**Name of the Journal: *World Journal of Gastroenterology***

**ESPS Manuscript NO: 30047**

**Manuscript Type: Original article**

***Basic Study***

**Thymoquinone suppresses migration of** **LoVo human colon cancer cells** **by** **reducing Prostaglandin E2** **induced COX-2 activation**

Hsu HH *et al*. Thymoquinone reducing PGE2 induced COX-2 activation

Hsi-Hsien Hsu, Ming-Cheng Chen, Cecilia Hsuan Day**,** Yueh-Min Lin, Shin-Yi Li, Chuan-Chou Tu, Viswanadha Vijaya Padma, Hui-Nung Shih, Wei-Wen Kuo, Chih-Yang Huang

**Hsi-Hsien Hsu,** Division of Colorectal Surgery, Mackay Memorial Hospital, Taipei 10449, Taiwan

**Hsi-Hsien Hsu,** Mackay Medicine, Nursing and Management College, Taipei 10449, Taiwan

**Ming-Cheng Chen,** Division of Colorectal Surgery, Taichung Veterans General Hospital, Taichung 40705, Taiwan

**Cecilia Hsuan Day,** Department of Nursing, MeiHo University, Pingtung 912, Taiwan

**Yueh-Min Lin,** Department of Pathology, Changhua Christian Hospital, Changhua 500, Taiwan

**Yueh-Min Lin,** Jen-Teh Junior College of Medicine, Nursing and Management, Miaoli 35664, Taiwan

**Shin-Yi Li, G**raduate Institute of Basic Medical Science, China Medical University, Taichung 40402, Taiwan

**Chuan-Chou Tu,** Division of Chest Medicine, Department of Internal Medicine, Armed Force Taichung General Hospital, Taichung 41152, Taiwan

**Viswanadha Vijaya Padma,** Department of Biotechnology, Bharathiar University, Coimbatore 641 046, India

**Hui-Nung Shih,** Graduate Institute of Chinese Medical Science, China Medical University, Taichung 41352, Taiwan

**Wei-Wen Kuo,** Department of Biological Science and Technology, China Medical University, Taichung 40402, Taiwan

**Chih-Yang Huang,** Graduate Institute of Chinese Medical Science, China Medical University, Taichung 41352, Taiwan

**Chih-Yang Huang,** Department of Health and Nutrition Biotechnology, Asia University, Taichung 41352, Taiwan

**Author contributions:** Hsu HH, Kuo WW and Huang CY contributed equally to this work; Hsu HH, ChenMC, DayCH, Li YM, LIN SY**,** TuCC, PadmaVV, Shih HN, Kuo WW and Huang CY designed the research; Hsu HH, ChenMC, DayCH, Li YM, Li SY, TuCC, PadmaVV, Kuo WW and Huang CY performed the research; Hsu HH, Shih HN wrote the paper.

**supported by (**in part) the Taiwan Ministry of Health and Welfare Clinical Trial and Research Center of Excellence, No. MOHW105-TDU-B-212-133019.

**Institutional review board statement:** The study did use of animal subjects from affidavit of approval of animal use protocol china medical university.

**Institutional animal care and use committee statement:** All protocols were reviewed and approved by the Institutional Review Board (IRB, ethical clearance number 104-223-N), Animal care and use committee of the China Medical University, Taichung, China, and the study was conducted in accordance with the principles of laboratory animal care.

**Conflict-of-interest statement:** We declare that there are no conflicts of interest to disclose.

**Data sharing statement:**No additional data are available.

**Open-Access:** This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

**Manuscript source:** Unsolicited manuscript

**Correspondence to:** **Chih-Yang Huang, PhD,** Graduate Institute of Chinese Medical Science, China Medical University, Taichung, Taiwan, No. 91, Hsueh-Shih Road, Taichung 40402, Taiwan. [cyhuang@mail.cmu.edu.tw](mailto:cyhuang@mail.cmu.edu.tw)

**Telephone:** +886-4-22053366-3313

**Fax:** +886-4-22032295

**Received:** September 6, 2016

**Peer-review started:** September 8, 2016

**First decision:** October 20, 2016

**Revised:** November 9, 2016

**Accepted:** December 16, 2016

**Article in press:**

**Published online:**

**Abstract**

***AIM***

to identify potential anti-cancer constituents in natural extracts that inhibit cancer cell growth and migration.

***METHODS***

Our experiments used high dose of thymoquinone (TQ) as an inhibitor to arrest LoVo (a human colon adenocarcinoma cell line) cancer cell line growth by cell proliferation assay and immunoblotting assay. The low dose of TQ was not significantly reduced LoVo cancer cell growth. Cyclooxygenase 2 (COX-2) is an enzyme that is involved in the transformation of arachidonic acid into prostaglandin E2 (PGE2) in humans. PGE2 can promote COX-2 protein and tumor cell proliferation and was used as a control.

