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Determination Cytotoxic effect of Thymoquinone and Expression analyses of Apoptotic and Autophagic related genes with Thymoquinone treatment in Colon Cancer Cells

Esra Tokay*[1]

Abstract

Thymoquinone (Tq; 2-isopropyl-5-methyl-1,4 benzoquinone) is the main ingredient that presents in the volatile oil of *Nigella sativa* (black seed). Recently, it has been reported that, Tq was inhibited cell proliferation of many cancer cell lines including ovarian, lung, breast, osteosarcoma, pancreatic, fibrosarcoma, lung cancer, squamous cell carcinoma and myeloblastic leukemias. In this study, the anti-cancer effect of Tq was investigated in human Colon Cancer Cells (HT-29) with MTT assay. Positive control was achieved with 5-fluorouracil (5-FU). Also, the invasion capability of HT-29 cells was determined in presence of Tq and 5-FU with scratch analyses. In addition, the autophagic and apoptotic effect of Tq were investigated by qRT-PCR method to find out the pathway of anti-proliferative effect in Tq-treated HT-29 cells. As a result, Tq was inhibited the cell proliferation of HT-29 cells in time dependent manner, with 118 μM for 24 h and 84 μM for 48 h, respectively. A concentration of 150 μM of Tq was able to significantly reduced the invasiveness of HT-29 cells approximately 8 fold compared with not treated cells at 6h. ATG-12, ATG-7 and LC3-II were significantly downregulated in presence of Tq. Conversely, the expression of all the autophagy genes were downregulated in presence of 5-FU. Moreover, pro-apoptotic gene Bax was significantly upregulated nearly 15 fold whereas the expressions of Bcl-2 and Bcl-XL (pro-survival genes) were decreased in presence of Tq. On the other hand, in 5-FU treated HT-29 cells, the expression of Bax, Bcl-2 and Bcl-XL genes and autophagy related genes were significantly downregulated.

Keywords: Tq, autophagy, apoptosis, HT-29

1. INTRODUCTION

Colon cancer which is the third frequent type of cancer globally, has accounted the second cause of cancer-associated death in western World and for one-third of all malignant tumors predominantly occured at the junction of the rectum and the sigmoid colon in the world [1].

5-Fluorouracil (5-FU) is usually used during the initial treatment of colorectal cancer. It inhibits the pyrimidine synthesis function of thymidylate

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synthase and destroyes the normal functions of DNA and RNA [2]. However, it has been reported that to be degraded in the liver because of bio-incompatible feature. This mean, higher doses are required for the treatment of the cancer. However, these treatments constitute a major challenge involved in lower efficiency and higher side effects. It is necessary to develop plant-based anti-cancer agents for treatment of Colon Carcinoma [2].

Thymoquinone (Tq; 2-isopropyl-5-methyl-1,4 benzoquinone) is the main ingredient that presents in the volatile oil of *Nigella sativa* (black seed). *Nigella* has demonstrated clinical therapeutic activity in many diseases, including bronchial asthma, diarrhea, headache, gastrointestinal illnesses, eczema, hypertension, and obesity [3]. The animal studies of Tq has been shown that it has anti-oxidant, anti-inflamatory, anti-platelet, anti-artherosclerotic, and anti-diabetic activity and also it has protective effects against heart, liver, and kidney damage [4]. Recently, it has been reported that, Tq inhibited cell proliferation of many cancer cell lines including ovarian, lung, colon, breast, osteosarcoma, pancreatic, fibrosarcoma and lung cancer, squamous cell carcinoma and myeloblastic leukemias [5]. Additionally, Tq showed anticancer activity through caspase 3 by down regulation of XIAP in mouse neuroblastoma (Neuro-2a) [6]. Especially, it has antitumor activity for pancreatic cancer [7].

There are restricted studies about Tq and colon cancer cells. Novel kinase target influenced by Tq were revealed with *in-silico* analysis of peptide array data obtained from in Tq-treated HCT116wt, DLD-1 and HT-29 cells by El-baba and his group. It was found that, AKT-MEK-ERK1/2 pathway proteins were upregulated >2 fold and oncogenic PAK-1 was promoted by Tq [8].

