MEDICAL SCIENCE MONITOR

LAB/IN VITRO RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2017; 23: 2096-2103
DOI: 10.12659/MSM.901183

Received: 2016.08.22
Accepted: 2016.10.06
Published: 2017.05.02

Capilliposide C Sensitizes Esophageal Squamous Carcinoma Cells to Oxaliplatin by Inducing Apoptosis Through the PI3K/Akt/mTOR Pathway

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Source of support: This study was supported by a grant from the National Natural Science Foundation of China (grant no. 81303274)

Background: Although platinum-based chemotherapy is the most effective strategy for esophageal cancer, toxicity and drug resistance limit the dose administration and the application of chemotherapy. Capilliposide C (CPS-C) is isolated from the Chinese herb *Lysimachia capillipes* Hemsl and is approved to be effective against carcinomas. However, the activity of CPS-C against esophageal cancer remains unclear. The present study was conducted to assess the chemosensitizing effects of CPS-C for enhancing the therapeutic efficacy of oxaliplatin in esophageal squamous carcinoma cells and explore the underlying mechanism.

Material/Methods: Human esophageal squamous cell carcinoma (ESCC) TE-1 and TE-2 were used. Several *in vitro* and *in vivo* analyses were carried out, including MTT, Annexin V/PI, Western blot, and TUNEL and immunohistochemistry in a xenograft model.

Results: CPS-C significantly enhanced the proliferative inhibition and apoptotic effect of oxaliplatin in ESCC cells. Oxaliplatin combined with CPS-C decreased the expressions of PI3K, phospho-Akt, phospho-mTOR, Bcl-2, and Bcl-XL, and increased the expression of Bax and caspase-3 significantly compared to oxaliplatin-only treatment. Furthermore, in the ESCC xenograft model, CPS-C significantly enhanced the anti-cancer effects and apoptosis of oxaliplatin.

Conclusions: The results indicated that CPS-C enhanced the anti-proliferative and apoptotic effect of oxaliplatin by modulating the PI3K/Akt/mTOR pathway on ESCC *in vitro* and *in vivo*.

MeSH Keywords: Apoptosis • Esophageal Neoplasms • Phosphatidylinositol 3-Kinases • Saponins

Full-text PDF: http://www.medscimonit.com/abstract/index/idArt/901183

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Background

Esophageal cancer (EC) is one of the most rapidly growing causes of cancer mortality and remains the 8th most common cancer and the 6th most common cause of death from cancer worldwide [1]. EC is mainly classified into two types: squamous cell carcinoma (SCC) and esophageal adenocarcinoma (EAC). SCC is the main histological type of EC and accounts for more than 90% of all cases [2]. Despite the developments in the management of esophageal squamous cell carcinoma (ESCC), more than 60% patients will suffer tumor recurrence and systemic metastasis within 5 years. The overall median survival is 9 months (95% CI: 8.9–9.1), with an overall 5-year survival rate of 15.5%. Patients with SCC have worse survival outcomes than those with EAC [3]. Chemotherapy is the main treatment for metastatic diseases and can improve quality of life. Cisplatin and 5-FU are the most effective agents and have been extensively studied in ESCC for decades. However, they are also associated with significant toxicity. Several trials have suggested that oxaliplatin is equally or more effective but less toxic than cisplatin [4,5]. Oxaliplatin is another promising agent and is used with increasing frequency in ESCC [6–10]. Although platinum-based chemotherapy inhibits ESCC cell growth, it leads to severe side effects including nausea, vomiting, diarrhea, and suppression of the hemopoietic system. Therefore, less toxic treatments and more effective strategies are needed. Natural products are suitable alternatives as sensitizers, and increasing numbers of research studies have focused on agents extracted from natural resources [11–16].

Capilliposide C (CPS-C) (Figure 1), isolated from the Chinese herb *Lysimachia capillipes* Hemsl *(L. capillipes)*, is a novel oleanane triterpenoid saponin and is approved to be effective against a variety of cancers, including gastric cancer, prostate cancer, non-small cell lung cancer, nasopharyngeal cancer, ovarian cancer, and so on[17–20]. As a natural plant extract, CPS-C has fewer side effects and its mechanism is related to inducing apoptosis, down-regulation of Bcl-2, JNK, and P38a/b, up-regulation of Bax, p-JNK, and p-P38, and increasing the PUMA-Bax pathway [17–20]. However, the mechanism underlying its anti-tumor effects in ESCC and how to obtain optimum therapeutic modality still remain unclear. Therefore, the aim of the present study was to investigate the synergism between CPS-C and oxaliplatin in ESCC and elucidate the underlying molecular mechanism. The results of our study suggested that CPS-C serves as a novel anti-tumor agent to sensitize ESCC cells to oxaliplatin and that the combination therapy of CPS-C and oxaliplatin may be beneficial for patients with ESCC.

