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Research Article

Thymoquinone exerts anti-tumor activities on human hepatocellular carcinoma cells: role of angiogenesis-related genes *VCAM*, *Grb2* and *EZH2*

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Abstract

Human hepatocellular carcinoma (HCC) is the most prevalent and recurrent type of primary adult liver cancer without any effective therapy. Thus, there is an increase demands for finding new drugs and treatment strategies with selective and potent effects towards HCC. Plant-derived compounds acting as anti-cancer agents can induce apoptosis through targeting several signaling pathways. Thymoquinone (TQ), the major biologically active compound of the black seed oil (*Nigella sativa*) has demonstrated inhibitory activities on various cancers by targeting several pathways. In the present study, we have evaluated the molecular mechanisms that underlie the anti-proliferative, anti-metastatic, and pro-apoptotic activities exerted by TQ on liver cancer cell line HepG2, a well-documented HCC *in vitro* model. Cell proliferation was determined by WST-1 assay, apoptosis rate was assessed by flow cytometry using annexin-V/TAAD staining, wound healing assay to investigate the metastasis, and the expression of target genes was assessed by Real-time RT-PCR analysis. We found that TQ significantly reduced HepG2 cell viability and induced apoptosis in a dose-dependent manner. Migration of HepG2 cells was suppressed in response to TQ. Moreover, TQ decreased the expression of several angiogenesis-related genes including versican (*VCAM*), growth factor receptor-bound protein 2 (*Grb2*), and the histone methyltransferase for lysine 27 of histone 3 (*EZH2*). The findings suggest that TQ exerts inhibitory effects on HCC most likely through targeting key genes involved in the invasiveness and metastatic activities of HCC cells and also suggest that TQ could be used in future as potential anti-HCC candidate in chemotherapy.

Keywords: Thymoquinone, Angiogenesis, Apoptosis, HepG2, Human hepatocellular carcinoma.

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1. Introduction

Hepatocellular carcinoma (HCC) is considered as the predominant

form of primary malignant liver tumor and accounts to 80–90% of all liver cancers [1-3]. Worldwide, HCC accounts to 5% of all human cancers and approximately one million cases of HCC are

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diagnosed, while more than 690,000 HCC patients die every year [4, 5]. Among tumors, HCC is the fifth most common type of cancer in men and eighth in women [6] and is also considered as the third cause which leads to cancer-related death globally [7]. HCC incidence is increasing dramatically in many countries due to the exposure to various environmental risk factors including chronic hepatitis B and C viral infections, obesity, and diabetes mellitus [8-10]. Most HCC cases are diagnosed at late stages where treatments become ineffective [11-13]. At the molecular level, HCC is a complex disease which is a major challenge facing the development of effective treatments [14-16]. Several molecular signaling mechanisms are involved in the development of liver cancer. Indeed, several studies have shown the contribution of multiple pathways in promoting the overexpression of various genes implicated in angiogenesis such as versican (*VCAN*), growth factor receptor-bound protein 2 (*Grb2*) and the histone methyltransferase for lysine 27 of histone 3 (*EZH2*) [5, 17, 18]. A cytoplasmic expression of *VCAN* protein was shown to be upregulated in HCC compared to matched normal tissues [19] and this overexpression was suggested as candidate biomarker for early stages of HCC [20]. In the same context, high expression of *EZH2* [21] and *Grb2* genes [22] were detected in HCC and also suggested as a promising diagnostic biomarkers of HCC.

The current treatment regimens have a number of side effects as well as HCC has low response and resistance to chemotherapy at advanced stages. Therefore, there is an increase need for developing new drugs and treatments with minimum side effects and high potency to treat HCC patients.

The Plant Kingdom is a rich resource for finding treatment for many illnesses and diseases. Many medicinal plants and their purified components such as thymoquinone (TQ) have shown beneficial therapeutic potentials in the recent years, and they are widely used as alternatives to chemical drugs [23]. TQ, the major bioactive component of the essential oil of black seeds (*Nigella sativa*) has shown promising inhibitory effects of on a large number of tumors including HCC through targeting several pathways [24-30]. Several studies investigated the inhibitory effects of TQ on HepG2 cells [31-33] but the underlying molecular mechanisms are still largely unknown [32].

Consequently, the aim of the present study was to evaluate the effect of TQ on the expression of key genes involved in the invasiveness and metastasis of HCC cells. Our results showed that TQ significantly reduced HepG2 cell viability, induced apoptosis and suppressed the migration of HepG2. TQ-induced inhibitory effects in HepG2 were associated with a significant decrease in the expression of several angiogenesis-related genes such as *VCAN*, *Grb2* and *EZH2*. The present findings suggest that TQ exerts inhibitory effects on HCC most likely through targeting key genes involved in invasion and metastasis, and also suggest that TQ could be used in future as potential anti-HCC candidate in

chemotherapy.

