

# Thymoquinone chemosensitizes colon cancer cells through inhibition of NF- $\kappa$ B

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**Abstract.** In the present study, the effects and molecular mechanisms of thymoquinone (TQ) on colon cancer cells were investigated. Cell viability was determined using a Cell Counting Kit-8 assay, and the results revealed that treatment with TQ significantly decreased cell viability in COLO205 and HCT116 cells in a dose-dependent manner. TQ treatment additionally sensitized COLO205 and HCT116 cells to cisplatin therapy in a concentration-dependent manner. To investigate the molecular mechanisms of TQ action, western blot analysis was used to determine the levels of phosphorylated p65 and nuclear factor- $\kappa$ B (NF- $\kappa$ B)-regulated gene products vascular endothelial growth factor (VEGF), c-Myc and B-cell lymphoma 2 (Bcl-2). The results indicated that TQ treatment significantly decreased the level of phosphorylated p65 in the nucleus, which indicated the inhibition of NF- $\kappa$ B activation by TQ treatment. Treatment with TQ also decreased the expression levels of VEGF, c-Myc and Bcl-2. In addition, the inhibition of NF- $\kappa$ B activation with a specific inhibitor, pyrrolidine dithiocarbamate, potentiated the induction of cell death and caused a chemosensitization effect of TQ in colon cancer cells. Overall, the results of the present study suggested that TQ induced cell death and chemosensitized colon cancer cells by inhibiting NF- $\kappa$ B signaling.

## Introduction

Cancer is a significant public health problem worldwide. According to the World Cancer Research Fund International, ~12.7 million cancer-associated mortalities (13% of all mortalities) occurred worldwide in 2008, with males accounting for 6.6 million mortalities and females accounting

for 6 million (1). In developed countries, colorectal cancer is one of the most commonly observed types of cancer, ranking 2nd and 3rd in women and men, respectively (1). The lifetime risk for colorectal cancer development in the general population is ~6%, and colorectal cancer is responsible for ~8% of all cancer-associated mortalities worldwide (1). Among the Chinese population, the incidence of colorectal cancer is increasing (2). At present, no optimal adjuvant chemotherapy exists for clinical use; therefore, developing rationally designed, novel adjuvant therapeutic tools for the treatment of colon cancer is a constant requirement (2). Previously, the use of natural substances, including curcumin, eicosapentaenoic acid, apple polyphenols, capsaicin and thymoquinone (TQ), for cancer chemoprevention has been investigated (3-5). TQ is the primary active ingredient of volatile *Nigella sativa* (black cumin) seed oil, which is used as a spice in countries with a low incidence of colorectal cancer, including Egypt, Pakistan and India (6). Traditional medicine has taken advantage of the anti-inflammatory, antioxidant and anticarcinogenic properties associated with TQ, which supports the hypothesis of TQ being a promising dietary chemopreventive agent (6). In the previous decade, the antitumor activity of TQ has been investigated in a number of studies (6-8). TQ was observed to induce antitumor effects in several types of cancer, including breast (9), lung (10), multiple myeloma (11), pancreatic (12), cervical (13), colon (14) and prostate cancer (15), as well as squamous (16) and hepatocellular carcinoma (17), acute lymphoblastic leukemia (18), glioblastoma (19), osteosarcoma (20), neuroblastoma (21), bladder (22), gastric (23) and ovarian cancer (24). Although the effect of TQ has been investigated in numerous types of cancer, the molecular mechanisms underlying its action remain to be elucidated. The present study investigated the effect of TQ on colon cancer cell growth and the underlying molecular mechanisms. In addition, the present study identified the effect and molecular mechanism of TQ action on the chemosensitivity of colon cancer cells to cisplatin (CisPt).

