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Apoptotic Activities of Thymoquinone, an Active Ingredient of Black Seed (*Nigella sativa*), in Cervical Cancer Cell Lines

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Abstract

Thymoquinone (TQ) is the main constituent of black seed (*Nigella sativa*, spp) essential oil which shows promising *in vitro* and *in vivo* anti-neoplastic activities in different tumor cell lines. However, to date there are only a few reports regarding the apoptotic effects of TQ on cervical cancer cells. Here, we report that TQ stimulated distinct apoptotic pathways in two human cervical cell lines, Siha and C33A. TQ markedly induced apoptosis as demonstrated by cell cycle analysis in both cell lines. Moreover, quantitative PCR revealed that TQ induced apoptosis in Siha cells through p53-dependent pathway as shown by elevated level of p53-mediated apoptosis target genes, whereas apoptosis in C33A cells was mainly associated with the activation of caspase-3. These results support previous findings on TQ as a potential therapeutic agent for human cervical cancer.

Key Words: apoptosis, caspase, cervical cancer, *Nigella sativa*, thymoquinone

Introduction

Cervical cancer is one of the most common malignancies of women in the world, accounting for 15% of all cancer-related deaths (7). It was estimated that almost 500,000 women died every year from cervical cancer worldwide, of which > 80% of the mortality occur in developing countries, where mortality from this disease is the highest among deaths caused by the neoplasm.[†] In the United States, there are an estimated 13,000 annual new cases of cervical cancer and 50,000 of advanced pre-cancerous conditions (38). Cervical cancer is the second most common cancer in women, next to breast cancer and is

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the most common gynaecological cancer in Malaysia. Furthermore, Chinese women have the highest ASR (Age-standardized Incidence Rate) of 23.2 per 100,000, followed by Indians with ASR of 16.4 and Malays of 8.7 per 100,000 (11). Infection by the human papillomavirus (HPV) is the major causal factor in the development of most cases of cervical cancer (21). In addition, high-risk HPVs, such as HPV16 and HPV18, have been detected in 94-100% of cervical precancerous lesions and cancer (9).

p53 gene is a specific gene that functions to suppress the growth of tumors. Loss of p53 functions has been attributed to the majority of human cancers (20). Indeed, study on the p53 status in a series of human cervical cancer cell lines revealed that mutation of the gene is responsible for the p53 dysfunction in HT-3 and C33A cell lines whereas in the HPV-positive cell lines such as Siha, Hela and CaSki, p53 expression is abolished by the HPV-E6 oncoprotein (36). HPV16 has been known to play a role in the pathogenesis of cervical cancer by overexpressing oncoprotein E6, which then immortalizes the host cells through degradation of p53 protein (30). The importance of HPV infection and the p53 status has also been considered in the development of agents to treat cervical cancer (39). Reactivation of p53-dependent apoptotic cell death by leptomycin B and actinomycin D was observed in HPV-positive, yet harboring wild-type p53, Siha, Hela and CaSki cell lines (19). More recently, Duncan *et al* demonstrated that the chymotrypsin-like serine protease inhibitor, succinyl-alaninealanine-proline-phenylalanine chloromethyl ketone (AAPF-CMK) induced apoptosis in Siha, but not in C33A cells which are expressing mutant p53 (14).

Generally, therapeutic measurements for treatment of invasive cervical cancer are surgery, radiotherapy and chemotherapy (24). However, these options have certain adverse side effects due to the non-specific cytotoxicity of drugs and resistance to treatment and represent a great problem in the cervical cancer management. In addition, many of these treatments present clear limited anti-cancer activities.[#] Current investigations search for potent, safe and effective anticancer agents to overcome resistance and reduce side effects; currently, many herbal therapeutics are recommended for the treatment of cancer as they have less side effects than conventional therapeutic procedures and are relatively less costly (10, 12). Many medicinal plants are used in folk and traditional medicine, and their biologically active derivatives are being increasingly used in clinical trials for anticancer activities, and are offering alternatives in cancer therapy (1, 22, 28, 32). Merghoub *et al*. (29) showed that the extracts from *Inula viscosa* (L.) Ait. produced marked cytotoxic effects on two human cervical cancer cell lines. An exotic mushroom extract was found to induce strong antiproliferative activities on cervical cancer cell lines (27). Clausine B, a carbazole alkaloid isolated from the stem bark of Clausena excavates, produced anticancer activities against four cervical cancer cell lines tested (40).

