Thymoquinone induces G2/M arrest, inactivates PI3K/Akt and nuclear factor-κB pathways in human cholangiocarcinomas both *in vitro* and *in vivo*

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Abstract. Cholangiocarcinoma (CCA) is a notoriously lethal tumor mostly due to the *de novo* or acquired resistance to traditional chemotherapy besides gemcitabine and, therefore, an increasing need for effective strategies to prevent and treat the poor prognosis of this tumor is required. Thymoquinone (TQ), a hopeful natural product derived from black cumin (*Nigella sativa*) has shown considerable antineoplastic properties. Whether TQ exerts antitumor effects on CCA cells *in vitro* and *in vivo* remains unknown. Examinations of cell viability assay, detection of cell cycle and apoptosis, electrophoretic mobility shift assay (EMSA), western blotting and immunohistochemistry were used in the present study. We demonstrated that TQ inhibited the growth of human CCA cell lines (TFK-1 and HuCCT1) in a dose- and time-dependent manner. Firstly, our results provided evidence that TQ not only inhibits the proliferation of CCA cells, induces cell cycle arrest and prompts cell apoptotic effect *in vitro*, but it also exhibits inhibitory effects of tumor growth and angiogenesis *in vivo*. The responsible mechanism is at least partially due to TQ inhibiting the growth of CCA cell lines induced by downregulation of PI3K/Akt and NF-κB and regulated gene products, including p-AKT, p65, XIAP, Bcl-2, COX-2, VEGF. Taken together, these results provide strong evidence of our hypothesis that TQ alone presents a promising therapeutic regimen for the treatment of CCA cells with better efficiency.

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Introduction

Cholangiocarcinoma (CCA) is an aggressive malignant tumor that originates from bile duct epithelial cells (1). This cancer is the second most common primary hepatic malignancy, after hepatocellular cancer (2). There is a significant geographical variation in the incidence of CCA, with the highest incidence occurring in eastern Asian countries including Thailand, Korea, China and Japan (3,4). Conditions that result in prolonged biliary inflammation, such as primary sclerosing cholangitis (PSC), liver fluke infestation, congenital abnormalities, such as choledochal cysts, and hepatitis B and C, predispose patients to developing CCA (5). CCA is often detected at an advanced stage, which has a very poor prognosis, and a 5-year survival rate of <5-10% (6). Additionally, CCA tends to remain clinically insidious until it has progressed to a terminal stage. Despite the limited clinical benefits, chemotherapy represents the cornerstone of disease management for patients with inoperable CCA (7). Although novel anticancer drugs and therapeutic regimens have been extensively explored, few treatment options have exhibited promising results in improving the outcome of this lethal disease (8,9). Therefore, the development of new therapeutic strategies is urgently needed.

Therapeutic agents, including gemcitabine, cisplatin or 5-FU, cause major side-effects when given at the maximum tolerated doses, and CCA fails to adequately respond to these drugs due to the acquisition of chemoresistance (1). In the current literature, there is no neoadjuvant therapy that is considered a standard approach for the treatment of patients with CCA (10). Therefore, we examined whether some natural medications that possess potent antitumor, anti-inflammatory and anti-angiogenic functions may be more efficient in treating CCA.

Thymoquinone (TQ) is a bioactive ingredient derived from black seed (*Nigella sativa*) oil (Fig. 1A) (11,12). In folk medicine, the seed is reportedly associated with anti-inflammatory effects and has been shown to protect against bronchial asthma, dysentery, headache and gastrointestinal problems (13). Furthermore, TQ has been reported to exhibit anticancer effects on cell lines derived from breast, colon, ovarian, larynx

and lung cancer, as well as myeloblastic leukemia and osteosarcomas, without any serious side-effects (14,15).

To corroborate the anti-inflammatory and antitumor activity of TQ, we sought to understand the activity of TQ in human CCA cell lines and in a CCA-inoculated mouse model and to elucidate the potential mechanism of action. Among the various molecular defects that allow CCA cells to evade drug-induced apoptotic signaling, the activation of the AKT and nuclear factor-κB (NF-κB) pathways is particularly obvious (16-18). Constitutive activation of the AKT and NF-κB pathways has been reported to play a key role in cell proliferation, invasion, angiogenesis, metastasis, suppression of apoptosis and chemoresistance of CCA (19,20). However, the potential efficacy of TQ in the human CCA cells and the possible mechanism of action remain unknown.

Materials and methods

Materials. The p-AKT, AKT, XIAP, COX-2, p65 and cyclin B1 antibodies were obtained from Cell Signaling. The Bcl-2 and Bax antibodies were purchased from Santa Cruz Biotechnology, and the anti-β-actin antibody was purchased from Sigma. Thymoquinone (Sigma) was dissolved in dehydrated alcohol to create a 20 mmol/l stock solution.