## Materials and methods

**Cell culture and materials.** TQ, PDTC, Tris, glycine, NaCl, sodium dodecyl sulfate (SDS), bovine serum albumin (BSA) and  $\beta$ -actin antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). TQ was dissolved in dimethyl sulfoxide (DMSO; Merck Millipore, Darmstadt, Germany)

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to make a 50 mM stock solution and stored at  $-20^{\circ}\text{C}$  until use in subsequent experiments. Additional dilutions were performed in cell culture medium (RPMI-1640; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) so that the final DMSO concentration was  $<0.1\%$ . Primary antibodies, including rabbit polyclonal anti-human p65 (catalog no., sc-101749; 1:500), rabbit polyclonal anti-human B-cell lymphoma 2 (Bcl-2; catalog no., sc-492; 1:2,000), rabbit polyclonal anti-human vascular endothelial growth factor (VEGF; catalog no., sc-507; 1:1,000), mouse polyclonal anti-human c-Myc (catalog no., sc-40; 1:500), and secondary antibodies, including goat anti-rabbit horseradish peroxidase (HRP)-conjugated (catalog no., sc-2054; 1:10,000) and goat anti-mouse HRP-conjugated (catalog no., sc-2005; 1:10,000) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Primary rabbit polyclonal anti-human  $\beta$ -actin (catalog no., sc-7210; 1:200) antibody was purchased from Sigma-Aldrich. The COLO205 and HCT116 colon cancer cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 2% penicillin/streptomycin (6 mg/ml penicillin, 10 mg/ml streptomycin; Sigma-Aldrich). The cells were cultured in tissue culture flasks ( $75\text{ cm}^2$ ; Corning Incorporated, New York, NY, USA) and incubated at  $37^{\circ}\text{C}$  in a humidified chamber containing 5%  $\text{CO}_2$ .

**Cell viability assay.** Cell viability was determined using a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). Briefly,  $2 \times 10^4$  cells per well were seeded into a 96-well plate (Corning Incorporated). Subsequent to 24 h culturing, cells were treated with cisplatin and TQ for 24 h or TQ alone for 48 h. Finally, 20  $\mu\text{l}$  CCK-8 solution was added to each well, and was incubated for 2 h at  $37^{\circ}\text{C}$ . The optical density (OD) of each well at 450 nm was measured using a VICTOR™ X multi-label reader (PerkinElmer, Inc., Waltham, MA, USA). The percentage cell viability was calculated as follows:  $(\text{OD}_{\text{drug}} / \text{OD}_{\text{control}}) \times 100$ . To analyze the role of nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) in TQ activity, cells ( $2 \times 10^4$  cells per well) were treated with 50  $\mu\text{M}$  pyrrolidine dithiocarbamate (PDTC) in combination with TQ for 12, 24 and 48 h. The percentage cell growth inhibition was calculated as follows:  $(\text{OD}_{\text{control}} - \text{OD}_{\text{drug}}) \times 100$ .

**Preparation of nuclear extract.** Nuclear extracts were prepared as previously described (25). Briefly, cells were harvested, washed twice with ice-cold phosphate buffered saline (PBS; Hyclone, Beijing, China) for 1 min and resuspended in 1 ml of ice-cold PBS. Cells were pelleted by centrifugation at  $12,000 \times g$  for 5 min, suspended in ice-cold buffer [10 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.5 mmol/l  $\text{MgCl}_2$ , 0.2 mmol/l KCl, 0.2 mmol/l phenylmethylsulphonyl fluoride, 0.5 mmol/l dithiothreitol], mixed by vortexing for 10 sec and centrifuged at  $12,000 \times g$  for 5 min. The nuclear pellet was washed in 1 ml buffer (20 mmol/l HEPES, 25% glycerol, 0.42 mol/l NaCl, 1.5 mmol/l  $\text{MgCl}_2$ , 0.2 mmol/l ethylenediaminetetraacetic acid), resuspended in 30 ml buffer, mixed by rotation for 30 min at  $4^{\circ}\text{C}$  and centrifuged at  $12,000 \times g$  for 20 min. Finally, the supernatants were used as nuclear extracts.

