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Cardamonin inhibited cell viability and tumorigenesis partially through blockade of testes-specific protease 50-mediated nuclear factor-kappaB signaling pathway activation

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ABSTRACT

Previous studies have shown that testes-specific protease 50 (TSP50), a pro-oncogene overexpressed in many types of tumors, could promote cell proliferation, invasion, tumorigenesis, and tumor metastasis, suggesting that it is a potential cancer therapeutic target in drug discovery. Here, a luciferase assay system driven by the TSP50 gene promoter was used to screen the inhibitor of expression of TSP50. The study found that cardamonin, a flavone compound, could efficiently inhibit the expression of TSP50 in both mRNA and protein levels. Further results revealed that cardamonin also efficiently inhibited the viability of TSP50 high-expressing cancer cells by inducing G2/M-phase arrest and mitochondrial-dependent apoptosis. Surprisingly, knocking down the expression of TSP50 gene had the same effects as treatment with cardamonin. Moreover, it has been found that cardamonin had an inhibitory potency on TSP50 high-expressing tumor growth in vivo. In contrast, overexpression of TSP50 greatly decreased the cell sensitivity to the inhibitory effect of cardamonin and reversed the decreased tumor-inhibitory effect of cardamonin. Additionally, both TSP50 interference and treatment with cardamonin could suppress p65 nuclear translocation, and overexpression of TSP50 reversed the suppressive effect of cardamonin on p65 nuclear translocation. Taken together, these results suggest that cardamonin inhibited cell viability and tumorigenesis at least partially via blocking the activation of TSP50-mediated nuclear factor-kappaB signaling pathway, and cardamonin may be a promising anticancer drug candidate in the development of a novel agent for TSP50 high-expressing cancer cells.

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1. Introduction

Testes-specific protease 50 (TSP50) gene, which was isolated from human breast cancer cells in 1999 as a novel threonine protease, plays a crucial role in mammalian spermatogenesis (Li et al., 2012; Shan et al., 2002; Yuan et al., 1999). Previous studies have indicated that TSP50 is specifically expressed in testes, but abnormally reactivated in many cancer biopsies associated with poor prognosis (Liu et al., 2014; Xu et al., 2004; Zheng et al., 2011). Our previous results have shown that overexpression of TSP50 in Chinese hamster ovary cells markedly increased cell proliferation, colony formation, and promoted tumor formation in nude mice. In contrast, TSP50 catalytic triad mutation significantly depressed cell proliferation and invasion in vitro and abolished the oncogenicity of TSP50 in vivo (Li et al., 2012; Song et al., 2011, 2014). In addition, downregulation of TSP50 induces apoptosis, reduces cell proliferation and colony formation in p19 cells (Zhou et al., 2010). Moreover, our previous studies have shown that alantolactone induced mitochondrial-dependent apoptosis in breast cancer cells via downregulation of TSP50 expression (Mi et al., 2014). All these results suggest that TSP50 plays a crucial role in tumorigenesis and could be a novel drug screening target for cancer therapy.

Cardamonin, a flavone compound, is first extracted from the fruits of large black cardamom (*Amomum subulatum*), and has been recently found in many other plant species, some of them are traditional Chinese medicinal (TCM) herbs (Goncalves et al., 2014; Katsori and Hadjipavlou-Litina, 2011; Wei et al., 2012). It has been reported that cardamonin possesses the anti-inflammatory activity

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in mice and rats by downregulating nuclear factor kappaB (NF-κB) signaling pathway and the serum levels of tumor necrosis factor-alpha (TNF)-α and interleukins (Israf et al., 2007; Wei et al., 2012). Furthermore, results from Yadav suggested that cardamonin has an “enhancement effect” on TNF-related apoptosis-induced ligand in human colon cancer cells (Yadav et al., 2012). Moreover, cardamonin inhibits the growth of various cancer cells including PC-3 and A549 (Park et al., 2013a; Pascoal et al., 2014; Tang et al., 2014).

In the present study, a firefly luciferase reporter screening system driven by TSP50 promoter was established, and over 300 purified compounds derived from TCM were screened. It was found that cardamonin could effectively inhibit the expression of TSP50. Further results have shown that cardamonin could induce G2/M-phase arrest and mitochondrial-dependent apoptosis in cancer cells that expressed high levels of TSP50, but had no significant effects on cancer cells that expressed low levels of TSP50. These results have demonstrated that TSP50 is a novel cancer therapeutic target, and cardamonin could be a potential antitumor drug for tumors expressing high levels of TSP50.

2. Materials and methods

2.1. Cell lines and cell culture

HEK293T (human embryonic kidney) cells, L02 (normal human hepatocyte) cells, HepG2 (human liver carcinoma) cells, and MDA-MB-231 (human breast cancer) cells were routinely grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Invitrogen, NY, USA). SMMC7721 (human hepatocarcinoma) cells, H7402 (human liver cancer) cells, SGC7901 (human gastric carcinoma) cells, $4T1^{luc}$ (mouse breast cancer) cells, and A549 (human lung carcinoma) cells were routinely grown in Rosewell Park Memorial Institute medium 1640 (RPMI 1640; Gibco, Invitrogen). All cells were supplemented with 10% fetal bovine serum (FBS; TBD Science, Hangzhou, China), penicillin (100 U/mL), and streptomycin (100 μg/mL; Ameresco) at 37 °C with 5% carbon dioxide.

