Thymoquinone inhibits metastatic phenotype and epithelial-mesenchymal transition in renal cell carcinoma by regulating the LKB1/AMPK signaling pathway

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Abstract. Thymoquinone, isolated from the seeds of Nigella sativa, has exhibited antitumor properties in a variety of cancer types. However, few studies have investigated the effect of thymoquinone (TQ) on migration and invasion in renal cell carcinoma (RCC). In the present study, our results confirmed that TQ significantly inhibited the migration and invasion of the human RCC 769-P and 786-O cell lines, as demonstrated by wound healing and Transwell assays. Additionally, TQ upregulated the expression of E-cadherin and downregulated the expression of Snail, ZEB1 and vimentin at the mRNA and protein levels in a concentration-dependent manner. Subsequently, the phosphorylation levels of liver kinase B1 (LKB1) and AMP-activated protein kinase (AMPK) were increased upon TQ treatment. To further validate the role of LKB1/AMPK signaling, we revealed that TQ-mediated increase of E-cadherin level and reduction of Snail level could be further enhanced by LKB1 overexpression. Furthermore, co-treatment with the AMPK inhibitor Compound C attenuated the anti-metastatic effect of TQ on RCC and partially abrogated the high expression of E-cadherin and the low expression of Snail mediated by TQ. In contrast, the AMPK activator AICAR demonstrated the opposite effect. Collectively, the present study revealed that TQ could markedly suppress the metastatic phenotype and reverse the epithelial-mesenchymal transition in RCC by regulating the LKB1/AMPK signaling pathway, indicating that TQ may be a potential therapeutic candidate against RCC.

Introduction

Renal cell carcinoma (RCC) is the most common type of kidney cancer worldwide (1). Despite the improvement of diagnostic techniques and therapeutic treatments, the majority of patients with RCC are diagnosed at an advanced stage or have already presented with metastasis. It is reported that in 30% of patients with RCC, metastatic lesions have already developed at the stage of diagnosis (2). In view of the poor response of patients with RCC to chemotherapy and radiotherapy, the treatment of metastatic unresectable RCC consists a great challenge for clinicians. Therefore, it is essential to find potential therapeutic agents for the treatment of RCC.

Thymoquinone (TQ), isolated from the seeds of Nigella sativa, is a natural polyphenolic compound (3). Numerous studies have reported that TQ has broad pharmacological effects. For example, TQ is regarded as an IRAK1 (interleukin-receptor-associated kinase 1) inhibitor with anti-inflammatory activities (4). In addition, TQ is reported to have antioxidant effects in activated BV-2 murine microglial cells (5). Additionally, previous studies revealed that TQ has...
antitumor activities such as suppression of proliferation, induction of apoptosis, inhibition of metastasis and enhancement of chemosensitivity (6-9). In RCC, TQ has been found to induce apoptosis by downregulating c-FLIP and Bcl-2 (10). However, few studies have been performed about the effect of TQ on migration and invasion in RCC.

Liver kinase B1 (LKB1), also known as serine/threonine kinase 11 (STK11), was first identified in Peutz-Jeghers syndrome (11). Studies revealed that LKB1 has been verified to regulate cell polarity and maintain energy balance (12,13). Additionally, it is well known that LKB1 directly phosphorylates the AMP-activated protein kinase (AMPK) at the Thr172 site. Accumulating evidence indicated that the role of LKB1 in tumor progression is vital and that the LKB1/AMPK pathway regulates the AMP-activated protein kinase (AMPK) at the Thr172 site. Accumulating evidence indicated that the role of LKB1 in tumor progression is vital and that the LKB1/AMPK pathway participated in the migratory and invasive process of various tumors, including colon, breast and lung cancer (14-16).

In the present study, we aimed to explore the correlation between TQ and metastasis in RCC and the underlying function mechanism of TQ against RCC.

Materials and methods

Reagents. TQ was obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO). Primary rabbit monoclonal antibodies (diluted at 1:1,000) against phosphorylated-LKB1 (3482), LKB1 (3050), phosphorylated-AMPK (9957), AMPK (9957), E-cadherin (3195), Snail (3879), ZEB1 (3396), vimentin (5741) and β-actin (4970) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Furthermore, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), the AMPK inhibitor Compound C (ComC) and the AMPK activator AICAR were obtained from Sigma-Aldrich.

Cell culture. HK2, a human renal tubular epithelial cell line and the human RCC cell lines 769-P and 786-O were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). These three cell lines were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 µg/ml streptomycin and 100 U/ml penicillin (Invitrogen, Carlsbad, CA, USA). All cells were cultured at 37°C in a humidified incubator with 5% CO₂ atmosphere.

