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Oridonin induces G2/M cell cycle arrest and apoptosis in human oral squamous cell carcinoma

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ABSTRACT

Oridonin, an active diterpeniod isolated from *Rabdosia rubescens*, has been reported for its anti-tumor activity on several cancers, however, its effect on oral squamous cell carcinoma (OSCC) remains unclear. In this study, we demonstrated for the first time that oridonin inhibited the growth of OSCC cells both *in vitro* and *in vivo*. Oridonin decreased the proliferation and clonal formation of cultured OSCC cells in a dose-dependent manner. Further study indicated that oridonin induced G2/M phase arrest in OSCC cells, which was associated with the down-regulation of proteins related to G2/M transition including cdc25C, cdc2 and cyclin B1, as well as the up-regulation of p53 and phosphorylated-cdc2. In addition, we discovered that oridonin induced OSCC cell apoptosis by activating the intrinsic apoptotic pathway, which was indicated by the increased expression of cleaved-caspase 3, cleaved-caspase 9 and proapoptotic protein Bax and reduced expression of caspase 9 and anti-apoptotic protein Bcl-xl. Finally, oridonin suppressed the growth of OSCC in an xenograft mouse model. Immunohistochemical analysis showed a reduction of cyclin B1-positive cancer cells and an increase of TUNEL-positive cancer cells in oridonin-treated mice. Therefore, oridonin may be a potentially effective agent for the treatment of OSCC in future.

1. Introduction

Head and neck cancer is the sixth most common cancer worldwide, of which the largest subset (~ 90%) is oral squamous cell carcinoma (OSCC) (Mignogna et al., 2004). There are ~ 300,000 new cases of OSCC annually worldwide, and > 50% of OSCC patients experience a relapse regardless of the therapeutic approach or location or stage of the disease (Nagata et al., 2015). Despite advances in multimodality therapy including surgery, radiation and chemotherapy in the 20th century, the overall survival rate has shown only a modest increase in recent years and still ranges from 45% to 53% (Blatt et al., 2017; Warnakulasuriya, 2009). Therefore, searching for more novel and effective OSCC therapeutic agents is of great importance.

Traditional Chinese herbal medicines have gained increasing attention worldwide in recent years for their advantages of low cost, abundant sources, high efficiency and great diversity (Hsiao and Liu, 2010). Oridonin, a diterpenoid extracted from the medicinal herb *Rabdosia rubescens*, has been previously reported to exhibit anti-bacterial and neuroprotective effects (Osawa et al., 1994; Zhang et al., 2013; Zhao et al., 2016). Oridonin also exerts anti-inflammatory effects in autoimmune diseases and organ transplantation *via* promoting the $CD4^+CD25^+$Treg cell differentiation and modulating the balance between Th1 and Th2 cells (Hu et al., 2008; Ku and Lin, 2013). Recently, the antitumor effect of oridonin has aroused great research interest. Oridonin has been shown to inhibit the *in vitro* or *in vivo* growth of several human malignancies such as leukemia (Huang et al., 2017), pancreatic cancer (Gui et al., 2017), gastric cancer (Wang et al., 2016), colon cancer (Wu et al., 2016b), and breast cancer (Wu et al., 2016a). Its antineoplastic mechanisms involved the activation of the ERK-p53 apoptotic pathway and inhibition of the PTK-Ras-Raf-JNK survival pathway *etc.* (Cheng et al., 2009; Hu et al., 2007). However, the effect of oridonin on OSCC cells has not yet been investigated.

In this study, to elucidate whether oridonin has any effects against OSCC cells and the underlying mechanism of action, we performed the cell proliferation assay, cell colony formation assay and cell cycle and apoptosis analysis. We also determined the expression of related proteins with the western blot assay. In addition, an xenograft mouse model of OSCC was established to identify the antitumor effect of oridonin *in vivo*.

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2. Materials and methods

2.1. Animals

Male BALB/c nude mice (4 weeks of age) were purchased from Shanghai Laboratory Animal Center (Shanghai, China) and maintained in the animal care facilities of the Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine under pathogen-free conditions. Animal welfare and experimental procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals (The Ministry of Science and Technology of China, 2006) and the related ethical regulations of the hospital. All efforts were made to minimize animal suffering and to reduce the number of animals used. All experimental procedures received approval by the Laboratory Animal Care and Use Committee of the hospital.

