Cardamonin reduces chemotherapy resistance of colon cancer cells via the TSP50/NF-κB pathway *in vitro*

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Abstract. It has previously been reported that cardamonin is able to regulate glycometabolism and vasodilation whilst also exhibiting anti-inflammatory and antitumor properties. The antitumor effect of cardamonin is multifaceted, and so it is necessary to investigate the antitumor mechanisms of cardamonin at the molecular level. Cardamonin alters chemotherapy-resistant colon cancer cell growth; however, the underlying mechanism is unknown. The present study was conducted to investigate the effect of cardamonin on chemotherapy-resistant colon cancer cells and the possible mechanisms of action. Cardamonin significantly suppressed the growth of chemotherapy-resistant colon cancer cells, induced apoptosis and promoted caspase-3/9 activity and Bax protein expression in 5-fluorouracil (5-FU)-resistant HCT-116 cells. Cardamonin significantly suppressed c-MYC, octamer-binding transcription factor 4, cyclin E, testes-specific protease 50 and nuclear factor-κB protein expression in 5-FU-resistant HCT-116 cells. The findings of the present study demonstrate that cardamonin suppresses chemotherapy-colon cancer cell via the NF-κB pathway *in vitro*.

Introduction

Colon cancer is a common malignant tumor of the gastrointestinal tract (1). Domestic studies following colorectal surgery suggest that the annual survival rate of patients with high risk stage II and stage III colon cancer is ~20-30% (2). The primary cause of mortality in patients with colon cancer is recurrence and metastasis (2). In recent years, the morbidity of colon cancer has increased (3). The main subtypes of colon cancer include adenocarcinoma, mucinous adenocarcinoma and undifferentiated carcinoma (3). The primary therapeutic method for treating colon cancer is surgical resection, which is accompanied by chemotherapy, immunotherapy and traditional Chinese medicine (TCM) (3). Metastasis is a common feature of aggressive colon cancer, with these metastases primarily affecting the liver (4); ~30% of patients exhibit pre- or postoperative liver metastasis (3). However, a minority of patients are suitable for excision and are susceptible to postoperative recurrence (5).

Distant metastasis of colon cancer is a topic that has been well researched in recent years (6). Although there are a large number of novel chemotherapeutics available, the toxic and adverse effects, as well as the tolerance generated by these therapies, mean that they are not completely satisfactory (7). Prior studies have demonstrated that patients with cancer succumb to drug-resistant disease induced by chemotherapeutics, which invalidates treatment (7). There is a lack of novel effective chemotherapeutics that can overcome this resistance (8), as the molecular mechanisms of chemotherapy resistance and the associated genetic changes are complicated and comprise multiple aspects, including drug transport, supersession, repair and regulation of apoptosis (8).

Apoptosis is regulated by a number of factors, including nuclear transcription factor-κB (NF-κB) (9), which combines with the fixed nucleotide sequences of certain gene promoters. NF-κB is activated by multiple stimuli, including cell adhesive attraction (10). The activation of NF-κB regulates cell apoptosis and inflammation (10). However, suppression of NF-κB activity is able to increase the occurrence of spontaneous apoptosis in cancer cells or increase apoptosis induced by cytotoxic drugs (9). On the basis of these data, it has been speculated that cell adhesion may regulate apoptosis genes and changes the sensitivity of cancer cells to drugs by activating NF-κB, to induce multi-cell drug resistance (10).

Cardamonin was originally extracted and separated from black cardamom, of the family Zingiberaceae (the fruit of *Amomum subulatum*; Fig. 1) in 1976 and is a monomeric alkaloid (11). A previous study revealed that cardamonin serves an important role in cell proliferation by regulating various signal transduction pathways (12). Cardamonin activates the mechanistic target of rapamycin complex 1 downstream target p70 ribosomal S6 kinase and eukaryotic initiation factor 4E binding protein 1 in smooth muscle cells to reverse insulin resistance (13). Furthermore, cardamonin is able to activate

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mitogen-activated protein kinase and NF-κB signal pathways in mononuclear cells, regulating inflammatory responses mediated by lipopolysaccharides (14). The monomer regulates cell surface receptors and Wnt/β-catenin signal pathways in the inflammatory response (14). The aim of the present study was to investigate the effect of cardamonin on chemotherapy-resistant colon cancer cell and its possible mechanism.