The black seed or "Habbatul-barakah" in Arabic (*Nigella sativa*) is a spice, and a herbaceous, flowering plant. It has been used for many centuries as a food flavor and natural remedy to promote health and to treat a broad array of diseases in many countries in the Middle East, South Asia and the Far East (18). Black seed is one of the most extensively studied plants both phytochemically and pharmacologically; numerous studies have shown that the seeds and oil of this plant are characterized by a very low degree of toxicity (3). Many studies have been reported for its antimicrobial, anti-hyperlipidaemic, anti-hyperglycaemic, diuretic and anti-oxidant effects (2) as well as anti-neoplastic activities in different types of cancer (10). The biological activities of *Nigella sativa* seed are related to the main active components, thymoquinone, or TQ in short, a crystalline substance that has been isolated from the essential oil and is considered the major component of the essential oil (3, 33). TQ has been considered as a potent, anti-carcinogenic, antioxidant and anti-mutagenic agent (5, 6, 24). TQ has been shown to exert anti-neoplastic effects both *in vitro* and *in vivo* (18, 24). The growth inhibitory effects of TQ is specific to cancer cells leading to improvements in therapeutic index while it is less toxic to and prevents non-tumor normal cells from sustaining chemotherapy-induced damaged (18). Regarding cervical cancer cells, Brewer *et al*. (8) found that selenomethione in combination with estrogen, lycopene and TQ caused cellular damage of Siha cells as evidenced by decreased proliferation rate. An ethanol extract from *Nigella sativa* has been found to inhibit proliferation and induces apoptosis in the human cervical cancer HeLa cell lines (15).

However, despite knowledge of these potential anti-neoplastic effects, the mechanism by which TQ induces apoptosis in cervical cancer still remains to be elucidated. The purpose of the present investigation was to explore the mechanism of TQ in inducing apoptosis on two different human cervical cell lines differing in HPV and p53 status. Siha is HPV-16-positive and carries wild-type p53; C33A cells are HPV-negative and carry mutant p53.

[#]Management of Cervical Cancer. The clinical practice guideline. Ministry of Health Malaysia. 2003, http://www.moh.gov.my/medical/HTA/cpg.htm (accessed 30 November 2012).

Materials and Methods

Cell Lines

Two human cervical cell lines, Siha and C33A, were used. The cells were cultured in DMEM (Gibco, Carlsbad, California, USA) medium and maintained in a humidified incubator with 5% CO_2 and 95% air at 37°C.

Preparation of TQ Solution

Thymoquinone was reconstituted in ethanol at 10 mM concentration and appropriate working concentrations were prepared in DMEM (Gibco, California, USA) supplemented with 10% fetal bovine serum (JRS, California, USA) immediately prior to the experiments.

Anti-Proliferation Assay (Cell Viability Assay)

Cellular proliferation was assessed by the ability of cells to convert soluble MTT (Sigma Aldrich, St. Louis, Missouri, USA) into an insoluble formazan precipitate. Exponentially growing cells were seeded in 96-well plates at an initial density of 2×10^4/well, treated with defined concentrations of TQ (10-100 μM) and maintained in culture for 22 h. Plates were centrifuged at 1,000 rpm to collect floating cells using a microplate swing rotor centrifuge. The media were then carefully removed from the wells without disturbing the cell pellets. The cells were then incubated in 30 μl MTT (Sigma-Aldrich, USA) at concentration of 5 mg/ml in phosphate buffer saline (PBS) for 2 h. The intracellular formazan complex was dissolved in DMSO and the absorbance was measured at 570 nm in a microplate reader. Cellular proliferation was expressed as a percentage of cell viability of TQ-treated cells relative to untreated controls.

Cell Cycle Analysis

Total populations of floating and adherent cells were harvested 24 h after treatment by centrifugation at 1,000 rpm. The cell pellets were fixed with 75% ethanol in PBS for 30 min, washed with PBS and then stained with Propidium Iodide solution (50 μg/ml) containing 200 μg/ml RNAse. DNA content and cell cycle profiles were analyzed using a laser scanning cytometer (LSC101; Olympus) as described previously (20).

Caspase Activity Assay

Specific proteolytic activities of the caspase-3 and caspase-8 in cells treated with TQ were determined by the ApoAlert Caspase Assay Kits (Clontech, California, USA). Cells were seeded in 6-well plates and treated with either control (0.1% ethanol in DMEM) or 40 μM TQ. Cells incubated with complete medium served as baseline controls. Cells were harvested at intervals after exposure to TQ up to 12 h. Selective caspase activities in cell lysates were measured and expressed as fold increase from the baseline controls.

