Bufalin induces apoptosis in human esophageal carcinoma ECA109 cells by inhibiting the activation of the mTOR/p70S6K pathway

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Abstract. The present study examined whether bufalin could induce human esophageal carcinoma ECA109 cells apoptosis via inhibiting the activation of mechanistic target of rapamycin (mTOR)/p70 S6 kinase (p70S6K) pathway is discussed in this article. The present study used the esophageal squamous cell carcinoma ECA109 cell line to assess the apoptosis-inducing effects of bufalin via inhibition of the mTOR/p70S6K pathways. A plasmid containing the wild-type mTOR gene (wtmTOR) was transfected into ECA109 cells. The levels of p70S6K, phosphorylated (p)-p70S6K, cellular inhibitor of apoptosis-1 (cIAP-1) and Bcl-2-associated death promoter (BAD) in ECA109 cells were examined by western blot analysis, and apoptosis was detected by flow cytometry analysis and Giemsa staining. The results revealed that the expression of p-p70S6K was increased as the time progressed (at 0, 12 and 24 h), and then decreased at 30, 36, 42 and 48 h after transfection. The expression of cIAP-1 was significantly decreased as time progressed following the addition of bufalin, whereas that of BAD was increased. The levels of p-p70S6K and cIAP-1 were significantly higher in the wtmTOR-transfected group than in the control and empty vector-transfected groups, and then reduced following addition of bufalin; however, BAD expression was significantly lower in the wtmTOR-transfected group. The results of flow cytometry revealed the cell cycle of ECA109 was arrested at G2/M phase and the apoptotic rate was significantly lower in the wtmTOR-transfected group than in the control and empty vector-transfected groups, and then increased following addition of bufalin. In conclusion, the findings of the present study demonstrated that bufalin induced apoptosis in esophageal carcinoma cells via the inhibition of the mTOR/p70S6K pathway and indicated that treatment with bufalin could be combined with chemotherapy to overcome the resistance of esophageal carcinoma cells to chemotherapeutic-induced apoptosis.

Introduction

Esophageal carcinoma, which including squamous cell carcinoma (SCC) and adenocarcinoma, is a common serious malignancy worldwide; it ranks eighth in incidence and fourth in mortality of all cancer types owing to its extremely aggressive nature and the poor survival rate of patients (1-3). The genesis of esophageal carcinoma is a gradual process that combines various carcinogenic factors and multiple stages, which includes various physical and chemical factors, the abnormal activation of signaling pathways, disequilibrium of apoptosis regulation and anti-apoptotic signaling. All of these changes result in the loss of normal regulatory mechanisms of cells, culminating in over-proliferation, which leads to the occurrence of cancer (4).

Bufalin is a cardiotonic steroid and a component of the traditional Chinese medicine, Chansu, which is obtained from the skin and parotid venom gland of toads (5). Owing to the similarity in the chemical structure between bufalin and digoxin, bufalin is expected to have a digoxin-like function (5,6). Bufalin has been shown to induce apoptosis in leukemia and solid tumor cells (5-10). In recent years, studies have revealed that bufalin could effectively inhibit the proliferation of tumor cells, and also assessed its possible molecular mechanism (6-8). The results of previous research also demonstrated that bufalin could inhibit the activation of ERK pathway, accordingly inhibit the proliferation and migration of esophageal carcinoma cells (11); however, the mechanism of inducing apoptosis has remained unclear.

Data has revealed that the RAC serine/threonine-protein kinase (Akt/mechanistic target of rapamycin (mTOR)/p70 S6 kinase (p70S6K) pathway broadly exists in every cell of organism (12,13). This pathway is often irregularly activated in the genesis and development of tumor (12-14). Aberration of upstream signaling causes the phosphorylation of p70S6K,
inhibits apoptosis, increases cell proliferation, produces abnormal translation of protein and induces tumor formation. Inhibition of mTOR can prevent the phosphorylation of p70S6K, inhibit the translation of protein, prevent cell cycle progression and induce cell apoptosis (15). On the basis of previous studies (15), the present study transfected human esophageal carcinoma ECA109 cells with wild-type mTOR (wtmTOR) and the changes to p70S6K, phosphorylated (p)-p70S6K, Bcl-2-associated death promoter (BAD) and cellular inhibitor of apoptosis-1 (cIAP-1) protein were detected. The present study investigated the biochemical mechanisms of apoptosis by bufalin in human esophageal carcinoma cells. The current study assessed the influence of bufalin on ECA109 cell apoptosis, and attempted to reveal the function of gene intervention on development of tumor, and provide novel guidance for the clinical treatment of esophageal carcinoma.