***RESULTS***

Our results show that 20 µmol/L TQ can significantly reduce human LoVo colon cancer cells proliferation. TQ treatment reduced the levels of p-PI3K, p-Akt, p-GSK3β, and β-catenin and thereby inhibited the downstream COX-2 expression. Results also show that the reduction in COX-2 expression translates to reduction in PGE2 levels and suppression of EP2 and EP4 activation. Further analysis show that TG treatment inhibited the nuclear translocation of β-catenin t in the LoVo cancer cell. The levels of the cofactors LEF-1 and TCF-4 were also decreased in the nucleus following TQ treatment in a dose-dependent manner. Treatment with Low dose TQ inhibited the COX-2 expression at transcriptional level and the regulation of COX-2 expression efficiently reduced LoVo cell migration. The results were further verified in in vivo models by confirming the effects of TQ and/or PGE2 using tumor xenografts in nude mice models.

***CONCLUSION***

TQ inhibited LoVo cancer cell growth and inhibited migration therefore the results highlight therapeutic advantage of using TQ in combination therapy against colorectal cancer.

**Key words:** Thymoquinone; LoVo cell; Cyclooxygenase 2; Prostaglandin E2; migration

**© The Author(s) 2016.** Published by Baishideng Publishing Group Inc. All rights reserved.

**Core tip:** prostaglandin E2 (PGE2)-induced migration in human LoVo colon cancer cells and the major activating mechanism in the p-Akt/p-PI3K/p-GSK3β/β-catenin/LEF-1/TCF-4 pathway that ultimately up-regulates Cyclooxygenase 2 (COX-2) expression. Thymoquinone (TQ) suppresses cancer cell migration and reveals a potential therapeutic target for colon adenocarcinoma metastasis. PGE2 activation of COX-2 and β-catenin to induce human LoVo colon cancer cell migration was blocked by TQ. Our experiments used cell proliferation assay, immunoblotting assay, immunofluorescence assay, nuclear extraction and in vivo experiments to examine the COX2 protein, which affects transfer performance in highly metastatic LoVo cancer cells treated with TQ.

Hsu HH, Chen MC, Day CH**,** Lin YM, Li SY, Tu CC, Padma VV, Shih HN, Kuo WW, Huang CY. Thymoquinone suppresses migration of LoVo human colon cancer cells by reducing Prostaglandin E2 induced COX-2 activation. *World J Gastroenterol* 2016; In press

**Introduction**

Colorectal cancer is one of the most universally diagnosed gastrointestinal cancers and among the main causes of cancer-related death in western developed countries[[1](#_ENREF_1),[2](#_ENREF_2)]. Despite advanced chemotherapeutic treatments, more than 130000 new cases of colon cancer occur every year[[3](#_ENREF_3)], causing more than 56000 deaths/year in America[[4](#_ENREF_4)]. TQ is a phytochemical compound isolated from *Nigella sativa* that possesses anti-carcinogenic activity that induces apoptosis in tumor cells and can interfere with cancer cell survival through different modes[[5](#_ENREF_5),[6](#_ENREF_6)]. Available treatments for cancer include surgical removal, chemotherapy and adjuvant chemotherapy for patients who are strong enough to undergo it. To date, the surgical removal of cancer tissue is considered the most appropriate way to address colon cancer. Our present study investigated the use of phytochemical drugs as a supplementary chemotherapy approach. Laboratory studies have shown that TQ significantly inhibits oral cancer through the p38β MAPK family[[7](#_ENREF_7)]. Among the hereditary colon cancers, hereditary non-polyposis colon cancer (HNPCC) patients present a particularly high risk for synchronous metastasis past the lymphatic system[[8](#_ENREF_8),[9](#_ENREF_9)]. In this study, we used the LoVo colorectal cancer cell line, which was surgically extracted from a 56-year-old colon cancer patient. Many previous studies have verified that PGE2 promotes cancer and have considered it a cancer marker; therefore, we used PGE2 used as a control[[10-12](#_ENREF_10)]. PGE2 seems to assist cell survival in colorectal cancer cells by augmenting Ras-MAPK signaling[[13](#_ENREF_13)].