2. Materials and Methods

2.1 Cell culture and treatment

Liver cancer cell line HepG2 was purchased from American Type Culture Collection, Manassas, VA, USA. Cells were maintained in DMEM (UFC-Biotech, Riyadh, SA) supplemented with 15% (v/v) fetal bovine serum (BioWhittaker, Lonza, Belgium), penicillin (100 U/ml) and streptomycin (100 µg/ml) (Sigma-Aldrich, St-Louis, MO, USA). Cells were maintained in a humidified incubator containing 5% CO₂ at 37°C. For all treatments, a 10 mM solution of TQ (Sigma-Aldrich, St-Louis, MO, USA) was prepared in 10% DMSO (Sigma-Aldrich, St-Louis, MO, USA) and appropriate working concentrations were prepared with cell culture medium. The final concentration of DMSO was always less than 0.1% in both control and treated conditions.

2.2 Cell proliferation assay

Cell proliferation was analyzed by a colorimetric cell proliferation assay using WST-1 Cell Proliferation Reagent Kit (Sigma-Aldrich, USA). For this purpose, HepG2 cells were seeded in 96-multiwell plates at a density of 10⁴/well and incubated overnight. Then, the cells were exposed to different concentrations of TQ for 24 h. Cell proliferation rate then was evaluated through a rapid WST-1 reagent. 10 µL of the WST-1 solution was added and incubated for an additional 3h at 37°C. Finally, the absorbance was read at 450 nm with a microplate ELISA reader (ELx800™ Biotek, USA) and the results were analyzed by the Gen5 software (Biotek, USA). The percentage of cell viability was calculated by assuming control (untreated) samples as 100 % viable.

2.3 Apoptosis Assay

To study the apoptosis, HepG2 cells were seeded in 6-well plates at a density of 2 x10⁵ cells/well, grown for 24h and exposed to different concentrations of TQ for 24h. Cell apoptosis rate was assessed using the Annexin V Binding Guava Nexin® Assay by capillary cytometry (Guava Easycyte Plus HP system, with absolute cell count and six parameters) following the manufacturer's recommendations for adherent cells (Guava Technologies Inc, Hayward, CA, USA). Guava Nexin® Assay utilizes annexin V-phycoerythrin (PE) to detect phosphatidylserine on the external membrane of apoptotic cells. The cell impermeant dye, 7-amino-actinomycin (7-AAD), is used in the Guava Nexin® Assay as an indicator of cell membrane structural integrity.

2.4 Wound healing assay

Wound healing assay was described elsewhere [34, 35]. Briefly, The HepG2 cells were seeded in 6-well plate at a density of 2x10⁴ cells/well. After an incubation of 24 h, the culture media was removed and a fresh culture media was added with 100 µM of TQ

for 24 h. Then, a scratch was made on the monolayered cells with a 10 μ l pipette tip in each well. Phase contrast microscope (Leica, Wetzlar, Germany) with 500X magnification was used to record the images at 0 h and the images of the same scratch were taken after an incubation of 24 h.

2.5 Real-time RT-PCR analysis

Real-time RT-PCR analysis was described elsewhere [36]. Briefly, the cells were treated with different concentrations of TQ for 24h. Total RNA was isolated and purified from HepG2 cells using the RNeasy kit (Qiagen, Hilden, Germany). Then, total RNA was subjected to reverse transcription using oligo dT and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was done with the LightCycler 480 SYBR Green I Master kit (Roche Diagnostics) and the Mastercycler Realplex apparatus (Eppendorf, Montesson, France). The results were normalized with RPL11 mRNA. The sequences of the primers for PCR amplification were: *VCAN* (sense: 5' - TTGGACTGATGGCAGCACACT-3' ; antisense: 5' - GGCCATTCTCATGCCAAATG -3'); *GRB2* (sense: 5' - CAGAAGAGAGGCCGAGGCTAA- 3' , antisense: 5' - AGCTCGTCGCTGCAGTAG-3'), *EZH2* (sense: 5' - TGTCTTACTTGTGGAGCCGCT -3' , antisense: 5' - TGGTGCCAGCAATAGATGCTT-3'), *RPL11* (sense: 5' - AGCCAAGTCTTGGAGCAGCTTA -3' , antisense: 5' - TTGGGCCTCTGACAGTACAGTGAACA -3'). Amplicons were size controlled on agarose gel and purity was assessed by analysis of the melting curves at the end of the RT-PCR reaction.