Cell culture. TFK-1 cells, which are moderately differentiated, and HuCCT1 cells, which are poorly differentiated, were kindly provided by the Cancer Cell Repository, Tohoku University, Japan. The cells were grown in monolayers in RPMI-1640 medium, supplemented with antibiotics, 10% fetal bovine serum (FBS) (all from Gibco) and 2 mmol/l glutamine at 37˚C with 5% CO_2 in a humidified atmosphere.

Cell viability assay. To assess cell viability, both the TFK-1 and HuCCT1 cells were seeded in 96-well plates at a density of $3x10^3$ cells/well and allowed to adhere and grow overnight in RPMI-1640 medium containing 10% heat-inactivated FBS. Cell Counting Kit-8 (CCK-8; Dojindo Lab-Orgemcitabineries, Kumamoto, Japan) was used to determine the viability of the cells. The cells were then cultured with TQ (0, 10, 20, 40, 60, or 80 μM) for 24, 48 or 72 h. Cell viability was measured using the CCK-8 kit according to the manufacturer's instructions, as previously described (22). Three independent experiments were performed.

Cell cycle analysis. The CycleTest™ Plus DNA Reagent kit (BD Biosciences, San Jose, CA, USA) was used to identify the percentage of cells in the G0-G1, S and G2-M phases of the cell cycle. Subconfluent CCA cells were plated at a density of $1x10^6$ cells/well in 6-well plates. After treatment with TQ (0-60 μM) for 48 h, the supernatant was discarded, and the cells were washed with phosphate-buffered saline (PBS), then centrifuged. The cells were washed twice with ice-cold PBS, and 3 ml of ice-cold 70% ethanol was added. The cells were then incubated for 1 h at 4˚C. The cells were washed twice with PBS, and 10 mg/ml RNase A was added. Propidium iodide (PI) was added to the tubes at a final concentration of 0.05 mg/ml, and the cells were incubated at 4˚C for 30 min in the dark. Flow cytometric analysis was performed using FACScan (Becton-Dickinson) to detect the percentage of cells in the various phases of the cell cycle, as previously described (22). The experiments were repeated three times.

Detection of cell apoptosis. The PI/Annexin V-FITC apoptosis detection kit (BD Biosciences) was used to assess the number of apoptotic CCA cells after treatment with TQ; the kit was used according to the manufacturer's instructions. Briefly, following treatment, the cells were harvested with trypsin, washed in PBS and counted. The cells were then resuspended in binding buffer, and 5 ml of Annexin V and 5 ml of PI were added. The cells were then incubated at room temperature for at least 15 min in the dark. The percentage of apoptotic cells was analyzed using flow cytometry (Epics Altra II; Beckman Coulter, USA) as previously described (23). The experiments were repeated three times.

Electrophoretic mobility shift assay (EMSA). Nuclear extract (5 μg) was incubated with 1 μg of poly(deoxyinosinic-deoxycytidylic acid) in binding buffer for 30 min at 4˚C. DNA-binding activity was confirmed using a biotin-labeled oligonucleotide bio-NF-κB probe (5'-AGTTGAGGGGACTTTCCCAGGC-3') using an EMSA kit according to the manufacturer's instructions (Viagene, Beijing, China). The probe was resolved on a 4% polyacrylamide gel containing 0.25X TBE (Tris/borate/EDTA) buffer and visualized using a Cool Imager imaging system (IMGR002; Viagene).

Western blot assay. The method was described previously (24). Briefly, the cells were washed twice in PBS, sonicated in lysis buffer and homogenized; alternatively, tumor tissues were excised, minced and homogenized in protein lysate buffer. Debris was removed by centrifugation. Samples (20 μg) of the total protein lysate were resolved on 12% polyacrylamide SDS gels and were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skim milk in TBST buffer (TBS plus 0.1% Tween-20), incubated with the appropriate primary Ab and subsequently incubated with an alkaline phosphatase-conjugated secondary Ab. The blots were developed using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Tiangen Biotech Co., Ltd., Beijing, China). The membranes were then washed, and the protein bands were visualized following exposure of the membrane to X-ray film. β-actin was used as a loading control.

Animal model and treatments. Surgical procedures and administration of drugs to the animals were in accordance with the Institutional Animal Ethics Guidelines. Tumors were fixed by subcutaneous injection of $4x10^6$ HuCCT-1 tumor cells into the flanks of the mice. The tumor volumes were approximated according to the formula: $\pi/6xa^2xb$, where a is the short axis, and b is the long axis. When the tumors reached ~100 mm^3 after approximately two weeks, the mice were randomly assigned to four groups, each containing seven mice. The mice received either daily doses of PBS or 2, 4 or 8 mg/mouse TQ given daily by intragastric intubation. The doses and methods were based on our preliminary experiments and previous reports (15). The treatments lasted for 20 days, during which time the sizes of the tumors were documented. The mice were euthanized three days after the last treatment, and the tumors

were excised, weighed and fixed in 10% buffered formalin for immunohistochemistry and apoptotic assays.

