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Research Article

Targeting Itch/p73 pathway by thymoquinone as a novel therapeutic strategy for cancers with p53 mutation

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Abstract

The tumor suppressor p73 is a member of p53 family and has a high degree of similarity with p53 function and structure. Like p53, p73 can also induce the expression of several genes involved in cell cycle and apoptosis. p73 expression is downregulated in many tumors by several mechanisms including the ubiquitination pathway. Thus, understanding the ubiquitin-proteasome pathway in p73 regulation will help in targeting this later and develop a new promising therapeutic strategy for cancer with p53 mutations. The aim of this study was to evaluate the effect of Thymoquinone (TQ), the major biologically active compound of the black seed oil on the expression of several E3 ubiquitin ligase enzymes known to be regulators of p73 and the related events in cancer cells with p53 mutation, such as the human acute lymphoblastic leukemia Jurkat cells, the human triple-negative breast cancer (MDA-MB-468 cells) and human promyelocytic leukemia HL60 cells. RNA-seq data showed that several E3 ubiquitin-ligase enzymes, well documented to be involved in the degradation of p73 including Itch, Pirh2, E3s Pin2, Mdm2, TRIM32 and SCFFBXO45 were downregulated in Jurkat cells. Among the target genes, Itch was significantly downregulated in TQ-treated Jurkat cells as compared with control cells. TQ-induced Itch downregulation was confirmed by real-time RT-PCR in Jurkat cells, MDA-MB-468 cells and HL60. Treating Jurkat cells with either TQ or the proteasome inhibitor MG132 induced an upregulation of p73. The present study indicates that TQ could be a promising inhibitor of the E3-ubiquitin ligase Itch leading to the upregulation of tumor suppressor p73 in cancers expressing mutant p53.

Keywords: Thymoquinone; Itch; p73; E3 ubiquitin-ligase enzymes; mutant p53, Jurkat cells.

1. Introduction

Silencing of tumor suppressor genes (TSGs) is a common characteristic in human cancer cells. Beside to genetic and epigenetic changes, the ubiquitination-dependent degradation through proteasome is one of the main mechanisms involved in the regulation of TSGs [1, 2]. p73 was shown to be targeted and ubiquitinated by several E3 ubiquitin-ligase enzymes such as Itch and Pirh2 causing the degradation of p73 through the proteasome

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[3-5]. Thus, p73 silencing observed in several tumors could be attributed in large part to the overexpression of Itch and Pirh2 in those cancers that leads to inhibit apoptosis and promote tumorigenesis. In agreement with this hypothesis, targeting E3 ubiquitin-ligase enzymes Itch and Mdm2 was shown to induce p73 upregulation with subsequent apoptosis [6, 7]. In the same way, the E3 ubiquitin-ligase Mdm2 has been also shown to promote p73 degradation in Hela cells [5, 8]. While Mdm2 overexpression decreased p73 expression, this effect was counteracted by the proteasome inhibitor MG-132 suggesting that ubiquitination-induced degradation of p73 in proteasome is a main cause of inhibition of apoptosis and promotion of cell proliferation in cancer [8].

p73 protein has a high degree of similarity in structure with p53 protein especially at the level of DNA-binding domain (79%). Unlike p53, which is silenced in 50% of human cancers [9], p73 is rarely mutated in cancer and its upregulation enables cancer cells to undergo apoptosis via p53-independent pathways rendering p73 a potent target for anti-cancer therapy in tumors with p53 mutation including acute lymphoblastic leukemia (ALL) [10-12], MDA-MB-468 breast cancer [13, 14] and human promyelocytic leukemia HL60 cells [15, 16]. Considering that p73 upregulation in response to pharmacological tools in cancers with p53 mutation allowing cancer cells to undergo apoptosis [10, 17-20], exploring the molecular mechanisms involved in p73 regulation will help us in targeting this tumor suppressor and develop a new promising therapeutic strategy for cancers with p53 mutation.