Cell proliferation assay. A modified MTT assay was used to detect the growth inhibition of TQ on RCC. Briefly, the 769-P and 786-O cells were seeded in 96-well plates with ~90% density and treated with ascending concentrations of TQ (0.5, 1, 2.5, 5, 10 and 20 µM) at different time-points (0, 24, 48 and 72 h). Subsequently, each well was mixed with 0.5 mg/ml MTT dye solution for another 4 h at 37°C. Subsequently, the culture medium was removed and 150 µl dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The optical density (OD) of each well was determined at 490 nm by a 96-well microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The inhibitory rate of cell growth was calculated as follows: [(OD 490control group - OD 490treated group) / OD 490control group] x 100.

Western blotting. Briefly, the RCC 769-P and 786-O cells were harvested after certain treatment and lysed on ice for 10 min in a lysis buffer [10 mmol/l Tris-HCl (pH 7.4), 150 mmol/l NaCl, 0.1% sodium dodecyl sulfate (SDS), 1 mmol/l ethylenediaminetetraacetic acid, 1 mmol/l ethylene glycol tetraacetic acid, 0.3 mmol/l phenylmethylsulfonyl fluoride, 0.2 mmol/l sodium orthovanadate, 1% NP-40, 10 mg/ml leupeptin and 10 mg/ml aprotinin]. Subsequently, the clarified protein lysates (about 40-60 µg) were separated by 10 or 15% sodium dodecyl

Quantitative real-time PCR assay. Following the treatment of the 769-P and 786-O cell lines with ascending concentrations of TQ (2.5, 5 and 10 µM), their total RNA was extracted using TRIzol reagent (Invitrogen). Complementary DNA (cDNA) was then synthesized using a PrimerScript RT reagent kit (Takara, Dalian, China). Then the relative levels of target gene messenger RNA (mRNA) were evaluated by quantitative real-time PCR assay (qRT-PCR) using FAST SYBR Green Master Mix. The primers are as follows: Human E-cadherin (119 bp) forward, 5'-CGAGAGCTACACGGTCGACGGG-3' and reverse, 5'-GGTGTCCAGGGAAATATGGG-3'; human Snail (140 bp) forward, 5'-TGAGAGCTCTTAATCCAGCGA-3' and reverse, 5'-AGATGAGCATTCCAGCGACG-3'; human ZEB1 (86 bp) forward, 5'-GATGAGTATCGCAGGAGTCG-3' and reverse, 5'-ACAGCAGTGTCTTGGTGTTGGTGGTGGT-3' and reverse, 5'-CATGTA CGTGTGATCCAGCCG-3' and reverse, 5'-CTCCATTAATGTCGACGACTATG-3'. The n-fold change in the expression of mRNA was analyzed according to the 2^−ΔΔCt method.

Transwell migration assay. Transwell migration assay was used to assess the effect of TQ on RCC cell migration. The cells (769-P, 5x10^4; 768-O, 4x10^4) with 200 µl serum-free medium were seeded into the upper chamber, while 10% fetal calf serum-containing medium was added to the lower chamber. Twenty-four hours later, the migrated cells on the bottom of the filter were fixed with 4% paraformaldehyde, followed by 0.1% crystal violet staining (Beyotime Institute of Biotechnology, Shanghai, China). The cells were then counted in five independent visual fields using an optical microscope (Olympus Corp., Tokyo, Japan) at a magnification x100.

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Wound healing assay. The RCC 769-P and 786-O cell lines were seeded onto 6-well plates. When the cell density reached up 90-100%, scratch wounds were created across the monolayer with the tip of a 200-µl pipette. Subsequently the wounded cultures were incubated in a serum-free medium upon TQ treatment at 0 and 24 h and images (magnification x100) were captured by an inverted microscope to evaluate the migratory property. The experiments were performed in triplicate.
sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Bedford, MA, USA). Subsequently, the membranes were incubated with antibodies against phosphorylated-LKB1, total-LKB1, phosphorylated-AMPK, AMPK, E-cadherin (E-Ca), Snail and β-actin overnight at 4˚C. The bands were then washed with TBST (Tris-buffered saline with Tween) buffer and incubated with horseradish peroxidase (HRP-)linked secondary antibody at room temperature (25˚C) for 1 h. Finally, the protein bands were detected by an enhanced chemiluminescence detection kit (Bio-Rad Laboratories) and exposed to Image Lab 4.0 (Bio-Rad Laboratories) imaging software.

Plasmid transfection. LKB1 cDNA was cloned into pcDNA3.1 vector. The cells were seeded onto 6-well plates and transfected with the corresponding plasmid using X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions.

Statistical analysis. All experimental data are presented as the means ± standard deviation (SD) and GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA) software was used for statistical analyses. Differences between two groups were analyzed using Student’s t-test (two-sided), while one-way ANOVA test was used for comparisons among multiple independent groups. P<0.05 was considered to indicate a statistically significant difference.