Materials and methods

Cell lines and cell culture. The human colon cancer HCT116 cell line was purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China) and cultured for 24 h in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all from Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in an atmosphere containing 5% CO_2. Fluorouracil (5-FU) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). 5-FU-resistant HCT116 cells were generated by continuous exposure to increasing concentrations (0, 1.5, 3.25, 6.5, 12.5, 25 μmol/l) of 5-FU for 6-8 months.

Cell viability assay. HCT116 cells ($5x10^3$ cells/well) were cultured in a 96-well plate and treated with cardamonin (0, 10, 20, 40 and 80 μM, Sigma-Aldrich; Merck KGaA) for 24, 48 and 72 h, as described previously (15). A total of 20 μl MTT (Sigma-Aldrich; Merck KGaA; 0.5 mg/ml) was added to each well for 4 h and the medium was removed. Next, 150 μl DMSO was added per well for 20 min to dissolve formazan crystals. Cell viability was measured using an ELISA reader at 492 nm.

Cell apoptosis analysis. HCT116 cells ($2x10^6$ cells/well) were cultured in a 6-well plate and treated with cardamonin (0, 20, 40 and 80 μM) for 72 h. Apoptosis was measured using an Annexin V-FITC/PI Cell Apoptosis Double Dye kit (BD Biosciences, Franklin Lakes, NJ, USA). Cells were resuspended with 1X binding buffer (BD Biosciences, Franklin Lakes, NJ, USA). The cell suspension was incubated with 5 μl Annexin V-FITC and 5 μl PI at room temperature for 15 min in the dark. Cell apoptosis was measured using a flow cytometer (C6; BD Biosciences) and analyzed using FlowJo software (version 7.6.1; FlowJo LLC, Ashland, OR, USA).

Caspase activity assay. HCT116 cells ($2x10^6$ cells/well) were cultured overnight in a 6-well plate and treated with cardamonin (0, 20, 40 and 80 μM) for 72 h. Cell lysates were prepared by the addition of 100 μl lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) for 15 min. Protein content was determined using a bicinchoninic acid assay. A total of 5-10 μg protein was incubated with caspase-3 colorimetric DEVD-pNA substrate (Beyotime Institute of Biotechnology) and caspase-9 colorimetric LEHD-pNA (Beyotime Institute of Biotechnology) at 37°C for 1 h. Caspase activity was measured using an ELISA reader at 405 nm.

Western blotting. HCT116 cells ($2x10^6$ cells/well) were cultured overnight in a 6-well plate and treated with cardamonin (0, 20, 40 and 80 μM) for 72 h. Cell lysates were prepared by the
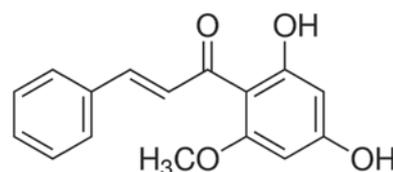
Figure 1. Chemical structure of cardamonin.
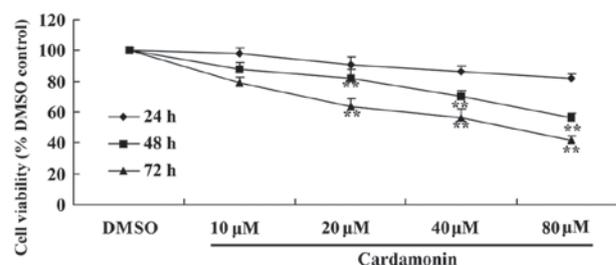
Figure 2. Effect of cardamonin on chemotherapy-resistant colon cancer cell growth. **P<0.01 vs. DMSO control group. DMSO, dimethyl sulfoxide.

addition of 100 μl lysis buffer for 15 min. Protein content was determined using the BCA method. A total of 50 μg protein per lane was separated by 8-10% SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes. The membranes were blocked with TBST containing 5% non-fat dried milk at 37°C for 1 h and incubated with specific primary antibodies targeted at B-cell lymphoma-associated X (Bax, sc-6236, 1:1,000), Myc proto-oncogene protein (c-MYC, sc-789, 1:1,000), octamer-binding transcription factor 4 (Oct4, sc-9081, 1:1,000), and cyclin E (sc-481, 1:1,000) (all from Santa Cruz Biotechnology, Inc.), testes-specific protease 50 (TSP50, ab181993, 1:1,000; Abcam), NF-κB (sc-101749, 1:1,000) and GAPDH (sc-25778, 1:1,000) (both from Santa Cruz Biotechnology, Inc.) overnight at 4°C. Following three washes with TBST, membranes were incubated with anti-rabbit or mouse horseradish peroxidase-conjugated secondary antibodies (sc-2004 or sc-2005, 1:5,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Signals were detected by enhanced chemiluminescence (ECL Plus detection system) and band density was analyzed using Carestream MI software (Carestream Health, Inc., Rochester, NY, USA).