Several *in vitro* and *in vivo* studies have shown that thymoquinone (TQ), which is the bioactive compound of the volatile oil derived from seeds of *Nigella sativa* plant, has potent pro-apoptotic activities against various cancer cells [10, 21-23]. TQ was shown to inhibit cell proliferation and induce apoptosis in the p53-deficient acute lymphoblastic leukemia cell line (Jurkat cells) via p73 upregulation through mechanisms that are not fully understood [10]. Consequently, the aim of the present study was to evaluate whether TQ could inhibit the E3 ubiquitin-ligase enzymes involved in the degradation of p73 leading to the stability of this later with subsequent apoptosis in cancer cells.

2. Methods and Materials

2.1 Cell culture and treatment

Human T lymphocyte cell line Jurkat, acute myeloid leukemia (AML) cell line-HL60 and the human cell line (MDA-MB-468 cells) were obtained from the America Type Culture Collection (Mannassa, VA, USA). Cells were maintained in RPMI1640 (Sigma-Aldrich, St-Louis, MO) media for Jurkat and HL60 and Dulbecco's modified Eagle medium (DMEM) (UFC-Biotech, Riyadh, SA) for MDA-MB-468 supplemented with 15% (v/v) fetal calf serum (FCS, Biowhitaker, Lonza, Belgium), 2 mM glutamine, penicillin (100 IU/ml) and streptomycin (Sigma St. Louis, MO (100µg/ml). For all treatments, a 10 mM solution of TQ (Sigma–Aldrich, Louis, MO, USA) was prepared in 10% DMSO (DiMethylSulfOxide; Millipore, Molsheim, France) 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. Proteasome inhibitor, MG132 was obtained from Sigma-Aldrich and from Gentaur Europe (Kampenhout, Belgium).

2.2 RNA-Seq, Differentially Expressed Genes, and Bioinformatics Analysis

Jurkat cells were treated with 20µM TQ for 24 h, in triplicates and three independent experiments were performed. The total RNA was extracted using RNeasy kit (Qiagen). RNA-Seq and Differentially expressed genes are determined were carried out as described elsewhere [24].

2.3 Real-time RT-PCR analysis

Total RNA was isolated and purified from Jurkat cells and MDA-MB-468 cells (treated with 5 and 10 µM TQ for 24 h) or HL60 (treated with 5 µM TQ for 24 h), using the RNeasy kit (Qiagen). 1 µg of total RNA was used to create cDNA libraries using Superscript III Reverse Transcriptase (Invitrogen) with specific primers and oligo dT. Real-time PCR was performed using SYBR Green qPCR (iQ SUPERMIX, BioRad) on ABI7500 system. The PCR reactions were performed for 40 cycles at 95°C for 15 sec, 60°C for 15 sec and 72°C for 1 min. The results were normalized with ribosomal protein L11 (RPL11) mRNA. The sequences of the primers for PCR amplification were: Itch (sense, 5′-GCACGGGCGAGTTTACTATG-3′; antisense, 5′-TGCTGCATTGCTCCTTGAAG-3′); RPL11 (sense, 5′-ATCCTTTGGCATCCGGAGAA-3′; antisense: 5′-GTCCAGGCCGTAGA TACCAA-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.

2.4 Western blot analysis

Jurkat cells were grown in RPMI medium and then treated with proteasome inhibitor MG-132 at 20 µM for 3 and 6 h or with proteasome inhibitor MG-132 at 10 µM and TQ at 20 µM for 24 h. The cells were then harvested, centrifuged to discard the RPMI medium, washed with cold PBS (phosphate buffered saline), resuspended in RIPA buffer (25 mM Tris, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS; Sigma–Aldrich, USA) containing protease inhibitors and incubated on ice for 15 min. Cell suspensions were sonicated three times for 30 s, and then were centrifuged at 10,000g for 16 min at 4°C. The supernatant was collected and the protein concentration was determined by the Bradford method (Bio-Rad, Marne la Coquette, France). Equal amounts of total protein were taken. After adding

the loading buffer (5% mercaptoethanol with laemmli), sample of proteins were placed in a water bath at 100°C. Then the proteins were separated on 10–12% polyacrylamide gels and electrophoretically transferred to a nitrocellulose membrane. After blocking with 5% BSA (Bovine Serum Albumin) and tween 20 in PBS. Membranes were then incubated with a mouse monoclonal anti-p73 (BD Biosciences Pharmingen), or mouse monoclonal anti β-actin antibody (Abcam, Paris, France), according to the manufacturer's instructions at 4°C overnight. The membranes were then washed three times with PBS for 10 min. Membranes were, thereafter, incubated with anti-mouse antibody (diluted to 1:10,000) at room temperature for 1h30. The membranes were then washed with PBS five times. Signals were detected by chemiluminescence using the ECL Plus detection system (Amersham, GE Healthcare UK).

