Physiology International 109 (2022) 3, 371–387
DOI: 10.1556/2060.2022.00163

AKADÉMIAI KIADÓ

Modulation of miR-192/NF-κB/ TGF-β/ E-cadherin by thymoquinone protects against diethylnitrosamine /carbon tetrachloride hepatotoxicity

HEND ASHOUR[1,2†*], MAHA EID FARGHALY[3†], AKEF ABDELHALIM KHOWAILED[2], BASMA EMAD ABOULHODA[4], LAILA AHMED RASHED[5], MOHAMED MAHMOUD ELSEBAIE[5] and SAFY SALAH GABER[3]

[1] Department of Medical Physiology, Faculty of Medicine, King Khalid University, Abha, Saudi Arabia

[2] Department of Medical Physiology, Faculty of Medicine, Cairo University, Cairo, Egypt

[3] Department of Medical Physiology, Faculty of Medicine, Beni-Suef University, Beni-Suef, Egypt

[4] Department of Anatomy and Embryology, Faculty of Medicine, Cairo University, Cairo, Egypt

[5] Department of Biochemistry, Faculty of Medicine, Cairo University, Cairo, Egypt

Received: September 25, 2021 • Revised manuscript received: February 26, 2022 • Accepted: May 16, 2022
Published online: August 12, 2022

ABSTRACT

Scientific efforts have been made for a better understanding of the pathogenesis of hepatocellular carcinoma (HCC). We investigated the possible role of miR-192/nuclear factor-κB (NF-κB)/transforming growth factor-β (TGF-β)/E-cadherin in hepatic tumorigenesis. We expected a modulatory impact of thymoquinone. Thirty adult male rats were assigned into 3 groups ($n = 10$); (1) Control group. Group (2): Experimental HCC induced by intraperitoneal injection of diethylnitrosamine (DENA) followed by carbon tetrachloride (CCl4). Group (3): Thymoquinone 20 mg kg^{-1}/oral supplementation starting from the model induction to the end of the 8th week. The HCC (DENA-CCL4) model was confirmed by elevated serum levels of alpha-fetoprotein and transaminases (ALT, AST) and by histopathological examination which

* Corresponding author. Department of Medical Physiology, Faculty of Medicine, Cairo University, Cairo, Egypt. Tel.: +20 966508133732. E-mail: hend.a.hassan@kasralainy.edu.eg; hiahmad@kku.edu.sa

† These authors contributed equally as first authors.

denoted marked cellular atypia and features of neoplasia. Suppressed hepatic miR-192 and E-cadherin expression were detected in the HCC (DENA-CCL4) group accompanied by elevated tumor necrosis factor (TNF-α), interleukin (IL6)/NF-κB & TGF-β1. Thymoquinone treatment protected the rat livers from hepatic tumorigenesis. Thymoquinone diminished ($P < 0.001$) alpha-fetoprotein and improved ALT, AST. It preserved hepatic miR-192 and normal E-cadherin expression. Thymoquinone-treated rats showed abrogated TNF-α, IL6/NF-κB/TGF-β. Thymoquinone increased cell apoptosis markers Bax/Bcl2 and diminished cellular atypia. Pearson's correlations revealed positive association between miR-192 expression and E-cadherin and Bax/Bcl2 as well, and it was negatively correlated to alpha-fetoprotein, NF-κB and TGF-β and the cellular atypia score. In conclusion, thymoquinone protected the liver tissues through preserving miR-192 and E-cadherin and aborting NF-κB & TGF-β signaling. The current results highlight a new role for thymoquinone in preventing hepatic tumorigenesis.

KEYWORDS

hepatocellular carcinoma, thymoquinone, microRNA-192, NF-κB, TGF-β, E-cadherin

INTRODUCTION

Liver cancer-related mortality is rising globally, increasing the worldwide economic burden. Therefore, understanding the pathogenesis of hepatocellular carcinoma (HCC) and searching for traditional medicine deserves special scientific attention. HCC is closely associated with chronic microenvironment inflammation and fibrosis, regulating tumor initiation and proliferation, which is known as the hepatic inflammation-fibrosis-cancer axis [1]. The nuclear factor-kappa-B (NF-κB) is the heart of the inflammatory process associated with HCC. Following activation, it promotes the survival of pre-cancerous hepatocytes and accelerates cancer growth [2]. Therefore, targeting the inflammatory process is important in the general anticancer approach. The loss of physiological cellular adhesion contributes to the distortion of tissue architecture and hence facilitates cancer progression. Epithelial cadherin (E-cadherin) is one of the cell surface glycoproteins involved in cell-to-cell adhesion. E-cadherin has been defined as a tumor suppressor factor, since its downregulation is documented in malignant epithelial tumors [3]. The loss of E-cadherin contributes to the increased invasion capabilities and the poor differentiation observed in HCC [4]. Meanwhile, overexpression of transforming growth factor beta (TGF-β) is correlated with hepatocyte carcinogenesis and the progression of HCC [5]. The activated hepatocytes release TGF-β during liver injury which acts as a crosstalk between pre-malignant hepatocytes and modification of the microenvironment favoring cancer initiation. Additionally, TGF-β mediates the epithelial to mesenchymal transition documented in HCC [6].

MicroRNAs (miRs) are a novel class of small non-coding single-stranded RNA molecules involved in various biological processes. The miRs may influence post-transcriptional gene regulation which affects mRNA stability and protein translation, apoptosis, development, proliferation, and differentiation [7]. MicroRNAs are also involved in the regulation of the inflammatory microenvironment in cancer [8]. Therefore, miRs play a fundamental role in tumorigenesis. Specifically, miR-192 is involved in tumor regulation, and its downregulation was reported in tumors such as renal [9], lung [10], osteosarcoma [11], as well as liver [12] cancers. Thereby, miR-192 may contribute to the pathogenesis of HCC. Recent evidence suggests an

association between TGF-β-mediated EMT signaling and miRNAs, which provides new insight into the microenvironmental disturbance of cancers [13], and it has been suggested that the TGF-β pathway can stimulate or inhibit miRNA maturation [14]. However, the relation of miR-192 to NF-κB and TGF-β/E-cadherin in HCC, in particular, is still unclear.

Thymoquinone, [TQ, 2-Isopropyl-5-methyl-1,4-benzoquinone] is the predominant bioactive constituent found in black seeds of *Nigella sativa*, particularly used as a condiment in the Middle East. Accumulating evidence has shown that TQ has anti-oxidant and anti-proliferative effects in many types of cancers [15] including liver cancer [12]. Recent investigations documented a valuable role of TQ in regulating the expression of several miRs in cancers [16], as it may have epigenetic modification characteristics responsible for cancer regulation [17]. However, limited variants of miRs were shown to be involved in the protective effect of thymoquinone against HCC, including miR-16 and miR-375 [18]. Meanwhile, the role of thymoquinone in regulating miR-192 in HCC is largely unknown.