RNA Extraction, cDNA Synthesis and Quantitative RT–PCR

Total RNAs were prepared using the TRI Reagent (Sigma-Aldrich, USA). Complementary DNAs were synthesized from 5 μg total RNA using the Omniscript Reverse transcriptase (Qiagen, Germany) according to the manufacturer's instructions. Real-time PCR was performed using the QuantiTect SYBR Green PCR Kit (Qiagen, Germany) per the manufacturer's recommendations. Amplification was carried out in a LightCycler (Roche, Germany) with SYBR green detection and melt curve analysis. The oligonucleotide primers used have been described previously, and are specific for *p53AIP1, Noxa* (20), and *caspase-3* (23). The amount of cDNA present in any given sample was normalized to the amount of DNA of the housekeeping gene β-actin (26). Reaction mixtures contained 2 μl cDNA, 2X QuantiTect SYBR Green RT-PCR Master Mix, 300 nM each of forward and reverse primer and diethyl nuclease-free water added to a final volume of 15 μl. All samples were investigated in triplicates and the melting curves obtained after PCR amplification confirmed the specificity of the SYBR green assays.

Statistical Analysis

The data obtained were expressed as the mean ± standard deviation (SD) that were interpreted using Microsoft Office Excel 2010® for Mac. The significance of differences was analyzed by Student's *t*-test. A value of $P < 0.05$ was considered significant.

Results

The present investigation examined the effects of TQ on the proliferation of Siha and C33A cells measured by the MTT assay. The cells were treated with different concentrations (10-60 μM) of TQ for 12 and 24 h. TQ was found to remarkably reduce the cells viability in a dose- and time-dependent manner (Fig. 1).

To determine the nature of cell death, cell cycle analysis was performed using a laser scanning cytometer. The cell cycle analysis revealed that TQ

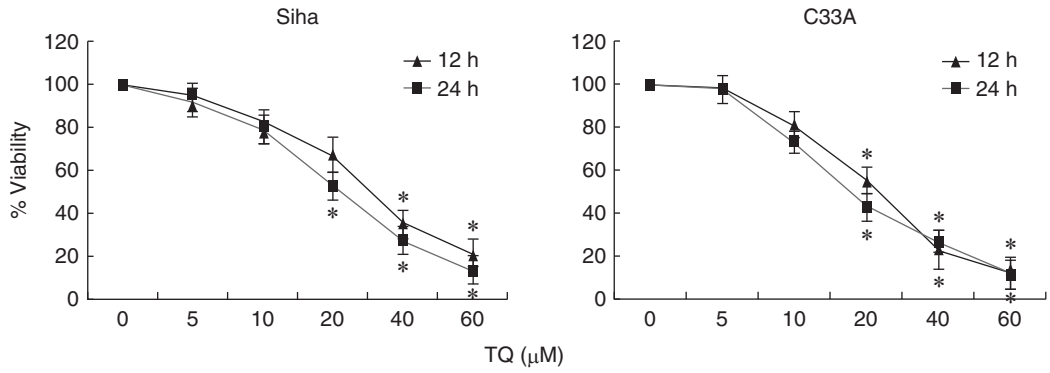

Fig. 1. TQ reduced the number of viable Siha and C33A cells in a concentration-dependent manner. Dose-response curves on the effect of TQ analyzed by MTT assay on Siha and C33A cell viability at 12 or 24 h of exposure. The vertical and horizontal axes display percentage of cell viability and sample concentration (μM), respectively. Data shown are means ± SD of three independent experiments each performed in triplicates. *$P < 0.05$ by the comparison with the respective controls (0.1% ethanol, EtOH).