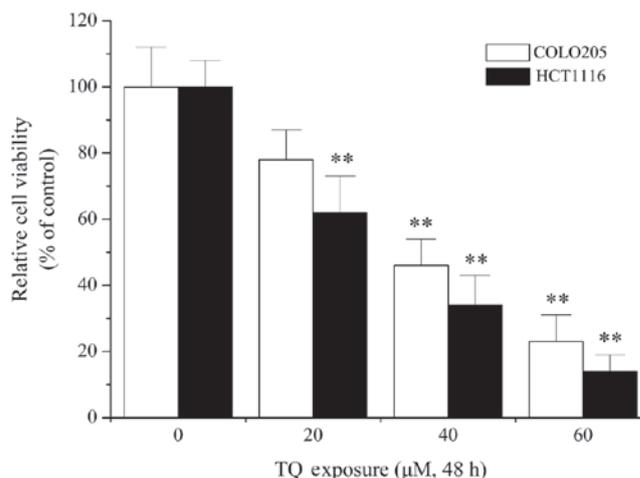


Figure 1. TQ induced death of colon cancer cells. Cultured cells ( $2 \times 10^4$  cells per well) were treated with various concentrations (0, 20, 40 and 60  $\mu\text{M}$ ) of TQ for 48 h. Cell viability was measured using Cell Counting Kit-8 and presented as a percentage of the control. Data are presented as the mean  $\pm$  standard deviation of five experiments. \*\* $P < 0.05$  vs. the respective control group. TQ, thymoquinone.

**Western blot analysis.** Using 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), whole cell lysates or nuclear extracts were electrophoresed, as previously described (26). The proteins were transferred onto a 0.4- $\mu\text{m}$  polyvinylidene difluoride membrane (EMD Millipore, Bedford, MA, USA) in transfer buffer (25 mM Tris, pH 8.5, 0.2 M glycine and 20% methanol). The membranes were blocked by 5% BSA in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h, washed twice with TBST for 10 min each and incubated with primary  $\beta$ -actin, p65, Bcl-2, VEGF and c-Myc antibodies at  $4^{\circ}\text{C}$  overnight. Subsequent to three washes with TBST for 10 min each, the membranes were probed with secondary peroxidase-conjugated antibody (dilution, 1:10,000; Sigma-Aldrich). Finally, the immunoreactive bands were visualized using an enhanced chemiluminescence detection kit (Immobilon WBKLS0500; Merck Millipore).  $\beta$ -actin was used as an internal control for all western blot analyses.

**Statistical analysis.** SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Values were expressed as the mean  $\pm$  standard deviation. Numeric variables were compared by one-way analysis of variance.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Effect of TQ on the viability of colon cancer cells.** Previous studies have shown the antitumor and anticarcinogenic activities of TQ in numerous types of cancer, including breast cancer, glioblastoma and lymphoma (27-29). In order to elucidate the molecular mechanisms underlying the function of TQ, the effect of TQ on colon cancer cells was examined in the present study. As shown in Fig. 1, 20  $\mu\text{M}$  TQ treatment resulted in a significant decrease in cell viability in HCT1116 cells when compared with the control ( $P = 0.013$ ). Furthermore, 40  $\mu\text{M}$  TQ treatment significantly