Material and Methods

Chemicals and reagents

CPS-C was obtained from Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Peking Union Medical College (Peking, China). Oxaliplatin was purchased from Sigma-Aldrich Co. (St. Louis, Missouri, USA). MTT was purchased from Sigma-Aldrich Co. (St. Louis, Missouri, USA). The Annexin V Apoptosis Detection kit was purchased from BD Biosciences (Franklin Lakes, New Jersey, USA). PI3 kinase p110α (C73F8) rabbit mAb (#4249), phospho-Akt (Ser473) (D9E) rabbit mAb (#4060), phospho-mTOR (Ser2448) (D9C2) rabbit mAb (#5536), phospho-mTOR (Ser2448) (49F9) rabbit mAb (IHC Specific) (#2976), Bcl-2 (124) mouse mAb (#15071), Bcl-xL (54H6) rabbit mAb (#2764), Bax (D2E11) rabbit mAb (#5023), and caspase-3 antibody (#9662) were purchased from Cell Signaling Technology (Danvers, Massachusetts, USA). Anti-GAPDH was purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). The In Situ Cell Death Detection Kit was purchased from Sigma-Aldrich Co. (St. Louis, Missouri, USA).

ESCC cell lines

Two cell lines (TE-1 and TE-2) that were established from a well or poorly differentiated human ESCC [21] and the human esophageal epithelial cell line Het-1A [22] were obtained from Shanghai Institute of Cell Biology (Shanghai, China). TE-1 and TE-2 cell lines were cultured in RPMI 1640 (GE Healthcare Life Sciences, Logan, Utah, USA) and Het-1A cells were cultured in BEGM (GE Healthcare Life Sciences, Logan, Utah, USA)

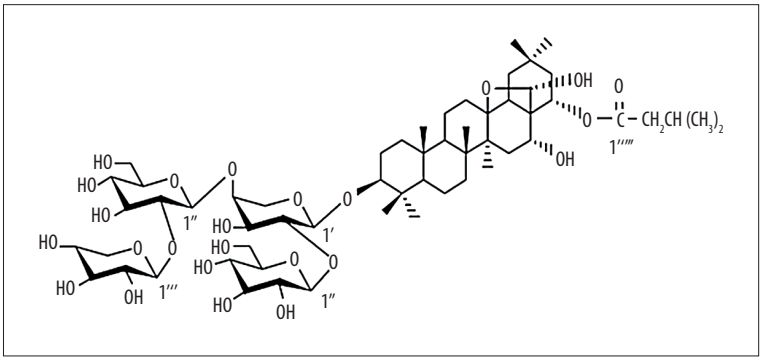

Figure 1. Chemical structure of CPS-C.

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supplemented with 10% fetal bovine serum (GE Healthcare Life Sciences, Logan, Utah, USA) at 37°C with humidified 5% CO_2.

MTT assay

The antiproliferative effects of CPS-C and oxaliplatin were assessed using a MTT assay. TE-1 and TE-2 cells were seeded and then treated with CPS-C (from 0.0625 to 1 μg/mL). Absorbances at a wavelength of 450 nm as the OD value were detected, and dose-dependent curves were generated. Further, TE-1 and TE-2 cells were treated with oxaliplatin (from 1 to 256 μM) and CPS-C (1 μg/mL) to generate curves and calculate 50% inhibition concentration (IC50) values.

Apoptosis assay

Apoptosis was quantified using the Annexin V/PI method. TE-1 and TE-2 cells were treated with oxaliplatin (16 μM) with or without CPS-C (1 μg/mL). Cells were harvested, trypsinized, and washed with phosphate-buffered saline (PBS) after treatment for 48 h. Then the cells were stained with the Annexin V Apoptosis Detection kit prior to being detected by flow cytometry (Beckman Coulter, Inc., Brea, California, USA).