Immunohistochemistry. Immunohistochemistry analysis was performed using an anti-Ki67 Ab. Briefly, after deparaffinization, rehydration and antigen retrieval, the permeabilized sections (4 μm) were blocked with 3% bovine serum albumin (BSA) for 2 h and incubated overnight with primary Abs. The sections were subsequently cultivated for 30 min with IgG, mounted and examined under a light microscope or a fluorescence microscope.

In situ detection of apoptotic cells. Histological analysis of DNA fragmentation was performed to identify the apoptotic cells. Tumor sections were stained with the TUNEL reagent (Roche, Shanghai, China), and the TUNEL-positive cells were counted in 10 randomly selected x400 high-power fields. The apoptosis index was calculated according to the following formula: The number of apoptotic cells/total number of nucleated cells x 100%.

Statistical analysis. The data obtained are expressed as the mean values ± standard deviation of at least three separate experiments. Statistical significance was determined using Student's t-test, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

TQ exerts antiproliferative effects in CCA cells in vitro. To determine the effects of TQ on cell growth, the CCA cell lines (TFK-1 and HuCCT1) were treated with varying concentrations (0-80 μM) of TQ for 24, 48 and 72 h, and cell viability was assessed using the CCK-8 assay. As shown in Fig. 1B and C, TQ treatment resulted in a dose- and time-dependent inhibition of cell growth in both the CCA cell lines tested. The inhibitory concentration IC_{50} values were ~62.91, 33.47 and 23.17 μM for the TFK-1 cells and 48.27, 27.51 and 20.28 μM for the HuCCT1 cells for the 24, 48 and 72 h treatments, respectively. Of note, TQ inhibited the poorly differentiated HuCCT1 cell line more than the moderately differentiated TFK-1 cell line. These results indicate that TQ has potent antiproliferative effects in CCA cell lines.

TQ induces G2/M phase cell cycle arrest and alters the expression of G2/M phase cell cycle-related proteins in CCA cells. Based on the preliminary assays in which we evaluated the effect of TQ on the growth of CCA cells, the 0, 20, 40 and 60 μM doses of TQ were selected for further *in vitro* mechanistic studies. To explore the underlying mechanism of the TQ-induced growth inhibition of the cells, the effect of TQ on the cell cycle distribution was studied using flow cytometric analysis of the cellular DNA content. As shown in Fig. 2, treatment with TQ for 48 h resulted in a significant dose-dependent arrest of the CCA cells in the G2/M phase of the cell cycle. The G2/M phase cell cycle distribution was 4.05, 9.40, 11.76 and 18.55% in the TFK-1 cells (Fig. 2A) and 5.51, 12.25, 16.14 and 24.02% in the HuCCT1 cells when treated with 0, 20, 40 and 60 μM TQ (Fig. 2B), respectively. This increase in the percentage of cells in G2/M phase was

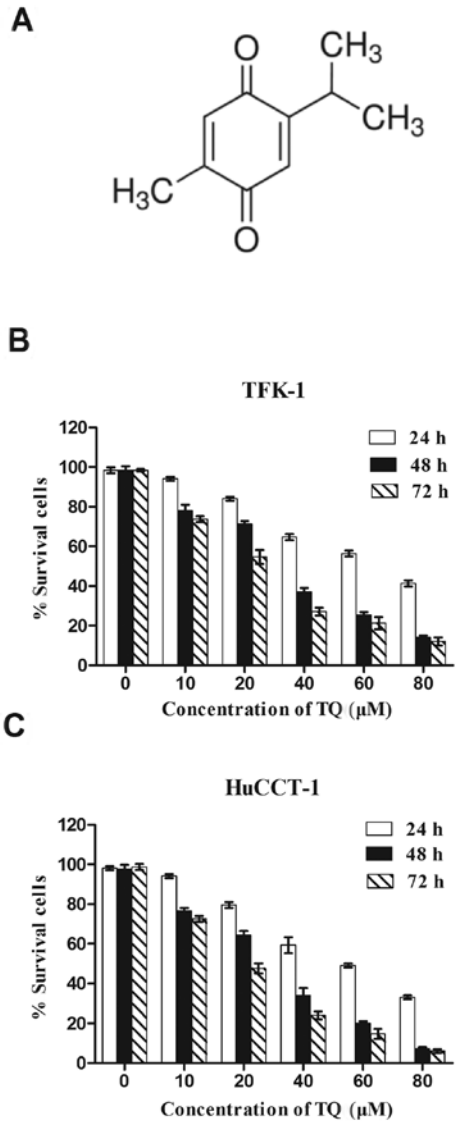

Figure 1. TQ inhibits CCA viability *in vitro*. (A) The structure of TQ. (B and C) CCA lines were incubated with increasing doses of TQ for 24, 48 or 72 h. CCK-8 assay was performed to assess the cell viability index. TQ, thymoquinone; CCA, cholangiocarcinoma.

accompanied by a concomitant reduction in the percentage of cells in G0/G1 phase.