Compared to normal intestinal tissues, COX-2 expression is 80%-90% higher in colorectal cancers. Cancers of the head, breast, cervix, bladder and gastrointestinal system have also shown high levels of COX-2 expression[[14-16](#_ENREF_14)]. The role of COX-2/PGE2 signaling affects cell physiology in multiple tumor types and maintains colorectal tumorigenesis[[12](#_ENREF_12),[17](#_ENREF_17)]. PGE2 as a proangiogenic factor is associated with transformed vascular permeability and angiogenesis[[18](#_ENREF_18)]. COX-2 expression is thought to contribute to the principal PGE2 metabolic product[[19](#_ENREF_19),[20](#_ENREF_20)]. Some non-steroidal anti-inflammatory drugs (NSAIDs) and vegetables produce anti-tumor effects that reduce PGE2 synthesis or inhibit COX-2[[21-24](#_ENREF_21)]. In our experiments, we sought to identify compounds similar to NSAIDs or adjunct drugs to increase the effectiveness of cancer chemotherapy. Our experimental drug, TQ, has promising anti-tumor effects: it inhibited the incidence of fore-stomach tumors and fibrosarcoma tumors and increased cellular longevity[[25](#_ENREF_25),[26](#_ENREF_26)]. We previously evaluated PGE2-induced migration in human LoVo cancer cells and the major activating mechanism in the p-Akt/p-PI3K/p-GSK3β/β-catenin pathway that ultimately up-regulates COX-2 expression (unpublished data). After the addition of TQ, the exact anticancer mechanism produced by PGE2 was determined. Previous studies have demonstrated that β-catenin translocation, which includes co-interaction with and activation of the promoters LEF-1 and TCF-4, subsequently modulates downstream gene expression[[27](#_ENREF_27)]. The nuclear cofactors LEF and TCF were triggered to initiate the transcription and translation of COX-2[[28](#_ENREF_28)]. Cell metastasis efficiency is a focus of our work because it correlates with COX-2 activity[[29](#_ENREF_29),[30](#_ENREF_30)]. Moreover, cell migration is promoted due to COX-2 expression[[31](#_ENREF_31)]. Numerous studies of animals treated with TQ have demonstrated that TQ is not toxic [[32-34](#_ENREF_32)].

Our experiments used immunoblotting assays, immunofluorescence assays, nuclear extraction and in vivo experiments to examine the COX2 protein, which affects transfer performance in highly metastatic LoVo cancer cells treated with TQ.

**MATERIALS AND METHODS**

***Cells, antibodies, reagents and enzymes***

The human colon cancer cell line LoVo was obtained from the American Tissue

Culture Collection (ATCC) (Rockville, MD, United States). LoVo cells were established from metastatic nodules that were resected from a 56-year-old colon adenocarcinoma patient.

We utilized antibodies against the following proteins: phospho-PI3K, phospho-Akt, COX-2, phospho-GSK3β, β-catenin, LEF-1, HADAC-1 (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, United States) and TCF-4 (Cell Signaling Technology, Inc. Beverly, Ma, United States). α-Tubulin and β-actin (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, United States) were used as loading controls. The following horseradish peroxidase-conjugated antibodies were purchased from Santa Cruz Biotechnology, Inc. (CA, United States): goat anti-mouse IgG, goat anti-rabbit IgG, and rabbit anti-goat IgG. Nude mice were purchased from the National Laboratory Animal Center (NLAC).

***Cell culture***

The LoVo colon cancer cell line from the American Type Culture Collection (ATCC) (Rockville, MD, United States) was cultured on 10-cm2 culture dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 100 µg/ml penicillin, 100 µg/ml streptomycin, 2 mmol/L glutamine, 1 mmol/L HEPES buffer, and 10% clontech fetal bovine serum in humidified air (5 % CO2) at 37 ˚C.

***Cell proliferation assay***

LoVo cells were seeded at a density of 1.5 × 104 cells per well in 24-well plates, and after 24 h the cells were treated with different concentration of TQ varying from 0 to 20 µmol/L (Sigma Aldrich, St Louis MO, United States) dissolved in DMSO. An MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay was used to determine living cells 24 h after treatment. Culture supernatants were removed, and MTT (Sigma, United States) in phosphate-buffered saline (PBS, pH 7.4) was added to each well. After 4 h of incubation at 37 ˚C, the MTT solution was removed, and DMSO was added to dissolve the resultant formazan crystals. Absorbance was read at 540 nm in a Flexstation 3 device (MDS Analytical Technologies, Canada). The percentage viability was calculated using the following equation: (test-background)/(control-background) × 100.

***Immunoblotting assay***

Cultured LoVo cells were washed with cold PBS and resuspended in lysis buffer (50 mmol/L Tris, pH 7.5, 0.5 mol/L NaCl, 1.0 mmol/L EDTA, pH 7.5, 10% glycerol, 1 mmol/L BME, 1% IGEPAL-630 and a proteinase inhibitor cocktail (Roche Molecular Biochemical)) to isolate total proteins. After incubation for 30 min on ice, the supernatant was collected by centrifugation at 12000 × *g* for 15 min at 4 ˚C. Protein concentration was then determined using the Bradford method. Samples containing equal protein amounts (60 µg) were loaded and analyzed using immunoblotting analysis. Proteins were separated using 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, Belford, MA, United States). The membranes were blocked with blocking buffer (5% non-fat dry milk, 20 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, and 0.1% Tween 20) for at least 1 h at room temperature. The membranes were incubated with primary antibodies in the above solution on an orbital shaker at 4 ˚C overnight. Following the primary antibody incubations, the membranes were incubated with horseradish peroxidase-linked secondary antibodies (anti-rabbit, anti-mouse, or anti-goat IgG). Detection was performed using ECL reagent and a digital imaging system.