Western blot analysis

TE-1 and TE-2 cells were treated with oxaliplatin (16 μM) with or without CPS-C (1 μg/mL) for 48 h. Cells were lysed. and total protein was extracted by cell lysis buffer containing Tris-HCl and Triton X-100. Cell lysate proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes (Millipore, Billerica, Massachusetts, USA). Membranes were blocked with 5% non-fat milk in TBST, and then incubated overnight at 4°C with primary antibodies (rabbit anti-rat PI3K, phospho-Akt, phospho-mTOR, Bcl-2, Bcl-xL, Bax, caspase-3 monoclonal antibodies, and mouse anti-rat GAPDH monoclonal antibody [1: 1,000]). Membranes then were reacted with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibodies for 30 min at room temperature. Proteins were visualized with enhanced chemiluminescence reagent and detected using ImageQuant LAS 4000 (Pittsburgh, Pennsylvania, USA).

Animal treatment

BALB/c nu/nu mice were obtained from and housed at the Laboratory Animal Center of Zhejiang University (Hangzhou, China).The animal care and experimental protocols were carried out according to the guidelines established by National Institutes of Health. The protocol was approved by the Hangzhou Cancer Hospital Research Ethics Committee. The mouse model of xenograft tumor was established by subcutaneously injecting TE-2 cells. Twenty-four tumor-bearing nude mice (male, aged 4 weeks, weighing 19–21 g) were randomly divided into four groups: control group (intraperitoneal injection with 0.1 mL of normal saline twice a week); CPS-C group (intraperitoneal injection with 0.1 mL of CPS-C [1 mg/kg] twice a week); oxaliplatin group (intraperitoneal injection with 0.1 mL of oxaliplatin [5 mg/kg] twice a week); and CPS-C + oxaliplatin group (intraperitoneal injection with 0.1 mL of CPS-C [1 mg/kg] and 0.1 ml oxaliplatin [5 mg/kg] twice a week). Tumor volume and weight were evaluated. and mice were sacrificed after 7 weeks. Tumor samples were obtained and fixed in 4% formalin fixative, and then were embedded in paraffin.

TUNEL analysis and immunohistochemistry

The terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) method was used to detect apoptosis with an *in situ* cell death detection kit according to the manufacturer's instructions. Reactions were examined under an Olympus BX61 fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Paraffin was removed from the paraffin sections by using xylene, 100% ethanol, 95% ethanol, 80% ethanol, and distilled water. After antigen retrieval by microwave heating in citrate buffer for a 5-min cycle, tissue sections were incubated with protein blocking solution and washed with PBS while switching from one step to other. The Expose Mouse and Rabbit Specific HRP/DAB kit was used to perform immunostaining according to the manufacturer's instructions (Abcam, Cambridge, UK). Tissue sections then were incubated with primary antibodies for 1 h at ambient temperature, followed by incubation with HRP conjugate, and then were incubated with the DAB substrate for 10 min and counterstained with Mayer's hematoxylin for 2 min.

Statistical analysis

Data were expressed as the mean ±SD. SPSS 19.0 (SPSS Inc., Chicago, Illinois, USA) was used to perform statistical analysis. Student's t-test was used to analyze two groups, and one-way ANOVA was used to analyze multiple groups. P values <0.05 were considered statistically significant.

Results

CPS-C enhances proliferative inhibition effect of oxaliplatin in ESCC cells

In order to explore the anti-tumor and chemosensitizing activities of CPS-C, the MTT assay was used to evaluate the effect of CPS-C in normal human esophageal epithelial cell line Het-1A and ESCC cell lines TE-1 and TE-2. The results showed

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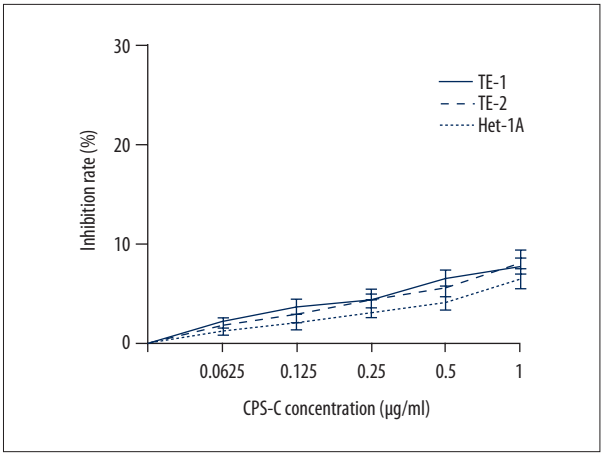

Figure 2. Anti-proliferative activity of CPS-C in normal human esophageal epithelial cell line Het-1A and ESCC cell lines TE-1 and TE-2.

