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Original Article

Celastrus orbiculatus Extracts Inhibit Human Hepatocellular Carcinoma Growth by Targeting mTOR Signaling Pathways*

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ABSTRACT **Objective**: To characterize the molecular mechanism underlying the antineoplastic activity of *Celastrus orbiculatus* Thunb. extracts (COE). **Methods**: The human hepatocellular carcinoma HepG2 cells with mammalian target of rapamycin (mTOR) knockdown expressed (HepG2/mTOR$^-$) were constructed using molecular biological technology. *In vitro*, the HepG2/mTOR$^-$ cells were treated with COE at various concentrations (10, 20, 40, 80, 160 and 320 μg/mL). Cell viability was determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays. According to the half-maximal inhibitory concentration (IC_{50}) value (140 mg/L), the concentrations of COE in the subsequent experiment was set to alleviate cytotoxicity. The HepG2/mTOR$^-$ cells were divided into 5 groups: negative control (untreated), COE treatment groups (40, 80, 120 mg/L COE) and positive control group (cisplatin, DDP, 2 mg/L), respectively. Wild-type HepG2 cells were used as a blank control. The effects of COE on the cell apoptosis were analyzed by flow cytometry and transmission electronic microscopy (TEM), respectively. The protein expression levels of mTOR signal pathways were determined by Western blotting. *In vivo*, HepG2/mTOR$^-$ cells (2×10^6 cell/mice) were subcutaneously injected into the right flank of nude mice. Thirty-six female nude mice were randomly assigned to 6 groups according to body weight (6 mice per group) as follows: solvent vehicle control, Banmao Capsule treated group (BM, 195 mg/kg), Tegafur, Gimeracil and Oteracil Potassium Capsules (10 mg/kg) treated group, and different dosages of COE (10, 20, 40 mg/kg) groups. Tumor growth was monitored and immunohistochemical staining was used to examine the expression of apoptosis-related proteins in tumor tissues. **Results**: COE inhibited the proliferation significantly in a concentration-dependent manner in HepG2/mTOR$^-$ cells (P<0.01). COE significantly induced the apoptosis of HepG2/mTOR$^-$ cells (P<0.01), and the apoptotic bodies can be observed under TEM. COE significantly inhibits the proteins expression of mTOR-related signal pathways. *In vivo*, COE significantly inhibited tumor growth in nude mice (P<0.01). Moreover, the results showed that COE down-regulated the expression of Bcl-2 and Bcl-xL, and up-regulated the levels of Bax and caspase-3 protein (P<0.01). **Conclusion**: COE was a potential chemotherapeutic drug in HCC treatments via targeting mTOR signal pathway.

KEYWORDS *Celastrus orbiculatus*, hepatocellular carcinoma, mammalian target of rapamycin, apoptosis

Celastrus orbiculatus is a member of the family Celastraceae and the genus Celastrus.[1] *Celastrus* and several of its defined constituents possess anti-cancer,[2-4] anti-inflammatory[5-7] and anti-oxidant properties.[8] Our previous studies demonstrated that the ethyl acetate extract of *Celastrus orbiculatus* (COE) has significant anti-tumor effects *in vitro*[9,10] and *in vivo*.[11,12] Hepatocellular carcinoma (HCC) is the fifth most common malignant tumor, third in mortality worldwide.[13] Despite multimodal therapy, including surgery, chemotherapy and radiotherapy, the curative effect on HCC patients is not as good as anticipated.[14]

The mTOR signaling pathway is a promising target in the light of its frequent dysregulation in

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hepatocellular carcinoma and its central role in regulating cell proliferation, migration, survival and angiogenesis.[15,16] Aberrant mTOR signaling was detected in up to 48% of hepatocellular carcinoma, and a correlation between poor outcome and mTOR signaling activation has been shown.[17] Preliminary results from our study suggested that COE could inhibit the activity of mTOR signaling pathway,[18] but the underlying molecular mechanism has not yet to be revealed completely. This study explored the effects of COE on the proliferation and apoptosis in the HepG2/mTOR$^-$ cells, which may shed new light on the clinical treatment for cancer characterized by mTOR activation.