2.2. Reagents

Oridonin (7,20-epoxy-ent-kauranes), dimethylsulfoxide (DMSO) and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] were purchased from Sigma (St Louis, MO, USA). Antibodies used for western blot analysis were bought from the following sources: cdc25C (5H9), cdc2 (POH1), phospho-cdc2 (Tyr15)(p-cdc2), caspase 8, caspase 9 and cleaved-caspase 3 (Cell Signaling Technology, Danvers, MA, USA); p53, cyclin B1, Bax and Bcl-xl (Proteintech Group, Wuhan, China); and β-actin (clone AC-40) (Sigma). IRDye 800CW goat anti-mouse secondary antibody and goat anti-rabbit secondary antibody were obtained from LI-COR Biotechnology (Lincoln, NE, USA). Propidium iodide (PI)/Rase staining buffer and the FITC Annexin V apoptosis detection kit were purchased from BD Pharmingen (San Diego, CA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibody and 3,3′-diaminobenzidine (DAB) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.3. Cell culture

Three human OSCC cell lines (WSU-HN4, WSU-HN6 and CAL27) were included in this study. WSU-HN4 and WSU-HN6 were kindly provided by the University of Maryland Dental School, and CAL27 was from the American Type Culture Collection. In the continuous treatment procedure, the OSCC cell lines were cultured in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA, USA) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Invitrogen). All cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.4. Cell proliferation assay

The MTT assay was modified and used to examine the cell proliferation. OSCC cells (2×10^3/well) were seeded in 96-well microplates. After 24 h, cells were exposed to different concentrations of oridonin and were cultured for 24, 48, 72, or 96 h. Cells treated with the same volume of culture medium containing vehicle were set as control. At each time point of detection, 20 μl/well of MTT solution (5 mg/ml) was added into the plate and cells were cultured for an additional 4 h at 37 °C. The supernatant was discarded and 150 μl DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at a wavelength of 570 nm using a microplate reader.

2.5. Cell clonogenic assay

Cells were seeded into 6-well plates at 800 cells/well in 2 ml culture medium. After 24 h, culture medium was replaced with fresh medium containing vehicle (control) or different concentrations of oridonin. The medium was changed every 3 days. After 3 weeks, the supernatants were discarded and cell clones were stained for 15 min with a solution containing 0.5% crystal violet and 25% methanol, followed by three rinses with tap water to remove the excess dye. Colonies consisting of > 50 cells were counted under a microscopy.

2.6. Cell cycle and apoptosis analysis

To analyze the cell cycle and apoptosis of the OSCC cells, flow cytometry was carried out. Tumor cells (2×10^5 cells/well) were seeded in 6-well plates. After 24 h, the medium was removed and replaced by fresh culture medium without (control) or with oridonin (10 or 20 μM). For cell cycle analysis, tumor cells were harvested 24 h after treatment, and washed and fixed with cold 70% ethanol. Incubation of the fixed and permeabilized cells with PI/Rase staining buffer resulted in quantitative PI binding with total cellular DNA, and the fluorescence intensity of PI-labeled cells was proportional to the DNA content. Cell cycle distribution was analyzed using a flow cytometer and the ModFit software (Becton Dickinson, San Jose, CA, USA). Apoptotic and necrotic cell death were analyzed 48 h after oridonin treatment by double staining with fluorescein-isothiocyanate (FITC)-conjugated annexin V and PI, in which Annexin V bound to the exposed phosphatidylserine on the surface of the apoptotic cells, and PI labeled the late apoptotic/necrotic cells with membrane damage. Staining was performed according to the instructions of the detection kit and the apoptotic data were processed with the FlowJo software.

2.7. Western blotting

The OSCC cells treated with different concentrations of oridonin (0, 10, 20 μM) were collected, and then lysed on ice in RIPA buffer [150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.5% deoxycholic acid, 0.1% SDS, 5 mM EDTA, 0.7% phenylmethylsulfonyl fluoride, 0.2% aprotinin, 0.2% leupeptin, and sodium metavanadate]. After centrifugation at 12,000*g* for 15 min, the protein content of the supernatant was determined by Enhanced BCA Protein Assay Kit (Beyotime, Haimen, China), and then samples were incubated at 100 °C for 10 min. Equal amounts of total protein were loaded onto SDS-polyacrylamide gels with different concentrations (8% for cdc25C, p53 and cyclin B1; 10% for cdc2, phospho-cdc2, Bax, Bcl-xl and β-actin; and 15% for caspase 8, caspase 9 and cleaved-caspase 3), and were then electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membrane was later blocked with blocking buffer (5% non-fat milk powder in Tris-buffered saline/Tween 20) for 1 h at room temperature and then incubated with primary antibodies at 4 °C overnight. Polyclonal secondary antibodies were used at a dilution of 1:10,000. Protein bands were finally detected by the Odyssey Infrared Imaging system (LI-COR Biosciences).

2.8. In vivo anti-tumor activity

For xenograft implantation, 2×10^6 WSU-HN6 cells/mouse were injected subcutaneously into the back next to the right hind limb, and permitted to grow until palpable. Then mice were randomly assigned into control and treated groups and treatment was initiated. The oridonin treated group received intraperitoneal injection of oridonin (30 mg/kg) every other day for a total of 17 days, whereas mice in the control group were administered vehicle in the same way. Tumors were measured every 3 days with a Vernier caliper and tumor volumes were calculated according to the following formula: tumor volume (mm^3) = $A \times B^2 \times 0.52$, where A is the longest diameter and B is the shortest diameter. At the end of the experiment, tumor-bearing mice were killed, and tumors were weighed after being separated from the surrounding muscles and dermis. Finally, the tumors were fixed with 4% phosphate-buffered paraformaldehyde and embedded in paraffin.