***Nuclear extraction***

Cytoplasmic and nuclear fractions were obtained using extraction reagent containing membrane lysis buffer (10 mmol/L HEPES (pH 8.0), 1.5 mmol/L MgCl2, 10 mmol/L KCl, 1 mmol/L DDT, and proteinase inhibitor) and nuclear lysis buffer [20 mmol/L HEPES (pH 8.0), 1.5 mmol/L MgCl2, 10 mmol/L NaCl, 1 mmol/L DDT, 0.2 mmol/L EDTA, 0.25 mol/L glycerol, and proteinase inhibitor]. Following treatment, the cells were resuspended in PBS, incubated with ice-cold membrane lysis buffer for 10 min, and centrifuged at 12000 rpm for 2 min to pellet nuclei. The supernatant was stored for use as a cytoplasmic fraction, and the nuclei pellet was lysed with nuclear lysis buffer to obtain a nuclear fraction.

***Immunofluorescence assay***

LoVo cells were treated with increasing TQ dosages (5, 10, and 20 µmol/L) for 24 h, and PGE2 (5 µmol/L) was added for the last six hours. An immunofluorescence assay was performed on the LoVo cells using a β-catenin antibody (1:250) directed against a target of interest. A fluorophore-conjugated secondary antibody (green) that was directed against the primary antibody was used for detection, and a blue fluorescent DAPI nuclear acid counterstain that preferentially stains dsDNA was included. Anti-β-catenin (green) merged with DAPI (blue) is also shown in the resultant confocal microscopy images.

***Migration assay***

A migration assay was performed using a 48-well Boyden chamber (Neuro Probe) plate with 8-µm pore size polycarbonate membrane filters[[35](#_ENREF_35)]. The lower compartment was filled with DMEM containing 10% FBS. LoVo cells were placed in the upper part of the Boyden chamber, which contained serum-free medium, and incubated for 4-8 h. After incubation, cells on the membrane filter were fixed with methanol and stained with 0.05% Giemsa for 1 h. The cells on the upper filter surface were removed with a cotton swab. The filters were then rinsed in double-distilled water for additional stain leaching. The cells were then air-dried for 20 min. Migratory phenotypes were determined by counting the cells that migrated to the lower side of the filter using microscopy at 200 × and 400 × magnification. Ten fields were blindly selected and counted as the mean cell number in each filter. The sample was assayed in triplicate.

***Study animals***

Twelve nude mice were bred in the animal care facility of the Chinese Medicine Library (Taichung, Taiwan) and werepurchased from The National Laboratory Animal Center. The animals received subcutaneous injections of LoVo colorectal cancer cells (1 × 106 cells per injection) in the back. Ambient temperature was maintained at 25 °C, and the mice were kept on an artificial 12-h light-dark cycle, with the light period beginning at 7:00 am. All of the protocols were approved by the Institutional Animal Care and Use Committee of China Medical University, Taichung, Taiwan, ROC.

LoVo cells were treated with PGE2 (5 µmol/L) for 24 h then injections of LoVo colorectal cancer cells (1 × 106 cells per injection) After one weeks. injected with various concentrations (0.5, 10 and 20 μmol/L) of TQ  three weeks (3 times per week) were applied to appropriate itration and cnoculated tumors into (*n* = 2 each) and assar used Immunoblotting assay. All protocols were reviewed and approved by the Institutional Review Board (IRB, ethical clearance number 104-223-N), Animal care and use committee of the China Medical University, Taichung, Republic of China, and the study was conducted in accordance with the principles of laboratory animal care[[36](#_ENREF_36)].

***Statistical analysis***

Each experiment was duplicated at least three times. The results are presented as the mean ± SE, and statistical comparisons were made using Student’s t test. Significance was defined at *p* < 0.05 or 0.01.

**RESULTS**

***Effects of TQ on cell viability in the LoVo cancer cell line***

We first tested the effect of TQ on cell viability in LoVo cells. Several TQ concentrations were used to evaluate cell viability after co-culture for 24 hr. The results indicated that 20 µmol/L TQ can significantly reduce LoVo cell proliferation (Figure 1).

***p-PI3K-, p-Akt-, p-GSK3β- and β-catenin-induced COX-2 expression was down-regulated by TQ***

Previous reports have demonstrated that PGE2 increases COX-2 expression via activation of the EP4/β-catenin pathway, suggesting that PGE2 regulates p-PI3K, p-AKT, and p-GSK-3β expression in LoVo cells. PGE2 has been reported to activate downstream signaling via EP2 and EP4 to elicit various biological responses. As shown in Figure 2, TQ treatment reduced the up-regulation of COX-2 expression by PGE2, and β-catenin had an obvious effect on EP2 and EP4 regulation by PGE2.

***Effect of TQ on β-catenin translocation***

β-catenin is a key protein that affects nuclear COX-2 transcription. We used an immunofluorescence assay to evaluate β-catenin protein levels in the cytosol and nucleus. Confocal microscopy confirmed that TQ treatment led to the translocation of β-catenin into the LoVo cell nucleus (Figure 3).

***Nuclear translocation inhibition of β-catenin in the LoVo cancer cell line***

Previous studies have demonstrated that the translocation of β-catenin involves co-interaction with and activation of the promoters LEF-1 and TCF-4, which subsequently modulate downstream gene expression. Evaluation of isolated LoVo cell nuclei showed that β-catenin translocation decreased in the nucleus, but its interactions with the cofactors LEF-1 and TCF-4 did not change. We observed a concentration-dependent decrease in the levels of β-catenin and the proteins LEF-1 and TCF-4 in the nucleus following TQ treatment (Figure 4).