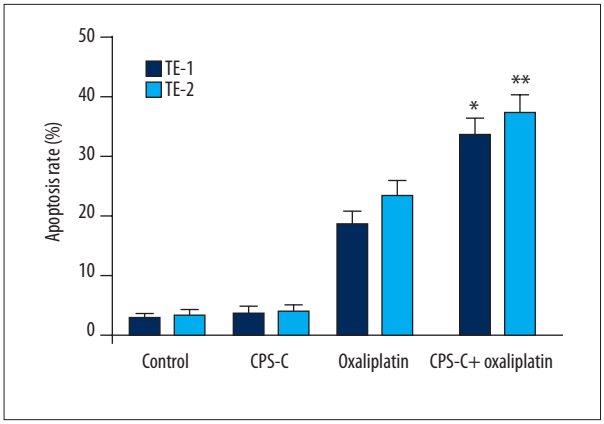

Figure 4. CPS-C enhances oxaliplatin-induced apoptosis in ESCC cell lines TE-1 and TE-2. *, ** Statistically significant difference (P<0.01) between the CPS-C + oxaliplatin treatment group and the oxaliplatin treatment group in TE-1 and TE-2, respectively.

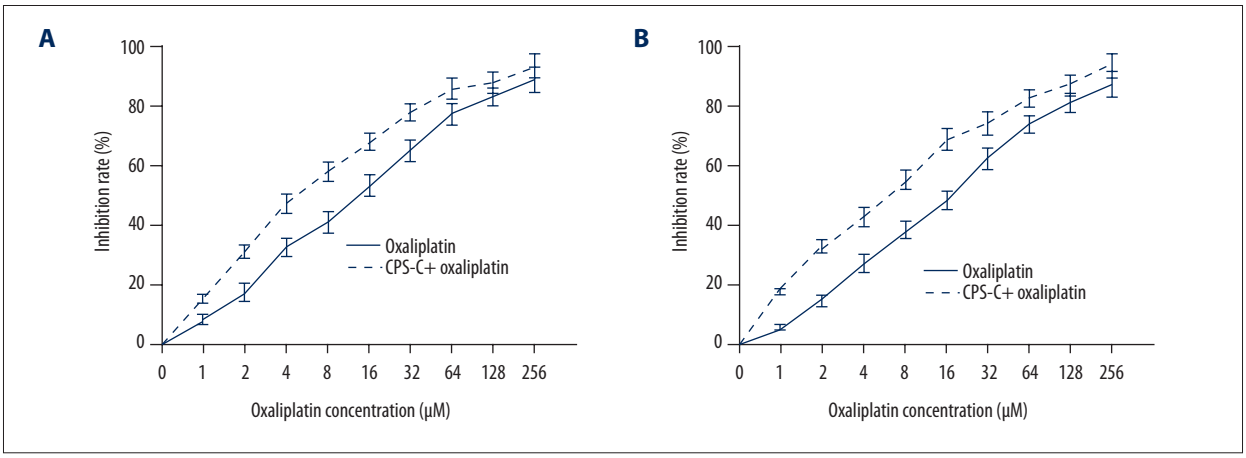

Figure 3. CPS-C enhances oxaliplatin sensitivity in ESCC cell lines TE-1 (**A**) and TE-2 (**B**), as determined by MTT assay. Inhibition rates were significantly increased in the CPS-C + oxaliplatin treatment group compared with the oxaliplatin group (P<0.01) at 48 h and were dose dependent.

that CPS-C did not have an anti-proliferative effect in esophageal epithelial cell line Het-1A with increasing concentrations. CPS-C also displayed no cytotoxicity in ESCC cell lines TE-1 and TE-2 at concentrations less than 1 μg/mL (Figure 2). TE-1 and TE-2, which were ESCC cell lines with different malignant potentials, were treated with CPS-C and oxaliplatin. The results showed that oxaliplatin (from 1 to 256 μM) inhibited the proliferation of ESCC cell lines in a dose-dependent manner (IC50 values for TE-1 and TE-2 cells at 48 h were 14.90±1.26 μM and 20.39±1.74 μM, respectively) and that the proliferative inhibition effect of oxaliplatin significantly increased when combined with CPS-C (1 μg/mL), with IC50 values decreasing to 5.43±0.63 μM and 6.64±0.91 μM for TE-1 and TE-2 cells at 48 h, respectively (Figure 3A, 3B).