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical analysis was performed using SPSS software (version 19.0; IBM Corp., Armonk, NY, USA). Statistical analysis was performed using one-way analysis of variance analysis, with post-hoc Student-Newman-Keuls test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of cardamonin on 5-FU-resistant colon cancer cell growth. To determine the anticancer effect of cardamonin on chemotherapy-resistant colon cancer cell growth, an MTT assay was used to measure the viability of HCT166 cells. As presented in Fig. 2, cardamonin reduced the viability of
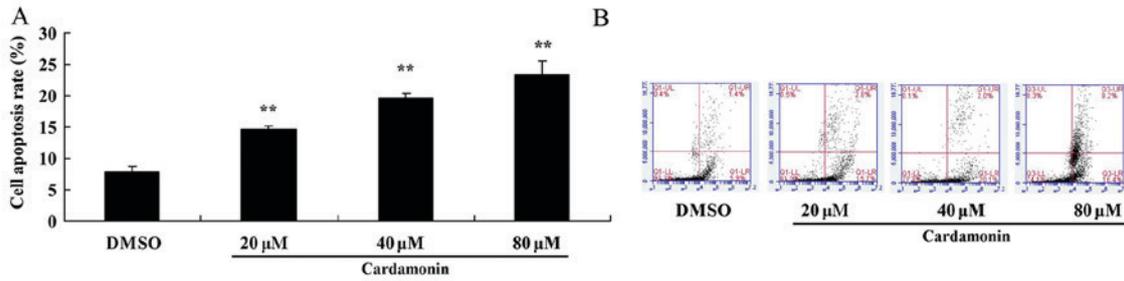
Figure 3. Effect of cardamonin on chemotherapy-resistant colon cancer apoptosis. Effect of cardamonin on chemotherapy-resistant colon cancer apoptosis rate using (A) statistical analysis, and (B) flow cytometer. **P<0.01 vs. DMSO control group. DMSO, dimethyl sulfoxide.
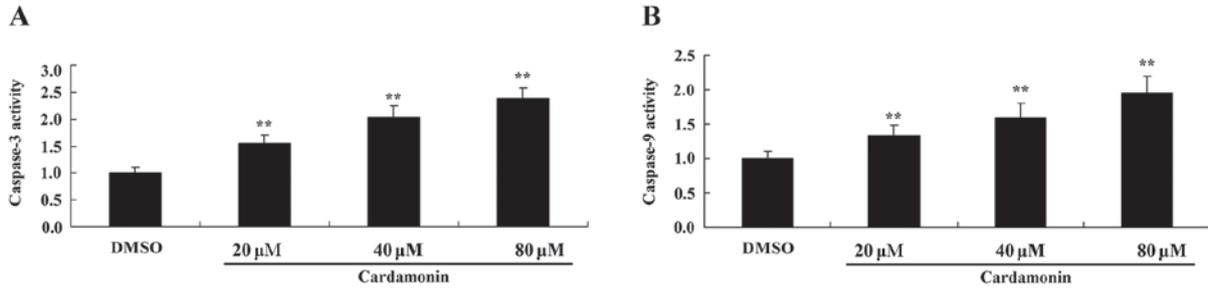
Figure 4. Effect of cardamonin on (A) caspase-3 and (B) caspase-9 activity in chemotherapy-resistant colon cancer cells. **P<0.01 vs. DMSO control group. DMSO, dimethyl sulfoxide.
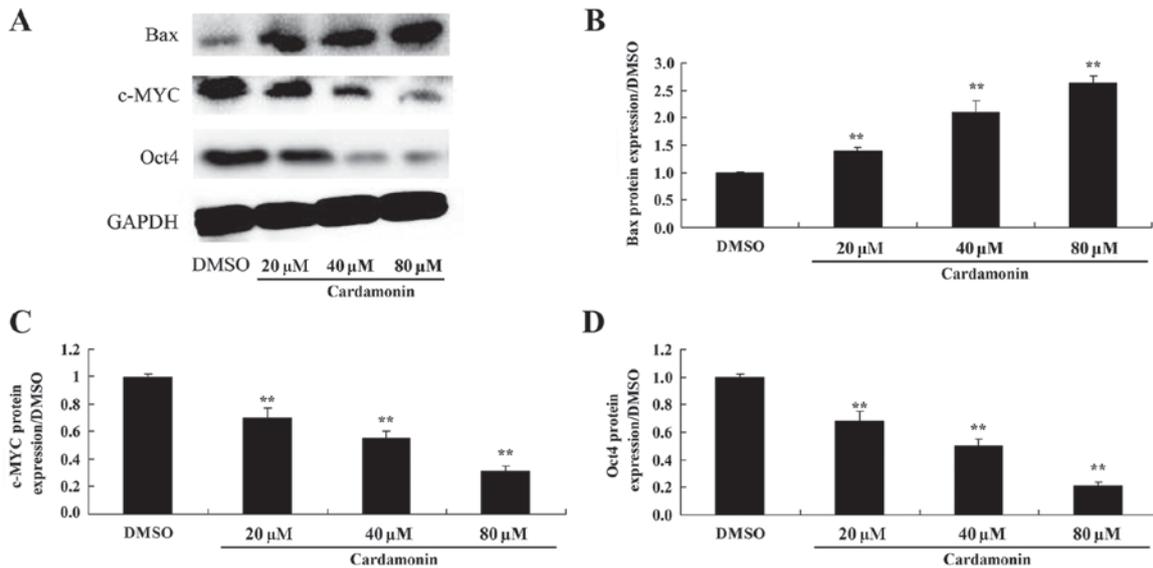
Figure 5. (A) Western blotting and densitometry analysis of the effect of cardamonin on (B) Bax, (C) c-MYC and (D) Oct4 protein expression in 5-fluo-rouracil-resistant colon cancer cells. **P<0.01 vs. DMSO control group. Bax, Bcl-2-associated X protein; c-MYC, Myc proto-oncogene protein; Oct4, octamer-binding transcription factor 4; DMSO, dimethyl sulfoxide.

