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

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Core tip: Prostaglandin E2 (PGE2) induces migration of human LoVo colon cancer cells, and the major mechanism involves the activation of the p-Akt/p-PI3Kβ-catenin/LEF-1/TCF-4 pathway that ultimately up-regulates cyclooxygenase 2 (COX-2) expression. Thymoquinone (TQ) suppresses cancer cell migration and represents a potential therapeutic target for colon adenocarcinoma metastasis. PGE2 activation of COX-2 and β-catenin in human LoVo colon cancer cell migration was blocked by TQ. Our study used cell proliferation assay, immunoblotting assay, immunofluorescence assay, nuclear extraction and in vivo experiments to examine the COX2 protein, which affects the metastasis of highly metastatic LoVo cancer cells treated with TQ.

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[1,2]. Despite advanced chemotherapeutic treatments, more than 130000 new cases of colon cancer are diagnosed each year[3], causing more than 56000 deaths/year in America[4]. Thymoquinone (TQ) is a phytochemical compound isolated from Nigella sativa that possesses anti-carcinogenic activity and induces apoptosis in tumor cells, and it can interfere with cancer cell survival through different mechanisms[5,6]. 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 p38MAPK family[7]. Among the hereditary colon cancers, hereditary non-polyposis colon cancer (HNPCC) patients present a particularly high risk for synchronous metastasis via the lymphatic system[8,9]. In this study, we used the colorectal cancer cell line LoVo, which was developed from a 56-year-old colon cancer patient. Many previous studies have verified that prostaglandin E2 (PGE2) promotes cancer development and have considered it a cancer marker; therefore, we used PGE2 as a control[10-12]. PGE2 seems to assist cell survival in colorectal cancer cells.
by augmenting Ras-MAPK signaling\textsuperscript{[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\textsuperscript{[14-16]}. COX-2/PGE2 signaling affects cell physiology in multiple tumor types and maintains colorectal tumorigenesis\textsuperscript{[12,17]}. PGE2 as a proangiogenic factor is associated with transformed vascular permeability and angiogenesis\textsuperscript{[18]}. COX-2 expression is thought to contribute to the principal PGE2 metabolic product\textsuperscript{[19,20]}. Some non-steroidal anti-inflammatory drugs (NSAIDs) and vegetables produce anti-tumor effects that reduce PGE2 synthesis or inhibit COX-2\textsuperscript{[21-24]}. 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, and it inhibited the incidence of fore-stomach tumors and fibrosarcoma tumors and increased cellular longevity\textsuperscript{[25,26]}. We previously evaluated PGE2-induced migration in human LoVo cancer cells, and the major mechanism involves the activation of the p-Akt/\(\beta\)-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 \(\beta\)-catenin translocation, which includes co-interaction with and activation of the promoters LEF-1 and TCF-4, subsequently modulates downstream gene expression\textsuperscript{[27]}. The nuclear cofactors LEF and TCF were triggered to initiate the transcription and translation of COX-2\textsuperscript{[28]}. Cell metastasis efficiency is a focus of our work because it correlates with COX-2 activity\textsuperscript{[29,30]}. Moreover, cell migration is promoted due to COX-2 expression\textsuperscript{[31]}. Numerous studies of animals treated with TQ have demonstrated that TQ is not toxic\textsuperscript{[32-34]}.

Our study used immunoblotting assays, immunofluorescence assays, nuclear extraction and in vivo experiments to examine the COX2 protein, which affects the metastasis of 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-GSK3\(\beta\), \(\beta\)-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). \(\alpha\)-tubulin and \(\beta\)-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 colon cancer cell line LoVo was cultured in 10-cm\(^2\) culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 \(\mu\)g/mL penicillin, 100 \(\mu\)g/mL streptomycin, 2 \(\mu\)mol/L glutamine, 1 \(\mu\)mol/L HEPES buffer, and 10% fetal bovine serum (FBS) in humidified air (5% CO\(_2\)) at 37 \(^\circ\)C.