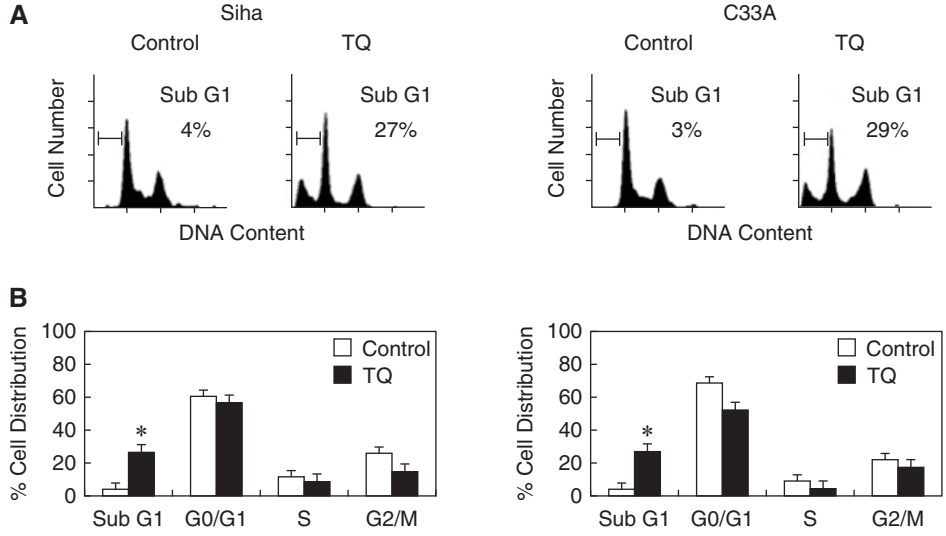

Fig. 2. TQ induced apoptosis in both Siha and C33A cell lines. (A) Representative images of Siha and C33A cell cycle profiles 24 h after treatment with either control (0.1% ethanol) or TQ at 40 μM as analyzed by LSC. The horizontal and vertical axes represent DNA content and cell number, respectively. Apoptotic cell population is indicated as a percentage of the sub-G1 fraction. (B) Cell cycle distribution analysis of cells in sub G1, G0/G1, S, and G2/M) from (A). Data are means ± SD of three independent experiments. *$P < 0.05$ by the comparison with the respective controls.

treatment at 40 μM induced apoptosis in Siha and C33A cells with sub-G1 populations of 27% and 29%, respectively (Figs. 2A and 2B). It has been known that the activation of caspase proteases, including caspase-3, is crucial to apoptotic cell death (25). Therefore, in the present study, we performed caspase-3 activity assay; we found that exposure to 40 μM TQ triggered caspase-3 activation in both cell lines (Fig. 3).

To confirm whether the apoptotic activities in these cells were mediated by p53, the transcriptional level of genes involved in p53-mediated apoptosis, namely *p53AIP1* and *Noxa*, was examined using quantitative RT-PCR. Indeed, TQ treatment upregulated *p53*-mediated pro-apoptotic target genes *p53AIP1* and *Noxa* in Siha cells (Fig. 4). In contrast, *p53AIP1* and *Noxa* mRNAs levels remained unchanged in C33A cells (Fig. 4). We also confirmed by caspase-3 activity assays (Fig. 3) that exposure to 40 μM TQ triggered caspase-3 activation in both cell lines. In agreement with this, upregulated transcript levels of these caspases in Siha and C33A were observed after 6 h TQ treatment as measured by real-time RT-PCR (Fig. 4), indicating that TQ effects on caspase-3 activation are at the transcriptional level.

Discussion

Cervical cancer is a malignant neoplastic disease

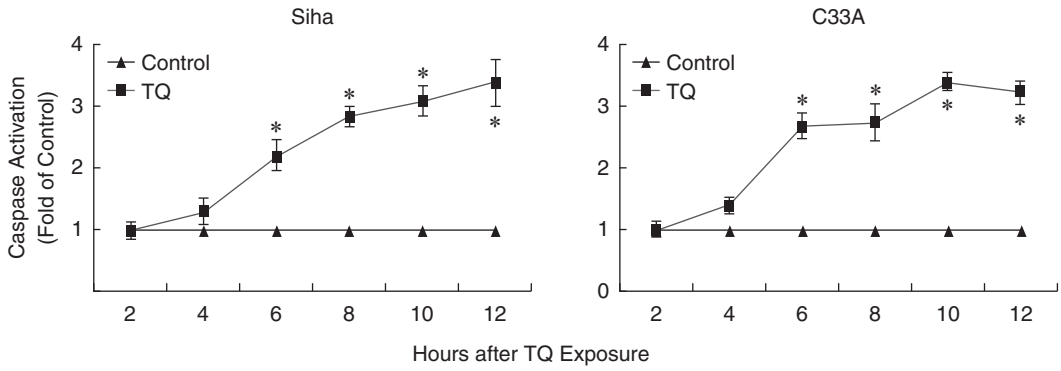

Fig. 3. Caspase-3 activity assays. The cells were treated with either 0.1 % EtOH (control) or with 40 μM TQ for up to 12 h, and caspases-3 activities were calorimetrically quantified. Each value are means ± SD of two separate experiments done in triplicate. *$P < 0.05$ by the comparison with the respective controls.