METHODS

Plant Material

The stems of *C. orbiculatus* plants (batch No. 070510) were purchased from Guangzhou Zhixin Pharmaceutical Co., Ltd. (Guangzhou, China) in 2007. Plant identification, extraction, and purification of COE were performed by the research group of Prof. Wang Qiang from China Pharmaceutical University.[19] The preparation and characterization of COE was performed from the Department of Chinese Materia Medica Analysis, China Pharmaceutical University (Nanjing, China). The chemical constituents of COE has been described previously.[9-12,18] The resultant COE micropowder was diluted in dimethyl sulfoxide (DMSO) to different concentrations before use. The final concentration of DMSO should not exceed 0.1% in the cell medium.

Chemicals and Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, cat No. M2128) was acquired from Sigma company (St. Louis, MO, USA). Cisplatin (DDP, cat No.160101) was obtained from Jiangsu Haosen Pharmaceutical Group Co., Ltd. Fetal bovine serum (FBS, cat No.10099-141) and DMEM (High glucose, cat No. 11965-084) were purchased from GIBCO-BRL (Gaithersburg, MD, USA). Antibodies against Bax (cat No.1063) and Bcl-2 (cat No.1017) were purchased from Epitomics (California, USA). Antibodies against Bcl-xL (cat No.2764), mTOR (cat No.2983), P70S6K (cat No.2708), phospho-P70S6K (cat No. 9204), 4EBP1 (cat No.9644), phospho-4EBP1 (cat No.2855), casepase-3 (cat No.9622) and β-actin (cat No.3700) were purchased from Cell Signaling Technology (Beverly, MA, USA). The enhanced chemiluminescence (ECL, cat No. RPN2232) kit was acquired from Amersham Life Science (Amersham, UK). Tegafur, Gimeracil and Oteracil Potassium Capsules (TJA, cat No. 23151214) was purchased from Qilu Pharmaceutical Co., Ltd (Shandong, China). Compound Banmao Capsule (复方斑蝥胶囊, BM, cat No.150902) was obtained from Guizhou YiBai Pharmaceutical Co., Ltd. (Guizhou, China).

Cell Culture

The human hepatocellular carcinoma cell line HepG2 was obtained from the Cell Bank of Shanghai Institutes for Biological Sciences (Shanghai, China). The GV248-mTOR-RNAi-EGFP lentivirus vector was constructed by our laboratory using molecular biological technology (Figure 1).[20] The mTOR-RNAi viruses infected HepG2 cells, and the obtained cells with low mTOR expression named HepG2/mTOR$^-$ cells. The HepG2 and HepG2/mTOR$^-$ cells were cultured in DMEM medium containing 10% FBS and incubated at 37 ℃ in a 5% CO_2 atmosphere.

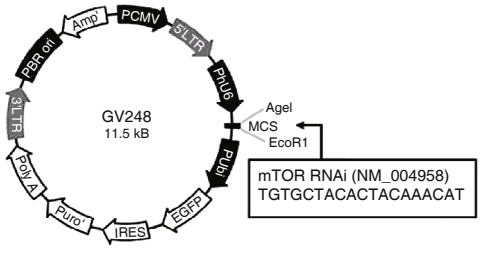

Figure 1. The Construction of GV248-mTOR RNAi Recombinant Plasmid

Note: The recombinant plasmid of Lentiviral vector GV248 carrying mTOR RNAi (NM_004958) gene

Cell Viability Assay

Cell viability was determined using MTT assays. The HepG2/mTOR$^-$ cells were seeded onto a 96 well plate at a density of 3000 cells/well and treated with COE at various concentrations (10, 20, 40, 80, 160 and 320 μg/mL). The concentration gradient was set according to the pharmacological effect of the preliminary experiment in triplicate to evaluate the effect of COE on cell viability. Then, each well was supplemented with 20 μL MTT and incubated at 37 ℃ for 4 h. The formazan crystals that formed were subsequently dissolved in 150 μL DMSO, and the optical density of the resultant reaction solution was read at 490 nm using a microplate reader (PerkinElmer EnSpire, USA). To evaluate the effect of COE on cellular growth, the half-maximal inhibitory concentration (IC_{50}) was calculated, and suitable test

concentrations were determined for further studies.