5-FU-resistant HCT166 cells in dose- and time-dependent manner. Particularly, 20-80 μM of cardamonin significantly reduced the viability of 5-FU-resistant HCT116 cells following treatment for 24, 48 and 72 h (Fig. 2; P<0.01).

Effect of cardamonin on 5-FU-resistant colon cancer cell death. The apoptosis rate in 5-FU-resistant HCT116 cells was assessed using flow cytometry. Increasing doses of cardamonin

was demonstrated to promote 5-FU-resistant HCT166 cell apoptosis in a dose-dependent manner, with 40-80 μM of cardamonin significantly increasing 5-FU-resistant HCT166 cell apoptosis at 72 h (Fig. 3; P<0.01).

The effect of cardamonin on caspase-3/9 activities levels in 5-FU -colon cancer cell. The apoptotic mechanism of 5-FU-resistant HCT166 cells treated with cardamonin was
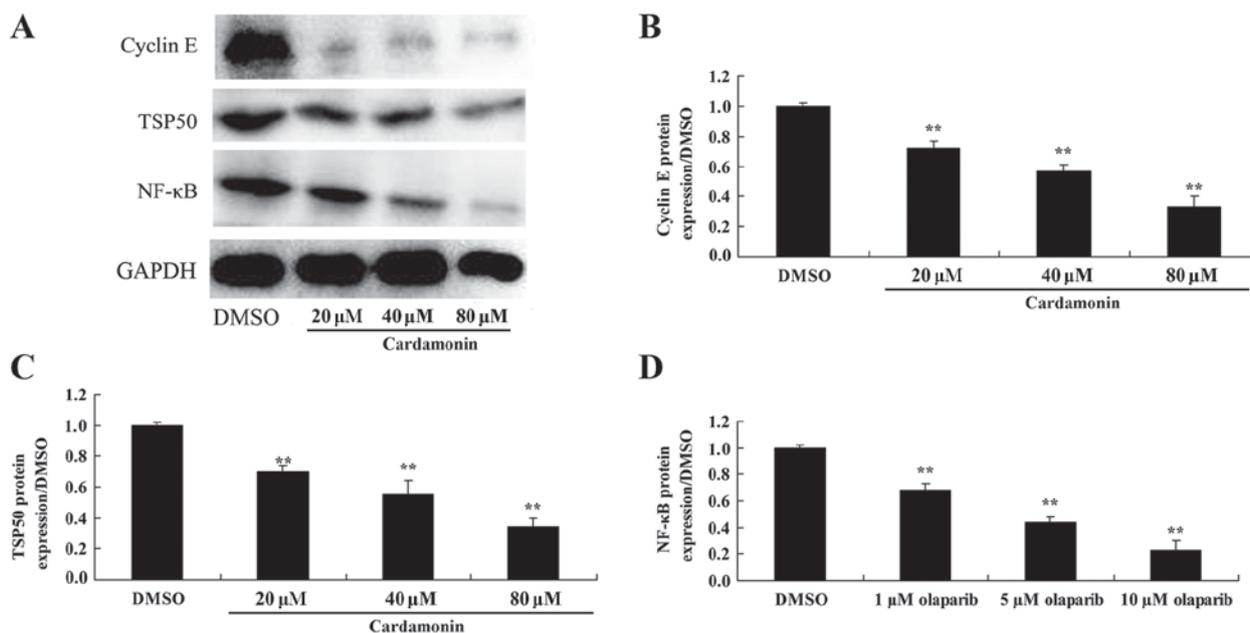
Figure 6. (A) Western blotting and densitometry analysis of the effect of cardamonin on (B) cyclin E, (C) TSP50 and (D) NF-κB protein expression in 5-fluorouracil-resistant colon cancer cells. **P<0.01 vs. DMSO control group. TSP50, testes-specific protease 50; NF-κB, nuclear factor-κB; DMSO, dimethyl sulfoxide.