Autophagy is an essential catabolic pathway for degradation of long-lived proteins and damaged unwanted organelles in the lysosome. It is necessary for recycling of cellular components to regular metabolism of the cell. In addition, it promoted cancer by allowing cells to survive under conditions of metabolic and genotoxic stress. [9]. Autophagy has important roles in various organism processes such as development, neurodegenerative diseases and cancer [10]. Autophagosome formation comprises several important protein complexes such as ATG-12, ATG-5, ATG-16L1 and ATG-8 (LC3). In the first ubiquitination like reaction involves ATG-7, ATG-10, ATG-12 and ATG-5 protein. In the second ubiquitination-like reaction, LC-3 is necessary for autophagic membrane elongation and vesicle completion. While free LC3 (also called LC3-I) is soluble, its lipid-conjugated form (LC3-II) is associated with growing and complete autophagosomes. For this reason, LC-3II is used as a molecular marker of autophagosome generation and accumulation. Also, Beclin1 (Ambra 1) which has recently been recognized to regulate autophagy, may decide cell death or survival via promoting the conversion between autophagy and apoptosis [11]. It was found that, cisplatin that is widely used as agent for chemotherapy, was induced autophagy in 58 types of cancers such as endometrial cancer cells, malignant mesothelioma cells, cervical cancer HeLa cells [12].

To the best of our knowledge, no study has been identified an association between autophagy and Tq-induced colon cancer cell death. Also, this is the first time, it is revealed the relation between autophagy and a chemotherapeutic agent namely 5-FU in colon cancer cells. Hence, in this study, the effect of Tq and 5-FU on Human Colon Cancer Cell line (HT-29) was investigated with MTT and Scratch assays. Also, Tq mediated autophagy and apoptosis mechanism was observed at mRNA level using qRT-PCR method. These findings will be important for understanding the relation Tq and autophagy-apoptosis mechanism.

2.MATERIAL-METHOD

2.1. Cell culture and Reagents

HT-29 cells were kindly a gift from Ege University/Prof. Dr. İsmet D. GÜRHAN. HT-29 cells were cultured using Dulbeccos's Modified Eagle Medium (High Clone) supplemented with 2 mM Glutamine (Sigma) and 5 % Fethal Calf Serum (Gibco) in a humidified incubator settings

of 5 % CO_2 and 37 °C. Thymoquinone and MTT reagent was taken from Sigma Aldrich.

2.2. MTT Analyses

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cells were plated at a density of 5×10^4 cells/well in 96-well tissue culture plates and subjected to different treatments (150μM, 75μM, 39 μM, 18 μM, 9 μM) of TQ and (50 ng/mL, 25 ng/mL, 12 ng/mL, 6 ng/mL, 3 ng/mL) 5-FU as positive control. Following a 24 h and 48 h incubation at 37°C in a humidified atmosphere containing 5% CO_2 and 95 % air. The cells were incubated for another 4 h with MTT reagent. The formazan product was dissolved in 0.004 M HCl supplemented isopropanol solution and read at 550 nm on Thermo Spectrophotometer. The percentage of cell viability was determined using this formula: (OD 550 sample/OD 550 control)*100. The data represented using Graphpad Prism 7 programme. IC_{50} values were calculated using Origin Pro 8.5 programme [13].

2.3. RNA Isolation and RT-Reaction

HT-29 cells were seed into 6-well plate per 500.000 cells/well. After 24 h of attachment, TQ was applied into wells indicated concentration (150 μM). Following 48h of incubation period, the cells were trypsinized and obtained pellet for RNA extraction. Total RNA was extracted from HT-29 cells using the GeneJET™ RNA Purification Kit (Thermo Scientific) according to the manufacturer's instructions. 1 µg of total RNA was reverse transcribed into cDNA using Revert Aid Reverse Transcriptase (20U/μL) and oligo(dT) as primer (100 pmol/uL)with fermentas cDNA synthesis protocol.

2.4. Quantitative Real Time-PCR (qPCR)

Real time PCR was performed with Light cycler 480 (Roche Diagnostics) using SYBR green detection in a total volume of 10 µL with 0.5 µL forward and reverse primers (100 ng/µL), 5 µL SYBR-Green Master Mix (1X), 1 µL cDNA (1 µg). Human beta microglobulin was used for normalization. The primers were listed in Table 1.

Cycling conditions were 95 ˚C for 10 min, 35 cycles of 95 ˚C for 15 s and 58 ˚C for 15 s 72˚C for 10 s and final extension of 72 ˚C for 1 min. Each sample was studied triplicate, and the Ct value was defined automatically by the instrument [12].