***TQ treatment inhibits LoVo cell migration***

Migration is an important step in LoVo colon cancer progression because this cancer metastasizes supraclavicular lymph nodes. We performed a Boyden chamber migration assay to determine the effects of TQ on LoVo cells and found that TQ inhibited LoVo cell migration in a dose-dependent manner (Figure 5).

***Effect of TQ on tumor growth in nude mouse xenografts***

To verify previous results on the growth inhibition and anti-metastatic effects of TQ in human LoVo colon cells, the therapeutic potential of TQ in a pre-subcutaneous LoVo cell model in nude mice was determined. TQ was administered for 30 consecutive days. The drug did not induce death or weight loss of more than 15% to 20% of original weight, which is a very sensitive parameter for perniciousness in mice. The effects of TQ on PGE2-associated protein markers for inflammation and metastasis in the nude mouse xenograft model were then determined. COX-2 and β-catenin protein levels in different tissue groups in the nude mice were downregulated by TQ treatment in a concentration-dependent manner (Figure 6).

**DISCUSSION**

TQ was shown to be an anticancer agent by virtue of its anti-proliferative potential and capacity to induce cell cycle arrest[[37](#_ENREF_37)]. In our previous studies, the COX-2 protein was found to be regulated by PGE2, resulting in cell proliferation and migration. Here, a 20 µmol/L concentration of TQ, which is 50% of the non-cytotoxic concentration determined by MTT assay, arrested cell migration in LoVo colorectal cancer cells. We tested TQ on LoVo colorectal cancer cells that were already induced by PGE2 (5 µmol/L); these cells exhibited significantly enhanced performance over the control group. Our experimental drug TQ inhibited the survival pathway proteins p-PI3K, p-Akt, p-GSK3β, β-catenin and COX-2.

When β-catenin is translocated from the cytosol into the nucleus, it plays a role in the transcription and translation of COX-2[[28](#_ENREF_28)]. The translocation of β-catenin from the cytosol into the nucleus also plays a role in the transcription and translation of COX-2.

Nuclear isolation techniques and immunofluorescence were used to determine the localization of β-catenin and the COX-2 transcription cofactors LEF-1 and TCF-4[[38](#_ENREF_38)]. Our experiments showed that TQ suppresses β-catenin translocation induced by PGE2, and the expression of both LEF-1 and TCF-4 was also suppressed. This result suggests that β-catenin loses its ability to translocate into the nucleus and bind to LEF-1 and TCF-4 following TQ treatment. These cofactors were also affected by TQ with a gradually declining, concentration-dependent trend. Immunofluorescence confocal microscopy showed fluorescent images of anti-β-catenin (green) and DAPI staining (blue) in the panel representing the cell nucleus. These results indicate that TQ treatment inhibited β-catenin translocation into the nucleus and subsequently reduced COX-2 activation.

We can observe the impact of TQ on COX-2 by assessing these translation- and transcription-associated proteins. COX-2 plays a central role in cell migration. These results showed that decreasing COX-2 levels will correspondingly reduce cell migration. In this context, we demonstrated that TQ significantly reduces metastasis in vivo. However, we investigated the therapeutic potential of TQ using a highly aggressive human LOVO cancer cell xenograft nude mouse model. p-Akt, β-catenin and COX-2 expression substantially decreased. TQ inhibited LoVo colorectal cancer cell growth and inhibited migration. In the future, we will evaluate the therapeutic advantage of combining chemotherapeutic agents for colorectal cancer treatment.

**Comments**

***Background***

Several types of phytochemicals are used for cancer chemotherapy. Our aim is to identify potential anti-cancer constituents in natural extracts that inhibit cancer cell growth and migration. Thymoquinone (TQ) is a phytochemical compound isolated from *Nigella sativa*. Previous data show that TQ suppresses the activation of AKT and inhibits cellular proliferation and the anti-oxidant/anti-inflammatory effect.

***Research frontiers***

prostaglandin E2 (PGE2)-induced migration in human LoVo colon cancer cells and the major activating mechanism in the p-Akt/p-PI3K/p-GSK3β/β-catenin/LEF-1/TCF-4 pathway that ultimately up-regulates cyclooxygenase 2 (COX-2) expression.

***Innovations and breakthrough***

TQ to suppress cancer cell migration and reveals a potential therapeutic target for colon adenocarcinoma metastasis. PGE2 activation of COX-2 and β-catenin to induce human LoVo colon cancer cell migration was blocked by thymoquinone.

***Application***

The results reveal that TQ can be considered as a potential treatment strategy in advanced stage colon cancer treatment.

***Peer-review***

The study described in this paper investigated the use of phytochemical drugs as a supplementary chemotherapy approach. For this purpose the authors used the high dose of TQ as an inhibitor to arrest LoVo (a human colon adenocarcinoma cell line) cancer cell line growth. The authors used immunoblotting assays, immunofluorescence assays, nuclear extraction and in vivo experiments to examine the COX2 protein, which affects transfer performance in highly metastatic LoVo cancer cells treated with TQ.

