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Curcumol induces cell cycle arrest in colon cancer cells via reactive oxygen species and Akt/ GSK3β/cyclin D1 pathway

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ABSTRACT

Ethnopharmacological relevance: Curcuma kwangsiensis S. G. Lee & C. F. Liang (Guangxi ezhu, in Chinese) belongs to the Zingiberaceae family, has been used as a traditionally Chinese medicine nearly 2000 year. Curcumol is one of the guaiane-type sesquiterpenoid hemiketal isolated from medicine plant Curcuma kwangsiensis S. G. Lee & C. F. Liang, which has been reported possesses anti-cancer effects. Our previous study found that the most contribution to inhibit nasopharyngeal carcinoma cell growth was curcumol.

Aim of the study: To assess the effect of curcumol on cell cycle arrest against human colon cancer cells (CRC) cells (LoVo and SW480) and explore its mechanism in vitro and in vivo.

Materials and methods: Curcumol was dissolved in absolute ethyl alcohol. The concentration of absolute ethyl alcohol in the control group or in experimental samples was always 1/500 (v/v) of the final medium volume. LoVo and SW480 cells were treated with different concentrations of curcumol (0, 53, 106, 212 and 424 μM). And then the cell cycle of each group was examined by flow cytometry. The protein levels of PI3K, p-Akt, cyclin D1, cyclin E, CDK2, CDK4 and GSK3β were determined by Western blot. The mRNA expression of PI3K, Akt, cyclin D1, CDK4, P27, p21, and P16 in the treated cells were analyzed by real-time RT-PCR. In addition, the antitumor activity of curcumol was evaluated in nude mice bearing orthotopic tumor implants.

Results: Curcumol induced cell cycle arrest in G1/S phase. RT-qPCR and Western blot data showed that curcumol enhanced the expression of GSK3β, P27, p21 and P16, and decreased the levels of PI3K, phosphorylated Akt (p-Akt), cyclin D1, CDK4, cyclin E and CDK2. Furthermore, curcumol induced reactive oxygen species (ROS) generation in LoVo cells, and ROS scavenger N-acetylcysteine (NAC) significantly reversed curcumol-induced cell growth inhibition. Besides, curcumol also prevented the growth of human colon cancer cells xenografts in nude mouse, accompanied by the reduction of PI3K, Akt, cyclin D1, CDK4, cycln E and significant increase of GSK3β.

Conclusions: Curcumol caused cell cycle arrest at the G0/G1 phase by ROS production and Akt/ GSK3β/cyclin D1 pathways inactivation, indicating the potential of curcumol in the prevention of colon cancer carcinogenesis.

1. Introduction

Colorectal cancer (CRC) is one of the most frequently diagnosed malignant tumors and the first leading cause of death in the digestive system of western countries (Siegel et al., 2014). Epidemiological studies have reported that the incidence of CRC has been increasing yearly in China (Li et al., 2011). Currently, radiotherapy, chemotherapy and surgery are three main therapeutic methods for human colorectal cancer. However, chemotherapy treatment of CRC is not satisfactory on clinic because of the toxic and side effects. And the 5-year survival of patients with CRC is approximately 8% (Ramasamy et al., 2015). Therefore, effective drugs with little adverse reaction need to be found for colorectal cancer therapy.

Curcumol, a guaiane-type sesquiterpenoid hemiketal was extracted from the roots of the herb *Rhizoma Curcumae*. Owing to its low side effects, more and more studies focus on its bioactivity. Thousands years usage and nearly researchers' studies all found that curcumol exhibits multi-bioactivity, including anti-tumor activity (Lü et al., 2011; Lu et al., 2012). Curcumol exerted its anticancer effect by inhibiting cell growth and inducing apoptosis (Qi-Ling Tang et al., 2015; Wei wei Zhang, 2011). At the molecular level, curcumol acts as an anticancer agent by up-regulating the expression of anti-oncogenes or down-

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regulating the expression of oncogenes. Our previous study showed that cucrumol can induce the apoptosis of colon cancer cells by P38 MAPK pathway, while there was few studies focus on the effect of cucumol on colon cancer' cell cycle. Owing to the significant effect of cell cycle phase to cancer cells, then we want to know whether curcumol has the same effect on colon cancers' cell cycle and its mechanism.

The phosphoinositide 3-kinase (PI3K)/Akt pathway is activated by many types of cellular stimuli and regulates fundamental cellular functions such as transcription, translation, proliferation, growth, and survival (M. Osaki, 2004). Notably, it has been shown that PI3K/Akt signaling was frequently disrupted and propagated in intracellular signaling cascades, which results in an imbalance between proliferation and apoptosis, and then causes tumorigenesis (M. Osaki, 2004). Evidence has shown that this pathway regulates cell cycle progression via affecting important cell cycle related molecules. These targets include cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors (Chang et al., 2003; Chen et al., 2011; Liao et al., 2014; Shimura, 2011; Zhang et al., 2015a). Aberrant activation of the PI3K/Akt signaling pathway is involved in many malignant tumors, including colorectal cancers, cervical cancer, ovarian cancer and so on (Agarwal et al., 2013; Francipane and Lagasse, 2013; M. Osaki, 2004; Ogino et al., 2014; Yasuda et al., 2007; Zhu et al., 2014). Consequently, targeting the PI3K/Akt pathway is an attractive therapeutic strategy for cancer treatment (Brugge et al., 2007; Chang et al., 2003; Lee et al., 2009; Xia et al., 2010). Some research groups have reported that the PI3K/Akt pathway can be activated by reactive oxygen species (ROS) (Lau et al., 2008). ROS, potentially harmful by-products of normal cellular metabolism that directly affect cellular functions, have been reported to lead to DNA lesions, protein oxidation and lipid peroxidation, which are crucial for cellular integrity and cell survival. It is well known that many antitumor agents exhibit anticancer activity via ROS-dependent way (Fang et al., 2007). It has been reported that curcumol can induce cancer cells apoptosis which related with ROS production increase (Xu LC, 2010). This prompted us to explore whether curcumol inactivates Akt pathway by inducing ROS generation in CRC cells.