Aim of the work

The current study was designed to investigate the role of miR-192 and TNF-α/IL6/NF-κB/TGF-β/E-cadherin signaling in HCC pathogenesis in a rat model of hepatic tumorigenesis. We suggested that thymoquinone could inhibit the initiation stage of hepatic carcinogenesis, and it may regulate the expression of miR-192 and E-cadherin and the TNF-α/L6/NF-κB TGF-β content in the liver tissue.

MATERIAL AND METHODS

Animals and ethical consideration

Thirty adult male Wistar albino rats (180–200 g) supplied by the animal care unit of the Faculty of Medicine, Cairo University were used for conducting this experiment. All animal procedures were approved by the Institutional Animal Care and Use Committee of Cairo University (# CU/III/F/20/19).

Model of hepatocellular carcinogenesis

Rats were housed for 2 days to be acclimatized before starting the experiment. The experimental model of HCC was induced as described in [19]. The rats received a single intraperitoneal injection of diethylnitrosamine (DENA) (# N0258 Sigma-Aldrich) freshly prepared in 0.9% saline in a dose of 200 mg kg^{-1} once, as an inducer for hepatocellular carcinoma. After two weeks, the animals received 6 doses of carbon tetrachloride (CCl_4) injections of 3 mL kg^{-1}/ s.c. /weekly to enhance the carcinogenic effect of DENA on HCC (DENA-CCl4). At the end of the 8^{th} week, we confirmed the model establishment by the elevated alpha-fetoprotein levels. Deaths were recorded as 20%, and finally we obtained 20 rats of an established HCC model.

Experimental design

The included rats were allocated into three experimental groups (10 rats each): 1) The control group received only saline as vehicle. 2) The experimental HCC model, HCC (DENA-CCl4).

3) HCC-Thymoquinone group, in which the rats were treated with thymoquinone (TQ, Sigma-Aldrich # 274666) in a dose of 20 mg.kg-1 orally [20] for 3 alternative days weekly starting from the beginning of the model induction to the end of the 8th week to assess the preventive effect of TQ on hepatic tumorigenesis.

Sample collection and preparation

At the end of the experimental study (8th week), rats were fasted overnight. Blood samples were withdrawn from the rat tail veins under pentobarbital anesthesia 50 mg kg^{-1} i.p. for serum preparation. A midline incision was made and the liver was harvested from each rat. Liver tissue specimens were prepared for histopathology examinations. To prepare 10% tissue homogenate, the liver specimens were placed in ice-cold saline. For every 5 mg tissue sample, 300 µL of ice-cold lysis buffer was added, and the samples were homogenized and centrifuged at 5,000 rpm for 20 min at 4 °C in a Hettich universal 32A (Germany) apparatus. The supernatants were collected in fresh tubes and preserved at −80 °C.

Biochemical analysis methods

Serum detection of α-fetoprotein (AFP). Quantitative detection of rat α-fetoprotein (AFP) serum levels followed a double antibody sandwich technique in which the rat ELISA kit (# MBS267612; MyBioSource, Egypt) was used. The procedures were conducted in accordance with the manufacturer's datasheet.

Estimation of the liver enzymes alanine transaminase (ALT) and aspartate transaminase (AST). Instructions were followed using the manufacturer's datasheets for serum ALT (Bio-Med Diagnostic-GPT, Egy-Chem), and for serum AST enzyme activity determination (BioMed Diagnostic-GOT, Egy-Chem).

Quantitative real-time PCR detection of E-cadherin and miR-192

RNA extraction and purification. Total RNA from each sample was extracted using the mirVana™ isolation Kit (Catalog number: AM1560; Thermoscientific, UK) according to the manufacturer's instructions. The primers used for E-cadherin and miR-192 are shown in Table 1. Real-time PCR was carried out using an Invitrogen kit. Primers for miR-192 were supplied by Applied Biosystems (TaqMan miRNA Assays ID # 002272, catalog # 4427975).

Table 1. Primers used in the PCR

Gene	Primer sequences
E-cadherin	Forward: 5′ TCCCAATACATCTCCCTTCACA3′
	Reverse: 5′ACCCACCTCTAAGGCCATCTTT3′
β-Actin	Forward: AGCCATGTACGTAGCCATCCA
	Reverse: TCTCCGGAGTCCATCACAATG
miR-192	Mature miR Sequence: CUGCCAAUUCCAUAGGUCACAG
U6	CGTTCCAATTTTAGTATATGTGCTGCCGAAGCGA

miR-192 expression values were normalized to small RNA U6, with high efficiency of miRs. Approximately 2 μg of total RNA were used for preparing a single strand of cDNA using a high-capacity cDNA reverse transcription kit (Superscript II; Gibco Life Technologies, Grand Island, NY). Real-time PCR amplification and analysis were performed using an Applied Biosystem instrument using software version 3.1 (StepOne™, Applied Biosystems, Lincoln Centre Drive, Foster City, CA). The reaction contained SYBR Green Master Mix supplied by Applied Biosystems (Lincoln Centre Drive, Foster City, CA). The relative expression of the target gene was determined with the help of the Applied Biosystem software using the $2^{-\Delta\Delta Ct}$ method.

Western blot detection of the protein levels of nuclear factor-kB (NF-κB), B cell lymphoma factor (Bcl2), and Bcl-associated X protein (Bax)

The NF-κB protein level in the liver homogenates was determined according to the manufacturer's recommendations. In short, proteins were extracted from the prepared tissue homogenates through ice-cold radio-immunoprecipitation assay (RIPA). The proteins were then transferred to polyvinylidene difluoride membranes (Pierce, Rockford, IL, USA). Membranes were blocked with 5% (w/v) skimmed milk powder for 1 h at room temperature. The following primary antibodies were used: NF-κB p65 rabbit polyclonal antibody (# Catalog # 51-0500, RRID: AB_2533893, Invitrogen, Thermoscientific, Illinois, USA); for Bax detection, rabbit anti-rat polyclonal antibody Catalogue # MA5-14003; and for Bcl-2, the rabbit anti-rat polyclonal antibody Catalogue # PA5-27094. The primary antibodies were incubated overnight at 4°C, followed by incubation for 1 h with the secondary antibody (Goat anti-rabbit IgG- HRP-1mg Goat, Novus Biologicals). Next, ClarityTM Western ECL substrate Bio-Rad cat#170-5060 was applied. Finally, band intensities were analyzed against the housekeeping protein beta actin on the ChemiDoc MP imager. The ratio between Bax/Bcl2 was then calculated, which indicates the degree of cellular deaths.

Determination of transforming growth factor (TGF-β1), tumour necrosis factor (TNF-α) and interleukin 6 (IL6). Activated rat TGF-β1 was quantitatively determined in the liver tissue homogenates using the sandwich ELISA technique. Following the manufacturer's instructions, the standard sandwich enzyme-linked immune-sorbent assay technology was applied using the kit # MBS175833 for TGF-β1, # MBS2568059 for TNF-α, and #MBS8800160 for IL6, supplied by MyBiosourse, Egypt.