Table 1. The name, sequence and product lenght information of Autophagy and Apoptosis related genes and internal control primers

Gene	Sequence of primers	Product Length
ATG12	FRD:TCTATGAGTGTTTT GGCAGTG REV:ATCACATCTGTTAA GTCTCTTGC	171 bp
ATG7	FRD:AGGAGATTCAACC AGAGACC REV:GCACAAGCCCAAG AGAGG	191 bp
ATG3	FRD:TCACAACACAGGT ATTACAGG REV:TCACCGCCAGCATC AG	240 bp
Beclin 1 (ATG6)	FRD:TGTCACCATCCAGG AACTCA REV:CTGTTGGCACTTTC TGTGGA	180 bp
LC3-II	FRD:GAGAAGCAGCTTC CTGTTCTGG REV:GTGTCCGTTCACCA ACAGGAAG	138 bp
Hβ-2	FRD:TTTCTGGCCTGGAG GCTATC REV:CATGTCTCGATCCC ACTTAACT	295 bp
Bcl-2	FRD:GAGGATTGTGGCCT TCTTG REV:ACAGTTCCACAAA GGCATCC	119 bp
Bcl-XL	FRD:CATGGCAGCAGTA AAGCAGA REV:TGCTGCATTGTTCC CATAGA	164 bp
Bax	FRD:ACCAGCCTGTTTGA GAGTGG REV:AGTGATGCAGCAT GAAGTCG	160 bp

2.5. Scratch Assay

Cells were plated in six well plates (500.000 cell/well). 150 µM of TQ was added at final concentration after 24 h of attchment. Scratch wound was made in each well using a 200 µL micropipette tip. The wound area was photographed after scratching for control. The number of cells migrating into the initial wound area were counted at 24 hour after scratching using Image J programme [14].

2.6. Statistical Analyses

Standard deviations and p-values were calculated by using Mini Tab 14 software. One way Anova analysis was applied in between pairs for statistical significance.

3. RESULTS

3.1. Cytotoxic and invasion effect of Tq on colon cancer cells.

As shown in Figure 1, five different Tq (150 µM, 75 µM, 39 µM, 18 µM and 9 µM) concentrations for 24 h and 48 h decreased cellular viability of HT-29 cells in a dose and time-dependent manner. The IC_{50} concentrations were 118 µM for 24 h and 84 µM for 48 h, respectively. However, treatment with 5-FU reduce the cell viabity at the concentration of 25ng/mL and 50 ng/mL for 48 h.

Scratch assay was performed to search invasion capability of Tq and 5-FU on HT-29 cells. The scratch area was calculated using image J wound healing tool. The Fold Area (%) was calculated with these formulas: The first step: Area (%)=(SampleArea/ControlArea)*100. The second step: Fold Area (%)= Sample Area (%)/ Control Area (%) [14].

The images were screened for visualization of scratch areas. As shown in Figure 2A, 150 µM of Tq was able to significantly reduce the invasiveness of HT-29 cells about ~8 fold compared with not treated cells at 6h. On the other hand, also 5-FU reduced the invasiveness of HT-29 cells at 50 ng/mL concentration about 4 fold but not as high as TQ.

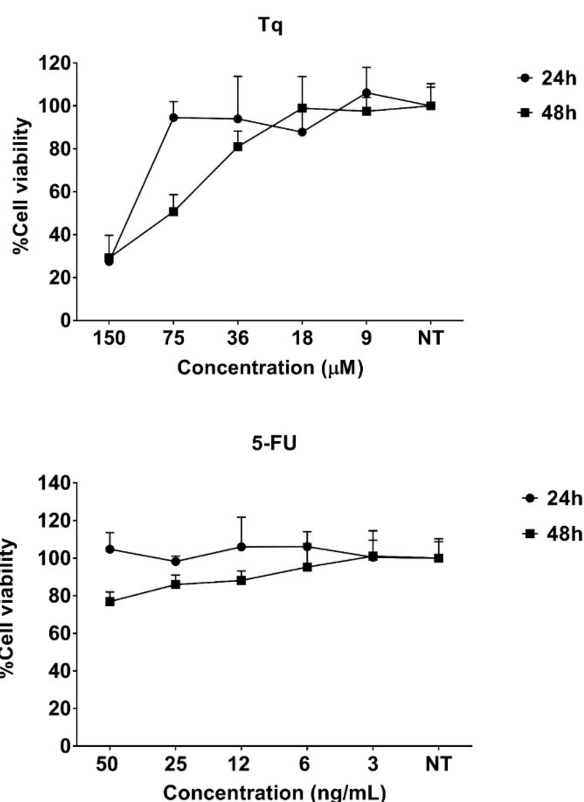

Figure 1. Cytotoxic effects of Tq and 5-FU at 24 h and 48h were revealed using MTT assay. HT-29 cells were plated at a density of 5×10^4 cells/well in 96-well tissue culture plates and subjected to five different treatments of Tq and 5-FU as positive control. NT (not-treated) contained 1 % of DMSO as control. The percent of cell viability was calculated using optic density values of 550 nM. 50 % inhibition concentration was determined using Origin pro 8.5.