Apoptosis Assay

Briefly, to assess the development of apoptosis induced by COE, the HepG2/mTOR$^-$ cells were stained with Annexin V-phycoerythrin (PE)/ 7-amino-actinomycin D (7-AAD). Cells were cultured in 6-well plates. Following overnight incubation, these cells were treated with COE at various concentrations (40, 80, 120 mg/L) for 24 h. Totally 2 mg/L DDP was used as a positive control. The concentration gradient is set according to the IC_{50} value. The maximum dose used was below the cytotoxic dose. Then the cells were collected by trypsinization, not containing EDTA. After being washed twice with 4 ℃ PBS, cells were re-suspended in 500 μL 1× binding buffer and then incubated for 15 min at room temperature in the dark after adding 5 μL 7-AAD and 1 μL PE additions. Samples were analyzed using a flow cytometer within 1 h of staining. For each analysis, 10,000 events were recorded. The number of early and late apoptotic cells were counted, respectively.

Ultrastructure Observation Using Transmission Electron Microscopy

The HepG2/mTOR$^-$ cells were treated with COE at various concentrations (40, 80, 120 mg/L) for 24 h. A total of 2 mg/L DDP was used as a positive control. The cells were collected by centrifugation at 4 ℃ and then fixed in 2.5% glutaraldehyde. For the next step, these specimens were rinsed with 0.1 mmol/L PBS, fixed in 1% osmium tetroxide for 1–2 h, dehydrated sequentially through a graded series of ethanol, and then processed for Epon™ embedding. A transmission electron microscope (TEM, CM100, Philips, The Netherlands) was used to observe ultrathin sections stained with uranyl acetate and lead citrate. The images of apoptotic bodies were recorded under electron microscope.

Western Blotting

The HepG2/mTOR$^-$ cells were seeded in a 6-well culture dish, treated with DMEM medium containing COE at concentrations of 40, 80, and 120 mg/L, and then cultured for 24 h. A total of 2 mg/L DDP was used as a positive control. Proteins were extracted from each group of treated cells and subsequently separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at a constant voltage of 120 V for 90 min. Then, the proteins were electro-transferred to a PVDF membrane. After incubation with 5% nonfat dried milk for 2 h at room temperature, the membrane was incubated in blocking buffer with appropriate primary antibodies overnight at 4 ℃, washed with Tris-buffered saline (pH 6.8) with Tween-20 (TBST) 3 times, incubated with the secondary antibody at room temperature for 2 h, and washed with TBST 3 times. Then, a gel imaging analysis system (Bio-Rad) was used to detect protein bands. The expression levels of apoptosis-related proteins and mTOR signaling pathway related proteins were analyzed.

In Vivo Tumor Growth Assay

Totally 36 six-week-old female nude mice were obtained from the Comparative Medicine Center of Yangzhou University [License No. SYXK (SU) 2012-0029] and cared for under specific pathogen-free (SPF) condition in accordance with the Institutional Animal Care and Use Committee. They were acclimatized for 1 week before use and had free access to food and water. The nude xenograft mice models are developed according to previous study.[19] The HepG2/mTOR$^-$ cells (2×10^6 cell/mice) were subcutaneously injected into the right flank of nude mice. When the tumor volume reached about 100 mm^3, the mice were randomly assigned to 6 groups (6 mice per group) according to body weight as follows: normal saline control, BM-treated group (195 mg/kg), TJA-treated group (10 mg/kg), and different dosages of COE (10, 20, 40 mg/kg, the concentration gradient was set according to the pre-experiment) treatment groups. The mice were treated by gavage once a day for 4 weeks. The tumors were measured twice per week using microcalipers and tumor volume (V) was calculated as follows: $V= (length\times width^2)/2$. Twenty-four hours after the last dose, the mice were sacrificed by euthanasia, and the tumors were weighed and photographed.

