Berberine displays antitumor activity in esophageal cancer cells in vitro

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Abstract

AIM

To investigate the effects of berberine on esophageal cancer (EC) cells and its molecular mechanisms.

METHODS

Human esophageal squamous cell carcinoma cell line KYSE-70 and esophageal adenocarcinoma cell line SKGT4 were used. The effects of berberine on cell proliferation were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For cell cycle progression, KYSE-70 cells were stained with propidium iodide (PI) staining buffer (10 mg/mL PI and 100 mg/mL RNase A) for 30 min and cell cycle was analyzed using a BD FACSCalibur flow cytometer. For apoptosis assay, cells were stained with an Annexin V-FITC/PI apoptosis detection kit. The rate of apoptotic cells was analyzed using a dual laser flow cytometer and estimated using BD ModFit software. Levels of proteins related to cell cycle and apoptosis were examined by western blotting.

RESULTS

Berberine treatment resulted in growth inhibition of KYSE-70 and SKGT4 cells in a dose-dependent and time-dependent manner. KYSE-70 cells were more...
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INTRODUCTION

Esophageal cancer (EC) is the sixth most common malignant gastrointestinal carcinoma worldwide. More than 50% of the global incidence of EC is in China[1]. A report published in 2016 shows that there are an 477,900 and 375,000 estimated new EC cases and deaths, respectively, in China[2]. Histologically, EC is divided into two major types: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). More than 90% of EC in China is ESCC. Although advances have been achieved in surgery and chemotherapy, the 5-year survival rate of EC in China is only 19.9%[3]. Esophagectomy is so far the only potentially curative approach for EC, but many patients are at an advanced stage of disease during initial diagnosis, thus ruling them out from surgery. Therefore, there is a critical need to develop alternative and novel approaches in EC therapy.

Berberine is a quaternary ammonium salt derived from Ranunculaceae and Papaveraceae families of plants. Apart from a broad range of bioactivities, such as anti-inflammatory, antibacterial and antidiabetic actions, accumulating studies have revealed that berberine exhibits antitumor properties by interfering with the multiple features of tumorigenesis and tumor development[4]. The antitumor activity of berberine is mainly mediated through the inhibition of cancer cell proliferation by inducing cell cycle arrest at the G1 or G2/M phases and initiation of apoptosis[5,6]. Previous studies have reported that berberine inhibits the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling cascades to inhibit cell proliferation in various cell lines derived from breast, lung, colon and liver cancer[7-12]. Berberine also activates AMP-activated protein kinase (AMPK), a major regulator of metabolic pathways, subsequently inhibiting mTOR, a downstream target of AMPK[12,13]. Although berberine possesses numerous anticancer activities in various cells, the effect of berberine on EC growth and its mechanism of action have not yet been fully elucidated. In this study, we reported that berberine inhibited EC cell growth by promoting cell cycle arrest at G2/M phase as well as apoptosis. The Akt, mTOR/p70S6K and AMPK signaling pathways were involved in the antitumor activity of berberine on EC.

MATERIALS AND METHODS

Reagents

Berberine hydrochloride was obtained from Ye-Yuan (Shanghai, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), propidium iodide (PI) cell cycle assay kit, Annexin V-FITC/PI apoptosis detection kit and western blot analysis ECL were purchased from Beyotime (Jiangsu, China). RPMI 1640 and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific (Waltham, MA, United States). All primary antibodies, including against p21, Akt, p-Akt (Ser473), mTOR, p-mTOR (Ser2448), p70S6K, p-p70S6K (Thr389), AMPK, p-AMPK (Thr172) and β-actin, were from Cell Signaling Technology (Danvers, MA, United States). All other common chemicals and buffers were from Boster (Wuhan, China).

Cell culture and maintenance

Human ESCC cell line KYSE-70 and EAC cell line...
SKGT4 were purchased from Kebai Technology (Nanjing, China). The culture medium for both cell lines was RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were incubated in a humidified atmosphere with 5% CO₂ at 37 °C.

Cell viability assay
Cell viability was measured by MTT assay. KYSE-70 (10⁴/well) and SKGT4 (5000/well) were seeded in 96-well culture plates and incubated overnight at 37 °C in a humidified 5% CO₂ incubator. On the following day, cells were treated with berberine hydrochloride at indicated concentrations for indicated durations. Then, 10 μL MTT dye was added to each well at a final concentration of 5 mg/mL. For an additional 4 h after incubation, blue MTT formazan crystals were dissolved in 100 μL/well of DMSO. The absorbance at 562 nm was measured on a Multiskan Spectrum microplate reader (Thermo Fisher Scientific). Cell viability was calculated by dividing the OD of samples by the OD of the control group. All experiments were repeated three times.