2.5 Statistical Analysis

All the data were presented as Mean ± S.E.M of triplicates done in the same experiment or an average of at least three separate experiments. Statistical analysis was performed using one way ANOVA followed by Tukey's post hoc test using GraphPad Prism 6 (Graph Pad Software, SanDiego, USA) or by Student's t-test (two-tailed) to compare the differences between two groups and the significant differences were indicated as **P<.05*, ***P<.01*, ****P<.001*, and *****P<.0001*.

3. Results

3.1 TQ decreases the expression of several E3 ubiquitin-ligase enzymes involved in the degradation of p73

Several E3 ubiquitin-ligase enzymes including Itch [6, 25, 26], E3s Pin2 [27], Pirh2 [5, 28, 29], Mdm2 [7, 8], TRIM32 [30] and SCF^{FBXO45} [31] have been shown to be involved in the degradation of p73 through the proteasome and that their inhibition in response to DNA damage results in p73 stabilization and activation. Thus, p73 silencing observed in several tumors could be attributed in large part to the overexpression of those E3 ubiquitin-ligase enzymes that leads to inhibit apoptosis and promote tumorigenesis. To know whether TQ can affect the expression of the E3 ubiquitin-ligase enzymes involved in the degradation of p73, we analyzed the gene expression after TQ exposure for 24 h. RNA-seq data showed that several E3 ubiquitin-ligase enzymes involved in the degradation of p73 including Itch, Pirh2, Mdm2, TRIM32 and SCF^{FBXO45} were downregulated (Table 1). Among the decreased E3 ubiquitin-ligase enzymes Itch was significantly down-regulated in response to TQ treatment. Interestingly, several pro-apoptotic genes known to be downstream targets of p73 such as BAX and PUMA were upregulated in TQ-treated Jurkat cells, while the anti-apoptotic gene BCL2, a well-documented downstream target of p73 was downregulated (Data not shown). As, Itch was significantly down-regulated in TQ-treated Jurkat cells, we then studied the effect of 5 and/or 10 µM TQ for 24 h on mRNA expression of Itch in Jurkat cells, MDA-MB-468 cells and HL60, using RT-qPCR. We found that mRNA expression of Itch gene was significantly decreased in a dose-dependent manner in Jurkat cells (Figure 1A) and MDA-MB-468 cells (Figure 1B) or HL60 (Figure 1C) treated with TQ at10 µM compared with control. These results suggest that the ubiquitination-mediated degradation of p73 in proteasome is a main cause of inhibition of apoptosis in cancers with p53 mutation and the inhibition of E3 ubiquitin-ligase enzyme Itch in response to TQ is a key event in the activation and stability of tumor suppressor p73 leading to apoptosis.

3.2 Inhibition of the ubiquitin-proteasome pathway is implicated in the upregulation of p73 by TQ

Considering the fact that p73 stability is regulated by the ubiquitin-proteasome pathway, we wanted to examine the effect of the proteasome inhibitor MG132 on the expression of p73 in Jurkat cells. Treating Jurkat cells using MG132 at 20µM induced an upregulation of p73 starting from 3h (Fig 2A). In the next step, we analyzed the effect of MG-132 as well as TQ on the expression of p73 after 24h (Fig. 2B). The results showed that treating Jurkat cells with either MG-132 at 10µM or TQ at 20µM induced an upregulation of p73 indicating that TQ mimics the effect of the proteasome inhibitor MG-132 on the expression of p73. Taken together, these findings suggest that the decreased p73 expression levels observed in Jurkat cells could be attributed; at least in part to its degradation in proteasome through ubiquitination-dependent process involves the E3 ubiquitin-ligases including Itch.