CPS-C enhances apoptosis induced by oxaliplatin in ESCC cells

To evaluate the effect of CPS-C on apoptosis induced by oxaliplatin in ESCC cells, the Annexin V-FITC assay was performed. The results showed that oxaliplatin induced significant apoptosis compared to the control group (18.6±2.43% and 23.4±2.60% for TE-1 and TE-2 cells, respectively, *vs.* 2.83±0.91% and 3.17±1.14%) and that the apoptosis rates increased significantly when oxaliplatin was combined with CPS-C 50 μg/mL (33.7±2.98% and 37.4±3.15% for TE-1 and TE-2 cells, respectively) at 48 h (Figure 4).

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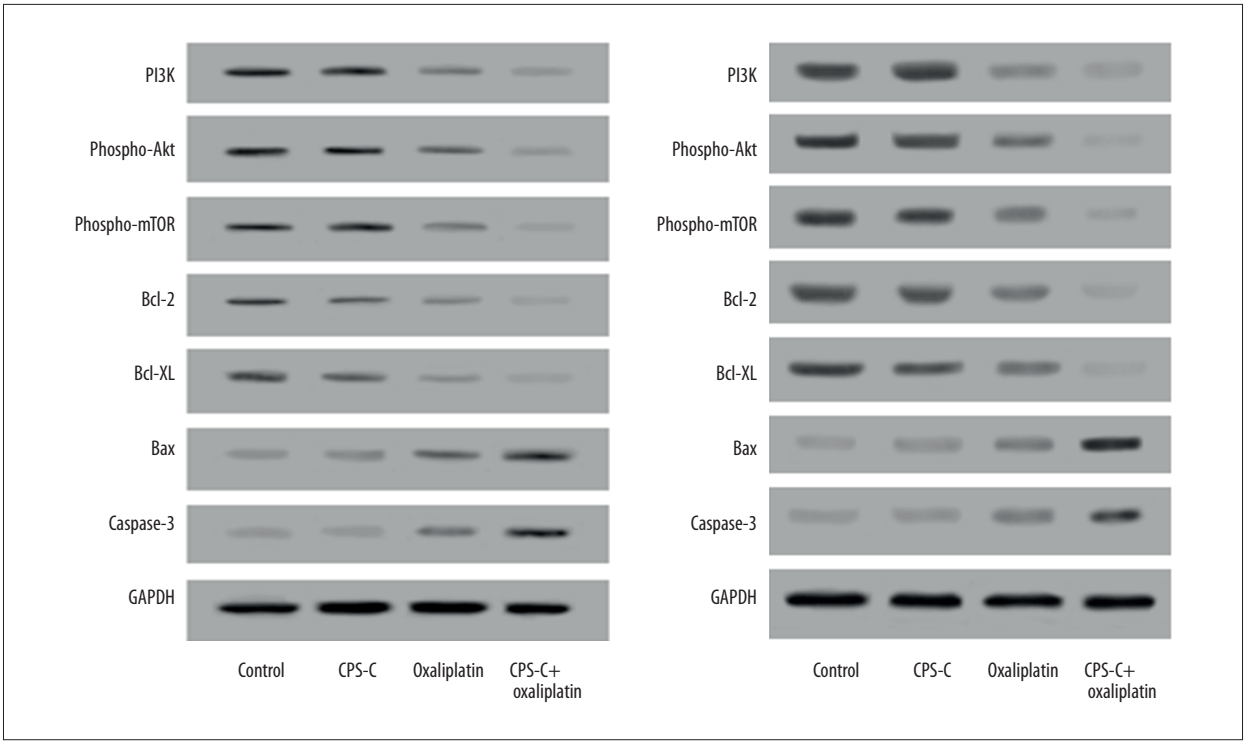

Figure 5. CPS-C increases oxaliplatin-induced changes in protein levels of the PI3K/Akt/mTOR pathway in ESCC cell lines TE-1 (**A**) and TE-2 (**B**). Protein levels were detected using Western blotting analysis. Oxaliplatin combined with CPS-C decreased the expressions of PI3K, phospho-Akt, phospho-mTOR, Bcl-2, and Bcl-XL, but increased the expression of Bax and caspase-3 significantly compared to oxaliplatin-only treatment, while no differences were observed in either the CPS-C group or the control group (P>0.05).