Tissue preparation for histological analysis

The liver specimens from each group were immediately placed in 10% formol saline. The specimens were then washed carefully and subjected to an ascending ethanol concentration series for tissue dehydration. The specimens were cleared in xylene and embedded in paraffin-forming blocks. Sections of about 5 μm thickness were cut and stained with hematoxylin and eosin.

Histopathological scoring

Semi-quantitative scoring of cellular atypia was graded as mild (grades 1-2), moderate (grade 3) or severe (grades 4-5) depending on the features of nuclear contour, hyperchromatism, and

nuclear cytoplasmic ratio [21] where mild atypia is described as a minimal difference, severe atypia characterized by prominent nuclear changes with marked pleomorphism and severe hyperchromatism, and moderate atypia as intermediate changes. Score (0) is given when no nuclear changes can be observed.

Statistical analysis

The data collected from the groups of this study were expressed as mean ± SD. Data were entered into the Statistical Package for the Social Sciences (SPSS, version 20). The Analysis of variance (ANOVA) test was applied to compare data of the different groups. Then, Tukey's post-hoc test was performed to determine the statistical difference amongst the groups. The histology scoring for cellular atypia was analyzed by Kruskal–Wallis and Mann–Whitney tests and was summarized as the median and interquartile range (50; 25–75%) and Pearson's correlation was applied to evaluate the relationship within the model and the treated groups. The correlation test was applied to detect the relation between miR-192 and other factors. Statistical significance was considered if the probability (p-value) was < 0.05.

RESULTS

Model confirmation by elevation and reduction of alpha-fetoprotein level by thymoquinone treatment

To confirm the establishment of the HCC model, we measured the serum alpha-fetoprotein level as a marker of hepatocyte carcinogenesis. There was a significant ($P < 0.001$) elevation in the alpha-fetoprotein level compared to the control group. Thymoquinone administration starting from the model induction abolished ($P < 0.001$) the elevated alpha-fetoprotein marker induced by HCC (DENA-CCl4) indicating the hepatoprotective effect of thymoquinone (Fig. 1A).

Elevation and reduction of liver enzymes by thymoquinone

Liver cell injury was determined by the release of aminotransferase enzymes to the circulation. The HCC group revealed elevated ($P < 0.001$) ALT & AST serum levels that are seen in Fig. 1B and C. Hepatocyte protection ($P < 0.001$) from HCC (DENA-CCl4) injury was obtained by thymoquinone administration. In the treated group HCC-thymoquinone, serum liver enzymes were diminished as compared to normal control data.

Changes in miR-192 expression among groups

The expression of the small non-coding microRNA (miR-192) in liver tissue was suppressed ($P < 0.001$) in the HCC (DENA-CCl4) rat group compared with the control. Effective ($P < 0.001$) preservation of miR-192 expression by HCC-thymoquinone treatment was recorded compared to the HCC (DENA-CCl4) rat values while it was still significant in the control group ($P = 0.02$) as shown by representative data in Fig. 1D.

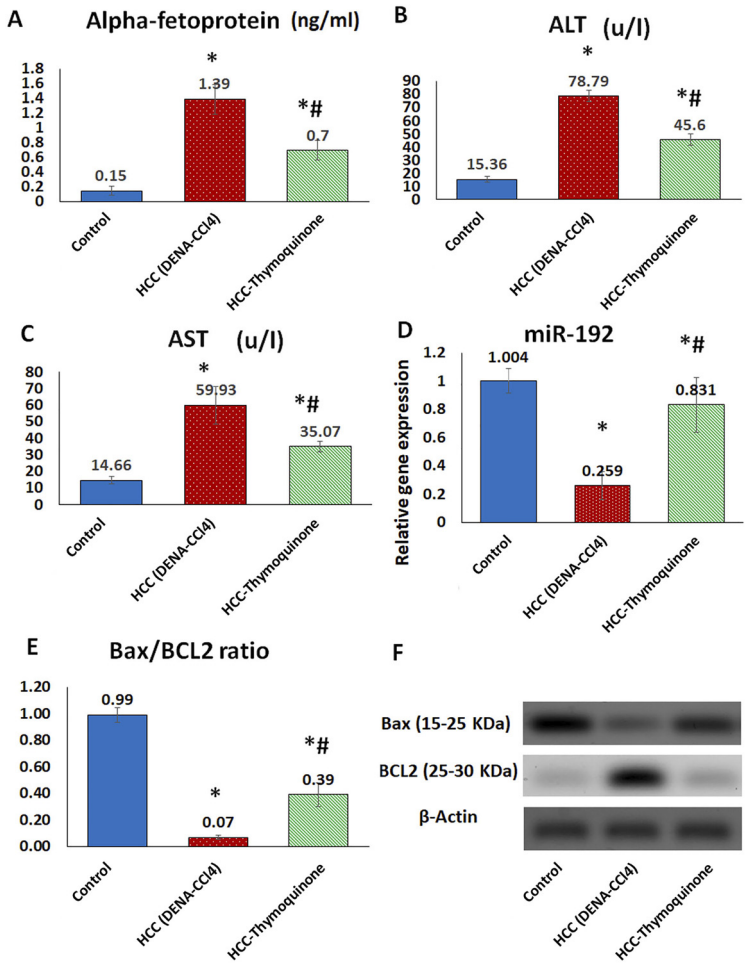

Fig. 1. Modulation of A) the marker of hepatic tumorigenesis alpha-fetoprotein (AFP), and the serum levels of hepatic transaminases, B) Alanine transaminase (ALT), and C) Aspartate transaminase (AST). D) Quantitative real-time PCR detection of the small noncoding microRNA-192 (miR-192). E) The calculated B cell lymphoma-associated X protein (Bax)/B cell lymphoma (Bcl2) protein ratio. Hepatocellular carcinoma (HCC) induced by diethylnitrosamine (DENA) administration in association with carbon tetrachloride (CCl_4). The HCC (DENA-CCl4) group showed decreased Bax and increased Bcl2, which denote cellular proliferation. Treatment with thymoquinone in the HCC-thymoquinone group improved the markers for liver cell injury ALT, AST and the indicator for tumorigenesis AFP compared to the HCC (DENA-CCl4) group. Thymoquinone preserved miR-192 and corrected the Bax/Bcl2 ratio. $n = 10$ in each group. *: denotes statistical significance compared to the control group and #: significant values when compared with the HCC (DENA-CCL_4) group. Significance was considered when ($P < 0.05$)

Determination of apoptosis using the estimation of Bax and Bcl2 protein levels

As demonstrated in Fig. 1E and F, the HCC (DENA-CCl4) group showed decreased Bax and increased Bcl2 levels ($P < 0.001$), which disturbed the balance between the apoptotic and anti-apoptotic proteins (Bax/bcl-2 ratio) and denoted excessive HCC cellular proliferation. HCC-thymoquinone treatment starting from the onset of the model induction prevented ($P < 0.001$) the Bax/Bcl2 disturbance and maintained normal cellular growth as revealed by histological examination.