analyzed. The activity of caspase-3 and -9 was measured using commercial kits. Caspase-3/9 activity was significantly increased in 5-FU-resistant HCT166 cells treated with 40-80 μM of cardamonin compared with the control group (Fig. 4; P<0.01).

The effect of cardamonin on Bax, c-MYC and Oct4 protein expression in 5-FU-resistant colon cancer cells. The influence of cardamonin on Bax, c-MYC and Oct4 protein expression in chemotherapy-resistant HCT116 cells was assessed. Compared with the control, 40-80 μM of cardamonin significantly increased Bax protein expression and suppressed that of c-MYC and Oct4 in 5-FU-resistant HCT166 cells (Fig. 5; P<0.01).

The effect of cardamonin on cyclin E, TSP50 and NF-κB protein expression in 5-FU-resistant colon cancer cells. Western blotting was used to investigate the possible mechanism by which cardamonin exhibits its anticancer effect. The results revealed that 40-80 μM of cardamonin significantly suppressed of cyclin E, TSP50 and NF-κB protein expression in 5-FU-HCT166 cell, compared with the control group (Fig. 6; P<0.01).

Discussion

Chemotherapy is an important treatment modality for patients with colon cancer; however, there are a number of associated negative side effects, including myelosuppression, gastrointestinal reaction and neurovirulence (16). As a result, the development of effective treatments without these effects is a frequently studied topic in the field of oncology (17). It has been reported that TCM exhibits favorable effects in colon cancer, improving symptoms and increasing the survival rate and patient quality of life (17). In addition, TCM avoids the side effects of chemotherapy by treating them (18). In the
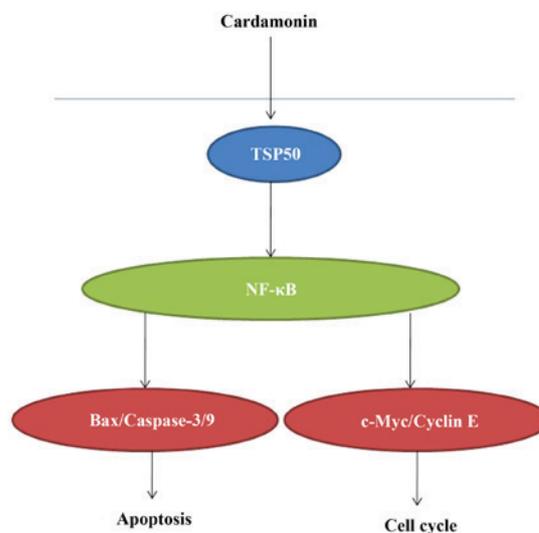
Figure 7. Cardamonin suppresses chemotherapy-resistance in colon cancer cells via the TSP50/NF-κB pathway *in vitro*. TSP50, testes-specific protease 50; NF-κB, nuclear factor-κB; Bax, Bcl-2-associated X protein; c-MYC, Myc proto-oncogene protein.

present study, cells were treated with 0, 10, 20, 40 and 80 μM of cardamonin for 24, 48 and 72 h, as previously described (15); the results indicated that cardamonin significantly reduced cell viability and induced apoptosis in 5-FU-resistant HCT116 cells. Jia *et al* (14) reported that cardamonin reduced chemotherapy-enriched breast cancer.

