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Original article

Oridonin inhibits breast cancer growth and metastasis through blocking the Notch signaling

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ABSTRACT

Background: Oridonin is a diterpenoid isolated from *Rabdosia rubescens* with potent anticancer activity. The aim of our study is to investigate the role of oridonin to inhibit growth and metastasis of human breast cancer cells. Methods: The effect of oridonin on proliferation was evaluated by MTT assay, cell migration and invasion were evaluated by transwell migration and invasion assays in human breast cancer cells. The inhibitive effect of oridonin *in vivo* was determined by using xenografted nude mice. In addition, the expression of Notch receptors (Notch 1–4) was detected by western blot. Results: Oridonin inhibited human breast cancer cells *in vitro* and *in vivo*. In addition, oridonin significantly induced human breast cancer cells apoptosis. Furthermore, the oridonin treatment not only inhibited cancer cell migration and invasion, but more significantly, decreased the expression of Notch 1-4 protein. Conclusion: Our results suggest that the inhibitive effect of oridonin is likely to be driven by the inhibition of Notch signaling pathway and the resulting increased apoptosis.

1. Introduction

Breast cancer is one of the most lethal malignancies among women (PDQ Adult Treatment Editorial Board, 2002–2016). Approximately 70% of patients are diagnosed at an advanced stage with lymph node metastasis (Naidoo and Pinder, 2016). 70% of patients were diagnosed with advanced lymph node metastases (Naidoo and Pinder, 2016). Only 25% of patients with early diagnosis, advanced breast cancer patients with 5-year survival rate of 20%–25% (Dai et al., 2016; Parsa et al., 2016). It is difficult to find early in the early years, mainly due to its inherent metastatic nature and poor prognosis (Xie et al., 2016; Bozorgi et al., 2015). The widespread use of chemotherapy has poor gastrointestinal toxicity, including severe nausea and vomiting, nephrotoxicity and neurotoxicity (Feng et al., 2016; Joo et al., 2009). More importantly, long-term use of chemotherapeutic drugs is bound to lead to drug resistance, which is a major obstacle to cancer chemotherapy (Kim et al., 2009). Then, to explore more effective methods to reduce side effects or to address drug resistance has become the most important issue for breast cancer.

Recently, natural products extracted from medicinal plants have received increasing attention in cancer treatment. Vitamin is extracted from Chinese herbal medicine and it is a natural compound of tetracycline diterpenoids (Wang et al., 2013; Gao et al., 2010) (structure shown in Fig. 1). Qi et al. (2012) reported that oridonin can effectively induce the apoptosis of pancreatic cancer cells. Oridonin nano-suspension is more effective than free lysine on G2/M cell cycle arrest and apoptosis in human pancreatic cancer PANC-1 cells. Gao et al. (2012) found oridonin induces apoptosis and senescence by increasing the consumption of hydrogen peroxide and glutathione in colorectal cancer cells. At the same time, some researchers report that autologous engulfed in the treatment of human breast cancer MCF-7 cells treated with oridonin (Cui et al., 2007). Among patients with lung cancer, oridonin also inhibits mTOR signaling and growth of lung cancer tumors, and mTORC1 inhibition may be an effective target for promoting clinical outcomes with oridonin treatment (Wang et al., 2014).

There are accumulating clinical evidences showing that, as a potential target, Notch could prevent the progress of tumor metastasis (Garcia and Kandel, 2012). Notch mediates vascular endothe-

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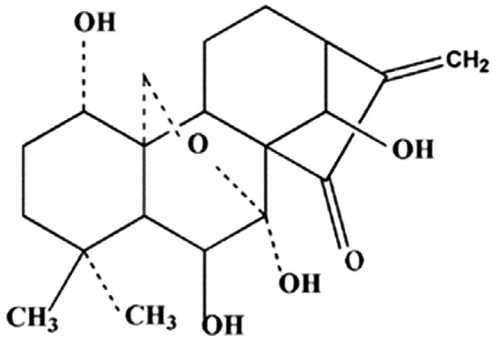

Fig. 1. Chemical structures of oridonin.

lial cells and tumor cells communication and promotes tumor angiogenesis of tumor (Villa et al., 2001). The tumor-related growth factors such as VEGF can induce jagged ligands in the microenvironment of tumor (Shawber and Kitajewski, 2004) and then activate Notch (Folkman, 2002) expressed in tumor endothelial cells. Surprisingly, Jagged 2 is the most Notch ligand associated with the overall and non-metastatic survival of breast cancer patients (Xing et al., 2011). Vascular endothelial cells express Notch receptors 1, 2 and 3, and Notch signaling is critical for the proper formation of functional vasculature (Iso et al., 2003). In addition, Notch activity was specifically upregulated in the tumor endothelium, suggesting that interference with Notch activity may have a negative effect on tumor neovascularization. Several Notch inhibitors such as RO4929097 (Tolcher et al., 2012) and MK-0752 (Krop et al., 2012) have been used in clinical trials. Thus, targeting the Notch pathway in endothelial cells may provide an effective strategy for antiangiogenic therapy (Zhou et al., 2007).