Immunohistochemical Staining

All tumor tissues were fixed in formalin, embedded in paraffin, cut into 4 μm sections dewaxed and rehydrated according to previous methods. The sections were washed with PBS for 5 min, microwaved in 10 mmol/L citrate buffer (pH 6.0 for 15 min) to unmask the antigens, and then immunostained using the avidin-biotinylated enzyme complex method with antibodies against caspase-3, Bax, Bcl-2, p70s6k, 4EBP1 and p-4EBP1 at a proper concentration and equivalent concentrations of

polyclonal nonimmune IgG controls. After incubation with the appropriate biotin-conjugated secondary antibody and then streptavidin solution, the color was developed using 3,3'-diaminobenzidine tetrahydrochloride as the chromogen. The sections were counterstained with Gill-2 hematoxylin (Thermo-Shandon, Pittsburgh, PA), and then dehydrated with increasing concentrations of ethanol and xylene.

Statistical Analysis

The data were analyzed using statistical package for the social sciences (SPSS) 22.0 statistical software. All statistical analyses were expressed as mean ± standard deviation ($\bar{x} \pm s$). The unpaired Student's *t*-test was used to determine *P*-values for the differences. Each experiment was repeated at least 3 times. $P<0.05$ was considered statistically significant.

RESULTS

COE Inhibited Proliferation of HepG2/mTOR$^-$ Cells

The green fluorescence was observed clearly under the microscope in HepG2/mTOR$^-$ cells (Figure 2A). Compared to the HepG2 wild type cells, the mTOR protein expression in HepG2/mTOR$^-$ cells was significantly decreased (Figures 2B and 2C). After adding different concentrations of COE (20, 40, 80, 160 and 320 mg/L), the proliferation ability was investigated by MTT at 24, 48 and 72 h, respectively. Compared with the untreated control group, the growth of HepG2/mTOR$^-$ cells was inhibited significantly in a concentration-dependent and time-dependent manner ($P<0.01$, Figure 2D). The IC_{50} of COE at 24 h was 140 mg/L. To decrease the cytotoxicity of the drug, the concentrations of COE were identified at 40, 80 and 120 mg/L for further studies.

Evaluation of Cell Apoptosis Using Annexin V-PE/7-AAD Dual Staining Assay

The HepG2/mTOR$^-$ cells were stained with annexin V-phycoerythrin (PE) and 7-AAD. The number of early apoptotic cells decreased up to 14.2% in HepG2/mTOR$^-$ cells, but the number of early apoptotic cells was 4.34% in wild-type HepG2 cells (Figure 3).

COE Regulated the Apoptotic-Related Proteins in HepG2/mTOR$^-$ Cells

The ultra-structures were investigated using TEM, showing bulging vesicular membrane and apoptotic bodies (Figure 4A). COE obviously reduced the Bcl-xL protein expression and the ratio of Bcl-2/Bax. Meanwhile, COE improved the caspase-3 protein expression in a concentration-dependent manner ($P<0.05$ or $P<0.01$; Figures 4B-E).

Effects of COE on mTOR Signaling Pathways in HepG2/mTOR$^-$ Cells

Compared with the untreated control group, the protein levels of mTOR and its downstream proteins, such as 4EBP1, p-4EBP1, P70S6k and p-P70S6k reduced significantly in a concentration-dependent manner ($P<0.05$ or $P<0.01$, Figure 5).