NF-κB serves an important role in the development and progression of tumors (19). NF-κB activation is associated with oncogenesis, angiogenesis, distant metastasis, anti-apoptosis and chemotherapy resistance (19). The association between NF-κB activation and chemotherapy resistance has previously been demonstrated (19). Cells overexpressing NF-κB

were insensitive to the chemotherapeutic bleomycin in B-cell lymphoma (20). In the present study, cardamonin significantly suppressed NF-κB protein expression in 5-FU-resistant HCT166 cells.

Apoptosis is an important physiological and pathological process (21). When external stimulating factors act on pro-apoptotic proteins, including caspase-3 and Bax, apoptotic pathways are activated (22). When external stimulating factors act on anti-apoptotic proteins, such as B-cell lymphoma 2, apoptosis is restrained and cell survival increases (22). Apoptosis is regulated by multiple factors, including NF-κB which is activated by a number of stimuli and suppressed apoptosis by regulating the expression of pro- and anti-apoptotic genes (23). The results of the present study indicate that cardamonin significantly increases caspase-3/9 activity and Bax expression in 5-FU-resistant HCT116 cell.

c-MYC is a nuclear protein, and can bind chromosomal DNA and regulates cell growth, differentiation and malignant transformation (24). In a number of human cancer cell lines, including myelogenous leukemia, retinoblastoma, neuroblastoma, breast cancer and lung cancer, the relevant sequences of c-MYC are amplified (25). Gene amplification of c-MYC is also observed in cell lines of humans' colon cancer (25,26). These findings also indicate that c-MYC has potential as an anti-tumor target. The expression of c-MYC is associated with cell growth and proliferation and is a potential inducible factor of apoptosis (27). The results of the present study demonstrate that cardamonin significantly suppresses c-MYC protein expression in 5-FU-resistant HCT166 cells. Park *et al* (28) demonstrated that cardamonin suppresses the proliferation of colon cancer cells by inhibiting the expression of cyclin D1 and c-MYC (28).

Oct4 regulates the cellular function of human embryonic stem cells (29). There are epithelial and melanin stem cells positive for Oct4 expression in human hair follicle tissues which are yet to differentiate into multiple cells (30). A previous study reported that Oct4 was abnormally expressed in malignant colorectal, lung and breast cancers (30). Oct4 expression is associated with the occurrence and development of tumors and the prognosis of patients (30). A prior study reported that Oct4 serves an important role in the drug resistance of malignant tumors, including prostate and liver tumors (30). Oct4 mediates chemotherapy resistance via the Oct4-AKT-ABCG2 pathway (31). Together, these studies suggest that cardamonin suppresses Oct4 protein expression in 5-FU-resistant HCT166 cells.

The expression of TSP50 promotes tumor development (32). TSP50 gene silencing in mouse teratocarcinoma P19 cells has been reported to suppress tumor cell proliferation, colony formation and migration, induce apoptosis and enhance sensitivity to doxorubicin (33). The mechanism by which this occurs is reported to be associated with the n E proteins in 5-FU-resistant HCT116 cells. Mi *et al* (15) reported that cardamonin inhibited cell viability via blocking the activation of the TSP50-mediated NF-κB signaling pathway in cancer cells (15). It is possible that repression of the NF-κB signaling and cyclin E proteins in 5-FU-resistant HCT116 cells. Mi *et al* (15) reported that cardamonin inhibited cell viability via blocking the activation of the TSP50-mediated NF-κB signaling pathway in cancer cells (15). It is possible that repression of the NF-κB signaling pathway is the mechanism by which cardamonin elicits its anticancer effect in chemotherapy-resistant colon cancer cells.

In conclusion, the results of the present study demonstrated that cardamonin significantly suppresses chemotherapy-resistant colon cancer cell growth, induces apoptosis and promotes the activation of caspase-3/9 and Bax expression. These data suggest that the anticancer effect of cardamonin in 5-FU-resitant HCT116 cells may be mediated via TSP50/NF-κB protein expression (Fig. 7). Cardamonin may therefore be a potential treatment for chemotherapy-resistant colon cancer and should be researched further.

Competing interests

The authors declare that they have no competing interests.

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