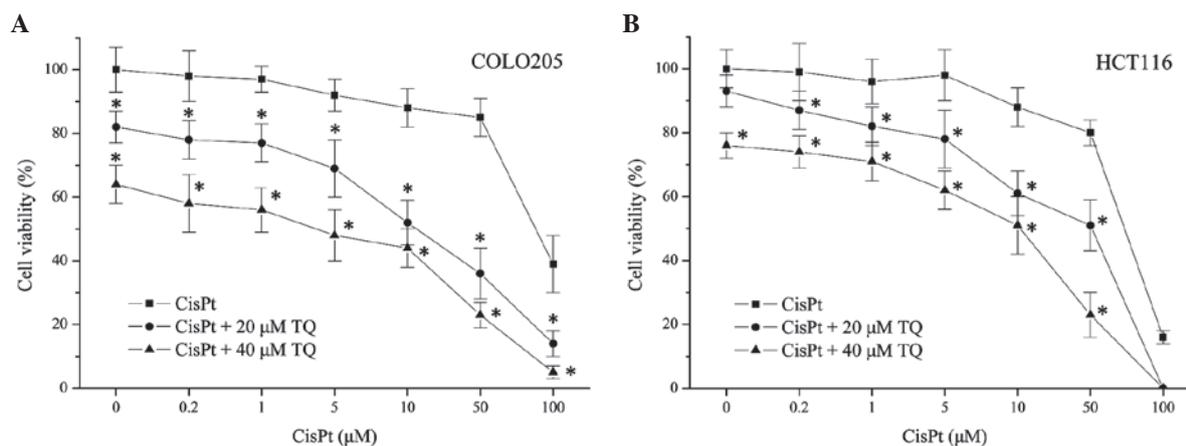


Figure 2. TQ increases the cytotoxicity of CisPt in colon cancer cells. (A) COLO205 cell line; (B) HCT116 cell line. Colon cancer cells were treated with 0.2, 1, 5, 10, 50 and 100  $\mu\text{M}$  CisPt, with or without 20 or 40  $\mu\text{M}$  TQ for 24 h. Cell viability was measured by a Cell Counting Kit-8 and is presented as a percentage of the control. Values are expressed as the mean  $\pm$  standard deviation of five experiments. \* $P < 0.05$  vs. cisplatin alone. TQ, thymoquinone; CisPt, cisplatin.

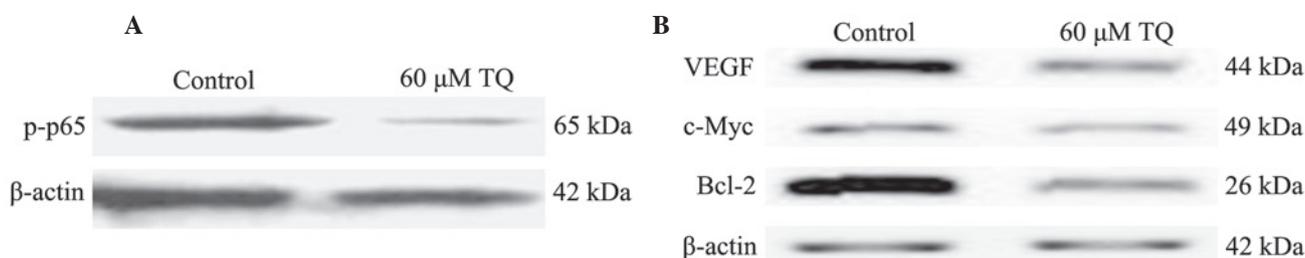


Figure 3. TQ inhibits the activation of NF- $\kappa$ B and downregulates its downstream gene expression. (A) TQ inhibited phosphorylation of the subunit of NF- $\kappa$ B, p65. COLO205 cells were treated with 60  $\mu\text{M}$  TQ for 18 h. Cells were harvested and the expression of phosphorylated p65 in the nucleus was evaluated by western blot analysis.  $\beta$ -actin was used as an internal control. A representative blot of three experiments with similar results is shown. (B) TQ downregulated NF- $\kappa$ B downstream gene products in COLO205 cells. Following 18 h of incubation, the levels of VEGF, c-Myc and Bcl-2 were determined by western blot analysis.  $\beta$ -actin was used as an internal control. A representative blot of three experiments with similar results is shown. TQ, thymoquinone; NF- $\kappa$ B, nuclear factor- $\kappa$ B; VEGF, vascular endothelial growth factor; Bcl-2, B-cell lymphoma 2.

decreased cell viability in COLO205 cells compared with the control ( $P=0.007$ ). Cytotoxicity assays indicated that TQ dose-dependently decreased cell viability of the COLO205 and HCT116 colon cancer cell lines (Fig. 1), which confirmed the antitumor activity of TQ, as previously reported (30,31).