Materials and methods

Cell lines. The human ESCC ECA109 cell line was purchased from Academia Sinica (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 (Sijiqing, Beijing, China) enriched medium containing 1% penicillin/streptomycin and 10% fetal bovine serum (FBS; Sijiqing, Beijing, China). Cell culture plates were maintained at 37°C in a 5% CO₂ incubator.

Reagents and antibodies. Bufalin was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany) and was dissolved in ethyl alcohol to make a 0.01 mol/l stock solution, which was kept at -20°C and diluted in phosphate buffer saline (PBS) when used. A plasmid containing wtmTOR was produced by Prof. Hao Jun (Department of Pathology, Hebei Medical University). Rabbit anti-human polyclonal antibodies p70S6K, phosphorylated (p)-p70S6K, cIAP-1, BAD and β-actin were purchased from Epitomics; Abcam (Cambridge, UK). The reagents of propidium iodide (PI) and RNAse for flow cytometry analysis were purchased from Sigma-Aldrich; Merck KGaA.

Transfection experiments. The transfection of wtmTOR plasmid (3 µg) and empty vector (3 µg) into ECA109 cells was performed using Lipofectamine 2000 (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and incubated for 24 h. The wtmTOR plasmid was kindly provided by Professor Haojun (Department of Pathology, Hebei Medical University, Shijiazhuang, China). The empty vector (pEGFP-C1) was purchased from Invitrogen: Thermo Fisher Scientific, Inc. The cells were divided into four groups: The control group (untransfected group), the empty vector-transfected group, the wtmTOR transfected group, and the group treated with add bufalin (60 nmol/l) after 24 h transfection.

Protein extraction and western blot analysis. Cells were harvested and lysed with lysis buffer (20 mM sucrose, 1 mM EDTA, 20 µM Tris-Cl, pH 7.2, 1 mM DTT, 10 mM KCl, 1.5 mM MgCl₂ and 5 µg/ml aprotime) for 30 min. Protein concentration was measured using a Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions. The total proteins (80 µg/lane) were separated on 10% SDS-PAGE gel and blotted onto a nitrocellulose membrane. The membrane was incubated with blocking buffer and then was incubated overnight with anti-p70S6K antibody (T2921; 1:5,000; Epitomics: Abcam anti-p-p70S6K antibody (ab2571; 1:2,000; Epitomics: Abcam), anti-cIAP-1 antibody (3302-1; 1:2,000; Epitomics: Abcam), anti-BAD antibody (1541-1; 1:2,000; Epitomics: Abcam) and β-actin antibody (ab8226; 1:500; Epitomics: Abcam) primary antibodies at 4°C. The membranes were washed three times with PBST and incubated for 1 h with a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG-HRP; SAB3700852; 1:5,000; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Blots were then developed with Super signal West Femto Maximum Sensitivity substrate (Pierce; Thermo Fisher Scientific, Inc.) on a FujiFilm LAS-3000 detection system (Fujifilm Corporation, Tokyo, Japan).

Giemsia staining. ECA109 cells were treated with 60 nmol/l bufalin for 48 h, and the cells were collected and put on the slides. The slides were then rinsed with sterile water and stained with freshly prepared Giemsa stain solution (Giemsa: phosphate=1.9; BDH Chemicals; Merck KGaA) for 5 min at room temperature. Following three washes in sterile water, the cells were examined for morphological changes using a light microscope at x400, magnification.

Flow cytometry analysis. ECA109 cells were collected for PI staining. Briefly, the cells were fixed in 70% ethyl alcohol at 4°C overnight, then washed with PBS and incubated with RNase (10 µg/ml) at 37°C for 30 min. Next the cells were incubated with PI (final concentration, 10 µg/ml) for 30 min in the dark. After incubating at 4°C for 30 min, flow cytometry was performed. MultiCycle AV software (Version 295, Beckman Coulter, Miami, FL, USA) was used to analyze the cell cycle and Expo32 ADC software (Version 1.2, Beckman Coulter, Miami, FL, USA) was used to analyze the apoptosis rate.