2.2. Plasmid constructs and transfection

The pRNAT-TSP50 shRNA#1, #2, #3, pcDNA3-TSP50, and pGL3-TSP50 P (pGL3-1237/+454) plasmids were prepared in the laboratory as described previously (Song et al., 2011; Wang et al., 2008). The pGL3-TSP50 plasmid was transfected using calcium phosphate cell transfection kit (Beyotime, Hainan, China), and other plasmids were transfected using lipofectamine 2000 (Invitrogen, NY), according to the manufacturer’s instructions.

2.3. Luciferase assay system

HEK293T cells were transfected with pGL3-TSP50 P and/or pCMV-β-galactosidase plasmids for 24 h. Later, the cells were plated onto the 96-well plates at a density of 5×10^3 cells/well. Later the cells were treated with natural compounds (5 μg/mL) in DMEM containing 3% FBS (v/v) (to reduce the complex interference caused by the composition of serum) for 24 h. Luciferase activity was measured as described previously (Sun et al., 2011; Zhang et al., 2010).

2.4. RNA extraction and reverse transcription-polymerase chain reaction

MDA-MB-231 cells were plated at a concentration of 5×10^5 cells/well in a six-well plate. At 24 h after plating, cells were treated with cardamonin (5 μg/mL) for another 24 h. Total RNA was extracted from the cells. Both RNA extraction and reverse transcription-polymerase chain reaction were performed as described previously (Wang et al., 2010).

2.5. Antibodies and western blotting assay

Mouse monoclonal antibodies against Bcl-2 (1:500), glyceraldehyde 3-phosphate dehydrogenase (1:2000), cyclin-dependent kinase 4 (CDK4; 1:800), CDK2 (1:800), cyclin E (1:1000), and p21 (1:1000) and rabbit polyclonal antibodies against Bax (1:800), p27 (1:1000), cyclin D (1:1000), and p65 (1:1000) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against cleaved caspase-9 (1:1000) and cleaved caspase-3 (1:1000) were obtained from Cell Signaling (Beverly, MA). Monoclonal antibody against TSP50 (1:500) was prepared in the laboratory (Liu et al., 2009). Horse radish peroxidase-conjugated secondary antibodies (goat anti-rabbit immunoglobulin [IgG] or goat anti-mouse IgG, 1:1000) were obtained from Cell Signaling. ECL-Plus kit was obtained from TransGen Biotech (Beijing, China). Cytosolic extracts were prepared and western blotting assay was performed as described previously (Lin et al., 2012).

2.6. 3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide assay

The cells were plated at 5×10^3 cells/well in 96-well plates for 24 h, and then treated with compounds for 48 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/mL; Sigma, St. Louis, MO) was added to each well and incubated for 4 h. After removing the fluid from wells, dimethyl sulfoxide (DMSO; 150 μL) was then added and shaken for 10 min. Cell viability was determined using microplate reader (Bio-Rad, Hercules, CA) at a wavelength of 570 nm.

2.7. Cell staining and TUNEL assay

The cells were plated at 1×10^5 cells/well in 24-well plates for 24 h, and then treated with compounds for 24 h. The cells were stained with DiI (Beyotime), 4′,6-diamidino-2-phenylindole (DAPI; Beyotime), and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (In Situ Cell Death Detection Kit; Fluorescein, Roche, Switzerland), according to the manufacturer’s instructions.

2.8. Cell cycle analysis

The treated cells were harvested, washed, and fixed in 70% ice-cold ethanol at 4 °C for 2 h. Later, the cells were stained with Cell Cycle and Apoptosis Analysis Kit (Beyotime), according to the manufacturer’s instructions. DNA was observed using flow cytometry (Beckman Coulter Epics, Hialeah, Florida, USA) to calculate the percentage of cells in G0/G1, S, and G2/M phases with MultiCycle software.

2.9. In vivo anticancer effect of cardamonin in 4T1 tumor-bearing mice

Cell suspensions (0.1 mL) containing approximately 5×10^6 $4T1^{luc}$ and $4T1\text{-pcDNA3-TSP50}^{luc}$ cells at exponential stage were injected subcutaneously into the backs of 14 BALB/c female mice. Treatment commenced when the diameter of the primary tumor reached about 6–8 mm (after 7 days of inoculation). The mice were randomly divided into three equal groups ($n = 8$/group) for treatment: negative control (physiological saline), cardamonin, and cardamonin + TSP50 groups. A dose of 100 μg/50 μL cardamonin or 50 μL physiological saline was injected into tumor in each group every 3 days for 15 days. Antitumor activity was evaluated

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