**TQ chemosensitizes colon cancer cells.** The present study aimed to investigate whether TQ affected the sensitivity of colon cancer cells to chemotherapy. Therefore, TQ and CisPt were combined to treat COLO205 and HCT116 colon cancer cell lines. As shown in Fig. 2, treatment with cisplatin alone induced COLO205 and HCT116 cell death in a dose-dependent manner. Combined treatment with cisplatin (0.2  $\mu\text{M}$ ) and TQ (20 or 40  $\mu\text{M}$ ) significantly decreased the cell viability of COLO205 ( $P=0.021$ , 20  $\mu\text{M}$  TQ;  $P=0.003$ , 40  $\mu\text{M}$  TQ) and HCT116 cells ( $P=0.038$ , 20  $\mu\text{M}$  TQ;  $P=0.004$ , 40  $\mu\text{M}$  TQ) when compared with 0.2  $\mu\text{M}$  cisplatin treatment alone. These results revealed that cell death induced by CisPt was enhanced by TQ in a concentration-dependent manner (Fig. 2), which indicated that TQ may potentiate the chemosensitivity of colon cancer cells.

**Role of NF- $\kappa$ B in TQ activity.** As demonstrated in the present results, TQ treatment may result in cell death and chemosensitizing of colon cancer cells. Therefore, the present study

aimed to investigate the underlying mechanisms of TQ action, including the role of NF- $\kappa$ B in the process. Western blot analysis was used to determine the effect of TQ on NF- $\kappa$ B activation. The results demonstrated that 60  $\mu\text{M}$  TQ significantly inhibited the phosphorylation of p65 protein, subunit of NF- $\kappa$ B (Fig. 3A). Furthermore, the expression levels of NF- $\kappa$ B-regulated genes that are involved in tumor angiogenesis, survival and apoptosis were measured. As demonstrated in Fig. 3B, protein levels of VEGF, c-Myc and Bcl-2 were markedly downregulated following 60  $\mu\text{M}$  TQ treatment.

**NF- $\kappa$ B inhibitor, PDTC, potentiates the activity of TQ.** As TQ inhibited NF- $\kappa$ B activation, the effect of NF- $\kappa$ B on TQ function was assessed. The NF- $\kappa$ B inhibitor, PDTC, was used to investigate the activity of TQ. As demonstrated in Fig. 4, combined treatment with 10  $\mu\text{M}$  cisplatin and 40  $\mu\text{M}$  TQ treatment for 24 or 48 h significantly inhibited cell growth inhibition when compared with 40  $\mu\text{M}$  TQ treatment alone ( $P=0.018$  and  $P=0.009$ , respectively) in COLO205 cells. Treatment with 50  $\mu\text{M}$  PDTC further potentiated the growth inhibition observed following 24 h ( $P=0.002$ ) and 48 h ( $P=0.031$ ) treatment when compared with 10  $\mu\text{M}$  cisplatin and 40  $\mu\text{M}$  TQ combined treatment. For HCT116 cells, following 12, 24 and 48 h treatment with 50  $\mu\text{M}$  PDTC, 10  $\mu\text{M}$  cisplatin and 40  $\mu\text{M}$  TQ significantly inhibited cell growth inhibition