Histological assessment of hepatic tissue architecture and cellular changes in the HCC model and the effect of thymoquinone

As demonstrated in Fig. 2, disturbed hepatic architecture with subcapsular neoplastic hepatocytes was detected in the liver specimens of the HCC (DENA-CCl4) group. Examination of the liver tissues revealed portal vein congestion with marked peri-portal inflammatory cell infiltration as well as nuclear pleomorphism and hyperchromatosis. The HCC-thymoquinone-treated group showed improved hepatic architecture with minimal cellular alterations, limited nuclear atypia, and mild portal vein congestion with very few peri-portal inflammatory cells. The scoring of cellular atypia in the specimens revealed severe atypia characterized by prominent nuclear changes (4; 3-4.25) in the HCC (DENA-CCl4) compared to the normal group score (0). In the HCC-thymoquinone-treated group there was marked improvement in the cellular atypia characteristics giving a score of (1.5; 1-2).

Analysis of the inflammatory markers tumour necrosis factor (TNF-α), interleukin 6 (IL6) and nuclear factor (NF-κB) in the liver samples

The pleiotropic transcription factor NF-κB and the inflammatory mediators TNF-α and IL6 were increased ($P < 0.001$) in the HCC (DENA-CCl4) rat model. However, the HCC-thymoquinone-treated rats revealed diminished ($P < 0.001$) hepatic NF-κB, TNF-α and IL6 levels as shown in Fig. 3A–C.

Disturbance of the fibrosis marker TGF-β and the cell adhesion factor E-cadherin in the HCC group and modulation by thymoquinone

The pro-fibrotic marker transforming growth factor (TGF-β) was increased ($P < 0.001$) in the liver homogenates of HCC (DENA-CCl4) rats. Meanwhile, cell adhesion disruption was detected ($P < 0.001$) by downregulation of E-cadherin expression. This facilitates hepatocyte tumorigenesis and invasion. This disturbed molecular milieu was fixed by thymoquinone treatment in the HCC-thymoquinone group. The treated group revealed diminished TGF-β ($P < 0.001$) and normalized E-cadherin levels ($P = 0.247$ compared to control group), which was accompanied by improved hepatic tissue architecture (Fig. 3D and F).

Correlation

Pearson's correlation was performed for further assessment of the association between the miR-192 expressed in the hepatic tissues and the disease progression, inflammation, and fibrosis markers, cellular atypia and cell death. Data are summarized in Table 2.

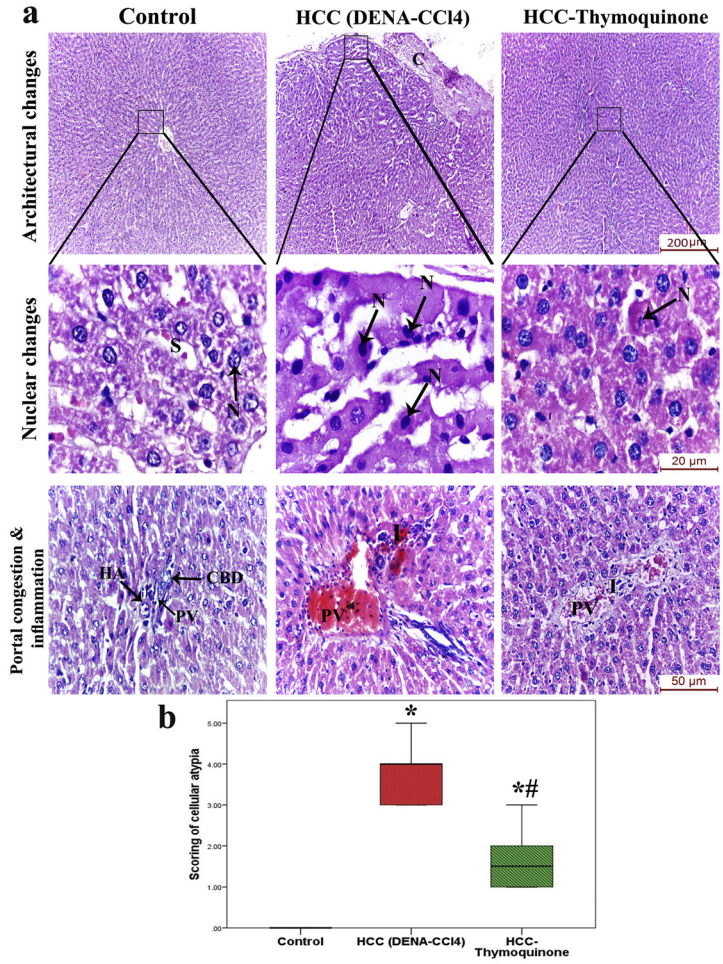

Fig. 2. Representative images of histopathological changes in the different study groups. **a)** The hepatocellular carcinoma (HCC) group displays highly irregular hepatic architecture with a subcapsular sheet arrangement of neoplastic hepatocytes beneath a markedly thickened capsule (C). Higher magnification of the HCC group displays nuclear pleomorphism and hyperchromatosis (N). The portal area shows portal vein congestion (PV) with marked periportal inflammatory cellular infiltration (I). The thymoquinone-treated group shows improved hepatic architecture with minimal cellular alterations, limited nuclear atypia (N) and mild portal vein congestion (PV) with very few periportal inflammatory cells (I). The control group shows the classical hepatic architecture with healthy hepatocytes radiating from the central vein and separated by endothelial-lined blood sinusoids (S), vesicular nuclei with prominent nucleoli (N). The portal area shows normal portal vein (PV), hepatic artery (HA), and common bile duct (CBD). **b)** Box-and-whisker plots for the cellular atypia score presented as the median and interquartile range (50; 25–75%). Score calculation and analysis of cellular atypia in the specimens revealed obvious atypia changes with nuclear characteristics in the HCC (DENA-CCl4) group compared to the normal control group. HCC-thymoquinone treatment denoted significant ($P < 0.001$) improvement. $n = 10$ in each group. *: denotes statistical significance compared to the control group and #: significant values when compared with the HCC (DENA-CCL4) group. Scoring for cellular atypia was analyzed by Kruskal–Wallis and Mann–Whitney tests. Significance was considered when ($P < 0.05$)