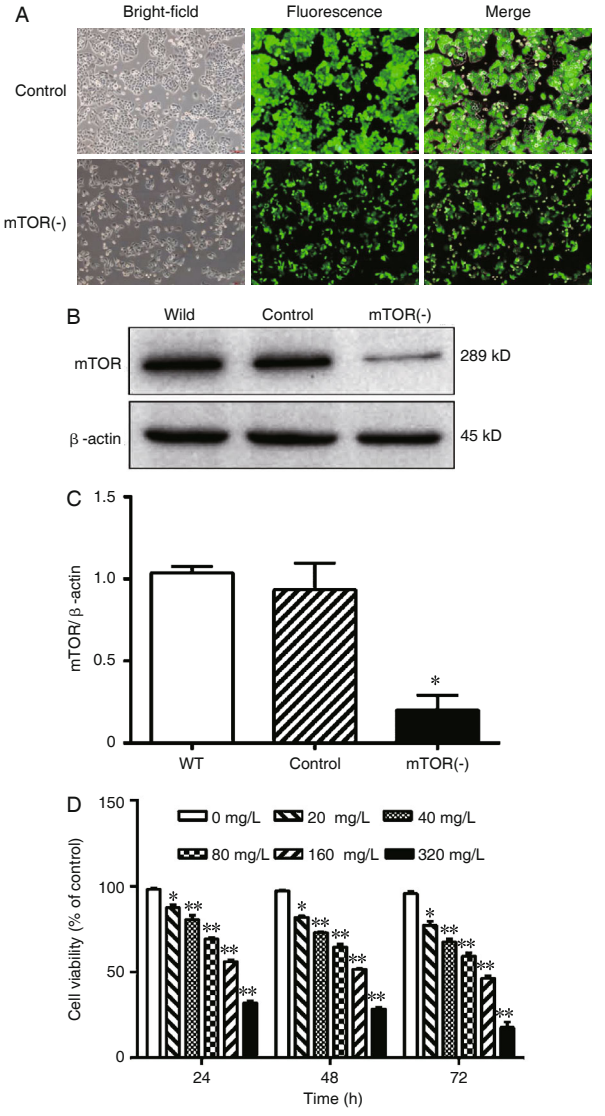

Figure 2. Effect of COE on the Proliferation of HepG2/mTOR$^-$ Cells

Notes: A: Green fluorescence were observed clearly under the microscope in HepG2/mTOR$^-$ cells (100×). B, C: The expression levels of mTOR protein in HepG2/mTOR$^-$ cells by Western blot. D: HepG2/mTOR$^-$ cell viability was measured by MTT assay. $^*P<0.05$, $^{**}P<0.01$, *vs*. 0 mg/LCOE.

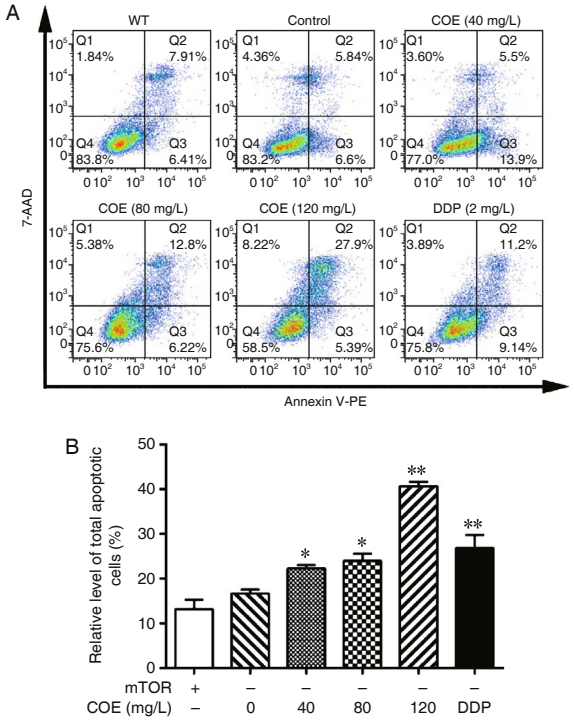

Figure 3. Effect of COE on the Apoptosis of HepG2/mTOR⁻ Cells

Notes: A: Flow cytometric analysis for detecting apoptotic cells in the early and late stage. Early apoptostic cells (Annexin V^+/7-AAD^-) and late apoptostic cells (Annexin V^+/7-AAD^+) were combined as the apoptotic cells. 7-AAD: 7-amino-actinomycin D; PE: phycoerythrin. B: COE induces the apoptosis in HepG2/mTOR⁻ cells following treatment for 24 h. $^*P<0.05$, $^{**}P<0.01$, *vs*. negative control.