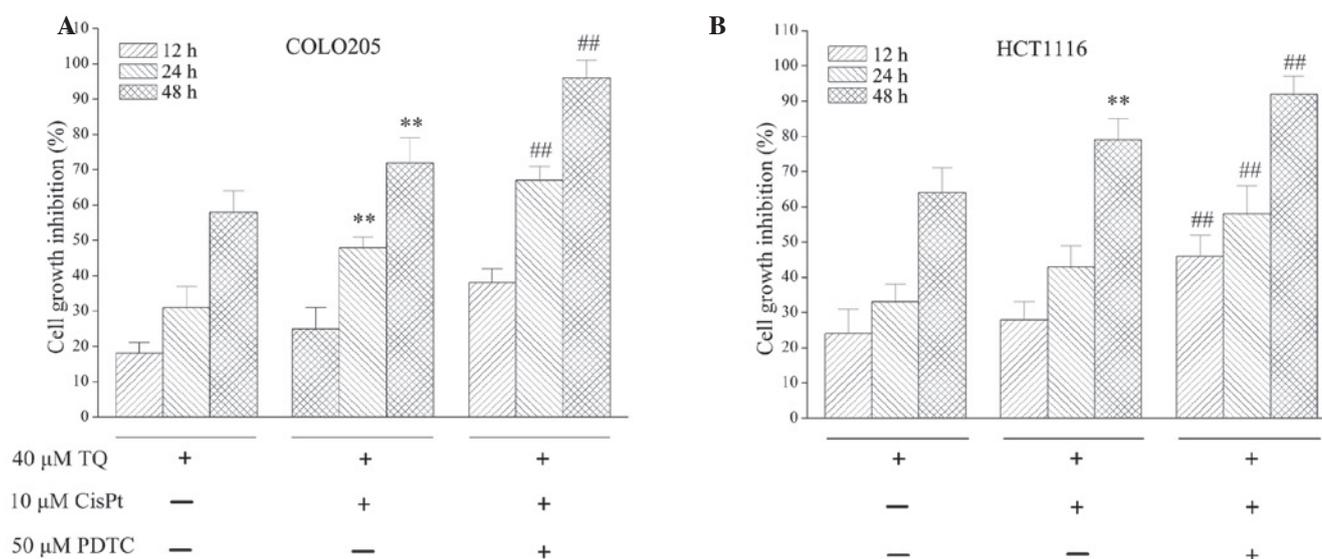


Figure 4. PDTC, a nuclear factor- $\kappa$ B inhibitor, potentiated the death-inducing effect of TQ on colon cancer cells. (A) COLO205 cell line; (B) HCT116 cell line. Cultured cells were seeded in a 96-well plate at a density of  $2 \times 10^4$  cells per well and treated with  $40 \mu\text{M}$  TQ, with or without  $50 \mu\text{M}$  PDTC. Following 12, 24 or 48 h incubation, cell viability was determined with a Cell Counting Kit-8. Cell viability is presented as a percentage of the control. Results are presented as the mean  $\pm$  standard deviation of at least three independent experiments. \*\* $P < 0.05$  vs. TQ treatment; ## $P < 0.05$  vs. TQ+CisPt treatment. TQ, thymoquinone; PDTC, pyrrolidine dithiocarbamate; CisPt, cisplatin.

( $P = 0.014$ ,  $P = 0.043$  and  $P = 0.027$ , respectively) when compared with combined  $10 \mu\text{M}$  cisplatin and  $40 \mu\text{M}$  TQ treatment. Furthermore, a chemosensitization effect of TQ on colon cancer cells was enhanced by  $50 \mu\text{M}$  PDTC treatment, which was demonstrated by increased cell growth inhibition (Fig. 4).

## Discussion

*Nigella sativa* is an annual herbaceous plant belonging to the Ranunculaceae family, which has been commonly used in traditional Middle Eastern folk medicine as a natural remedy for various ailments for  $>2,000$  years (8). *Nigella sativa* is additionally used as a food additive and flavoring in numerous countries (8). TQ, or 2-isopropyl-5-methyl-1,4-benzoquinone, is a *Nigella sativa* essential oil that is known to be the principal active compound of the seed, and is responsible for a number of its antioxidant and anti-inflammatory effects (6,8).

In 2003, Shoieb *et al* (32) reported *in vitro* experimental results that revealed TQ was able to inhibit growth and induce apoptosis in cancer cell lines. Since then, increasing numbers of studies have focused on TQ in cancer therapy. Numerous *in vitro* and *in vivo* studies have investigated the antitumor activity of TQ in several types of cancer (6,14,19,27-29,33). In tumor protein p53-null myeloblastic leukemia HL-60 cells, TQ induced apoptosis through an intrinsic signaling pathway (34). In human multiple myeloma cells, TQ inhibits proliferation, induces apoptosis and exerts a chemosensitization effect through suppressing signal transducer and activator of transcription 3 (acute-phase response factor) activation (35), and decreases F-actin polymerization and Bcl-2/Bcl-2 like 1 expression (36). TQ-induced apoptosis has been indicated to be mediated by reactive oxygen species generation (29,33,37) and the mitogen-activated protein kinase 14 signaling pathway (33). In 2004, Gali-Muhtasib *et al* (38) reported that TQ triggered apoptotic cell death in human colorectal cancer cells via a p53-dependent mechanism, and in 2008, Gali-Muhtasib *et al* (31) demonstrated

that TQ triggered inactivation of the stress response pathway sensor checkpoint kinase 1 and contributed to apoptosis in colorectal cancer cells. Gali-Muhtasib *et al* (30) additionally indicated that TQ may inhibit colon tumor cell invasion (30), which was confirmed by additional studies (39-41).