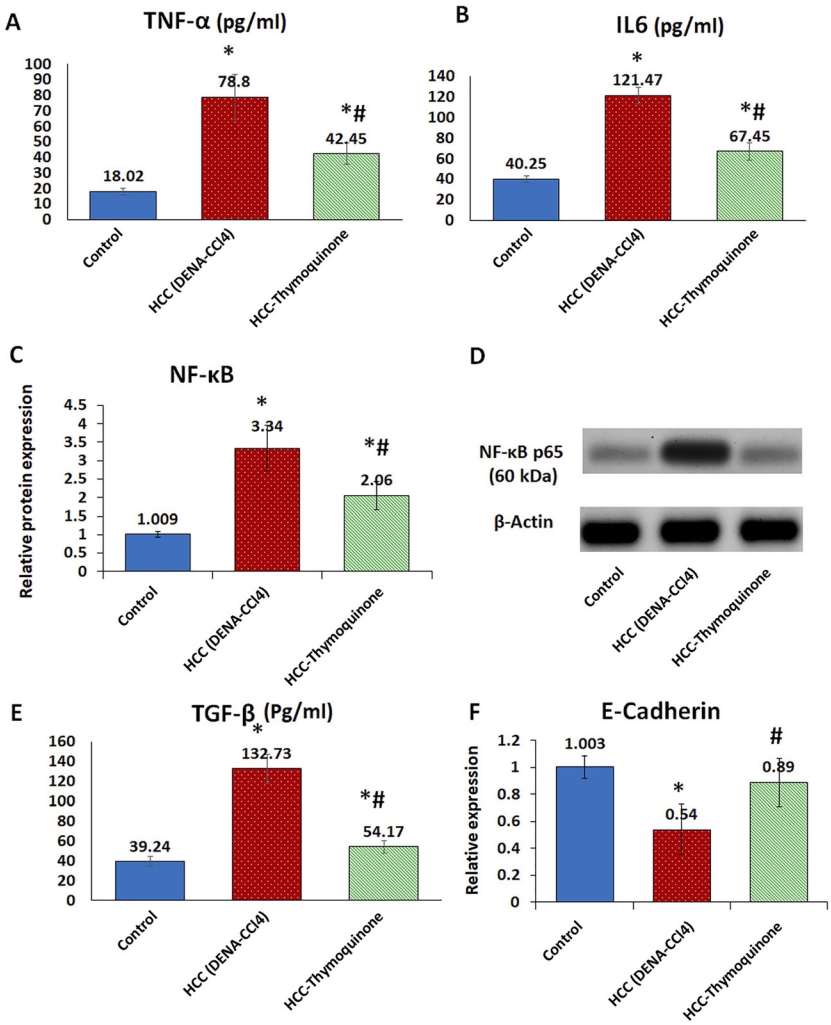

Fig. 3. Representation of the ELISA assessment of protein levels. A) tumor necrosis factor (TNF-α), B) interleukin 6 (IL6), C) the western blot protein levels of the nuclear factor (NF-κB) in the liver homogenate. D) Western blot bands of NF-κB. E) Transforming growth factor (TGF-β) protein and F) mRNA gene expression of endothelial adhesion molecule (E-cadherin) disturbance in the hepatic tumor model, and their modulation by thymoquinone. Hepatocellular carcinoma (HCC) induced by diethylnitrosamine (DENA) administration in association with carbon tetrachloride (CCl_4). Thymoquinone prevented the elevation of TNF-α, IL6, NF-κB, TGF-β in the HCC group and reversed the diminished the E-cadherin in the HCC group. $n = 10$ in each group. *: denotes statistical significance compared to the control group and #: significant values when compared with the HCC (DENA-CCL_4) group. Significance was considered when ($P < 0.05$)

Table 2. Assessment of the correlation coefficient between miR-19 and the measured factors using Pearson's correlation

	HCC (DENA-CCl4)	HCC-Thymoquinone
miR-192-Alpha-fetoprotein	−0.718*	−0.877**
miR-192-NF-κB	−0.944**	−0.839**
miR-192-E-Cadherin	0.823**	0.945**
miR-192-TGF-β	−0.857**	−0.725*
miR-192-Bax/BCL2 ratio	0.846**	0.823**
miR-192-cellular atypia score	−0.696*	−0.810**

Hepatocellular carcinoma (HCC) induced by diethylnitrosamine (DENA) administration in association with carbon tetrachloride (CCl_4). Nuclear factor-κB (NF-κB), transforming growth factor (TGF-β1), B cell lymphoma factor (Bcl2), and the Bcl-associated X protein (Bax) protein levels. * Significant correlation when ($r < 0.05$), ** Data are significant when ($r < 0.001$).

DISCUSSION

The present study presents miR-192 and NF-κB/TGF-β/E-cadherin signaling as a new target of hepatic tumorigenesis in the induced HCC rat model. Thymoquinone supplementation inhibits the initiation stage of hepatic carcinogenesis. The anti-proliferative effect of thymoquinone is mediated through preserving the anti-tumor miR-192 to prevent HCC.

Our model of hepatic carcinogenesis was established by the widely used diethylnitrosamine (DENA). The DENA-induced rat HCC model provides a reliable experimental histopathological picture similar to human HCC. DENA-induced lipid peroxidation of hepatocyte membranes is responsible for the leakage of liver transaminases [1]. Physiologically, the glycoprotein alpha-fetoprotein (AFP) is synthesized during the early stages of fetal liver development and its synthesis reduces after birth. AFP is a specific biomarker for HCC and assists the progression of HCC [22]. Elevated AFP plays an important role in hepatocarcinogenesis. It regulates cell proliferation, inhibits apoptosis and autophagy, and acts as an immunosuppressive agent [23].

The small non-coding RNA molecules (miRs) are functionally linked to normal stem cell functions as well as hepatic carcinogenesis [7]. The base–base interaction between miRs and mRNA play a critical role in the functional modification of RNA [16]. Specifically, miR-192 is explored as a tumor suppressor molecule. It possesses a remarkable role in the pathogenesis and regulation of HCCs [24]. Normally, miR-192 is abundant in liver tissues, and its down-regulation is accompanied by a poor prognosis in HCC patients. miR-192 is significantly diminished in HCC, which may represent a key event for HCC development [25]. On the other hand, miR-192 expression suppressed the features of HCC *in vitro* and *in vivo* [26].

The underlying genetic alterations observed in HCC resulted in an imbalance in the apoptotic machinery with enhanced anti-apoptotic Bcl and suppressed pro-apoptotic Bax [4]. This disturbance denotes excessive HCC cellular proliferation characterized by obvious atypia. Once the cancer model has been established, the dysregulated microenvironment contributes to the tumor behavior and progression [2]. The interaction between injured hepatocytes and the inflammatory cells appears to be central for both early and late stages of carcinogenesis [27]. This inflammatory response is mediated by TNF-α and IL-6 in rats treated with DENA [28]. Under normal physiological conditions, NF-κB is trapped and inactivated in the cytoplasm by

binding to its inhibitor IκB proteins. Meanwhile, in response to inflammatory mediators in tumor cells, IκB disassociates from NF-κB and permits the nuclear translocation of NF-κB to activate oncogenes and inactivate tumor suppressor genes resulting in the development of cancer [29]. In the same context, a significant increase in the NF-κB levels in the liver of DENA/CCl4-induced rats has been demonstrated [30].