Flow cytometric analyses of cell cycle and apoptosis
KYSE-70 cells (8 × 10⁴/well) were seeded in six-well plates in complete culture medium. After incubating for 12 h, cells were treated with berberine hydrochloride (50 μmol/L). Cells were harvested separately at 12 and 24 h later, and immediately fixed with 75% ethanol. For the cell cycle progression analysis, cells were stained with PI staining buffer (10 mg/mL PI and 100 mg/mL RNase A) for 30 min, and fluorescence intensity was measured by BD FACSCalibur (BD Biosciences, San Jose, CA, United States). For apoptosis analysis, cells were stained with the Annexin V-FITC/PI apoptosis detection kit. The rate of apoptotic cells was analyzed using a dual laser flow cytometer and estimated using the ModFit software (BD Biosciences).

Western blot analysis
Cell lysates were prepared with RIPA lysis buffer (50 mM Tris-HCl, 150 mmol/L NaCl, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, 100 μmol/L leupeptin, and 2 μg/mL aprotinin, pH 8.0). Protein extract (20 μg) was subjected to SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, United States). After blocking with 5% nonfat dry milk, membranes were incubated at 4 °C overnight with each of the following primary antibodies: p21, pAKT (Ser473), AKT, p-mTOR (Ser2448), mTOR, pp70S6K (Thr389), p70S6K, p-AMPK (Thr172), AMPK (all 1:1000 dilution) and β-actin. Membranes were washed with phosphate buffered saline plus Tween (PBST) buffer and incubated with horseradish peroxidase-conjugated secondary antibodies. After incubation, the membranes were washed three times with PBST and immersed in a SuperSignal West Pico Chemiluminescent Substrate from the detection kit (Thermo Fisher Scientific). Chemiluminescent detection of western blots was performed using an Amersham Imager 600 System (GE Healthcare Bio-Sciences, Pittsburgh, PA, United States).

Statistical analysis
Data were analyzed using Student’s t-test, and all data were expressed as mean ± SE of the mean. P < 0.05 was considered statistically significant.

RESULTS
Growth suppressive effect of berberine on human EC cells
To examine the biological consequences of berberine, we first examined its effect on the proliferation of ESCC and EAC cells. We observed that berberine significantly suppressed KYSE-70 proliferation after treatment with different concentrations (20, 40, 60
and 80 μmol/L at all tested time points (12, 24 and 48 h) (Figure 1A). Berberine had significantly suppressive effects on SKGT4 cell proliferation when tested at 24 and 48 h after treatment with berberine at 20, 40, 60 or 80 μmol/L. At the 12-h time point, berberine did not significantly inhibit SKGT4 cell proliferation until the concentration reached 80 μmol/L (Figure 1B). Upon comparison of the proliferation inhibitory effects of berberine against the two cell lines, KYSE-70 was more sensitive than SKGT4 to the dose-dependent and time-dependent suppressive effects of berberine. Therefore, we focused further on KYSE-70 cells in the following experiments.

**Cell cycle arrest effect of berberine on human EC cells**

To clarify whether impairment of cell cycle involved in the reduction of KYSE-70 growth was induced by berberine, KYSE-70 cells were treated with 50 μmol/L berberine for 48 h, stained with PI, and subjected to cell cycle progression analysis using flow cytometry. As shown in Figure 2A and B, when compared with the controls, it is evident that the fraction of G2/M cells was increased after berberine treatment (9.77% vs 25.94%, P < 0.01), whereas in parallel, we did not observe significant changes in cell numbers in G0/G1 phase (54.06% vs 51.06%). To explore further the molecular signals involved in berberine-induced G2/M phase arrest, Western blot analysis was used to determine the expression of p21; a key cell cycle negatively regulated protein. As shown in Figure 2C, after application of berberine at 50 μmol/L for 24 h, p21 level was increased. This indicates that berberine-induced cell cycle arrest at G2/M phase in KYSE-70 cells is mediated through p21 down-regulation.

**Apoptotic effect of berberine on EC cells**

To evaluate whether the antiproliferative activity of berberine was related to its apoptotic effect, KYSE-70 cells were treated with 50 μmol/L berberine, and flow cytometric analyses were performed by double staining with Annexin-V FITC/PI. As shown in Figure 3, berberine significantly increased KYSE-70 cell apoptosis (0.15% vs 43.73% at 12 h, P < 0.05; 0.83% vs 81.86% at 24 h, P < 0.05). We next evaluated the effect of berberine on KYSE-70 cell morphology. Phase contrast imaging (Figure 4) showed that untreated control KYSE-70 cells were epithelial-like adherent cells, with a flat and polygonal shape, that grew homogeneously and showed strong refraction. When treated with berberine, the cells showed reduced refraction and shrunk to a round shape. The treated cells grew in a scattered way, resulting in loss of intercellular conjunction. Consistent with the data in Figure 1, phase contrast imaging showed...
that berberine suppressed proliferation and promoted apoptosis.