4. Discussion

The tumor suppressor p73 was shown to be ubiquitinated by several E3 ubiquitin-ligase enzymes causing its degradation in the proteasome [3-5]. Interestingly, in cancer cells having defective p53 including Jurkat cells and MDA-MB-468 breast cancer, always when p73 is upregulated through the use of anti-cancer agents, several pro-apoptotic genes are activated with subsequent induction of apoptosis [10, 13, 14, 32, 33]. Therefore, it is of interest to find new natural compounds that can inhibit the E3 ubiquitin-ligase enzymes involved in the degradation of p73 which could be a new therapeutic strategy for cancers with p53 mutation. We found that TQ inhibits several E3 ubiquitin-ligase enzymes including Itch, Pirh2, Mdm2, TRIM32 and SCFFBXO45 known to be involved in the degradation of p73 (Table 1).

p73 silencing observed in several tumors could be attributed in large part to the overexpression of E3 ubiquitin-ligase enzymes that leads to inhibit apoptosis and promote tumorigenesis. In agreement with this hypothesis, inhibiting E3 ubiquitin-ligase enzymes Itch and Mdm2 was shown to induce p73 upregulation with subsequent

apoptosis [6, 7]. Mdm2 has been also shown to promote p73 degradation in Hela cells involving Itch [5, 8]. While Mdm2 overexpression decreased p73 expression, this effect was contracted by both the proteasome inhibitor MG-132 and Itch knockdown indicating that Mdm2 needs the presence of Itch to inhibit p73 [8]. The present study also showed that TQ significantly inhibits Itch as well as Mdm2 (Table 1). Interestingly, the present study showed that TQ significantly decreased mRNA expression of Itch gene in the three cancer cells expressing p53 mutant: Jurkat cells, MDA-MB-468 and HL60. By analyzing the data obtained from RNA-seq, we found several E3 ubiquitin-ligase enzymes known to be inhibitors of p73 are downregulated in TQ-treated Jurkat cells compared to control cells (Table 1) suggesting that p73 degradation observed in tumors with p53 mutation including leukemia and breast cancer may be attributed to the overexpression of E3 ubiquitin-ligase enzymes. Itch was found to be the significantly downregulated E3 ubiquitin-ligase and this observation is in line with previous studies which have reported Itch as inhibitor of p73 through ubiquitination-mediated process [6, 25, 26]. Other E3 ligases, E3s Pin2, Mdm2, TRIM32 and SCFFBXO45 are also found to be decreased upon TQ treatment of Jurkat cells (Table 1).