COE Attenuated Growth of Solid Hepatic Carcinoma in Nude Mice

Growth of subcutaneous tumors were significantly inhibited by COE in a concentration-dependent manner. The simultaneously determined tumor weight of the nude mice and tumor volume curves are shown in Figure 6. There was an obvious difference in the weight of nude mice and the control group after 30 days of COE treatment, meanwhile the tumor volume was significantly smaller than that of the negative control group ($P<0.05$). As the positive controls, 10 mg/kg TJA and 195 mg/kg BM also showed obvious anticancer activities ($P<0.01$). Compared with the positive controls, COE showed no significant difference ($P>0.05$).

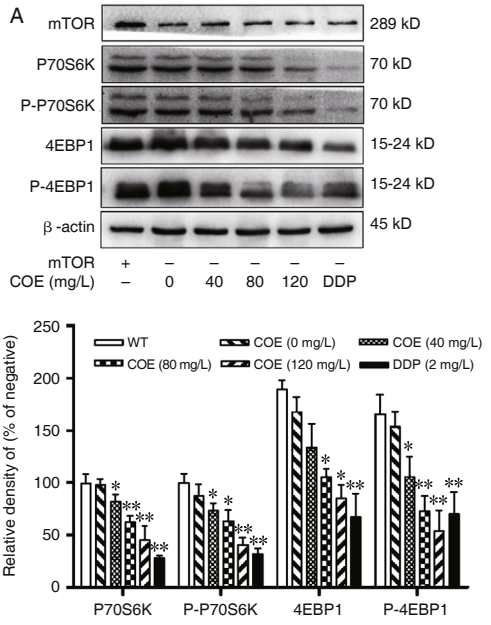

Figure 5. Effect of COE on Protein Expressions of mTOR, P70S6K, p-P70S6K, 4EBP1 and p-4EBP1 Determined by Western Blot in HepG2/mTOR⁻ Cells

Notes: A: 24 h after the treatment of COE (40, 80, 120 mg/L) and 2 mg/mL DDP, the proteins expression correlated mTOR signal pathways including mTOR, P70S6K, phospho-P70S6K, 4EBP1, phospho-4EBP1 were determined.. B: The relative gray values of protein bands. $^*P<0.05$, $^{**}P<0.01$ *vs*. COE (0 mg/L).

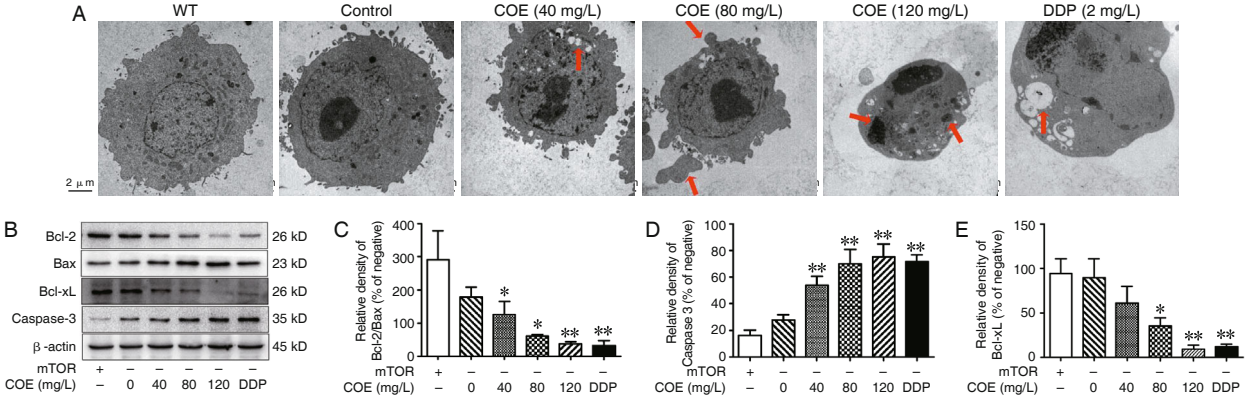

Figure 4. Effect of COE on Ultrastructures of HepG2/mTOR⁻ Cells and Protein Expressions of Bcl-2, Bax, Bcl-xL and Caspase-3

Notes: A: 24 h after the treatment of COE (40, 80, 120 mg/L) and 2 mg/mL DDP, the ultra-structures were investigated using transmission electron microscopy in HepG2/mTOR⁻ cells (5,800×; red arrow, apoptotic bodies). B-E: Expressing levels of apoptotic-related proteins, such as Bcl-2, Bcl-xL, Bax and caspase-3, detected by Western blot in HepG2/mTOR⁻ cells. $^*P<0.05$, $^{**}P<0.01$ *vs*. control.