2.6 Statistical Analysis

All the data were presented as mean \pm SE of triplicates done in the same experiment or an average of at least three separate experiments. The differences between the control and the treated cells were analyzed by Student's t-test (two-tailed) using GraphPad Prism 6 (Graph Pad Software, SanDiego, USA) and the significant differences were indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3. Results

3.1 TQ reduced HepG2 cell viability in a dose-dependent mechanism

To assess the anti-proliferative effect of TQ in HepG2 cells, cells were incubated with increasing concentrations of TQ for 24 h. We found that TQ significantly inhibited cell proliferation from 10 μ M compared to the control. Cell proliferation inhibition reached 60% and 80% at 30 μ M and 50 μ M of TQ respectively (Fig. 1). These findings indicate that TQ induces a dose-dependent inhibition of HepG2 cell proliferation.

3.2 TQ induced dose-dependent apoptosis in HepG2 cells

To investigate the apoptotic effect of TQ in HepG2 cells, cells were exposed to increasing concentrations of TQ for 24 h (Fig 2). We found that TQ significantly induced early apoptosis at 10 μ M by 12%, and this percentage reached 42% and 44% at 30 μ M and 50 μ M of TQ respectively (Fig. 2B). TQ had no effect on the number of apoptotic cells in late stage and dead cells (Fig. 2C). These findings indicate that TQ induces a dose-dependent early apoptosis in HepG2 cells.

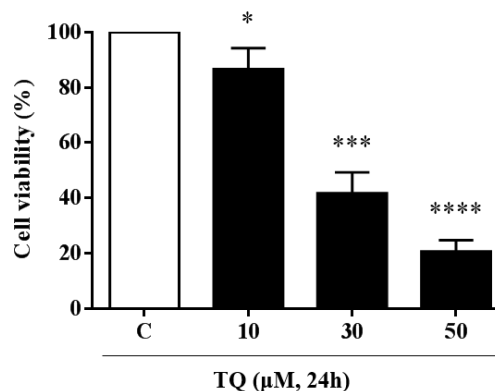


Figure 1: Dose-dependent effects of TQ on HepG2 cell viability. Cells were exposed to increasing concentrations of TQ for 24 h. Cell viability rate was assessed by WST-1 assay. The data are representative of three different experiments (n= 3). Values are shown as mean \pm SE. (n = 3); * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ versus respective control.

3.3 TQ inhibited wound healing in HepG2 cells

To investigate the effects of TQ on HepG2 cells motility, we performed a scratch/wound assay. The results displayed an obvious wound at 0 h that was healed in the control cells after 24 h (data not shown). Interestingly, the addition of TQ to HepG2 cells at 100 μ M resulted in a significant inhibition of cellular migration and wound healing (data not shown). These findings indicate TQ has promising potentials in controlling the HepG2 cell migration, and suggest that it could be a potent candidate to regulate metastasis in hepatocellular carcinoma.

3.4 TQ decreased the expression of the angiogenesis-related genes *VCAN*, *Grb2* and *EZH2* in a dose-dependent manner

As *VCAN*, *Grb2* and *EZH2* genes are upregulated in HCC and are known to be involved in the angiogenesis, cell proliferation, metastasis and inhibition of apoptosis [5, 17, 18], we studied the effect of TQ at 10, 30 and 50 μ M TQ for 24 h on mRNA expression of *VCAN* (Fig. 3A), *Grb2* (Fig. 3B) and *EZH2* (Fig. 3C) in HepG2 using RT-qPCR. We found that mRNA expression of all target genes *VCAN*, *Grb2* and *EZH2* was significantly decreased in a dose-dependent manner in HepG2 in response to TQ treatment;

suggesting a significant role for these genes in TQ-induced cell proliferation and metastasis inhibition and apoptosis in HepG2 cells.