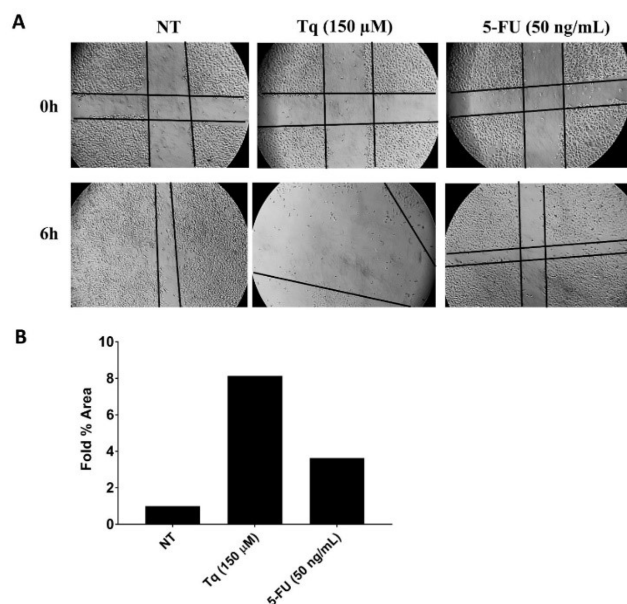

Figure 2. The invasion capability of HT-29 cells were determined using scratch assay in presence of Tq and 5-FU. NT was represented as control included 1 % DMSO. A. 1 % DMSO, 150 µM of Tq and 50 ng/mL of 5-FU treated HT-29 cells were screened at 0 h and 6 h using Nicon T 2000 camera. B. Fold % Area was calculated using Image J programme wound healing tool.

3.2. mRNA expression analyses of autophagy and apoptosis related genes in presence of TQ and 5-FU.

mRNA expression analyses were performed with qPCR method using specific autophagy and apoptosis related gene primers in presence of Tq and 5-FU. ATG-3, ATG-12, ATG-7, Beclin-1 and LC3-II primers for autophagy and Bcl-2, Bcl-XL and Bax primers for apoptosis were added into PCR reaction. As seen in Figure 3A, ATG-12, ATG-7 and LC3-II were significantly downregulated in presence of TQ. Conversely, the expression of all the autophagy genes were downregulated in presence of 5-FU (Figure 3A). In Figure 3B, pro-apoptotic gene Bax was significantly upregulated 15-fold whereas the expressions of Bcl-2 and Bcl-XL (pro-survival genes) were decreased in presence of Tq. On the other hand, In 5-FU treated HT-29 cells, Bax, Bcl-2 and Bcl-XL expressions were significantly downregulated (Figure 3B).

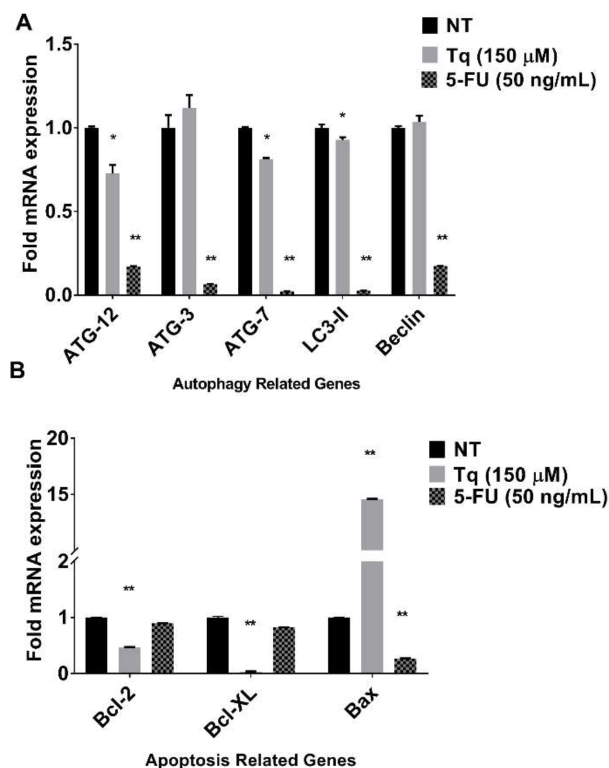

Figure 3. Fold mRNA expression of autophagic and apototic genes was determined with qPCR in presence of Tq and 5-FU at 48 h. Fold mRNA expression was calculated using ΔΔCt method.