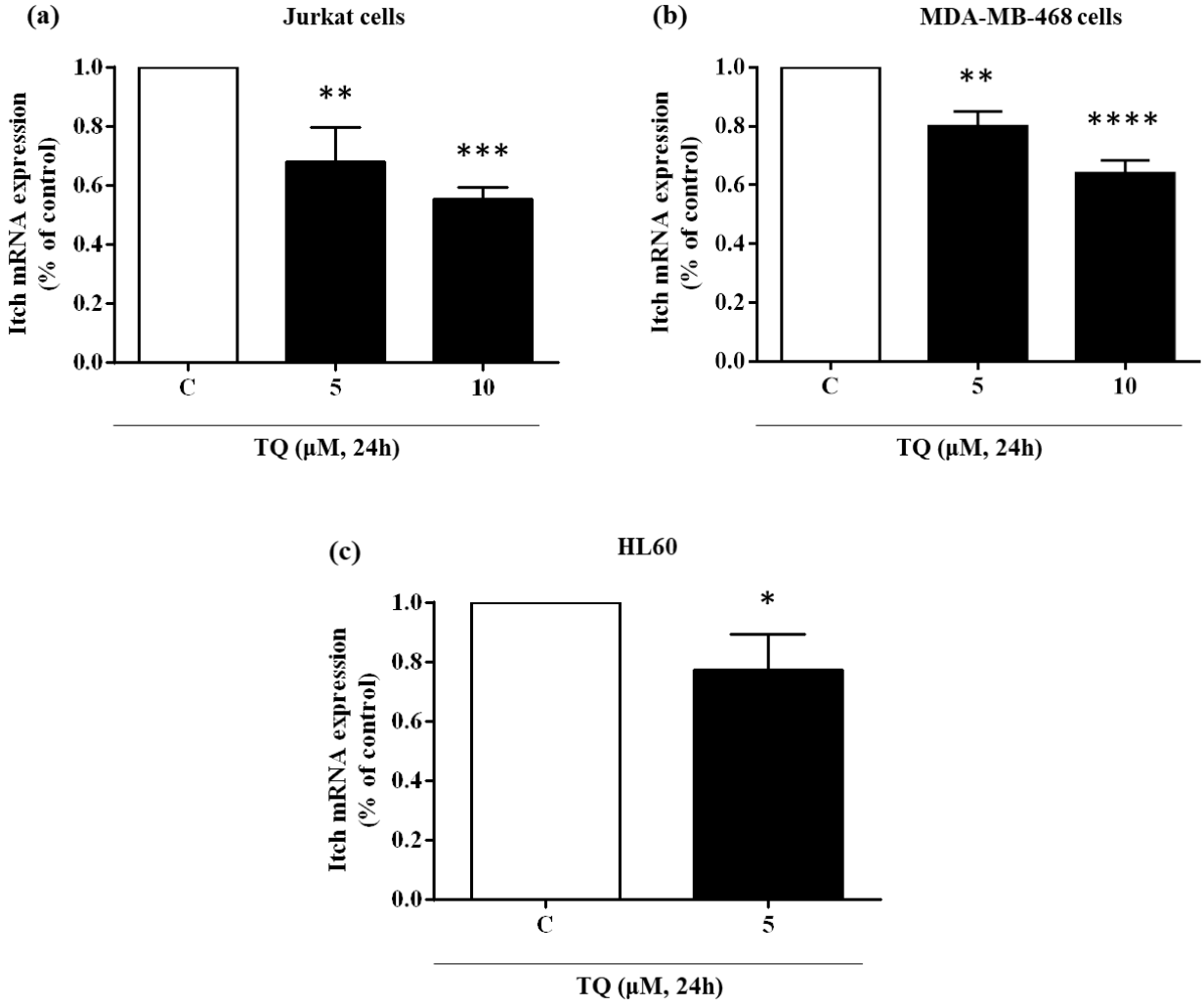

Figure 1: TQ induces the transcription downregulation of Itch in cancer cells. Jurkat cells (A), MDA-MB-468 cells (B) and HL60 cells were treated with 5 or 10 µM TQ for 24 h. The histograms show the quantification data of mRNA expression of Itch as assessed by real-time RT-PCR. Data are shown as mean ± SE. S.E.M. (n = 3); (**P<.05*, ***P<.01*, ****P<.001*, and *****P<.0001)* versus respective control.