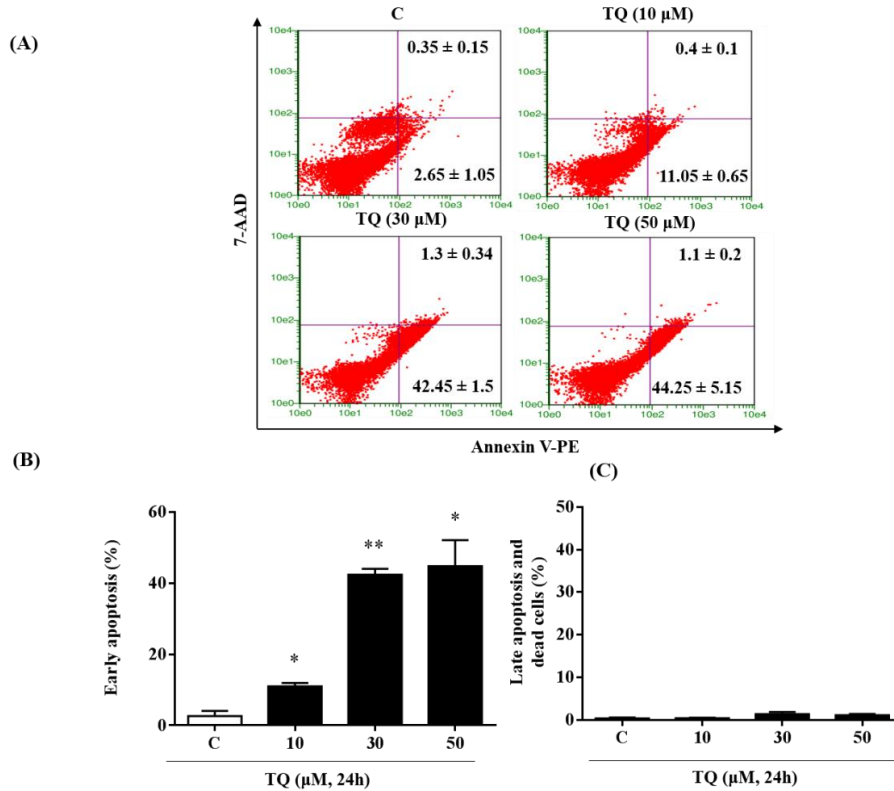


Figure 2: TQ dose effect on apoptosis in HepG2 cells. Cells were exposed to increasing concentrations of TQ for 24 h. Apoptosis in HepG2 cells was assessed by flow cytometry using the annexin V-PE and 7-AAD staining assay. (A) Lower-left quadrant: viable cells (annexin V-PE negative and 7-AAD negative cells). Lower-right quadrant: cells in the early stage of apoptosis (annexin V-PE positive and 7-AAD negative cells). Upper right quadrant: cells in the late apoptotic or dead (annexin V-PE positive and 7-AAD positive cells). Upper-left quadrant: mostly nuclear debris (Annexin VPE negative and 7-AAD positive). The number of apoptotic cells in early stage (B) and late apoptosis and dead cells (C) is expressed as percent relative to the total cell number. Values are shown as mean ± SE (n = 3); * $p < 0.05$, ** $p < 0.01$ versus respective control.

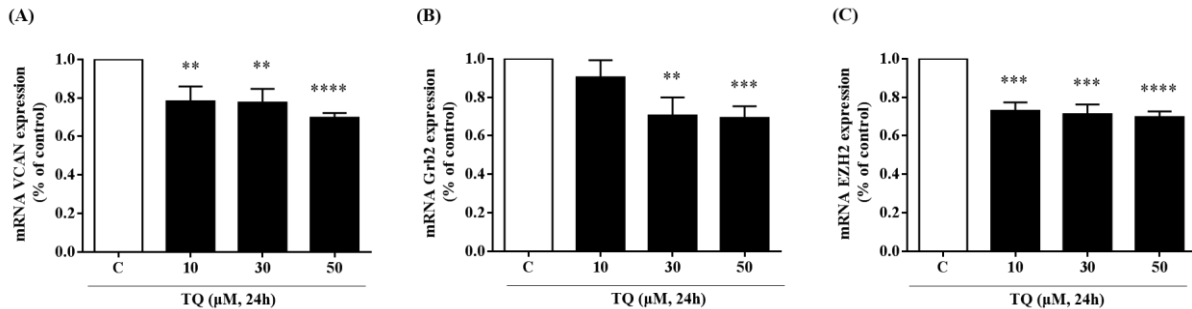


Figure 3: Dose-dependent effects of TQ on the expression of VCAN, Grb2 and EZH2 mRNA levels in HepG2 cells. Cells were exposed to increasing concentrations of TQ for 24 h. The histograms show the quantification data of mRNA expressions of VCAN (A) and Grb2 (B) and EZH2 (C), as assessed by real-time PCR. Results are means of three separate experiments performed in triplicate. Values are shown as means ± S.E.M. (n = 3); ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus respective control.