Statistical analysis. All data are presented as the mean ± standard deviation. Significant differences among the groups were determined by one-way ANOVA followed by Newman-Keuls method of post-hoc comparison. All results presented were obtained from at least three independent experiments. The statistical analyses were performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Transfection with wtmTOR plasmid influences the expression of p70S6K and p-p70S6K protein in ECA109 cells by western blot analysis. The wtmTOR plasmid was amplified successfully and transfected into esophageal carcinoma ECA109 cells. The expression of p70S6K and the activation of p70S6K at 0, 12, 24, 30, 36, 42 and 48 h were examined by western blot analysis. The expression of p70S6K was not significantly different at 0, 12, 24, 30, 36, 42 or 48 h (0.599±0.011, 0.594±0.013, 0.606±0.012, 0.608±0.010, 0.592±0.017, 0.599±0.021, 0.600±0.036, respectively; P>0.05). Therefore, p-p70S6K levels increased along with the time (at 0, 12 and 24 h, levels
were 0.389±0.013, 0.411±0.019 and 0.609±0.016, respectively), and then decreased at 30, 36, 42 and 48 h following transfection (0.573±0.015, 0.394±0.013, 0.383±0.006 and 0.262±0.018, respectively; P<0.05). The expression of p70S6K was the highest at 24 h. The difference was statistically significant. We selected 24 h as the optimal transfection time (Fig. 1).

Examination of cIAP-1 and BAD expression by western blot analysis following bufalin treatment. To confirm that bufalin induced ECA109 cell apoptosis, western blot analysis was performed to assess the expression of cIAP-1 and BAD following incubation with 60 nmol/l bufalin. The result showed that the expression of cIAP-1 was gradually decreased as time progressed for 2, 6, 12, 24, 36, and 48 h (0.542±0.003, 0.517±0.007, 0.455±0.002, 0.414±0.004, 0.369±0.026, 0.218±0.015, respectively; P<0.05), whereas the expression of BAD was gradually increased following addition of bufalin for 2, 6, 12, 24, 36, and 48 h (0.456±0.009, 0.659±0.042, 0.750±0.023, 0.813±0.019, 0.937±0.013 and 1.047±0.013, respectively; P<0.05) (Fig. 2).

Detection of p70S6K, p-p70S6K, cIAP-1 and BAD levels by western blot in different groups. The experiment was divided into four groups: The control group, empty vector transsected group, wtmTOR transsected group, and the bufalin/wtmTOR transsected group. Western blot analysis revealed that the level of p70S6K was not significant different in the control, compared with empty vector-transsected, wtmTOR-transsected, and the bufalin/wtmTOR-transsected groups (0.901±0.045, 0.914±0.023, 0.900±0.020, 0.898±0.022, respectively; P>0.05) (Fig. 3A). However, p70S6K was significantly higher in wtmTOR-transsected group compared with the control and empty vector groups, and then reduced following addition of bufalin for 2 h (0.761±0.085, 0.766±0.068, 0.952±0.059, 0.762±0.019; P<0.05; Fig. 3A).

The level of cIAP-1 was significantly higher in wtmTOR-transsected group compared with in control group, empty vector group, and addition of bufalin for 24 h (0.721±0.019, 0.731±0.248, 0.840±0.010 and 0.742±0.021, respectively; P<0.05). On the contrary, the expression of BAD was significantly lower in wtmTOR-transsected group than in the control and empty vector group, and then increased following addition of bufalin for 24 h (0.929±0.046, 0.944±0.060, 0.779±0.182 and 1.029±0.049, respectively; P<0.05; Fig. 3B).

Morphological analysis of ECA109 cells by Giemsa staining. To confirm that bufalin induced the morphological of apoptosis in ECA109 cells, ECA109 cells was treated with 60 nmol/l bufalin for 48 h and then Giemsa staining was performed. The apoptotic morphology was evident in bufalin-treated cells under a microscope at x400 magnification, including cytoplasmic shrinkage, nuclear condensation and the formation of apoptotic bodies (Fig. 4). However, no apoptotic morphology was observed in the control-treated cells.