In this work, we studied the effect of curcumol on cell cycle arrest against human colon cancer LoVo and SW480 cell lines, and examined whether inactivation of Akt/GSK3β/cyclin D1 pathways were involved in the anticancer activity of curcumol. In addition, we observed the ROS-generation and the relationship with Akt/ GSK3β/cyclin D1 pathways. We also investigated the antitumor effect of curcumol in vivo by a xenograft CRC mouse model.

2. Materials and methods

2.1. Materials and cell culture

Curcumol was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) (^{1}H NMR spectra in Fig. 1*b*). The 85 mM stock was prepared by dissolving the compounds in absolute ethyl alcohol which was purchased from Xilong Chemical Industry (Guangdong, China). Human colon cancer cells LoVo and SW480 were obtained from Guilin medical university (Guilin, China). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Auckland, New Zealand), RIPA, BCA protein assay kit, and PMSF were purchased from Beyotime Institute of Biotechnology (Shanghai, China). DCFH-DA (2′,7′- dichlorodihydrofluorescein diacetate) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Trizol was purchased from TIANGEN (Beijing, China). Antibodies against PI3K, p-Akt, cyclin D1, cyclin E1, cyclin-dependent kinase 2 (CDK2), cyclin-dependent kinase 2 (CDK4), and GSK3β were from Wanlei Technology (Shenyang, China). The concentration of absolute ethyl alcohol in the control group or in experimental samples was always 1/500 (v/v) of the final medium volume.

2.2. Cell Cycle Analysis

Cell cycle distribution was analyzed by flow cytometry as follows. After treatment of curcumol, cells were harvested and washed with PBS, and then fixed in 70% ice-cold ethanol for about 24 h at −20 °C. After that fixed cells were washed gently with cold PBS twice, incubated with 0.5 μg/mL RNase A for 30 min at 37 °C, and then stained with 50 μg/mL propidium iodide in the dark at room temperature for 30 min. The stained cells were calculated using a FAScan laser flow cytometer equipped with Cell Quest software (Becton Dickinson, San Jose, CA, USA).

2.3. Quantitative real-time PCR

Total RNA was extracted from the cultured cells using TRIzol reagent (TIANGEN) as recommended by the manufacturer's instructions. After detection, total RNA (5 μg) was reverse-transcribed into cDNA using M-MLV First Strand Kit (Cat#C28025-032, Invitrogen, USA). The PCR reactions were quantified by ABI 7500 fast real time PCR system. Specific PCR primers used in this experiment are listed in Table 1. Quantitative real-time PCR (RT-qPCR) was performed with QuantiTect SYBR Green PCR Kit (REF#4367659, Applied Biosystems, USA). Each sample was tested in triplicate. The relative fold change in gene expression was analyzed as $2^{-\Delta\Delta CT}$ by the software equipped with ABI 7500 fast (Applied Biosystems, California, Carlsbad, USA).

2.4. Western blotting

After treatment for 48 h, the cells were harvested and washed with ice-cold PBS twice, then exposed to RIPA lysis buffer with protease inhibitors (1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstain,

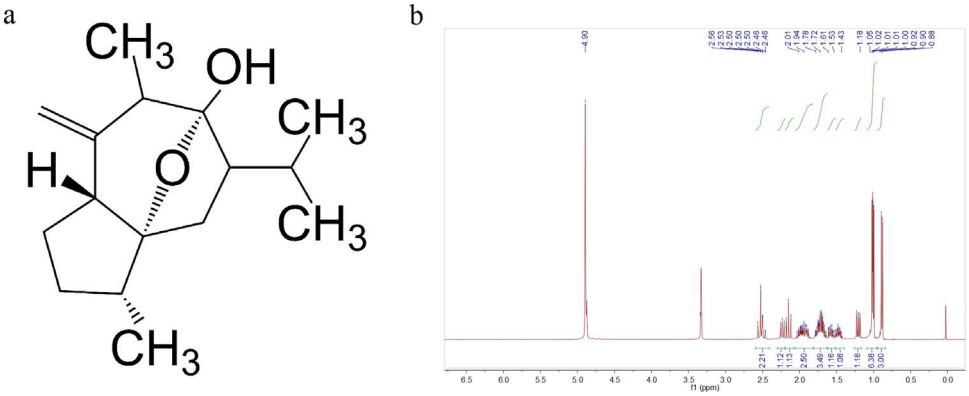

Fig. 1. ^{1}H NMR spectra of curcumol in CD_3OD. (a) The structure of curucumol. (b) The ^{1}H NMR spectra of curcumol.