**References**

1 **Gali-Muhtasib H**, Ocker M, Kuester D, Krueger S, El-Hajj Z, Diestel A, Evert M, El-Najjar N, Peters B, Jurjus A, Roessner A, Schneider-Stock R. Thymoquinone reduces mouse colon tumor cell invasion and inhibits tumor growth in murine colon cancer models. *J Cell Mol Med* 2008; **12**: 330-342 [PMID: 18366456 DOI: 10.1111/j.1582-4934.2007.00095.x]

2 **Lin TY**, Fan CW, Maa MC, Leu TH. Lipopolysaccharide-promoted proliferation of Caco-2 cells is mediated by c-Src induction and ERK activation. *Biomedicine (Taipei)* 2015; **5**: 5 [PMID: 25705585 DOI: 10.7603/s40681-015-0005-x]

3 **Jemal A**, Thomas A, Murray T, Thun M. Cancer statistics, 2002. *CA Cancer J Clin* 2002; **52**: 23-47 [PMID: 11814064]

4 **Jemal A**, Tiwari RC, Murray T, Ghafoor A, Samuels A, Ward E, Feuer EJ, Thun MJ. Cancer statistics, 2004. *CA Cancer J Clin* 2004; **54**: 8-29 [PMID: 14974761]

5 **Woo CC**, Kumar AP, Sethi G, Tan KH. Thymoquinone: potential cure for inflammatory disorders and cancer. *Biochem Pharmacol* 2012; **83**: 443-451 [PMID: 22005518 DOI: 10.1016/j.bcp.2011.09.029]

6 **Towhid ST**, Schmidt EM, Schmid E, Münzer P, Qadri SM, Borst O, Lang F. Thymoquinone-induced platelet apoptosis. *J Cell Biochem* 2011; **112**: 3112-3121 [PMID: 21688304 DOI: 10.1002/jcb.23237]

7 **Abdelfadil E**, Cheng YH, Bau DT, Ting WJ, Chen LM, Hsu HH, Lin YM, Chen RJ, Tsai FJ, Tsai CH, Huang CY. Thymoquinone induces apoptosis in oral cancer cells through p38β inhibition. *Am J Chin Med* 2013; **41**: 683-696 [PMID: 23711149 DOI: 10.1142/S0192415X1350047X]

8 **Kalady MF**. Surgical management of hereditary nonpolyposis colorectal cancer. *Adv Surg* 2011; **45**: 265-274 [PMID: 21954693]

9 **Wu Z**, Zhang S, Aung LH, Ouyang J, Wei L. Lymph node harvested in laparoscopic versus open colorectal cancer approaches: a meta-analysis. *Surg Laparosc Endosc Percutan Tech* 2012; **22**: 5-11 [PMID: 22318051 DOI: 10.1097/SLE.0b013e3182432b49]

10 **Chang SH**, Liu CH, Conway R, Han DK, Nithipatikom K, Trifan OC, Lane TF, Hla T. Role of prostaglandin E2-dependent angiogenic switch in cyclooxygenase 2-induced breast cancer progression. *Proc Natl Acad Sci U S A* 2004; **101**: 591-596 [PMID: 14688410 DOI: 10.1073/pnas.2535911100]

11 **Eruslanov E**, Kaliberov S, Daurkin I, Kaliberova L, Buchsbaum D, Vieweg J, Kusmartsev S. Altered expression of 15-hydroxyprostaglandin dehydrogenase in tumor-infiltrated CD11b myeloid cells: a mechanism for immune evasion in cancer. *J Immunol* 2009; **182**: 7548-7557 [PMID: 19494278 DOI: 10.4049/jimmunol.0802358]

12 **Greenhough A**, Smartt HJ, Moore AE, Roberts HR, Williams AC, Paraskeva C, Kaidi A. The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. *Carcinogenesis* 2009; **30**: 377-386 [PMID: 19136477 DOI: 10.1093/carcin/bgp014]

13 **Kaidi A**, Qualtrough D, Williams AC, Paraskeva C. Direct transcriptional up-regulation of cyclooxygenase-2 by hypoxia-inducible factor (HIF)-1 promotes colorectal tumor cell survival and enhances HIF-1 transcriptional activity during hypoxia. *Cancer Res* 2006; **66**: 6683-6691 [PMID: 16818642 DOI: 10.1158/0008-5472.CAN-06-0425]

14 **Voutsadakis IA**. Pathogenesis of colorectal carcinoma and therapeutic implications: the roles of the ubiquitin-proteasome system and Cox-2. *J Cell Mol Med* 2007; **11**: 252-285 [PMID: 17488476 DOI: 10.1111/j.1582-4934.2007.00032.x]

15 **Sano H**, Kawahito Y, Wilder RL, Hashiramoto A, Mukai S, Asai K, Kimura S, Kato H, Kondo M, Hla T. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. *Cancer Res* 1995; **55**: 3785-3789 [PMID: 7641194]