TGF-β is an eminent profibrogenic cytokine regulating liver inflammation and fibrosis and mediating the progression into hepatocellular carcinoma. Once a tumor has initiated, TGF-β exerts a promoting effect on the tumor itself and stromal cells to enhance cell growth, invasion, and metastasis [31]. TGF-β mediates hepatocyte destruction and hepatic stellate cell activation, myofibroblast generation resulting in extracellular matrix deposition, architectural disruption, aberrant hepatocyte regeneration [32]. Following treatment with DENA, increased TGF-β1 expression was demonstrated through immunohistochemical and western blot analysis of liver tissues [1]. TGF-β1 promotes epithelial/mesenchymal transition (EMT). It induces loss of epithelial cell markers, such as E-cadherin, in addition to the expression of mesenchymal cell markers, which correlates with increased invasiveness and metastasis [4]. Since the transmembrane glycoprotein E-cadherin is needed for the physiological cell to cell adhesion and contact inhibition of cellular proliferation [3], its physiological level contributes to cancerous inhibition and suppression of invasion [33]. Therefore, loss of E-cadherin is the hallmark of EMT: the junction among tumor cells is disrupted, facilitating facilitates cell detachment and metastasis [3].

The comprehensive biological functions of E-cadherin have been partly attributed to the interaction between E-cadherin and NF-κB signaling. In quiescent cells, E-cadherin mediates the association of NFκB-p65 with β-catenin, and thus NF-κB transcriptional activity is inhibited [34]. Meanwhile, NF-κB-p65 overexpression mediates degradation of E-cadherin, resulting in a loss of intercellular junctions, increasing cellular proliferation and migration capabilities [35]. Furthermore, the upregulated TGF-β represses the transcription of E-cadherin by inhibiting the promoter of the corresponding gene [36].

A direct relationship has been identified between miR-192 and the inflammation/fibrosis axis. Low expression of miR-192 was correlated with fibrogenesis [37], where TGF-β inhibits miR-192 expression in the renal cells. miR-192 seems to be associated with the maintenance of E-cadherin expression [14]. This interplay explains the negative correlation reported in the current study between miR-192 and TGF-β expression, and the positive relationship detected between miR-192 and the E-cadherin data. These findings highlight the potential of preventing miR-192 suppression to counteract the development of HCC [38].

Cancer development and progression are largely targeted through natural compounds, and studying the role of miRs is a recent research interest. Specifically, our target miR-192 was previously investigated in non-small cell lung cancer, in which it showed upregulation by curcumin. Application of curcumin induced cancer cell apoptosis, which was mediated by miR-192-5p/215 [39]. Here, our data demonstrate the preventive effect of thymoquinone on HCC development through abrogating the microenvironmental disturbance associated with miR-192 downregulation. Thymoquinone supplementation prevented the increase in hepatic enzymes and suppressed the elevated AFP shown in the model, together with improved liver histological architecture, suggesting that thymoquinone may have potential protective effects against DENA-induced liver damage [19]. A major concept is the anti-oxidative/anti-inflammatory potential of TQ that preserves normal tissue functions and reduces hyperplasia. Several studies investigated the role of thymoquinone in NF-κB/TGF-β/E-cadherin signaling in the prevention and

treatment of a diversity of cancers. The anticancer property of thymoquinone could derive initiated from the inhibition of NF-κB signaling. Several mechanisms have been proposed starting from inhibition of TNF-α and IL6-induced NF-κB activation to the prevention of its nuclear translocation and carcinogen production [40]. Scientific evidence suggests that the anticancer characteristics of thymoquinone come from epigenetic modifications by suppressing undesired epigenetic changes without DNA sequence alterations [17]. This may be achieved through regulation of miRs to counteract cancerous cell development. This process may be conducted depending on NF-κB signal suppression [16]. Thymoquinone upregulated miR-603 in breast cancer [41] and miR-29b in leukemia [42] by blocking NF-κB signaling and eventually reducing the rate of tumor growth. Others investigated the anti-proliferative effects of thymoquinone on miRs without involving NF-κB. It mitigated breast cancer cells via miR-34a upregulation [43] and diminished the proliferation of hepatic stellate cells by upregulating the tumor suppressor miR-30a to prevent epithelial to mesenchymal transition [44]. However, we believe this study is unique as it investigates the relation of thymoquinone to miR-192-related pathophysiology in HCC management. As represented in Fig. 4, we identified a novel mechanism of

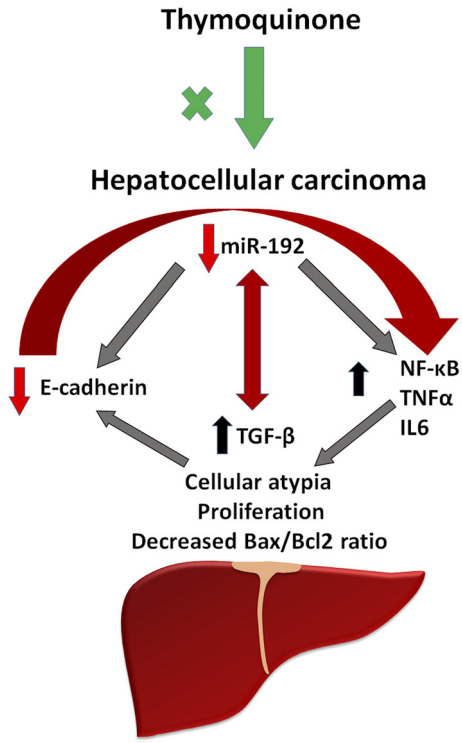

Fig. 4. Diagrammatic representation for the alterations in the levels of the liver microRNA-192 (miR-192), nuclear factor (NF-κB), tumor necrosis factor (TNF-α) interleukin (IL6), transforming growth factor (TGF-β), and the endothelial adhesion molecule (E-cadherin), furthermore the B cell lymphoma-associated X protein (Bax)/B cell lymphoma (Bcl2) ratio in hepatocellular carcinogenesis and its modulation by thymoquinone

thymoquinone for the early prevention of hepatic tumorigenesis through maintenance of miR-192/E-cadherin and aborting TNF-α, IL6/NF-κB//TGF-β1 levels in the hepatic tissue.

Limitation of the study

The relationship between miR-192 and NFκB in HCC is unclear, therefore we recommend further studies investigating cellular signaling pathways to fill in this research gap.

Conclusion

The results of the current study support the association of miR-192 and the dysregulated NF-κB/TGF-β1/E-cadherin in the pathogenesis of the HCC rat model. Thymoquinone was previously known as an anti-proliferative agent through its anti-inflammatory and antioxidant properties. Thymoquinone is believed to regulate several miRs variants. Here we emphasize its effect in preserving the hepatic miR-192 to prevent HCC. The current results highlight a new insight in which thymoquinone treatment prevents hepatic tumorigenesis.

Conflict of interest: The authors declare no conflict.

Funding information: King Khalid University # RGP.1/163/43.