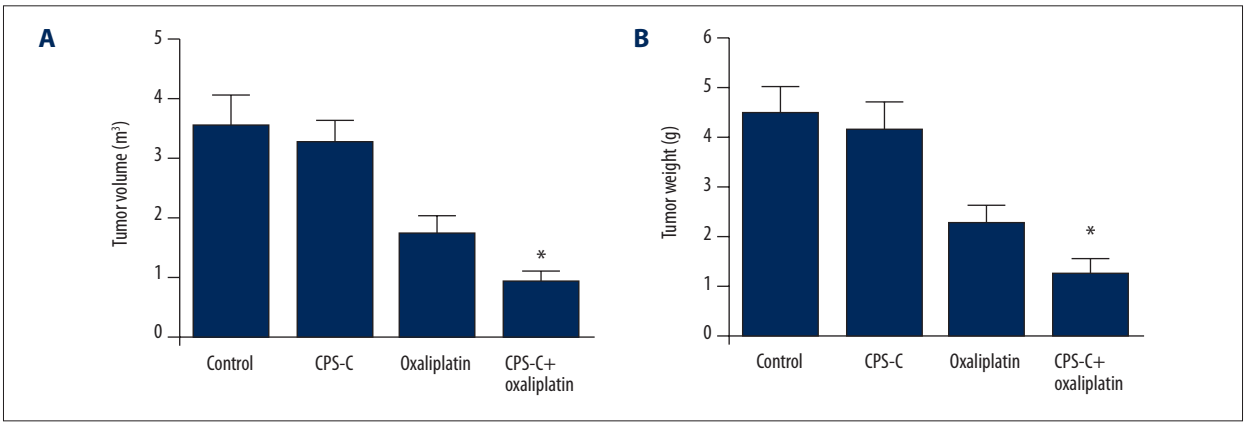

Figure 6. The antitumor effects of oxaliplatin combined with CPS-C on tumor growth in the xenograft model in nude mice of TE-2 cells. Tumor volumes (**A**) and weight (**B**) in the model mice. * Statistically significant difference (P<0.01) between the CPS-C + oxaliplatin group and oxaliplatin-only group. No differences were observed in either the CPS-C group or the control group (P>0.05).

CPS-C enhances proliferative inhibition and apoptosis effects of oxaliplatin by modulating the PI3K/Akt/mTOR pathway

The expression levels of key regulator proteins in the PI3K/Akt/mTOR pathway including PI3K, phospho-Akt, phospho-mTOR, Bcl-2, Bcl-XL, Bax, and caspase-3 in ESCC cells treated by oxaliplatin with or without CPS-C for 48 h were detected by Western blotting. The results showed that oxaliplatin combined with CPS-C decreased the expressions of PI3K, phospho-Akt, phospho-mTOR, Bcl-2, and Bcl-XL, but increased the expression of Bax and caspase-3 significantly compared to oxaliplatin-only