16 **Eberhart CE**, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology* 1994; **107**: 1183-1188 [PMID: 7926468]

17 **Brown JR**, DuBois RN. COX-2: a molecular target for colorectal cancer prevention. *J Clin Oncol* 2005; **23**: 2840-2855 [PMID: 15837998 DOI: 10.1200/JCO.2005.09.051]

18 **Form DM**, Auerbach R. PGE2 and angiogenesis. *Proc Soc Exp Biol Med* 1983; **172**: 214-218 [PMID: 6572402]

19 **Pugh S**, Thomas GA. Patients with adenomatous polyps and carcinomas have increased colonic mucosal prostaglandin E2. *Gut* 1994; **35**: 675-678 [PMID: 8200564]

20 **Rigas B**, Goldman IS, Levine L. Altered eicosanoid levels in human colon cancer. *J Lab Clin Med* 1993; **122**: 518-523 [PMID: 8228569]

21 **Hanif R**, Pittas A, Feng Y, Koutsos MI, Qiao L, Staiano-Coico L, Shiff SI, Rigas B. Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. *Biochem Pharmacol* 1996; **52**: 237-245 [PMID: 8694848]

22 **Elder DJ**, Halton DE, Hague A, Paraskeva C. Induction of apoptotic cell death in human colorectal carcinoma cell lines by a cyclooxygenase-2 (COX-2)-selective nonsteroidal anti-inflammatory drug: independence from COX-2 protein expression. *Clin Cancer Res* 1997; **3**: 1679-1683 [PMID: 9815550]

23 **Chan TA**, Morin PJ, Vogelstein B, Kinzler KW. Mechanisms underlying nonsteroidal antiinflammatory drug-mediated apoptosis. *Proc Natl Acad Sci USA* 1998; **95**: 681-686 [PMID: 9435252]

24 **Ciou SY**, Hsu CC, Kuo YH, Chao CY. Effect of wild bitter gourd treatment on inflammatory responses in BALB/c mice with sepsis. *Biomedicine (Taipei)* 2014; **4**: 17 [PMID: 25520930 DOI: 10.7603/s40681-014-0017-y]

25 **Badary OA**, Gamal El-Din AM. Inhibitory effects of thymoquinone against 20-methylcholanthrene-induced fibrosarcoma tumorigenesis. *Cancer Detect Prev* 2001; **25**: 362-368 [PMID: 11531013]

26 **Badary OA**, Al-Shabanah OA, Nagi MN, Al-Rikabi AC, Elmazar MM. Inhibition of benzo(a)pyrene-induced forestomach carcinogenesis in mice by thymoquinone. *Eur J Cancer Prev* 1999; **8**: 435-440 [PMID: 10548399]

27 **Kriegl L**, Horst D, Reiche JA, Engel J, Kirchner T, Jung A. LEF-1 and TCF4 expression correlate inversely with survival in colorectal cancer. *J Transl Med* 2010; **8**: 123 [PMID: 21092222 DOI: 10.1186/1479-5876-8-123]

28 **Nuñez F**, Bravo S, Cruzat F, Montecino M, De Ferrari GV. Wnt/β-catenin signaling enhances cyclooxygenase-2 (COX2) transcriptional activity in gastric cancer cells. *PLoS One* 2011; **6**: e18562 [PMID: 21494638 DOI: 10.1371/journal.pone.0018562]

29 **Xu L**, Stevens J, Hilton MB, Seaman S, Conrads TP, Veenstra TD, Logsdon D, Morris H, Swing DA, Patel NL, Kalen J, Haines DC, Zudaire E, St Croix B. COX-2 inhibition potentiates antiangiogenic cancer therapy and prevents metastasis in preclinical models. *Sci Transl Med* 2014; **6**: 242ra84 [PMID: 24964992 DOI: 10.1126/scitranslmed.3008455]

30 **Emenaker NJ**, Zudaire E, St Croix B. Chemoprevention of metastasis. *Oncotarget* 2014; **5**: 6556-6557 [PMID: 25179303]

31 **Zhao L**, Wu Y, Xu Z, Wang H, Zhao Z, Li Y, Yang P, Wei X. Involvement of COX-2/PGE2 signalling in hypoxia-induced angiogenic response in endothelial cells. *J Cell Mol Med* 2012; **16**: 1840-1855 [PMID: 22050691 DOI: 10.1111/j.1582-4934.2011.01479.x]

32 **Kirui PK**, Cameron J, Benghuzzi HA, Tucci M, Patel R, Adah F, Russell G. Effects of sustained delivery of thymoqiunone on bone healing of male rats. *Biomed Sci Instrum* 2004; **40**: 111-116 [PMID: 15133944]

33 **Hosseinzadeh H**, Parvardeh S. Anticonvulsant effects of thymoquinone, the major constituent of Nigella sativa seeds, in mice. *Phytomedicine* 2004; **11**: 56-64 [PMID: 14971722 DOI: 10.1078/0944-7113-00376]