Changes to ECA109 cells apoptotic rate and cell cycle detected by flow cytometry. Flow cytometry results showed that the apoptotic rate was gradually increased following addition of bufalin at concentrations of 0, 20, 40, 60, 80 and 100 nmol/l for 24 h (3.01±0.317, 3.67±0.306, 6.74±0.198, 7.59±0.340, 18.22±0.651 and 28.60±1.737%, respectively; P<0.05) (Fig. 5). The cell cycle of ECA109 was arrested at G2/M phase, and the cell percentage of G2/M is increased from 9.24±1.919 to 70.5±2.934% following addition of bufalin from 0 nmol/l to 100 nmol/l; this difference was statistically significant (Fig. 6).

The apoptotic rate of the cells in the different groups were examined, the results of which revealed that the apoptotic rate was significantly lower in wtmTOR-transsected group compared with in the control group, empty vector groups, and
addition of bufalin for 24 h group (5.60±0.411, 5.46±0.341, 4.19±0.210, 10.12±0.325%, respectively; all P<0.05; Fig. 7).

Discussion

Esophageal carcinoma is a high-incidence malignancy in China, which also has a high morbidity and mortality rate in the rest of the world (16). The genesis and development of this disease is a complicated process, including the accumulation and interaction of several factors, phases and multiple gene variations. Patients are primarily treated with surgery combining with radiotherapy, chemotherapy or immunotherapy; however, the 5-year survival rate remains low and the prognosis of patients is poor (2,5). The genesis and development of tumor is closely associated with abnormalities to signaling pathways (14,17). The apoptosis signaling pathway activated by bufalin has attracted considerable attention (7-9); however, there is little research on esophageal carcinoma and the exact mechanism by which this pathway is activated is unclear.

Apoptosis is a cell defense mechanism to eliminate malignant cells and has a notable role in preventing tumor development. In fact, a number of anticancer drugs function primarily to induce apoptosis through regulating apoptosis-associated signaling (18,19). Bufalin is a member of a class of toxic steroids purified from the traditional Chinese medicine Chansu. Previous studies have revealed that bufalin also exhibited antitumor effects; it induced the differentiation and apoptosis of leukemia HL-60 cells, and induce the apoptosis of gastric cancer cells, colon cancer cells and breast cancer cells (20-22). Bufalin could therefore induce cell cycle arrest and/or apoptosis as concentration increased. The cell cycle is divided into G0/G1, S, and G2/M phases, which refers to the presynthetic phase, synthesis period, and post-synthetic phase of DNA, respectively (23). The cell cycle is a useful
index for judging the status of cell proliferation. At present, there are several reports concerning bufalin-induced cell cycle retardation. A prior study revealed that bufalin mainly induced G2/M phase arrest in leukemia ML1 cells (24). Another study reported that bufalin could induce G0/G1 phase arrest in endometriosis matrix cell (25). Together, these results demonstrated that the influence of bufalin on cell cycle varied in different cells. However, the present study assessed whether bufalin could induce apoptosis in esophageal carcinoma cells and arrest the cell cycle. In the current study, flow cytometry analysis demonstrated that as bufalin concentration increased (0, 20, 40, 60, 80, 100 nmol/l) the apoptotic rate of ECA109 increased from 3.01±0.317% (bufalin, 0 nmol/l) to 28.60±1.737% (bufalin, 100 nmol/l) at 24 h of treatment with bufalin. The percentage of cells in G2/M phase was 70.5±2.934% compared with that in control group, which was 9.24±1.919%. The expression of cIAP-1 protein, which is a member of the IAP family, gradually decreased as the time of bufalin incubation increased effecting on esophageal carcinoma cells ECA109.

The expression of the apoptosis-promoting gene BAD, which belongs to the Bcl-2 family, gradually increased as the time of incubation increased. These results revealed that bufalin might induce cell cycle arrest at G2/M phase, and affect apoptosis of ECA109 cells on in a time- and dose-dependent manner.