TQ potentiates the apoptosis-inducing effect in CCA cells in vitro. In addition to the cell cycle arrest, morphological observation of the TQ-treated CCA cells indicated that the TQ-induced growth inhibition may also be associated with the induction of apoptosis. Therefore, the apoptosis-inducing effect of TQ in CCA cells was assessed. The cells were treated with various concentrations of TQ (0-60 μM). Then, the cells were stained with Annexin V/PI and subjected to flow cytometry to determine the amount of apoptotic cells. As shown in Fig. 3, treatment with TQ for 48 h resulted in a significant dose-dependent enhancement in both the number of early and late apoptotic CCA cells. The percentages of apoptotic cells were 8.5, 18.0, 26.1 and 67.3% in the TFK-1 cells (Fig. 3A) and 5.9, 21.7, 31.9 and 75.7% in the HuCCT1 cells treated with 0, 20, 40 and 60 μM TQ (Fig. 3B), respectively. Taken together,

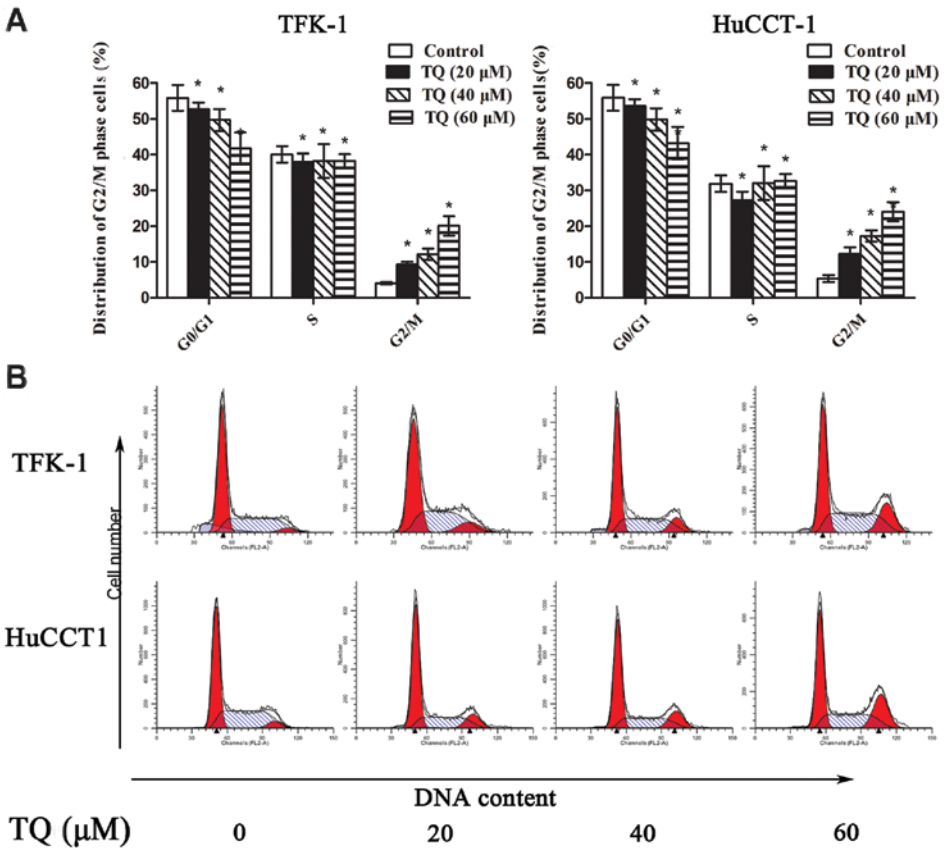

Figure 2. Cell cycle progression *in vitro*. (A) The representative flow cytometry histograms of cell cycle distribution for CCA cells treated for 48 h with solvent control or TQ (20, 40 and 60 μM). (B) The data from cell cycle distribution are representative of at least three independent experiments. *P<0.05, compared with control. CCA, cholangiocarcinoma; TQ, thymoquinone.