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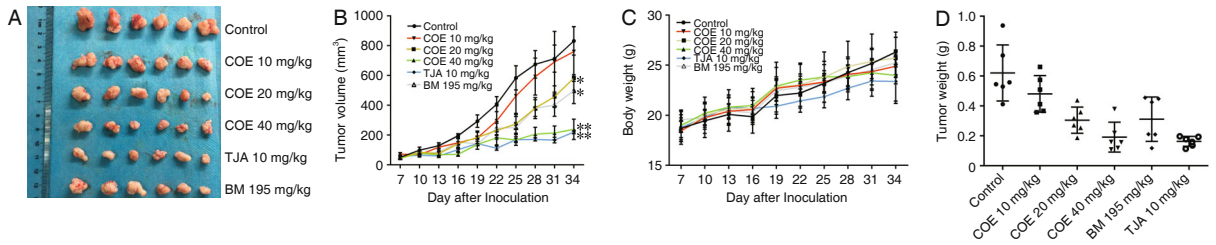

Figure 6. Effect of COE on Growth of Solid Hepatic Carcinoma in Nude Mice

Notes: A,B: The volume of the xenograft tumors. C: The body weights of the nude mice. D: The weight of the xenograft tumors. $^{*}P<0.05$, $^{**}P<0.01$, *vs*. negative control.

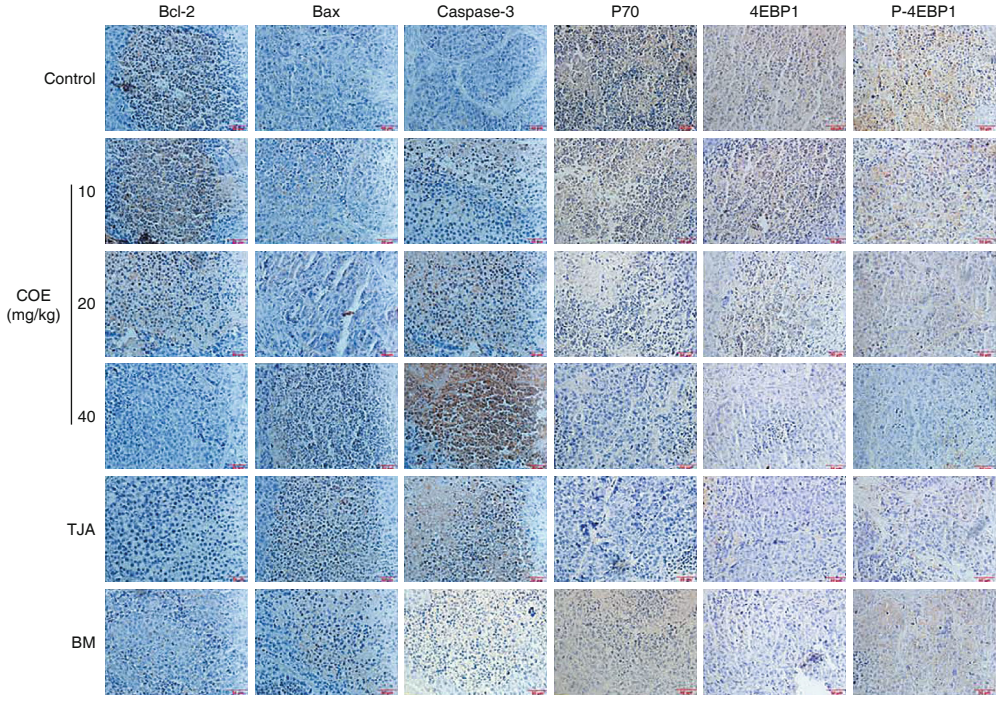

Figure 7. Effects of COE on Protein Expression of Apoptosis-Related Proteins and mTOR Signaling Pathway *in vivo*

Notes: Immunohistochemistry of tumor sections for protein expression detecting. Tumor sections were stained with antibodies against P70S6K, 4EBP1, phospho-4EBP1, Bcl-2, Bax, and caspase-3. Positive cells stained brown (400×).