A variety of signaling pathways are abnormally activated in the development of tumor; one such pathway, the PI3K/Akt/mTOR pathway, has been demonstrated to serve an essential role in the genesis of esophageal carcinoma. The PI3K/Akt/mTOR pathway is a notable signal transduction pathway that mediates tumor cell apoptosis. Activation of this pathway could inhibit apoptosis, increase cell cycle progression, and accordingly improve the survival and proliferation of tumor cells (12,15,26). mTOR contributes to the genesis and development of a variety of malignancies, including breast cancer (27) and lung cancer (28). mTOR accelerates the translation and expression of protein, alters the cell cycle distribution, affects apoptosis and participates in adjusting multiple types of physiological and pathological changes.

Figure 5. Flow cytometry analysis of the apoptotic rate of ECA109 cells treated with different concentrations of bufalin. The apoptotic rate gradually increased following addition of 0, 20, 40, 60, 80 or 100 nmol/l bufalin for 24 h. Group 1, 0 nmol/l; group 2, 20 nmol/l; group 3, 40 nmol/l; group 4, 60 nmol/l; group 5, 80 nmol/l; group 6, 100 nmol/l. *P<0.05 vs. control.
in the organism, mainly through phosphorylating the main signal factors p70S6K and Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) downstream (13,29). Activated mTOR can positively regulate the translation of S6K1, which phosphorylates p70S6 downstream, and promotes protein synthesis; this process promotes the synthesis of protein (15,29). 4E-BP1 is another notable regulation pathway downstream of mTOR; it combines with the cap-binding protein in its dephosphorylated state, inhibiting the origin of translation. Activated mTOR can phosphorylate 4E-BP1, and eukaryotic translation initiation factor 4E is released and form compound with other translation original factors, initiating translation and accelerating the expression of proteins associated with growth and differentiation (30). Therefore, the PI3K/Akt/mTOR pathway is considered to be an essential signaling pathway in protein synthesis that participates in the regulation of cellular proliferation, differentiation and apoptosis.

The present study successfully transfected the active mTOR plasmid wtmTOR into the ECA109 cell line. Previous studies revealed that the consisting activated mTOR signaling pathway could promote the transformation of normal cells to tumor cells (9,31). If the tumor cells could be reverted to the non-transformation form in vitro following inhibition of the mTOR signal transduction pathway, this reversion could have a role in inhibiting tumor development. Accordingly, the present study used bufalin in different treatment groups and assessed the changes in activation of the mTOR signaling pathway and the apoptosis-associated proteins cIAP-1 and BAD. The levels of p-p70S6K level in the wtmTOR-transfected group increased as the transfection period increased, and reached the maximum value at 24 h, and then gradually decreased; however, the
expression of p70S6K did not evidently change. For different treatment groups, the level of p70S6K activated (that is, p-p70S6K) in wtmTOR-transfected group was evidently higher than in the control, empty vector-transfected and bufalin-treated group. The transfection of plasmid makes the activation of p70S6K increase, whereas bufalin could inhibit this process. The expression of cIAP-1 was higher in the wtmTOR-transfected group than the others, despite the lower expression of BAD than in the other three groups, which revealed that transfection with this plasmid inhibited cell apoptosis, whereas bufalin promotes cell apoptosis. The results of the present study indicated that bufalin could inhibit the development of esophageal carcinoma by inhibiting the mTOR pathway and inducing cell apoptosis.

In summary, the results of the present study indicate that bufalin induces cell apoptosis through inhibiting the activation of the mTOR/p70S6K signaling pathway. These results indicated that bufalin could be used for clinical treatment and provided an experimental basis and future direction for the treatment of esophageal carcinoma. Bufalin may represent a novel antitumor drug, meaning that its feasibility and clinical utility degree should be thoroughly investigated.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors’ contributions
YD performed cell culture and was a major contributor in writing the manuscript. WL performed the flow cytometry analysis. XW was mainly responsible for the analysis of flow cytometry and revision of the paper. LZ performed western blotting experiments and analysis. MZ performed Giemsa
staining. HD performed transfection experiments. YL was responsible for the analysis of data and the revision of the paper. All authors read and approved the final manuscript.

**Ethics approval and consent to publish**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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