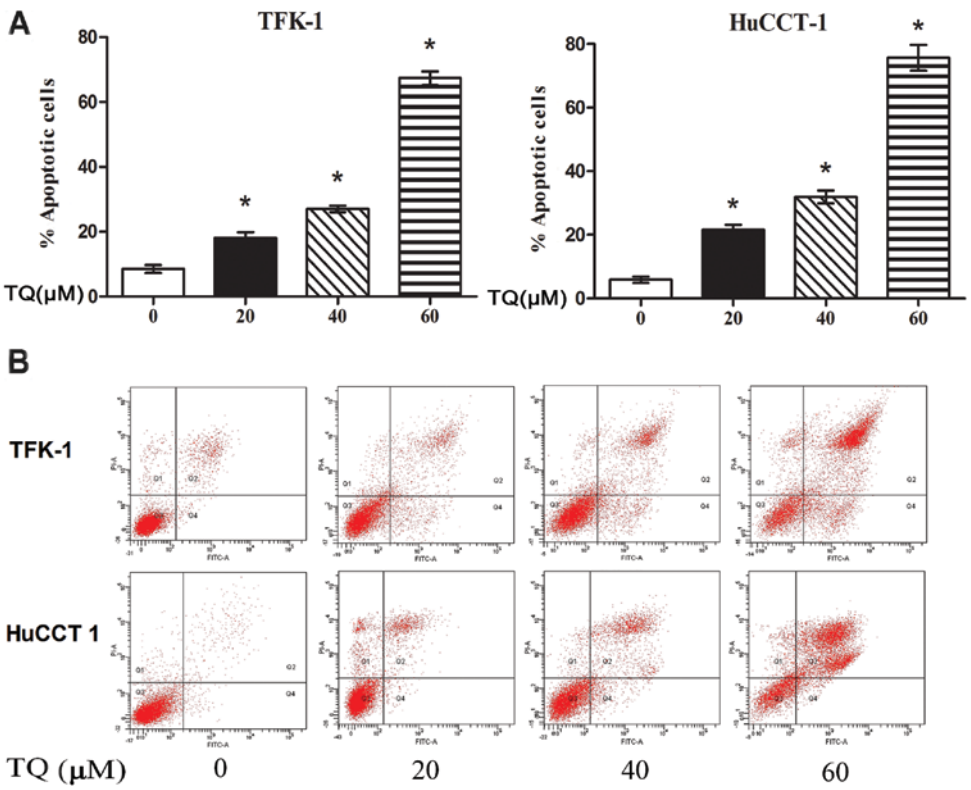

Figure 3. Cell apoptosis *in vitro*. (A) Histograms represent the apoptosis rates of treated HCC cells measured by flow cytometry. Data are presented as the means ± SD of three independent experiments. (B) The representative flow cytometry histograms of cell apoptosis for CCA cells treated for 48 h with solvent control or increasing doses of TQ. *P<0.05, compared with control. CCA, cholangiocarcinoma; TQ, thymoquinone.

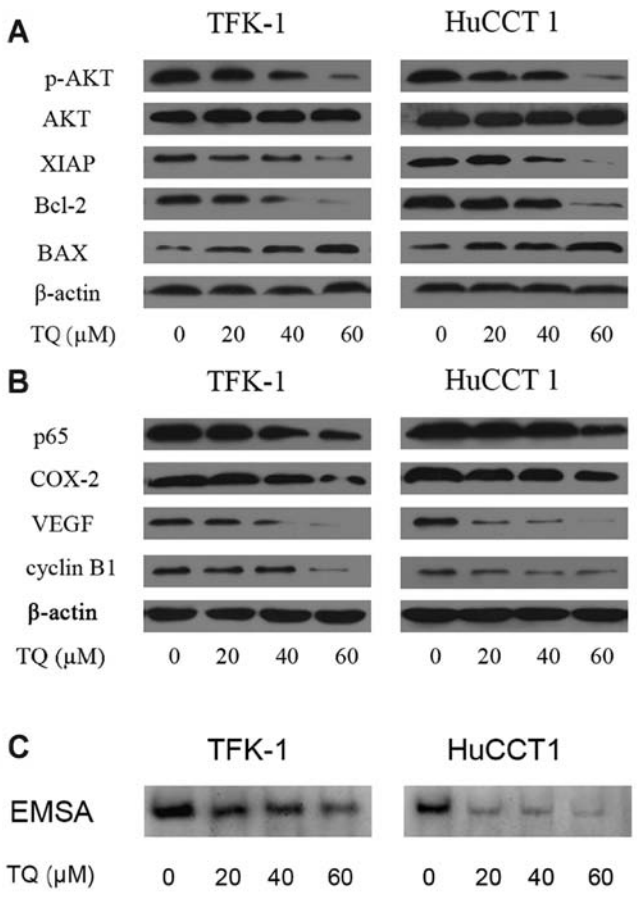

Figure 4. Activation of AKT and NF-κB pathways and the expression of NF-κB regulated gene *in vitro*. (A) Cells were treated with TQ and the protein extracts were analyzed by western blotting using antibodies specific for AKT and phospho-AKT. β-actin served as an internal control. (B) Western blot analysis for p65 and NF-κB-regulated gene products, with β-actin as protein internal control. (C) Nuclear extracts were extracted and subjected to EMSA to measure NF-κB DNA-binding activity. TQ, thymoquinone.

our results suggest that the TQ-induced growth inhibition was partially due to the induction of apoptosis.