4. DISCUSSION

The roles of autophagy is very complex mechanism because of tumor types, staging and stress factors in cancer development. Some studies suggest that autophagy process has dual behavior in cancer progression. Because of its role in cancer, inhibition or activation of the autophagic process is important for improving effective anticancer treatment strategies. For this reason, searching of new phytochemicals could be useful for treatment of several cancer types over autophagy mechanism [15].

Recently, there are some studies in using natural products/derivatives to target autophagy for cancer treatment such as lysosomal inhibitors chloroquine (CQ) and hydroxychloroquine (HCQ). Recent evidence has suggested that these molecules can regulate autophagy and can modulate the autophagic process both in vitro and in vivo by acting on several cellular signaling pathways and transcription factors [16].

In this study, Tq which is an active component of the *Nigella sative* seed oil, was searched for cytotoxicity and invasion cabability in HT-29 cells. At the same time, the effect of Tq was searched on autophagy and apoptosis mechanisms. qRT-PCR analyses were performed using specific primers to find relation between Tq with autophagy and apoptosis involved in the death mechanism of the cells.

In a result of cytotoxic analysis, TQ was inhibited 50 % of HT-29 cell proliferation with 85 µM concentration at 48 h. When we compared this activity with 5-FU as a chemotherapeutic agent, cytotoxic effect on HT-29 cells started from 25 ng/mL concentration. In the same way, Najjar and his group demonstrated that Tq-induced HT-29 cell death were ir-reversable when specific ERK and JNK inhibitors were used and also they mentioned about pro-oxidant effect with its apoptotic effects [17].

As a result of cytotoxic analysis, qRT-PCR studies were performed using specific autophagic and apoptotic primers to determine whether Tq-induced death was a stress-induced mechanism or programmed cell death. Macroautophagy system consists of 5 steps: 1.initiation, 2.nucleation, 3.elongation and closure, 4.fusion, 5.degradation and recycling. The initiation and nucleation steps require two protein complexes the Atg1/Ulk1 complex, which includes Ulk1, Atg13, FIP200, and Atg17, and the Vps34 complex, which includes the class III phosphatidylinositol 3 kinase Vps34, Vps15/P150, Beclin-1/Atg6, and Atg14. Elongation and closure steps involve Atg16L complex consisting of of Atg16L1-Atg12-Atg5, and LC3-II (microtubule-associated protein light chain 3-II). Additionally, ATG-3 and ATG-7 are essential for comprising of ATG12-ATG5 complex in elongation and closure steps [18]. q-RT-PCR analyses showed that, the expression of Bax, the pro-apoptotic gene, increased in Tq-treated HT-29 cells. However Bcl-2 and Bcl-XL pro-survival genes and also autophagy related ATG-7, ATG-12 and LC3-II gene expressions were decreased in presence of Tq. It mean that, Tq-dependent death mechanism is due to apoptosis whereas not autophagy system. The expression of apoptotic and autophagic genes were downregulated in 5-FU treated cells similar with the cytotoxic results.

Scratch assay was performed for searching invasion capability of TQ-treated HT-29 cells. The percent of wound area was increased about 8 fold in presence of 150 µM of TQ when compared with control group. Hence, it was found that, TQ was inhibited invasion capability of HT-29 cells. At the same time, 5-FU was increased the percent of wound area in HT-29 cells about 4 fold but not as TQ showed. In literature, also, it was found that TQ effectively inhibited the metastatic capacity of Renal Cell Carcinoma cells by inducing autophagy via AMPK/mTOR signaling pathway.in vitro, which was also verified in a xenograft model [19].

In a conclusion, Tq was significantly inhibited cell proliferation and invasion capability of HT-29 cells. Also, it was upregulated Bax expression whereas downregulated Bcl-2 and Bcl-XL expression. However, the expression of autophagy genes were decreased in presence of Tq. This is the first time, the relation between Tq, 5-FU and autophagy was declared in this paper. In future, Tq can be useful supplement for treatment of colon cancer with additional *in-vivo* experiments in detail.

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