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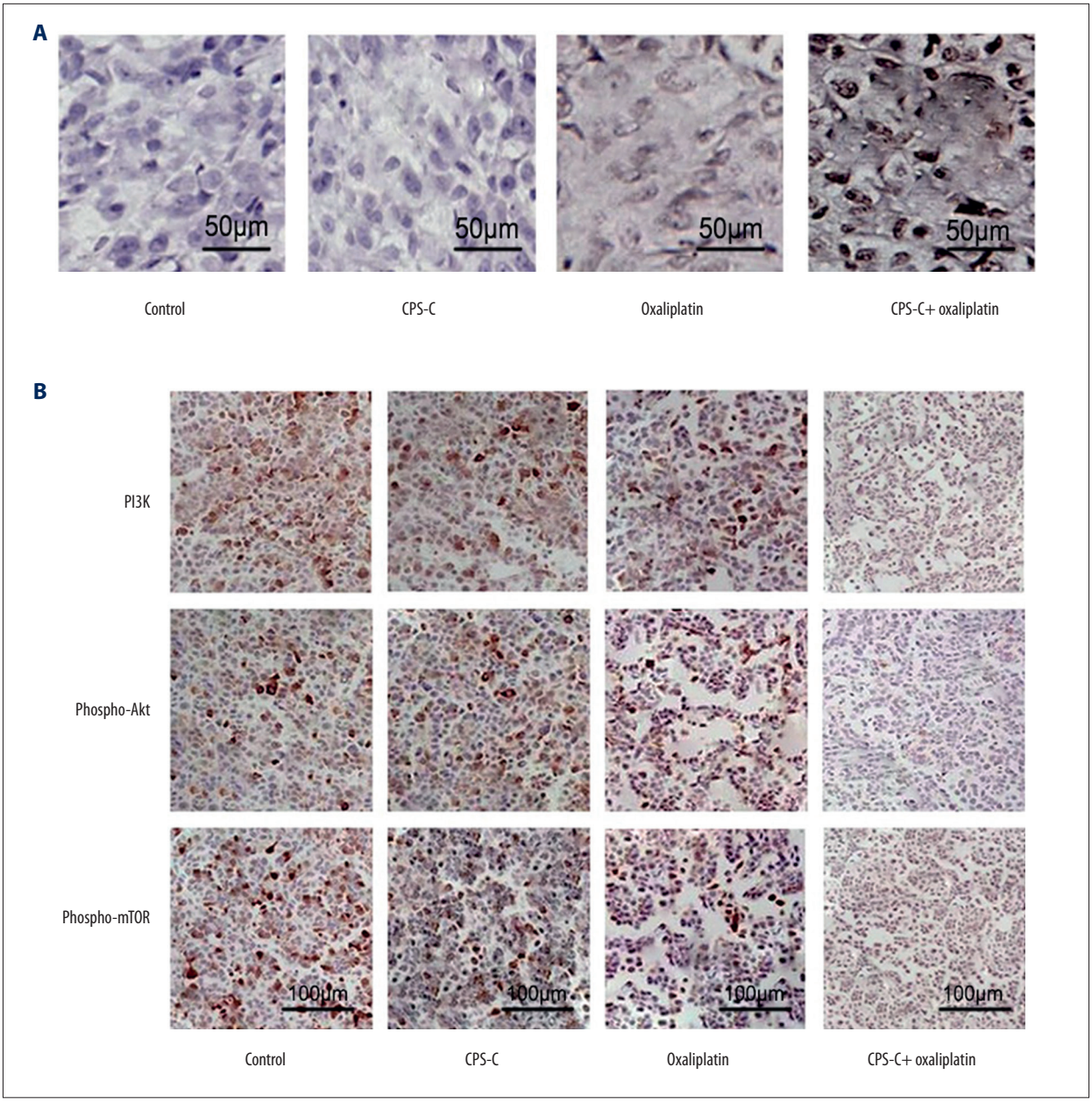

Figure 7. The effects of oxaliplatin combined with CPS-C on apoptosis and PI3K/Akt/mTOR protein expressions in tumor tissues. (**A**) Representative examples of TE-2 tumor xenografts stained with apoptosis by TUNEL assay (400× magnification). (**B**) Representative examples of immunohistochemistry staining for PI3K, phospho-Akt, and phospho-mTOR protein from TE-2 xenografts (400× magnification).

treatment, while no differences were observed in either the CPS-C group or the control group in TE-1 (Figure 5A) and TE-2 (Figure 5B) cells, respectively.

CPS-C enhances the anti-cancer effects and apoptosis of oxaliplatin in the ESCC xenograft model

We further determined the activity of β-elemene combined with oxaliplatin in TE-2 cells *in vivo*. The results showed that tumor volumes and weight in the CPS-C + oxaliplatin group were reduced significantly compared to the oxaliplatin-only group, while no differences were observed in either the CPS-C group or the control group (Figure 6A, 6B). TUNEL was used to analyze the apoptosis induced by CPS-C and oxaliplatin *in vivo*. Positively labeled cells were counted to determine the distribution of apoptotic cells in random microscopic fields. The apoptotic index of the control group was 0.48±0.17 and that of the CPS-C group was 0.65±0.44, while the apoptotic index of the

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oxaliplatin group was 4.81±0.63 and that of the CPS-C + oxaliplatin group was 8.60±0.52. There was a statistically significant difference between the oxaliplatin group and the CPS-C + oxaliplatin group ($P<0.01$) (Figure 7A). Immunohistochemical analysis was performed to verify the molecular mechanism of CPS-C in sensitizing the anti-cancer activity of oxaliplatin through the PI3K/Akt/mTOR pathway *in vivo*. The expressions of PI3K, phospho-Akt, and phospho-mTOR were decreased significantly in the CPS-C + oxaliplatin group compared to the oxaliplatin-only group (Figure 7B).