TQ suppresses PI3K and Akt activation in CCA cells in vitro. To determine whether the antitumor effect was associated with PI3K/Akt signaling, the TFK-1 and HuCCT-1 cells were treated as described above for 48 h and were then subjected to western blot analysis. As shown in Fig. 4, treatment with TQ resulted in the downregulation of p-Akt in both of the CCA cell lines, whereas the levels of total Akt protein were not altered (Fig. 4A). Furthermore, the downregulation of p-Akt was associated with the downregulation of XIAP and Bcl-2, as well as the upregulation of BAX, which is further evidence of the induction of apoptosis in cells exposed to TQ for 48 h (Fig. 4B). These results suggest that PI3K/Akt signaling was, at least partially, involved in the effect.

TQ inhibits the effects of NF-κB and NF-κB-regulated gene products in CCA cells in vitro. To investigate whether TQ abrogates the constitutively active NF-κB in the CCA cells, we used a DNA binding assay to determine whether NF-κB, a downstream target of the PI3K/Akt signaling pathway, was also involved in the effect. After the above treatments, nuclear extracts were obtained and the NF-κB DNA-binding activity was determined using an EMSA assay; additionally, total protein extracts were used to determine the expression of the downstream NF-κB genes by western blot analysis. As shown in Fig. 4C, treatment with TQ resulted in constitutive NF-κB activity. In both cell lines, TQ treatment significantly reduced the DNA-binding activity of NF-κB, and this decrease was correlated with the increased inhibitory effect on cell viability and increased apoptosis. To confirm our hypothesis, we tested whether the inhibitory effect of TQ on NF-κB may result in the downregulation of NF-κB regulated genes. As shown in Fig. 4B, in both cell lines, TQ treatment resulted in significantly decreased expression of COX-2, VEGF and cyclin B1, which are known to be regulated by NF-κB. These results are consistent with the increased growth inhibition and apoptosis-inducing effects, suggesting that *in vitro* TQ inhibits NF-κB DNA-binding activity and the expression of its downstream gene products, which are believed to be partially responsible for the enhanced cell killing.

TQ exerts antitumor, antiproliferative, pro-apoptotic and anti-angiogenic effects in HuCCT1 xenografts in nude mice. To evaluate the role of TQ in tumor proliferation *in vivo*, we examined the ability of TQ to suppress the growth of HuCCT-1 xenografts in nude mice. HuCCT-1-derived xenograft tumors were allowed to develop and grow to a size of ~100 mm^3; once the tumors had reached this size, TQ was administered at daily doses of 2, 4 or 8 mg/mouse by intragastric intubation for 20 days. As shown in Fig. 5A, the tumors in the mice treated with various concentrations of TQ decreased in volume after 20 days of treatment, and this reduction in volume was significantly different from that of the control tumors. These results suggested that TQ may largely inhibit the growth of the tumor xenografts. In general, the tumors in the control group grew continuously during the experimental period, whereas tumor growth in the TQ-treated mice was suppressed significantly (Fig. 5C). However, there was no apparent change in liver (Fig. 5J), spleen (Fig. 5K) or body weight (Fig. 5B) in the animals, indicating that TQ is a potential therapeutic agent for the treatment of CCA and is relatively non-toxic to mice. Ki-67 staining for cell proliferation was performed in the tumors that were removed from the animals. The relative number of Ki-67-positive tumor cells was substantially less in the tumors from mice treated with TQ than in the control tumors (Fig. 5G and H). Additionally, as shown in the representative images, the tumor xenografts from the TQ-treated mice showed a marked increase in the number of TUNEL-positive apoptotic cells compared to the control group (Fig. 5G and I). Quantification of TUNEL-stained samples showed a 2- to 3-fold increase ($P<0.05$) in the number of TUNEL-positive cells in the TQ-treated groups compared to the control group.

TQ inhibits PI3K/Akt activation in CCA xenografts in nude mice. We next evaluated the expression of p-Akt in the HuCCT-1 xenografts from the control and TQ-treated mice. The expression of p-Akt and its downstream gene products was assessed in the tumor tissues using western blot analysis, and the results showed that TQ treatment resulted in a decrease in the expression of p-Akt, XIAP and Bcl-2, as well as an increase