34 **Attoub S**, Sperandio O, Raza H, Arafat K, Al-Salam S, Al Sultan MA, Al Safi M, Takahashi T, Adem A. Thymoquinone as an anticancer agent: evidence from inhibition of cancer cells viability and invasion in vitro and tumor growth in vivo. *Fundam Clin Pharmacol* 2013; **27**: 557-569 [PMID: 22788741 DOI: 10.1111/j.1472-8206.2012.01056.x]

35 **Hsieh YC**, Hsieh SJ, Chang YS, Hsueh CM, Hsu SL. The lipoxygenase inhibitor, baicalein, modulates cell adhesion and migration by up-regulation of integrins and vinculin in rat heart endothelial cells. *Br J Pharmacol* 2007; **151**: 1235-1245 [PMID: 17592510 DOI: 10.1038/sj.bjp.0707345]

36 . Laboratory animal welfare; proposed U.S. government principles for the utilization and care of vertebrate animals used in testing, research and training. *Fed Regist* 1984; **49**: 29350-29351 [PMID: 11655723]

37 **Jrah-Harzallah H**, Ben-Hadj-Khalifa S, Almawi WY, Maaloul A, Houas Z, Mahjoub T. Effect of thymoquinone on 1,2-dimethyl-hydrazine-induced oxidative stress during initiation and promotion of colon carcinogenesis. *Eur J Cancer* 2013; **49**: 1127-1135 [PMID: 23131834 DOI: 10.1016/j.ejca.2012.10.007]

38 **Daniels DL**, Weis WI. Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. *Nat Struct Mol Biol* 2005; **12**: 364-371 [PMID: 15768032 DOI: 10.1038/nsmb912]

**P-Reviewer:** Kunac N **S-Editor:** Gong ZM

**L-Editor:** **E-Editor:**

**Specialty type:** Gastroenterology and hepatology

**Country of origin:** Taiwan

**Peer-review report classification**

Grade A (Excellent): 0

Grade B (Very good): B

Grade C (Good): 0

Grade D (Fair): 0

Grade E (Poor): 0

圖片4.tif

**Figure 1 thymoquinone affects cell viability in LoVo colon cancer cells.** To explore the effects of thymoquinone (TQ) on the viability of human LoVo colon cancer cells, we first treated LoVo cells with various concentrations (2.5, 5, 7.5, 10 and 20 µmol/L) of TQ for 24 h and subsequently measured cell viability by MTT assay. The results showed a significant reduction of cell viability of approximately 60% following treatment (20 µmol/L) for 24 h. Each value represents the mean S.E. a*p* < 0.05; b*p* < 0.01.

D:\20160906 discussion\Thymoquione-NEW\Fig. 2.tif

**Figure 2 thymoquinone inhibited the expression of PI3K, Akt, p-GSK3β, β-catenin and COX-2 proteins in LoVo cells.** LoVo cells were cultured in medium that was treated with TQ (5, 10 and 20 µmol/L) for 24 h; PGE2 (5 µmol/L) was added for the last 6 h. Following this, the cells were immunoblotted with the indicated antibodies. The cells were lysed, and the extracts were separated by 10% SDS-PAGE, transferred to PVDF membranes and immunoblotted with antibodies against p-PI3K, p-Akt, p-GSK3β, β-catenin and COX-2.

**Fig. 3.tif**

**Figure 3 thymoquinone inhibited nuclear translocation in the LoVo cancer cell line.** Nuclear isolation showed a decrease in β-catenin translocation into the nucleus. The cofactors LEF-1 and TCF-4 decreased in the nucleus following TQ treatment in a dose-dependent manner. TQ: thymoquinone.

**D:\20160906 discussion\Thymoquione-NEW\Fig. 4.tif**

**Figure 4 thymoquinone treatment inhibited β-catenin translocation into LoVo cell nuclei.** An immunofluorescence assay was performed on LoVo cells using a β-catenin primary antibody and a secondary antibody (1:250) producing green fluorescence; DAPI (blue fluorescence) was include to stain cell nuclei. Merged β-catenin and DAPI (green and blue, respectively) signals are shown. The indicated treatments were assessed. PGE2: prostaglandin E2; TQ: thymoquinone.

D:\20160906 discussion\Thymoquione-NEW\Fig. 5.tif

**Figure 5 thymoquinone efficiently inhibited LoVo cell migration.** LoVo cells were pretreated with increasing dosages (5, 10, and 20 µmol/L) of TQ for 24 h. A Boyden chamber migration assay was performed to assess cell migration ability. The responses to different treatments were analyzed *via* microscopy. PGE2: prostaglandin E2; TQ: thymoquinone.

**D:\20160906 discussion\Thymoquione-NEW\Fig. 6.tif**

**Figure 6** **Impact of thymoquinone and/or prostaglandin E2 administration on tumor growth in nude mice xenografts.** Tumor tissues were harvested from nude mice and lysed. Protein content was quantified and analyzed by immunoblotting. p-Akt, β-catenin and COX-2 levels in LoVo cells were detected. PGE2: prostaglandin E2; TQ: thymoquinone.