Results

The anti-proliferative effect of TQ on RCC cells. The chemical structure of TQ is depicted in Fig. 1A. Firstly, we detected the effect of TQ on the normal renal tubular epithelial HK2 cell line. The results demonstrated that there was no significant change in cell growth upon TQ treatment for 24 h, while a slight decrease in cell growth with TQ treatment for 48 and 72 h was observed, indicating a low cytotoxic effect of TQ on normal epithelial cells (Fig. 1B). Subsequently, in order to confirm the effect of TQ on RCC cell migration and invasion, it was essential to determine the concentration-dependent effect of TQ on cell viability. Human RCC 769-P and 786-O cells with 90% density were exposed to TQ treatment (0.5, 1.0, 2.5, 5.0, 10, 15, 20 µM) at different time-points (0, 24, 48 and 72 h), which revealed a gradually decreasing cell proliferation in a concentration- and time-dependent manner (Fig. 1C and D). Lower doses of TQ (up to 10 µM) exhibited a less than 10% inhibitory rate of cell growth, while higher doses of TQ (beyond 10 µM) exhibited a significant inhibition of cell proliferation (Fig. 1E and F). In view of this, the concentration of 10 µM at 24 h was chosen to explore the anti-metastatic potential of TQ on RCC cells.

The anti-metastatic effect of TQ on RCC cells. A wound healing assay was used to explore the effect of TQ on cell migration. The migration speed of the 769-P cell line was significantly reduced in the presence of TQ compared to the
untreated cells (Fig. 2A). Similar results were observed in the TQ-treated 786-O cell lines (Fig. 2B). Subsequently, the RCC 769-P and 786-O cell lines were treated with TQ for the Transwell migration assay and a significant reduction in cell migration was observed under the TQ treatment compared with that in the untreated group. The number of migrated cells on the lower surface of the chamber is demonstrated in Fig. 2C and D. The results indicated that TQ plays a crucial role in inhibiting the migration of RCC cells.

Subsequently, to confirm the inhibitory effect of TQ on the invasion of 769-P and 786-O cells, a Matrigel invasion assay was used for the detection of the cell invasion ability. After treatment with 10 µM TQ for 24 h, the invasiveness of the 769-P cell line was significantly inhibited compared with the negative control group (Fig. 2C). Similar results were observed in the 786-O cell line (Fig. 2D). Collectively, these data revealed that TQ could suppress the invasion of RCC cells.

The anti-metastatic effect of TQ is mediated by the LKB1/AMPK signaling pathway. A previous study revealed that the LKB1/AMPK signaling is implicated in cancer metastasis (18). Firstly, western blot analyses were performed to evaluate the expression of LKB1 and AMPK. As depicted in Fig. 4A, an increase in the phosphorylation levels of LKB1 and AMPK was observed in the 769-P cell line upon TQ treatment, while the total LKB1 and AMPK exhibited no change under TQ treatment. In addition, following 24 h of treatment, we observed a concentration-dependent increase in phosphorylated-LKB1 and phosphorylated-AMPK in 786-O cells treated with TQ, which was in accordance with the above-mentioned results (Fig. 4B). The results of western blot analysis demonstrated that TQ significantly induced the phosphorylation of LKB1 and AMPK in RCC cells. To further validate whether LKB1 participated in the inhibitory effect of TQ on 769-P and 786-O cell lines, LKB1 was overexpressed by plasmid transfection. The results revealed that overexpression of LKB1 further enhanced the expression of E-cadherin, while it reduced the expression of Snail in the TQ-treated 769-P and 786-O cell lines (Fig. 4C and D). In addition, phosphorylated-AMPK, the downstream kinase of LKB1, was upregulated under the overexpression of LKB1. Collectively, these results confirmed the role of LKB1 in the regulation of EMT in RCC.

To further explore whether LKB1/AMPK signaling plays a vital role in TQ-inhibited cell migration and invasion, Compound C (ComC, AMPK inhibitor) and AICAR (AMPK activator) were used in combination with TQ for the subsequent experiment. The findings revealed that co-treatment with ComC attenuated the anti-metastatic effect of TQ on 769-P and 786-O cells, as revealed by the Transwell migration assay and the Matrigel invasion assay (Fig. 5A and B). Additionally,
TQ-mediated upregulation of E-cadherin and downregulation of Snail were partially abolished by the synergistic treatment with ComC (Fig. 5C and D). In contrast, the anti-metastatic effect of TQ on RCC 769-P and 786-O cell lines was further reinforced by AICAR (Fig. 6A and B). In addition, AICAR further strengthened the expression of E-cadherin, while...
weakened the expression of Snail in 769-P and 786-O cells upon TQ treatment (Fig. 6C and D). These data strongly supported that TQ exhibited anti-metastatic effect through the activation of AMPK phosphorylation.