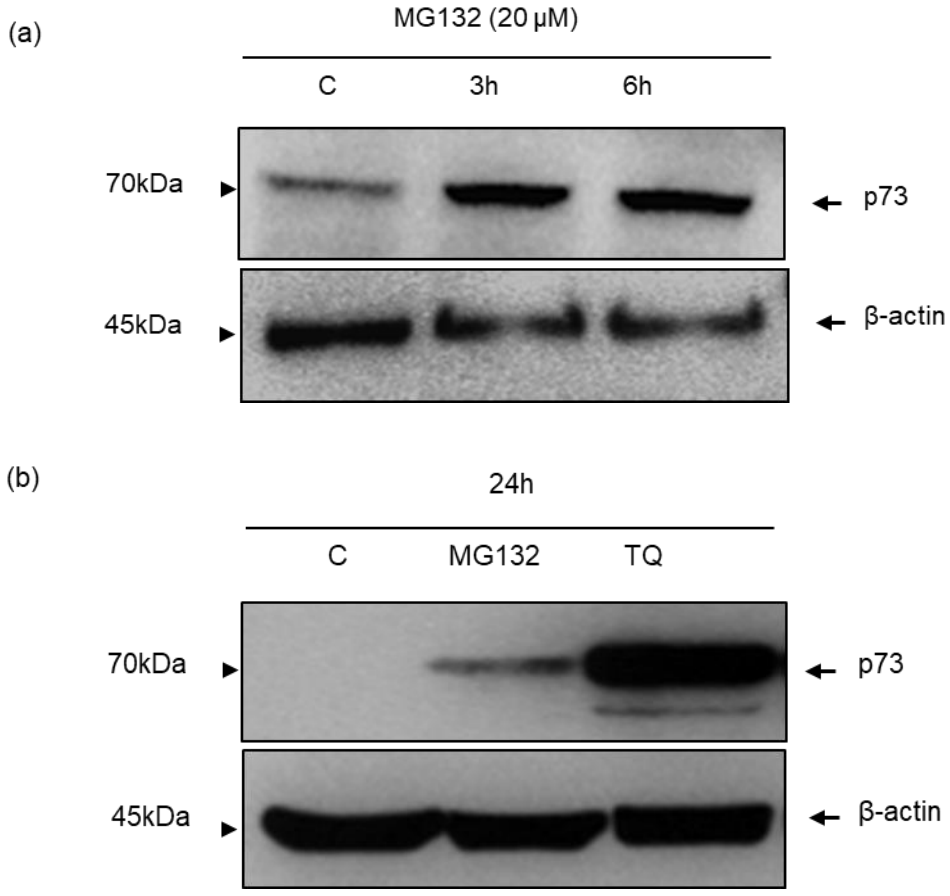

Figure 2: Effect of MG-132 and TQ on the expression of p73 in Jurkat cells. (a): Jurkat cells were treated with proteasome inhibitor MG-132 at 20 µM for 3 and 6 h. (b): Jurkat cells were treated with either proteasome inhibitor MG-132 at 10 µM or TQ at 20 µM for 24 h. Western blot was then performed using anti-p73 and antibody as described in materials and methods.

Table 1: Downregulation of E3 ubiquitin-ligase enzymes involved in the regulation of p73 in TQ-treated Jurkat cells as compared with untreated cells.

Gene	Gene Symbol	Gene Function	logFc*	P-value
Itch	Itch	E3 ubiquitin-ligase	-1.711967767	0.0072
FBXO45	FBXO4	E3 ubiquitin-ligase	-0.8247864812	0.3157
Mdm2	Mdm2	E3 ubiquitin-ligase	-0.77722801	0.348
TRIM32	TRIM32	E3 ubiquitin-ligase	-0.24379	0.9915
Pirh2	RCHY1	E3 ubiquitin-ligase	-0.2640004048	0.9527

We observed that TQ induced an upregulation of p73 as well as the pro-apoptotic genes BAX and PUMA and triggered a significant decrease in the expression of the anti-apoptotic gene Bcl 2 (data not shown) which are all known to be downstream targets of p73 suggesting that TQ exerts inhibitory effects on proteasome to prevent the degradation of p73. In agreement with these observations, TQ has been shown to induce a selective proteasome inhibition that results in the upregulation of p53 and BAX with subsequent induction of apoptosis [34]. Like TQ, the proteasome inhibitor was also able to induce an upregulation of p73 in Jurkat cells suggesting that ubiquitination-induced degradation of p73 in proteasome is a main cause of inhibition of

apoptosis in leukemia.

In conclusion, our study reveals that TQ induces an upregulation of the tumor suppressor p73 with a concomitant downregulation of several E3 ubiquitin-ligase enzymes known to be inhibitors of p73 in cancer. Itch could be the most likely E3 ubiquitin-ligase concerned with TQ-induced p73 upregulation with subsequent induction of apoptosis. The current study provides new insights into the regulation of p73 expression in cancers with p53 mutation in response to treatment with natural anti-cancer drugs and it suggests that inhibiting E3 ubiquitin-ligases by TQ could be a promising tool for cancer therapy especially for those with p53 mutation.

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Conflict of interest

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

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