4. Discussion

HCC is one of the most aggressive tumors with high complexity at the molecular level, which represents a major cause that limits the development of effective treatments. Therapies that target angiogenesis-related genes have shown promising results in different cancer therapies. *VCAN*, *Grb2* and *EZH2* genes are overexpressed in HCC and involved in angiogenesis, cell proliferation, metastasis and inhibition of apoptosis. However, due to adverse side effects of current cancer therapies and the resistance to these therapies in advanced stages of HCC cancer, there is an increasing need to find new inhibitors of those genes and other genes that might be involved in cancer progression. In the present study, we have evaluated the molecular mechanisms underlying the anti-proliferative, anti-metastatic and pro-apoptotic activities exerted by TQ on liver cancer cell line HepG2, a well-established HCC model. We found that TQ significantly inhibited HepG2 cell proliferation, migration, and apoptosis in a dose dependent manner; suggesting the specificity of TQ effects since it was used in its pure form. However, how physiological concentrations found naturally in black seeds would correlate with the current findings, and how the variable time points would affect these observations are yet to be tested.

TQ-induced inhibitory effects were also associated with a significant decrease in the expression of *VCAN*, *Grb2* and *EZH2* genes in HepG2. High expression levels of *VCAN* are known to promote proliferation, invasion, and metastasis of a large number of human cancer cells, including HCC [20, 37]. The knockdown of *VCAN* was shown to inhibit glioblastoma cells proliferation and migration most likely through targeting transforming growth factor TGF-beta 2 (TGF- β 2) [38]. Considering the fact that TQ was shown to repress the metastasis of prostate cancer cells through the downregulation of TGF- β [39], TQ-induced *VCAN* downregulation in the present study could be orchestrated by TGF- β without excluding other pathways. Additionally, our results showed that TQ significantly decreased the expression of *EZH2* in HepG2 suggesting that TQ can inhibit HCC angiogenesis by targeting *EZH2*. This hypothesis is supported by the fact that *EZH2* was shown to have a key role in the regulation of tumor angiogenesis and the decrease in its expression levels inhibited metastasis suggesting *EZH2* as a potential therapeutic target to reverse the migration and invasion of cancer cells [40-44]. Like *VCAN* and *EZH2* genes, several studies have shown that *Grb2* has an important role in proliferation and angiogenesis of several tumors including HCC through its contribution in the signaling pathway of various angiogenic factors [17, 22, 45, 46]. The overexpression of miR-564 was shown to inhibit the proliferation, migration and invasion of HCC cell lines through a direct effect on *Grb2* gene [46]. In the present study we found that TQ induces the downregulation of *Grb2* in HepG2 with subsequent cell proliferation

and metastases inhibition and apoptosis induction indicating that the downregulation of *Grb2* is a main event to the inhibition of angiogenesis and induction of apoptosis in HCC in response to natural products. In accordance with this hypothesis, the natural product, sinulariolide has been shown to inhibit HCC cell migration and invasion by suppressing several angiogenic factors including *Grb2* [47].

5. Conclusions

The present findings indicate that TQ exerts inhibitory effects on HCC most likely through targeting key genes (*VCAN*, *Grb2* and *EZH2*) involved in the invasiveness and metastasis of HCC cells (Fig. 4). These results also suggest that TQ could be used in future as potential anti-HCC candidate in chemotherapy.

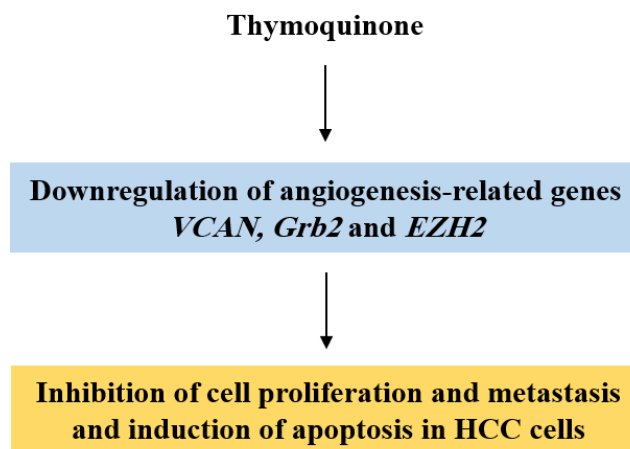


Figure 4: Schematic representation of molecular mechanisms underlie the anti-tumor activities induced by TQ in HCC cells.

Declaration of Conflicting Interests

The authors declare that they have no financial conflict of interest.

Author Contributions

MA, SFZ and SH designed the project and wrote the paper. MYA, RAS, MAH, and AAK performed research and analyzed data.

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