COE Regulated Apoptosis Signaling Pathways *in vivo*

To verify the activity of COE on apoptosis *in vivo*, the paraffin sections of tumor tissues were detected by immunohistochemical analysis (Figure 7). Compared with the untreated control group, the express levels of 4EBP1, p-4EBP1, P70S6k, and Bcl-2 protein reduced as the COE concentration increased. Meanwhile, the Bax protein expession increased.

DISCUSSION

Chinese medicine (CM) is widely used as effective therapies for patients with cancer in terms of suppressing tumor proliferation, inducing apoptosis and preventing complications.[21] CM also plays an important role in reducing side effects and improving the quality of life for cancer patients.[21-24] Previous results had shown that the COE had a significant ability against various human tumor cells via inhibiting the proliferation and inducing the apoptosis.[9-12]

mTOR is a class of non-conserved evolutionary protein kinase, and is involved in a variety of physiological and pathological processes, such as cell proliferation,[25] differentiation,[26] autophagy,[27] and angiogenesis,[28] etc. Accumulated evidence supports that there are mutations, amplifications or deletions of mTOR signaling pathways in many tumors. These proteins can cause over-activation of mTOR pathways, leading to abnormal proliferation of tumor cells.[29,30] Decreasing the level of mTOR expression in tumor cells may promote tumor apoptosis and inhibit tumor proliferation.

In the present study, we explored the effects of COE on HepG2/mTOR^{-} cells and the molecular

mechanism underlying the COE-initiated programmed death of HepG2/mTOR$^-$ cells. This study revealed that COE exhibited obvious inhibitory effects on HepG2/mTOR$^-$ cell proliferation. Moreover, the results of this study suggested that COE increased the early and late apoptosis of HepG2/mTOR$^-$ cells. Apoptosis is a defensive mechanism for the body to eliminate malignant cells, and it has a significant role in preventing cancer. Notably, the predominant function of numerous antitumor drugs is to induce apoptosis in tumor cells via various apoptosis-associated signaling pathways.[31-33] In the present study, Western blot analysis demonstrated that COE could not only reduce the expression of Bcl-2 and Bcl-xL protein, but also increase the expression of Bax and caspase 3 total protein, decreasing the ratio of Bcl 2/Bax. COE acted as a role of pro-apoptosis through activating Bcl-2, Bax and caspase 3 mediated signaling pathways. Furthermore, the results of these experiments demonstrated that COE obviously inhibited cell proliferation and promoted cell apoptosis. To further confirm the role of COE in inhibiting proliferation and promoting apoptosis, flow cytometry was performed to analyze cell apoptosis. We found that COE significantly promoted cell apoptosis. The number of apoptotic cells increased with the increasing concentrations of COE, which was consistent with the MTT assay and TEM analysis. Experiments *in vivo* provided additional support for the important function of COE in the process of HepG2/mTOR$^-$ cell proliferation and apoptosis. COE also inhibited the growth of xenograft tumor in nude mice, accelerated necrosis and apoptosis of tumor cells, which was consistent with *in vitro* results.

In conclusion, cell apoptosis induced by COE and low-expression of mTOR could inhibit the growth of HCC synergistically. Moreover, COE had a significant apoptosis-inducing effect on HepG2/mTOR$^-$ *in vitro* and *in vivo* via the apoptotic pathway by down-regulating the expression level of Bcl-2, Bcl-xL and up-regulating the level of Bax and caspase-3. Altogether, these findings revealed that COE might be a promising agent in treating hepatocellular carcinoma.

Conflict of Interest

The authors declare that they have no competing interests.

Author Contributions

Qian YY and Liu YQ designed the research; Li WY, Yan Y, Zhao XY, Yang T, Fang CC and Hou JJ performed the research; Li WY, Yan Y and Fang CC analyzed the data; and Qian YY and Li WY wrote the paper.

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