further activate caspase3 downstream to induce apoptosis of esophageal cancer cells.

Autophagy is an evolutionally-conserved lysosomal catabolism process [27]. LC3 of microtubule-associated protein 1 is closely associated with autophagy. LC3 has two forms, LC3I and LC3II. During the formation of autophagosome membranes, LC3I in the cytoplasm combines with phosphatidylethanolamine and converts to LC3II [28, 29]. LC3II is the first protein identified to be associated with autolysosome membranes in mammals, and is an important marker of autophagy [30]. Its level reflects the state of autophagy in cells. Using transmission electron microscopy and western blots, we found that CUR induced autophagy in esophageal cancer cells and increased expression of autophagy-related proteins in cells. The number of autophagosomes in esophageal cancer cells increased distinctly in the DC group. In addition, autophagy-related proteins LC3II, beclin1 and Atg7 increased while p62 decreased significantly. This result showed that CUR combined with DTX induced autophagy in esophageal carcinoma cells.

Studies found that the PI3K/AKT/mTOR signaling pathway is activated in a variety of tumor cells, leading to the growth, proliferation and drug resistance of tumor cells [31-33]. As a result, this pathway has become a target for tumor therapy. Multiple antitumor drugs induce cell apoptosis and autophagy by inhibiting the PI3K/AKT/mTOR signaling pathway [31-33]. The expression of p-PI3K, P-AKT and p-mTOR in esophageal cancer cells treated with DC decreased distinctly, suggesting that the PI3K/AKT/mTOR pathway was inhibited.

The PI3K pathway inhibitor LY294004 further inhibited the pathway when combined with DC. Consequently, we hypothesized that DC induced apoptosis and autophagy in esophageal cancer cells by inhibiting the PI3K/AKT/mTOR pathway.

Autophagy has a dual role in the process of tumor development. It inhibits tumor proliferation and gene instability by removing damaged cells and organelles. It also promotes tumor growth by providing essential cell precursors for biosynthesis and dynamic energy balance [28, 29]. A commonly used inhibitor of early autophagy is 3MA. We found that 3MA had no significant inhibitory effect on the activity of esophageal cancer cells. However, the combination of DC and 3MA enhanced the proliferation inhibition by CUR and DTX on esophageal cancer cells and promoted apoptosis, suggesting that the autophagy induced by DC may play a protective role in esophageal cancer cells. The application of autophagy inhibitor 3MA could reduce the protective effect of autophagy, and enhance the antitumor effects of DTX and CUR on esophageal cancer cells. CUR inhibits the activity of gastric cancer cells and induces autophagy, showing a protective effect on gastric cancer cells [34]. In contrast, CUR inhibits autophagy of prostate cancer cells after radiotherapy and enhances the radiotherapy sensitivity of prostate cancer, which may be partly related to the epigenetic activation of miR-143 and autophagy inhibition mediated by miR-143 [35]. The different effects of CUR on different tumors may be related to CUR concentration, which increases reduced glutathione in tumor cells at low doses and decreases it at high doses. When CUR reaches a certain concentration, the combination of DTX may cause changes in glutathione metabolism, lipid metabolism and carbohydrate utilization, resulting in different effects [36]. The relationship between autophagy and apoptosis in tumor cells is extremely complex. Our results suggested that inhibition of autophagy induced by DC promoted apoptosis of esophageal cancer cells. CUR has been found to inhibit the proliferation and apoptosis of hepatocellular carcinoma by downregulating the GPC3/Wnt/β-catenin signaling pathway mediated by autophagy [37, 38]. Treating a glioblastoma cell line with melatonin alone does not cause cell apoptosis. However, pretreating with autophagy inhibitor in combination with melatonin significantly induces apoptosis of glioblastoma cells [39]. From these findings, we hypothesized that autophagy induced by CUR and DTX might have protective effects on esophageal cancer cells. Autophagy inhibitor 3MA combined with DC may be a new strategy for the treatment of esophageal cancer.

Conclusion

This study suggested that CUR and DTX improved the antitumor effect of DTX in ESCC, possibly by inducing esophageal cancer cell apoptosis and autophagy through the PI3K/AKT/mTOR signaling pathways. DTX and CUR induced esophageal cancer cell autophagy, which may be protective for esophageal cancer cell proliferation and apoptosis. The combination of autophagy inhibitor 3MA, DTX, and CUR may become a new strategy for esophageal cancer treatment.

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Disclosure of conflict of interest

None.

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