### Cell proliferation assay

LoVo cells were seeded at a density of 1.5 \(\times\) 10\(^4\) cells per well in 24-well plates, and after 24 h the cells were treated with different concentrations of TQ varying from 0 to 20 \(\mu\)mol/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 \(^\circ\)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 as (test-background)/(control-background) \(\times\) 100.

### Immunoblotting assay

Cultured LoVo cells were washed with cold PBS and resuspended in lysis buffer [50 \(\mu\)mol/L Tris (pH 7.5), 0.5 mol/L NaCl, 1.0 \(\mu\)mol/L EDTA (pH 7.5), 10% glycerol, 1 \(\mu\)mol/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 \(\times\) g for 15 min at 4 \(^\circ\)C. Protein concentration was then determined using the Bradford method. Samples containing equal protein amounts (60 \(\mu\)g) 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 \(\mu\)mol/L Tris-HCl, pH 7.6, 150 \(\mu\)mol/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 \(^\circ\)C overnight. Following the primary antibody incubations, the membranes were incubated with horseradish
To explore the effect 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 ± SE. *P < 0.05; **P < 0.01.

Figure 1  Thymoquinone affects the viability of LoVo colon cancer cells.

To obtain a nuclear fraction, the nuclear pellet was lysed with nuclear lysis buffer [10 mmol/L HEPES (pH 8.0), 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 1 mmol/L DTT, 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 nuclear pellet was lysed with nuclear lysis buffer to obtain a nuclear fraction.

RESULTS

Effect of TQ on viability of LoVo cells

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

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.
LoVo cells were cultured in

LEF-1 and TCF-4 in the nucleus following TQ treatment decrease in the levels of not change. We observed a concentration-dependent its interactions with the cofactors LEF-1 and TCF-4 did subsequently modulate downstream gene expression.

Evaluation of isolated LoVo cell nuclei showed that
treatment led to the translocation of and nucleus. Confocal microscopy confirmed that TQ

Previous studies have demonstrated that the 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).

Effect of TQ on β-catenin translocation

β-catenin is a key protein that affects nuclear COX-2 transcription. The expression 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).

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).

DISCUSSION

TQ was shown to be an anticancer agent by virtue of its anti-proliferative potential and capacity to induce cell cycle arrest[37]. In our previous studies, 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 the migration of 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[38]. 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]. 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
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| Cytosol | Nuclear |
|---------|---------|
| PGE2   | -       | +       | +       | +       | +       | -       | +       | +       | +       |
| TQ     | -       | 5       | 10      | 20      | -       | 5       | 10      | 20      |

| µmol/L |
|--------|
| 92 kDa |
| 54 kDa |
| 79 kDa |
| 55 kDa |

**Figure 3** Thymoquinone inhibits β-catenin 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.

**Figure 4** Thymoquinone treatment inhibits 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 included 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.
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.

**COMMENTS**

**Background**

Several types of phytochemicals are used for cancer chemotherapy. The aim was 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, inhibits cellular proliferation, and shows anti-oxidant/anti-inflammatory effects.

**Research frontiers**

Prostaglandin E2 (PGE2) induces migration of human LoVo colon cancer cells, and the major mechanism involves the activation of the p-Akt/p-PI3K/p-GSK3β/β-catenin/LEF-1/TCF-4 pathway that ultimately up-regulates cyclooxygenase 2.

**Figure 5** Thymoquinone efficiently inhibits 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.

**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.
(COX-2) expression.

Innovations and breakthroughs
TQ suppresses cancer cell migration and represents 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.

Applications
The results reveal that TQ can be considered a potential treatment strategy for 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) cell growth. The authors used immunoblotting assays, immunofluorescence assays, nuclear extraction and in vivo experiments to examine the COX2 protein, which affects the metastasis of highly metastatic LoVo cancer cells treated with TQ.

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