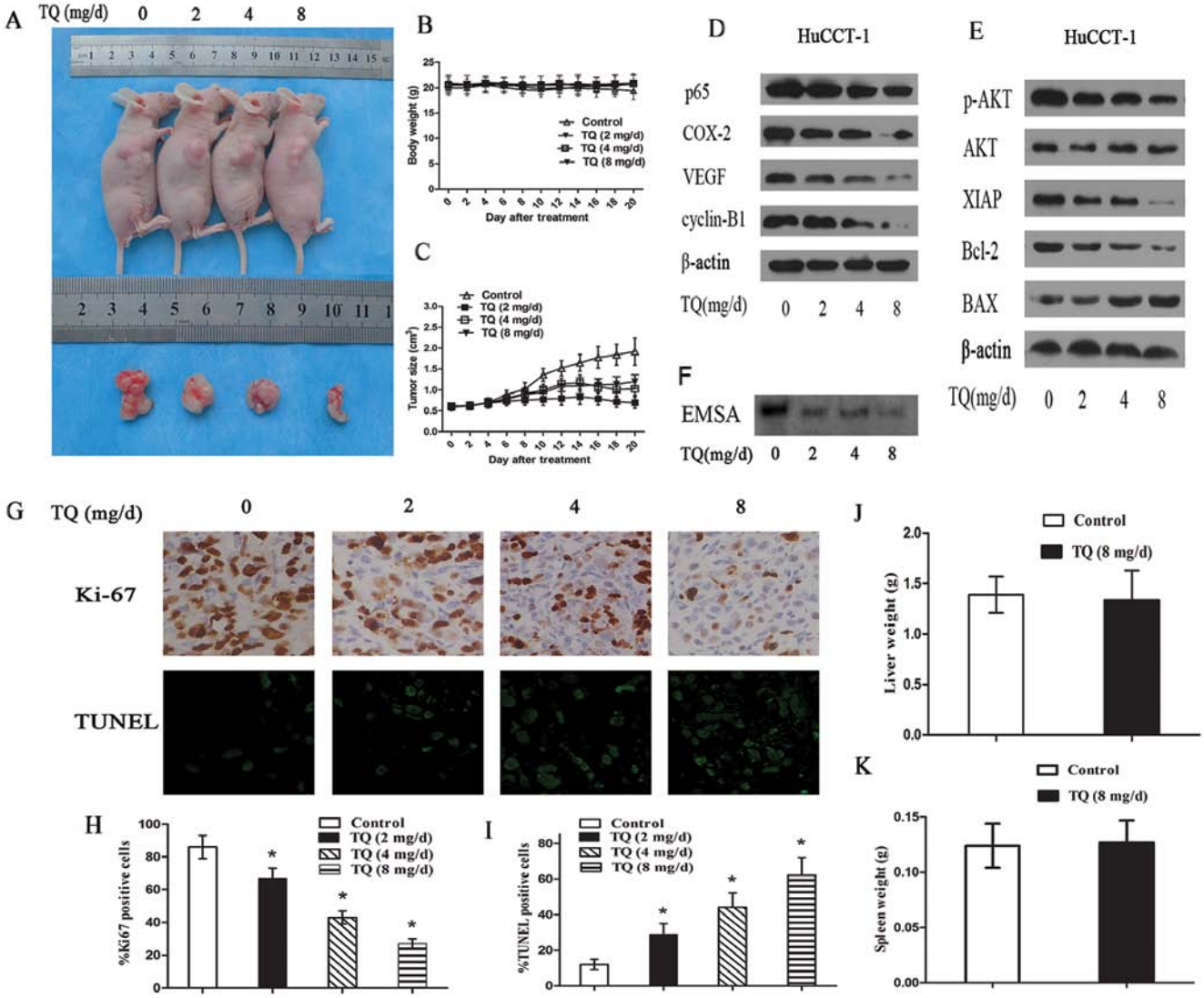

Figure 5. TQ inhibits CCA cancer tumor xenograft growth *in vivo*. (A) HuCCT-1 cells were injected to the flanks of nude mice and palpable tumors were allowed to develop for 14 days. Subsequently, PBS or 2, 4 or 8 mg/mouse TQ were given daily by intragastric intubation for 20 days. Three days later, tumors were excised and subjected to further analyses. Tumor volumes in TQ administered mice were smaller than those of control mice. (B) There was no significant difference in body weight between TQ-treated animals and controls. (C) Tumor size was measured every two days. There was a significant reduction in relative tumor volume from TQ-treated animals when compared with untreated controls. (D) Western blot analysis was performed to detect the expression of p65, COX-2, VEGF, cyclin B1 in tumor tissues. (E) The expression of AKT, phospho-AKT, XIAP, Bcl-2 and BAX in CCA tumor tissue was also measured by western blotting, with β-actin as protein internal control. (F) EMSA analysis of NF-κB DNA-binding activity in tumor tissues. (G) Immunohistochemical analysis of Ki-67 for cell proliferation and TUNEL analysis of apoptotic cells in tumor tissues. (H) Ki-67-positive cells were counted to calculate the proliferation index. (I) TUNEL-positive cells were counted to record the apoptosis index. (J) Liver weight of the nude mice in 8 mg/day TQ-treated and control group. (K) Spleen weight of the nude mice in 8 mg/day TQ-treated and control group. *P<0.05, compared with control. TQ, thymoquinone; CCA, cholangiocarcinoma.

in the expression of BAX (Fig. 5E). These results suggest that the PI3K/Akt signaling pathway is partially involved in the effects of TQ on CCA cells *in vivo*.