In the present study, we used human breast cancer cell line 4T1, and explored the mechanism of oridonin therapy. Furthermore, we identified Notch signaling pathway as a direct target of oridonin, and oridonin exerted the tumor suppressive effect via inhibiting Notch receptors expression in breast cancer cells.

2. Material and methods

2.1. Agents and cell lines

Oridonin (purity $\geq$ 98%) was purchased from Sigma-Aldrich China Inc. (Shanghai, China). The human breast cancer cell line 4T1 were obtained from the American Type Culture Collection (Rockville, Maryland, USA). Cell counting kit-8 (CCK-8) was provided by Dojindo Laboratories (Kumamoto, Japan). Hoechst 33258, the ApoDETECT annexin V–fluorescein isothiocyanate (FITC) apoptosis detection kit, the Light Shift chemiluminescent electrophoretic mobility shift assay (EMSA) kit, and NE-PER nuclear and cytoplasmic extraction reagents were obtained from ThermoFisher Scientific Inc. (Waltham, Massachusetts, USA). The cells were cultured in DMEM medium (Hyclone, Beijing, China) enriched with 10% fetal bovine serum (Hyclone) at 37 °C and 5% CO_2.

2.2. MTT assay

The cells were cultured in 96-well cell culture plates and treated as shown on the second day. According to the manufacturer's protocol, MTT assay (Promega) was used to assess the number of viable cells treated with drugs. At a reference wavelength of 630 nm, the absorbance (A) was measured at 570 nm.

2.3. Tumor inhibition assay in vivo

The protocol of the animal study was approved by the Animal Ethics Committee of Sichuan University. All experiments were conducted in accordance with institutional guidelines. Briefly, 56 male BALB/C athymic nude mice (Shanghai Laboratory Animal Center, Shanghai, China) weighing between 18 and 23 g (4–6 weeks old) were subcutaneously engrafted with 1×10^7 4T1 cells. Tumors were allowed to establish and grow to a volume of 100–150 mm^3. The rodents were then randomly divided into seven groups of eight mice; they received the following treatments by an intraperitoneal injection: 0.9% saline, two doses of oridonin (10 and 20 mg/kg). The treatment for each group was administered once daily for 21 days, with a 1-day interval every 6 days. Tumor size was measured every 3 days starting on the day of first treatment (day 0) and ending 28 days after the first treatment (day 28) according to the published literature (27). At day 28, the animals were killed and tumor weight was determined.

2.4. Flow cytometry

Quantification of apoptotic cells by flow cytometry was performed using an ApoDETECT annexin V–FITC apoptosis detection kit according to the manufacturer's instructions. Briefly, 1×10^5 4T1 cells subjected to the various treatments were harvested, washed twice with cold PBS, and then resuspended in 0.5 ml of binding buffer. Subsequently, 10 μl of FITC-labeled annexin V and 10 μl propidium iodide (PI) solution (20 μg/ml) were added to 190 μl of cell suspension. After incubation in the dark for 10 min at RT, the cells were analyzed on a flow cytometer (model FACS Calibur; BD Biosciences, Franklin Lakes, New Jersey, USA). Early apoptotic cells were defined as $FITC^+/PI-$ cells and late apoptotic cells were defined as FITC+/PI+ cells. Assays were repeated independently three times.

2.5. Cell migration and invasion assays

For the transwell migration assay, 5 × 104 BGC-823 cells were placed in the upper chamber (Corning). For the invasion assay, 1 × 105 BGC-823 cells were placed in the upper chamber containing 40 μl of matrigel. A medium supplemented with 20% FBS was added to the lower chamber. After incubation for several hours, the cells which were attached to the lower surface were stained with crystal violet for 20 min. The cells were counted in 5 random magnitudes of 100× magnification.

2.6. RNA isolation and quantitative real-time PCR (qRT-PCR)

Trizol reagent (Invitrogen) was used to isolate total RNA from tissue and cell samples. The spectrophotometry and electrophoresis were used to determine total RNA concentration and quality. The SuperScript III first-strand synthesis system was used to synthesize cDNA. The ABI 7900 system using SYBR Premix Ex Taq was applied to conduct RT-PCR. The 2-ΔΔCT method was used to calculate relative expression levels of miR-338-3p and Notch1.

2.7. Transient transfection

The pEGFP-Notch 1 plasmid was generated using the following primers: forward, 5′-CGCAGTTGTGCTCCTGAA-3′ and reverse, 5′-ACCTTGGCGGTCTCGTAGCT-3′. The Lipofectamine 2000 (Invitrogen) was used to conduct transfection based on the manufacturer's protocol. The qRT-PCR was conducted to monitor the transfection efficiency.