A recent study reported that TQ exerted an antitumor effect through the interruption of pro-survival mitogen-activated protein kinase kinase 7-mitogen-activated protein kinase 1 signaling in colorectal cancer (14). TQ exerted a direct antitumor effect, and also sensitized cancer cells to other therapies (23,42-44). In general, cancer cells may be initially susceptible to chemotherapy; however, over time they may develop resistance through certain mechanisms, including DNA mutations and metabolic changes that promote drug inhibition and degradation. Drug resistance has been a challenge for clinical cancer treatment (45). Velho-Pereira *et al* (42) reported that TQ may radiosensitize human breast carcinoma cells. Jafri *et al* (43) indicated that in lung cancer, TQ treatment may overcome resistance and sensitize lung cancer cells to CisPt. Previous studies have revealed the chemosensitization and radiosensitization effect of TQ in pancreatic (44), lung (43), gastric (23) and breast cancer (42). However, the mechanism by which TQ affects the sensitivity of colon cancer to chemotherapy has not been investigated. In the present study, the results confirmed the antitumor activity of TQ in colon cancer cells and provided novel evidence that TQ sensitizes colon cancer cells to CisPt by suppressing NF- $\kappa$ B activation.

NF- $\kappa$ B is an ubiquitous transcription factor, consisting of p50, p65 and nuclear factor- $\kappa$ B inhibitor  $\alpha$  (I $\kappa$ B $\alpha$ ), which is present in the cytoplasm and is activated in response to certain inflammatory stimuli, environmental pollutants, prooxidants, carcinogens, stress and growth factors (46). Following activation, NF- $\kappa$ B translocates from the cytoplasm to the nucleus, binds DNA and induces gene transcription. A number of kinases have been associated with the activation of NF- $\kappa$ B, including I $\kappa$ B $\alpha$  kinase. This activation has been shown to

result in the expression of a number of gene products that regulate apoptosis, proliferation, chemoresistance, radioresistance, invasion, angiogenesis, metastasis and inflammation (47,48). In numerous human cancers NF- $\kappa$ B is constitutively activated (49-51). NF- $\kappa$ B activation has been associated with various aspects of oncogenesis, including control of apoptosis, cell cycle, differentiation and cell migration (52,53). In addition, the activation of NF- $\kappa$ B in cancer cells by chemotherapy or radiation may hinder the ability of cancer therapy to induce cell death; therefore, NF- $\kappa$ B has been used as a target for tumor therapies (52,53). In colon cancer, via the regulation of numerous genes differentially expressed and implicated in tumorigenesis, NF- $\kappa$ B activation participates in the promotion and progression steps of colon cancer (54). The present study investigated the effect of TQ on NF- $\kappa$ B activation, and additionally examined the effect of TQ on NF- $\kappa$ B-regulated gene products. The results of the present study revealed that TQ treatment inhibited the phosphorylation of p65 protein in the nucleus of colon cancer cells and decreased the expression of NF- $\kappa$ B-regulated genes, including VEGF, c-Myc and Bcl-2. In addition, the inhibition of NF- $\kappa$ B with a specific inhibitor, PDTC, may potentiate the cell death induction and chemosensitization effect of TQ in colon cancer cells.

In conclusion, the present study demonstrated that TQ may result in cell death in colon cancer cells and sensitize colon cancer cells to CisPt therapy by suppressing NF- $\kappa$ B activation. TQ may be a positive option for adjuvant chemotherapy in the treatment of colon cancer.

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