TQ inhibits NF-κB and its downstream gene products in nude mice. We investigated whether the antitumor effect of TQ in mice was associated with the inhibition of NF-κB activation. The EMSA results showed that TQ had an effect on NF-κB activation in the tumor samples (Fig. 5F). Accordingly, we observed the expression of downstream NF-κB target genes (as mentioned above) in these tumors samples. As shown in Fig. 5D, TQ treatment resulted in a significant decrease in the expression of all these proteins in the HuCCT-1 xenografts compared to the control treatment.

Discussion

CCA is a lethal cancer in Asia and throughout the world. Patients often suffer from inoperable conditions when the diagnosis is made at a late stage, and they require systemic chemotherapy to combat the disease (25). However, no agent has been shown to provide excellent benefits in controlled clinical trials, and the treatment outcome remains poor due to the acquisition of drug resistance and dose-limiting toxicities to normal cells. Emerging evidence has shown that a loss of viability and the induction of apoptotic cell death are two major mechanisms by which chemotherapeutic agents kill cancer (26,27). In the present study, the results showed that TQ potently inhibits the proliferation of CCA cells and induces cell cycle arrest and

apoptosis *in vitro* and *in vivo*. Additionally, the key finding from the present study is that TQ exhibits a chemopreventive potential against human CCA cells by inhibiting the constitutive activation of proinflammatory transcription factors, including both PI3K/Akt and NF-κB, and their target genes that are involved in tumor cell survival and proliferation, both of which are associated with the pathogenesis and therapeutic resistance of CCA.

The results of the present study showed that TQ caused a G2/M phase cell cycle arrest in both TFK1 and HuCCT-1 cells *in vitro* and resulted in decreased expression of the G2/M checkpoint protein cyclin B1. However, the cellular and molecular bases of this phenomenon have not yet been clearly defined. It is conceivable that TQ prevented the progression of the cell cycle through G2/M phase, resulting in apoptosis. The results indicate that the cell cycle arrest is likely one of the major mechanisms of the anticancer activity of TQ.

With the goal of understanding how TQ inhibits cell viability and induces apoptosis, we observed that TQ treatment may effectively induce apoptosis in CCA by downregulating several anti-apoptotic proteins. The PI3K/Akt signaling pathway is associated with early carcinogenesis, and disruption of this pathway results in antiproliferation, anti-survival, anti-angiogenic and pro-apoptotic effects (28,29). Upregulation of this pathway, through the phosphorylation of Akt, plays a major role in carcinogenesis and drug resistance in numerous types of cancer, including CCA. Notably, the activation of Akt signaling has been observed in 40-50% of CCA tumors, indicating the importance of Akt signaling in CCA (30). Thus, the PI3K/Akt pathway is an attractive target in CCA (31). However, increasing evidence shows that NF-κB activation is a result of the underlying inflammation or is a consequence of the formation of an inflammatory microenvironment during malignant progression; thus, NF-κB provides a critical link between inflammation and cancer (32). Furthermore, previous reports have indicated that PI3K/Akt can trigger NF-κB activation by enhancing the transcriptional activity of the p65 subunit (33). Meanwhile, emerging evidence also indicates that overexpression of p-AKT, XIAP, Bcl-2, COX-2 and VEGF is associated with poor prognosis. Additionally, the EMSA results showed that TQ effectively inhibits the activation of the NF-κB pathway in the TFK1 and HuCCT-1 cell lines at an estimated concentration of 40 μM. Consistent with our hypothesis, we present important evidence documenting a significant reduction in tumor size *in vivo*, which is associated with the inhibition of the above anti-apoptotic proteins. In the present study, a marked suppression of tumor growth in mice xenografts was observed following TQ treatment.

Additionally, a conspicuous suppression of proliferation was observed from the Ki-67 and TUNEL immunostaining, which showed that there were an increasing number of apoptotic cells in the TQ-treated animals. However, further studies are needed to confirm and extend the present study to determine whether TQ can be used as an effective therapy for CCA. These features are of significant value in predicting the improved therapeutic outcome and warrant further investigation. In particular, absorption and analysis of the pharmacokinetic properties of TQ still need to be investigated in future studies; however, the results of our preliminary studies indicate that TQ exhibits low toxicity in the liver and spleen and allowed the mice treated with TQ to maintain a normal weight gain.

In conclusion, we have presented evidence that TQ treatment results in the downregulation of anti-apoptotic and pro-survival proteins that are transcriptionally regulated by the PI3K/Akt and NF-κB pathways, resulting in a loss of CCA cell survival and proliferation. Our *in vitro* findings, along with the *in vivo* results, support the further development of TQ as a novel therapeutic regimen for the targeted inactivation of the PI3K/Akt and NF-κB pathways for the treatment of human CCA. The combination of TQ and other conventional chemotherapeutic drugs may produce a greater therapeutic effect, as well as reduce the toxicity of the conventional chemotherapeutics. Further studies should investigate these possibilities.

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