Discussion

Chemotherapy has been applied to treat EC for more than 30 years. Although novel targeted drugs have been shown to be active against EC, platinum-based regimens remain the most active standard protocol and the mainstay of typical combination chemotherapy. Cisplatin is the most effective agent for EC, but its high toxicity leads to limitations in dose administration. Further, its efficacy also is limited owing to the development of drug resistance in tumor cells. Oxaliplatin [(trans-R,R)1,2-diaminocyclohexaneoxalatoplatinum (II), $C_8H_{14}N_2O_4Pt$] is equally or more effective but less toxic than cisplatin, and is a promising agent for combination chemotherapy in treating ESCC. However, the side effects of chemotherapy and drug resistance still seriously limit its wide application.

CPS-C has been shown to have comprehensive anti-tumor effects on numerous cancer types and low toxicity in patients, although its molecular mechanism remains unclear. Recently, there has been increasing interest in this agent for treatment of carcinomas. Previous studies showed that CPS-C inhibited prostate cancer cell lines PC3 and DU145 by inducing apoptosis through activating the MAPK pathway, decreasing P38 and JNK, but increasing p-P38, p-JNK, and ROS [17]. Another study demonstrated that CPS-C inhibited and induced the apoptosis of CNE-2 cells via increasing the PUMA-Bax pathway [18]. Also, CPS-C exerts an anti-tumor effect on non-small cell lung cancer cell lines through inducing ROS generation, cell cycle arrest, and apoptosis [19].

In the present study, we evaluated the combination effect of CPS-C and oxaliplatin on ESCC *in vitro* and *in vivo*. The results indicated the pharmacologic advantage of CPS-C in enhancing oxaliplatin-induced cytotoxicity, but found little effect of CPS-C on ESCC and normal human esophageal epithelial cells. We investigated the mechanisms by which CPS-C increases oxaliplatin sensitivity in ESCC and found that CPS-C promoted the effect of oxaliplatin by inducing apoptosis significantly and modulating the PI3K/Akt/mTOR pathway. Apoptosis is one of the most important processes in cell proliferation and a key molecular mechanism of anticancer therapies. Therefore, modulation of apoptosis is critical for the pharmacological strategy of chemotherapeutic agents. The PI3K/Akt/mTOR signaling pathway is involved in cell proliferation, differentiation, survival, apoptosis, and metastasis [23,24]. Overexpression of PI3K and the PI3K signaling pathway may cause cancer [25,26]. Akt is also a key regulator of cell growth that can regulate antiapoptotic proteins and cell proliferation [27]. Akt increases the phosphorylation of Bax and inactivates Bad, leading to inhibition of apoptosis [28]. mTOR increases protein translation by activation of the Akt and the Akt/mTOR signaling pathway through a multistep mechanism [29]. The Bcl-2 family including anti-apoptotic proteins, such as Bcl-2, Bcl-xL, and p-Bad, and pro-apoptotic proteins, such as Bad and Bax, is the main controller and regulates mitochondria-dependent apoptosis [30]. The Bcl-2/Bax ratio is considered to be a critical factor for resistance to apoptosis [31]. Caspases are cysteine proteases that mediate apoptotic cell death, and they are activated during apoptosis. Caspase-3, which is a downstream caspase, represents the most important member in the caspase family. Caspase-3 activates Bax and then releases cytochrome C, leading to the cleavage of caspase-9 [32]. Interaction between caspase-9 and caspase-3, caspase-6, and caspase-7 activates the latter capases, and finally cell apoptosis [33]. Our results showed that CPS-C promoted oxaliplatin-induced apoptosis by decreasing the expressions of PI3K, phospho-Akt, phospho-mTOR, Bcl-2, and Bcl-XL and increasing the expression of Bax and caspase-3.

Conclusions

We found that CPS-C enhanced the anti-proliferative and apoptotic effect of oxaliplatin by modulating the PI3K/Akt/mTOR pathway on ESCC *in vitro* and *in vivo*. This therapeutically effective combination is a novel, safe, and practical form of chemotherapy and results in a more powerful anti-ESCC effect.

Conflicts of interest

The authors declare no conflict of interest.

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