Figure 5. Inactivation of AMPK by Compound C (ComC) attenuates the anti-metastatic effect of TQ on RCC cells. (A and B) Transwell migration assay and Matrigel invasion assay were used to detect the change of cell migration and invasion in 769-P and 786-O cells with different treatments (negative control, TQ, ComC and both TQ and ComC). Five random fields were observed by microscopy. All the experiments were performed in triplicate. (C and D) Western blotting was performed to explore the expression of phosphorylated-AMPK, E-cadherin, Snail and β-actin in 769-P and 786-O cells with different treatment (negative control, TQ, ComC and both TQ and ComC). Representative bands of three experiments are shown. RCC, renal cell carcinoma; TQ, thymoquinone.

Figure 6. Activation of AMPK by AICAR further decreases migration and invasion, and reverses EMT in TQ-treated RCC cells. (A and B) Using Transwell migration assay and Matrigel invasion assay, the migrated and invaded 769-P and 786-O cells were assessed through different treatments (negative control, TQ, AICAR and both TQ and AICAR). (C and D) 769-P and 786-O cells treated with TQ or AICAR were immunoblotted for phosphorylated-AMPK, E-cadherin, Snail and β-actin. Representative protein bands from three independent experiments are shown. EMT, epithelial-mesenchymal transition; RCC, renal cell carcinoma; TQ, thymoquinone.
In conclusion, these results indicated that the LKB1/AMPK signaling pathway is involved in TQ-regulated migration, invasion and EMT properties in RCC.

Discussion

It has been widely reported that TQ exerts anti-metastatic activity in various cancers. TQ was found to inhibit the metastasis of melanoma through the downregulation of NLRP3 (NACHT, LRR and pyrin domain-containing protein 3) inflammasome (19). In addition, TQ suppressed the metastatic phenotype of glioblastoma cells, accompanied by the reduction of FAK (focal adhesion kinase), MMP2 (matrix metalloproteinase-2) and MMP9 (20). In the present study, we confirmed that TQ markedly suppressed migration and invasion of human RCC for the first time, as evidenced by the results of the wound healing and Transwell assays. EMT is a complicated process through which cells lose their epithelial properties and tight cell-cell junction, while they gain mesenchymal characteristics. Studies have revealed that EMT is closely correlated with cancer metastasis (21,22). It has been demonstrated that TQ could reverse EMT by reducing the mRNA expression of Twist1 (23). Our findings indicated that TQ drastically increased the expression of E-cadherin, while it decreased the expression of Snail, ZEB1 and vimentin at the mRNA and protein levels, which indicated a strong anti-metastatic activity of TQ on RCC.

A variety of signaling pathways are involved in tumor migration and invasion (24-26). LKB1/AMPK signaling has gained great attention in recent years. Studies indicated that LKB1 deficiency impaired the polarity of mammary epithelial cells, leading to an increase of the migratory and invasive capacity of epithelial cells (27). Furthermore, LKB1 is reported to be a well-known tumor suppressor. It can induce apoptosis and cell cycle arrest, to inhibit tumor progression (28,29). Therefore, dysregulation of LKB1 is closely associated with tumor initiation and progression. The loss of LKB1 was found to upregulate Snail expression, an EMT marker (30). Similarly, ZEB1 was upregulated in LKB1-deficient lung adenocarcinoma cells (31). In the present study, TQ upregulated phosphorylation levels of LKB1 and AMPK in RCC 769-P and 786-O cell lines. In addition, TQ-mediated high expression of E-cadherin and low expression of Snail could be further enhanced by overexpression of LKB1, indicating a critical role of LKB1 in TQ-inhibited EMT of RCC. In addition, AMPK, the downstream of LKB1, is closely associated with tumor growth and neovascularization of cancer cells (32). Furthermore, studies revealed that α-enolase promotes metastasis of colorectal cancer by negatively regulating AMPK signaling pathway (33). Our results demonstrated that co-treatment with AMPK inhibitor ComC could impair the anti-metastatic effect of TQ on RCC and reverse TQ-mediated upregulation of E-cadherin and downregulation of Snail. Conversely, the AMPK activator AICAR had the inverse effects.

In conclusion, the present study confirmed that TQ could inhibit metastatic phenotype and reverse EMT in RCC by regulating the LKB1/AMPK signaling pathway. These results indicated that TQ may be an optional therapeutic method for the treatment of RCC. Furthermore, LKB1/AMPK signaling may be a potential therapeutic target against RCC.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

BK, QK and BM conceived and designed the study. BK, JZha, BS and YY performed the experiments and analyzed the data. BK and JL drafted the paper. BK, QK, JL, JZhou and WL drafted, reviewed and edited the manuscript, and were also involved in the conception of the study. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

No applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

References