ACKNOWLEDGMENT

The authors express their gratitude to the Deanship of Scientific Research at King Khalid University for supporting this work through the small research group grant # RGP.1/163/43. We are grateful to Dr. Mariam Al-Ani from Face Studio Clinic, 273 Hagley Road, Birmingham, B16 9NB, UK for proofreading the manuscript.

REFERENCES

1. Ding YF, Wu ZH, Wei YJ, Shu L, Peng YR. Hepatic inflammation-fibrosis-cancer axis in the rat hepatocellular carcinoma induced by diethylnitrosamine. J Cancer Res Clin Oncol 2017; 143(5): 821–34. https://doi.org/10.1007/s00432-017-2364-z.
2. Hernandez-Gea V, Toffanin S, Friedman SL, Llovet JM. Role of the microenvironment in the pathogenesis and treatment of hepatocellular carcinoma. Gastroenterology 2013; 144(3): 512–27. https://doi.org/10.1053/j.gastro.2013.01.002.
3. Wong SHM, Fang CM, Chuah LH, Leong CO, Ngai SC. E-cadherin: Its dysregulation in carcinogenesis and clinical implications. Crit Rev Oncol Hematol 2018; 121: 11–22. https://doi.org/10.1016/j.critrevonc.2017.11.010.
4. Fabregat I. Dysregulation of apoptosis in hepatocellular carcinoma cells. World J Gastroenterol 2009; 15(5): 513–20. https://doi.org/10.3748/wjg.15.513.
5. Dooley S, ten Dijke P. TGF-beta in progression of liver disease. Cell Tissue Res 2012; 347(1): 245–56. https://doi.org/10.1007/s00441-011-1246-y.

6. Giannelli G, Koudelkova P, Dituri F, Mikulits W. Role of epithelial to mesenchymal transition in hepatocellular carcinoma. J Hepatol 2016; 65(4): 798–808. https://doi.org/10.1016/j.jhep.2016.05.007.
7. Zhao FL, Dou YC, Wang XF, Han DC, Lv ZG, Ge SL, et al. Serum microRNA-195 is down-regulated in breast cancer: A potential marker for the diagnosis of breast cancer. Mol Biol Rep 2014; 41(9): 5913–22. https://doi.org/10.1007/s11033-014-3466-1.
8. Guo L, Zhang Y, Zhang L, Huang F, Li J, Wang S. MicroRNAs, TGF-beta signaling, and the inflammatory microenvironment in cancer. Tumour Biol 2016; 37(1): 115–25. https://doi.org/10.1007/s13277-015-4374-2.
9. Khella HW, Bakhet M, Allo G, Jewett MA, Girgis AH, Latif A, et al. miR-192, miR-194 and miR-215: A convergent microRNA network suppressing tumor progression in renal cell carcinoma. Carcinogenesis 2013; 34(10): 2231–9. https://doi.org/10.1093/carcin/bgt184.
10. Feng S, Cong S, Zhang X, Bao X, Wang W, Li H, et al. MicroRNA-192 targeting retinoblastoma 1 inhibits cell proliferation and induces cell apoptosis in lung cancer cells. Nucleic Acids Res 2011; 39(15): 6669–78. https://doi.org/10.1093/nar/gkr232.
11. Wang Y, Zhang S, Xu Y, Zhang Y, Guan H, Li X, et al. Upregulation of miR-192 inhibits cell growth and invasion and induces cell apoptosis by targeting TCF7 in human osteosarcoma. Tumour Biol 2016; 37(11): 15211–20. https://doi.org/10.1007/s13277-016-5417-z.
12. Bimonte S, Albino V, Barbieri A, Tamma ML, Nasto A, Palaia R, et al. Dissecting the roles of thymoquinone on the prevention and the treatment of hepatocellular carcinoma: An overview on the current state of knowledge. Infect Agent Cancer 2019; 14(1): 10. https://doi.org/10.1186/s13027-019-0226-9.
13. Grelet S, McShane A, Geslain R, Howe PH. Pleiotropic roles of non-coding RNAs in TGF-beta-mediated epithelial-mesenchymal transition and their functions in tumor progression. Cancers (Basel) 2017; 9(7): 75. https://doi.org/10.3390/cancers9070075.
14. Wang B, Herman-Edelstein M, Koh P, Burns W, Jandeleit-Dahm K, Watson A, et al. E-cadherin expression is regulated by miR-192/215 by a mechanism that is independent of the profibrotic effects of transforming growth factor-beta. Diabetes 2010; 59(7): 1794–802. https://doi.org/10.2337/db09-1736.
15. Imran M, Rauf A, Khan IA, Shahbaz M, Qaisrani TB, Fatmawati S, et al. Thymoquinone: A novel strategy to combat cancer: a review. Biomed Pharmacother 2018; 106: 390–402. https://doi.org/10.1016/j.biopha.2018.06.159.
16. Homayoonfal M, Asemi Z, Yousefi B. Targeting microRNAs with thymoquinone: A new approach for cancer therapy. Cell Mol Biol Lett 2021; 26(1): 43. https://doi.org/10.1186/s11658-021-00286-5.
17. Khan MA, Tania M, Fu J. Epigenetic role of thymoquinone: impact on cellular mechanism and cancer therapeutics. Drug Discov Today 2019; 24(12): 2315–22. https://doi.org/10.1016/j.drudis.2019.09.007.
18. Bashir AO, El-Mesery ME, Anwer R, Eissa LA. Thymoquinone potentiates miR-16 and miR-375 expressions in hepatocellular carcinoma. Life Sci 2020; 254: 117794. https://doi.org/10.1016/j.lfs.2020.117794.
19. Farghaly ME, Khowailed AA, Aboulhoda BE, Rashed LA, Gaber SS, Ashour H. Thymoquinone potentiated the anticancer effect of cisplatin on hepatic tumorigenesis by modulating tissue oxidative stress and endoplasmic GRP78/CHOP signaling. Nutr Cancer 2022; 74(1): 278–87. https://doi.org/10.1080/01635581.2021.1879880.
20. Hassan H, Serag H, Abdel-Hamid N, Amr M. Synergistic curative effect of chicory extract and cisplatin against thioacetamide-induced hepatocellular carcinoma. Hepatoma Res 2015; 1(3): 147–54. https://doi.org/10.4103/2394-5079.167376.
21. Quaglia A, Jutand MA, Dhillon A, Godfrey A, Togni R, Bioulac-Sage P, et al. Classification tool for the systematic histological assessment of hepatocellular carcinoma, macroregenerative nodules, and dysplastic nodules in cirrhotic liver. World J Gastroenterol 2005; 11(40): 6262–8. https://doi.org/10.3748/wjg.v11.i40.6262.