**Berberine inhibited cell proliferation through Akt/mTOR/p70S6k and AMPK signaling pathways**

Previous studies have indicated that inhibiting Akt/mTOR/p70S6K signaling and activating AMPK contribute to berberine-induced loss of cell viability\(^{(9,14)}\). To address whether these signaling molecules are related to the biological consequences of berberine in KYSE-70 cells, western blot analyses were performed to examine the phosphorylation levels of these signaling molecules. Cells were treated with 50 µmol/L berberine for 6, 12 or 24 h, in comparison with control cells at each time point. Berberine markedly reduced phosphorylation of Akt at Ser473, mTOR at Ser2448 and p70S6K at Thr389, starting as early as 6 h after treatment and sustaining a reduced level for 24 h. Berberine clearly enhanced AMPK phosphorylation at Thr172 after 6 h treatment, and maintained increasing levels for 24 h.

Figure 3  Berberine promotes apoptosis in KYSE-70 cells. A: KYSE-70 cells were treated with 50 µmol/L berberine for 12 and 24 h. Apoptotic rates were measured using flow cytometry; B: Apoptotic cell values are expressed as mean ± SE of three experiments. \(^*P<0.05, \dagger P<0.01\) vs controls.

Figure 4  Berberine treatment induced morphological changes of KYSE-70 cells. Control cells and 50 µmol/L berberine-treated cells were observed under a phase contrast microscope at 12, 24 and 48 h after treatment. Bar represents all images equal to 200 µmol/L.
be considered as a potential source of drugs for the treatment of EC patients.

Cell cycle arrest and apoptosis are closely linked to cell proliferation in mammalian cells\(^{[17]}\). The major regulatory mechanism of cell growth, the cell cycle dictates the timing of DNA synthesis, and is divided into four distinct phases: M phase (chromosome segregation and mitosis), G1 phase (before DNA replication), S phase (DNA replication) and G2 phase (before mitosis). The cell cycle process includes mechanisms to warrant error amendment, and if not, the cells commit apoptosis, which is one of the most important contributors to the suppression of malignant transformation and elimination of tumors. Control of cell numbers is determined by a complicated balance of cell proliferation and death.

Previous studies have shown that berberine induces cell cycle arrest in various human cancer cells\(^{[9-12]}\). To determine whether berberine prompts cell cycle arrest of KYSE-70 cells, the cell cycle distribution was analyzed by flow cytometry after application of berberine. Our results demonstrated that berberine significantly blocked KYSE-70 cells at the G2/M phase of the cell cycle, suggesting that berberine inhibits KYSE-70 cell proliferation by inducing G2/M cell cycle arrest. These data are in agreement with previous studies in human breast cancer cells and liver cancer cells\(^{[5,11]}\). Appropriate control over cell cycle progression depends on many factors, such as cyclin-dependent kinase inhibitor p21 facilitating cell cycle arrest in response to a variety of stimuli. Our results showed that berberine augmented p21 level in KYSE-70 cells, indicating that berberine-induced cell cycle arrest in G2/M phase may be through regulation of cell cycle protein p21.

The PI3K/Akt/mTOR signaling pathway plays a crucial role in controlling cell proliferation and apoptosis\(^{[18]}\). Constitutive activation of this pathway is considered to be important in cell growth and homeostasis\(^{[19]}\). Specifically, activated mTOR directly phosphorylates many downstream targets including p70S6K to promote protein synthesis\(^{[20]}\). As a major regulator of cellular energy metabolism, AMPK is a negative regulator of the mTOR pathway\(^{[20,21]}\). Berberine regulation of cell proliferation and survival has been shown to involve Akt, mTOR/p70S6K and AMPK signaling pathways\(^{[10-12]}\). Our results showed that berberine treatment inhibited the phosphorylation of Akt and mTOR, as well as mTOR downstream target p70S6K, but enhanced the phosphorylation of AMPK. A previous study reported that, in breast cells, berberine transiently activated AMPK and inhibited Akt, but did not inhibit mTOR activity\(^{[22]}\). Our results showed that treatment with berberine induced sustained alterations (6-24 h) of increased levels of Akt and mTOR phosphorylation in KYSE-70 cells or increased level of AMPK phosphorylation in KYSE-70 cells. These results suggest that berberine alters Akt, mTOR and AMPK

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**Figure 5** Effects of berberine on AMPK and AKT/mTOR/p70S6K activities. KYSE-70 cells were treated with 50 μmol/L berberine for 6, 12 and 24 h, and protein expressions of p-AKT, AKT, p-mTOR, mTOR, p-p70S6K, p70S6K, p-AMPK and AMPK were analyzed by western blotting.
activity in an individual cell-dependent manner.

In conclusion, it is suggested that berberine inhibits EC cell growth by promoting cell cycle arrest at G2 phase and the apoptotic process. The Akt, mTOR/ p70S6K and AMPK signaling pathways are involved in the antitumor activity of berberine on EC. We have shown that berberine is an inhibitor of human EC cell growth and could be considered as a potential source of drugs for the treatment of EC patients.

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