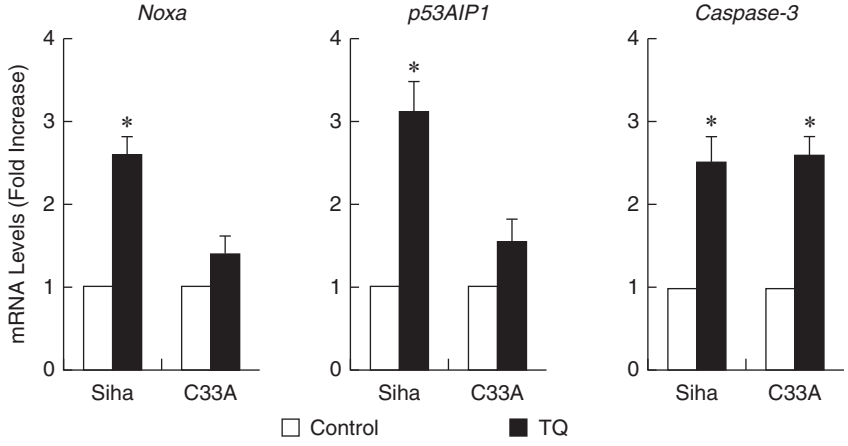

Fig. 4. TQ stimulates distinct apoptotic pathway in Siha and C33A cells determined by Quantitative RT-PCR. Expression levels of *Noxa, p53AIP1* and *Caspase-3* mRNAs in the cells were quantified 6 hours following treatments with either control or with 40 μM TQ. Relative mRNA values depicted are means ± SD of two independent experiments each performed in triplicates. *$P < 0.05$ compared with respective controls.

that tends to arises in the transitional zone between squamous and columnar cell epithelia (13). It is now established that infection by genital HPV is the major cause of this lesion (4). Practically, all malignant tumors are caused by HPV infections, with HPV types 16 and 18 responsible for about 70% of all cases of cervical carcinomas (37). The current treatments for most stages of cervical cancer includes surgical ablation, chemotherapy and radiotherapy, and are usually associated with complications such as recurrence of cancer after treatment, rectal dysfunction and vaginal strictures cystitis, and problems with sexual, bowel and urological complications (34).

The current work used established cell culture models to study the anticancer mechanisms of TQ using HPV-16-positive cervical cancer cell line Siha that carries wild-type p53 and the HPV-negative C33A cell line that harbors a mutant p53 gene. Investigation herein revealed that TQ significantly reduced the viability of both the C33A and Siha cells; these observations were dosage- and time-dependent. Recently TQ was found to induce apoptosis associated with elevated levels of p53 expression level in Siha cells with wild-type p53 gene (31). On the other hand, in p53-mutant cancer cells such as myeloblastic leukemia HL60 (16) and human osteosarcoma cells MG63 and MNNG/HOS cells (35), TQ-induced apoptosis through the activation of caspases-3, -8, and -9. These findings are in agreement with Ledgerwood and Morison (25) who suggested that activation of caspase proteases is fundamental to triggerting apoptotic cell death. Among those caspases, caspase-3 has been proposed as an important factor among the components of the apoptotic machinery by orchestrating DNA fragmentation (17). Consistent with this, we observed in our study activation of caspase-3 in two cervical cancer cell lines.

To elucidate the mechanism by which the black

seed induces growth inhibition, the present study examined the effects of TQ on apoptosis induction, cell-cycle progression and DNA synthesis in both cell lines, and found that TQ induced apoptosis in both the HPV-positive Siha and HPV-negative C33A cell lines. Apoptosis induction by TQ in Siha cell line possessing wild-type p53 apparently occurs as a result of reactivation p53-mediated apoptosis pathways as marked by upregulated transcription of the apoptosis-specific target genes, *Noxa* and *p53AIP1*. Activation of caspase-3 was also detected which could be explained by the fact that caspase-3 activation is crucial in the apoptotic cell death both by extrinsic and intrinsic pathways, irrespective of their p53 status (17). Further studies would be needed to address the mechanism on how TQ reactivates the p53 activity in HPV-positive cervical cancer cells. On the other hand, apoptosis in the C33A cell line was very likely due to activation of caspase (s). Involvement of p53 in TQ-induced apoptosis could be ruled-out since the cells were expressing mutant p53 in C33A. Collectively, these findings thus provide evidence that TQ induced apoptosis on Siha through activation of p53-mediated apoptosis target genes, whereas in p53-mutated C33A cells, apoptosis is associated with the activation of caspase-3.

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