22. Wong GL, Chan HL, Tse YK, Chan HY, Tse CH, Lo AO, et al. On-treatment alpha-fetoprotein is a specific tumor marker for hepatocellular carcinoma in patients with chronic hepatitis B receiving entecavir. Hepatology 2014; 59(3): 986–95. https://doi.org/10.1002/hep.26739.
23. Zheng Y, Zhu M, Li M. Effects of alpha-fetoprotein on the occurrence and progression of hepatocellular carcinoma. J Cancer Res Clin Oncol 2020; 146(10): 2439–46. https://doi.org/10.1007/s00432-020-03331-6.
24. Zheng XF, Liu KX, Wang XM, Zhang R, Li X. MicroRNA192 acts as a tumor suppressor in colon cancer and simvastatin activates miR192 to inhibit cancer cell growth. Mol Med Rep 2019; 19(3): 1753–60. https://doi.org/10.3892/mmr.2019.9808.
25. Gu Y, Wei X, Sun Y, Gao H, Zheng X, Wong LL, et al. miR-192-5p silencing by genetic aberrations is a key event in hepatocellular carcinomas with cancer stem cell features. Cancer Res 2019; 79(5): 941–53. https://doi.org/10.1158/0008-5472.CAN-18-1675.
26. Lian J, Jing Y, Dong Q, Huan L, Chen D, Bao C, et al. miR-192, a prognostic indicator, targets the SLC39A6/SNAIL pathway to reduce tumor metastasis in human hepatocellular carcinoma. Oncotarget 2016; 7(3): 2672–83. https://doi.org/10.18632/oncotarget.6603.
27. Capece D, Fischietti M, Verzella D, Gaggiano A, Cicciarelli G, Tessitore A, et al. The inflammatory microenvironment in hepatocellular carcinoma: A pivotal role for tumor-associated macrophages. Biomed Res Int 2013; 2013: 187204. https://doi.org/10.1155/2013/187204.
28. Fathy M, Nikaido T. In vivo modulation of iNOS pathway in hepatocellular carcinoma by Nigella sativa. Environ Health Prev Med 2013; 18(5): 377–85. https://doi.org/10.1007/s12199-013-0336-8.
29. Xia L, Tan S, Zhou Y, Lin J, Wang H, Oyang L, et al. Role of the NFkappaB-signaling pathway in cancer. Onco Targets Ther 2018; 11: 2063–73. https://doi.org/10.2147/OTT.S161109.
30. Mahmoud AM, Mohammed HM, Khadrawy SM, Galaly SR. Hesperidin protects against chemically induced hepatocarcinogenesis via modulation of Nrf2/ARE/HO-1, PPARgamma and TGF-beta1/Smad3 signaling, and amelioration of oxidative stress and inflammation. Chem Biol Interact 2017; 277: 146–58. https://doi.org/10.1016/j.cbi.2017.09.015.
31. Hong S, Lee HJ, Kim SJ, Hahm KB. Connection between inflammation and carcinogenesis in gastrointestinal tract: focus on TGF-beta signaling. World J Gastroenterol 2010; 16(17): 2080–93. https://doi.org/10.3748/wjg.v16.i17.2080.
32. Gupta DK, Singh N, Sahu DK. TGF-Beta mediated crosstalk between malignant hepatocyte and tumor microenvironment in hepatocellular carcinoma. Cancer Growth Metastasis 2014; 7: 1–8. https://doi.org/10.4137/CGM.S14205.
33. Mendonsa AM, Na TY, Gumbiner BM. E-cadherin in contact inhibition and cancer. Oncogene 2018; 37(35): 4769–80. https://doi.org/10.1038/s41388-018-0304-2.
34. Solanas G, Porta-de-la-Riva M, Agusti C, Casagolda D, Sanchez-Aguilera F, Larriba MJ, et al. E-cadherin controls beta-catenin and NF-kappaB transcriptional activity in mesenchymal gene expression. J Cell Sci 2008; 121(Pt 13): 2224–34. https://doi.org/10.1242/jcs.021667.
35. Zhou W, Wang Q, Xu Y, Jiang J, Guo J, Yu H, et al. RMP promotes epithelial-mesenchymal transition through NF-kappaB/CSN2/Snail pathway in hepatocellular carcinoma. Oncotarget 2017; 8(25): 40373–88. https://doi.org/10.18632/oncotarget.16177.
36. Lombaerts M, van Wezel T, Philippo K, Dierssen JW, Zimmerman RM, Oosting J, et al. E-cadherin transcriptional downregulation by promoter methylation but not mutation is related to epithelial-to-mesenchymal transition in breast cancer cell lines. Br J Cancer 2006; 94(5): 661–71. https://doi.org/10.1038/sj.bjc.6602996.
37. Krupa A, Jenkins R, Luo DD, Lewis A, Phillips A, Fraser D. Loss of MicroRNA-192 promotes fibrogenesis in diabetic nephropathy. J Am Soc Nephrol 2010; 21(3): 438–47. https://doi.org/10.1681/ASN.2009050530.

38. Chung AC, Huang XR, Meng X, Lan HY. miR-192 mediates TGF-beta/Smad3-driven renal fibrosis. J Am Soc Nephrol 2010; 21(8): 1317–25. https://doi.org/10.1681/ASN.2010020134.
39. Ye M, Zhang J, Zhang J, Miao Q, Yao L, Zhang J. Curcumin promotes apoptosis by activating the p53-miR-192-5p/215-XIAP pathway in non-small cell lung cancer. Cancer Lett 2015; 357(1): 196–205. https://doi.org/10.1016/j.canlet.2014.11.028.
40. Gomathinayagam R, Ha JH, Jayaraman M, Song YS, Isidoro C, Dhanasekaran DN. Chemopreventive and anticancer effects of thymoquinone: Cellular and molecular targets. J Cancer Prev 2020; 25(3): 136–51. https://doi.org/10.15430/JCP.2020.25.3.136.
41. Kabil N, Bayraktar R, Kahraman N, Mokhlis HA, Calin GA, Lopez-Berestein G, et al. Thymoquinone inhibits cell proliferation, migration, and invasion by regulating the elongation factor 2 kinase (eEF-2K) signaling axis in triple-negative breast cancer. Breast Cancer Res Treat 2018; 171(3): 593–605. https://doi.org/10.1007/s10549-018-4847-2.
42. Pang J, Shen N, Yan F, Zhao N, Dou L, Wu LC, et al. Thymoquinone exerts potent growth-suppressive activity on leukemia through DNA hypermethylation reversal in leukemia cells. Oncotarget 2017; 8(21): 34453–67. https://doi.org/10.18632/oncotarget.16431.
43. Bhattacharya S, Ahir M, Patra P, Mukherjee S, Ghosh S, Mazumdar M, et al. PEGylated-thymoquinone-nanoparticle mediated retardation of breast cancer cell migration by deregulation of cytoskeletal actin polymerization through miR-34a. Biomaterials 2015; 51: 91–107. https://doi.org/10.1016/j.biomaterials.2015.01.007.
44. Geng W, Li C, Zhan Y, Zhang R, Zheng J. Thymoquinone alleviates liver fibrosis via miR-30a-mediated epithelial-mesenchymal transition. J Cell Physiol 2020; 236(5): 3629–40. https://doi.org/10.1002/jcp.30097.