



Cytotoxicity, Apoptosis induction and change of *p53*, *PARP*, *p21* and *Bcl-2* genes expression in the human anaplastic thyroid carcinoma cells line (SW-1736) with curcumin

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DOI: 10.31383/ga.vol5iss1pp10-17

Abstract

Anaplastic thyroid carcinoma is highly invasive with a poor response to a treatment. In this study, curcumin bioavailability and its effects on apoptosis induction and selected genes expression of the human anaplastic thyroid carcinoma cell line (SW-1736) were examined. SW-1736 cells were incubated for 24 and 48 hours with different concentrations of curcumin 2.5, 5, 7.5 and 10 μ M to examine bioavailability, and for 24, 48 and 72 hours with concentrations of 2.5, 5, 7.5, 10 μ M and 2.5, 5 and 10 μ M respectively to examine apoptosis and the expression of *p53*, *PARP*, *p21* and *Bcl-2* genes. Then, bioavailability was analyzed by MTS kit, apoptosis was analyzed by flow- cytometry using Annexin V-FITC/PI kit and the expression of *p53*, *PARP*, *p21* and *Bcl-2* genes were analyzed by Real Time PCR. ANOVA test and SPSS 16 software were used for statistical analysis. The results indicate that curcumin at the concentration of 7.5 μ M has significantly decreased bioavailability in anaplastic thyroid cells in comparison with other treatments at both incubation periods. Induction of apoptosis with increasing concentration of curcumin in dose and time dependent manner increased in this cell line. Also, treatment with curcumin significantly decreased the expression of *Bcl-2* gene and increased the expression of *p53*, *PARP* and *p21* genes in some experimental groups compared to the control group. Curcumin inhibited the growth, proliferation and invasion of anaplastic thyroid cancer cells through altering the expression of the genes involved in the apoptosis process.

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Received

March, 2021

Accepted

May, 2021

Published

June, 2021

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Keywords

Curcumin, *Apoptosis*, *Cytotoxicity*, *Gene expression*, *SW-1736*

Introduction

Anaplastic thyroid carcinoma (ATC) is one of the most malignant and lethal solid tumors known in humans (Agarwal et al., 2003). These tumors are fast

growing, quickly invade the tissues around the throat and have a poor response to a treatment. About 90% of patients are more than 50 years old, because the disease usually occurs between 65-75 years of age. The risk in women is three times higher than in men

with the median survival prognosis of 4-12 month since the primary diagnosis (Spires et al., 1988; Tallroth et al., 1987; Ain, 1998; Inazawa et al., 2004). The ATC causes have not been well known. ATC may result from a goiter disease in persons living in the areas where goiter is endemic, or even occur in people who haven't got any clinical history of thyroid gland disease (Besic et al., 2010). The cytogenetic studies on malignant anaplastic cells by genomic hybridization method or identification of genetic deletion and addition in some regions of chromosome (Inazawa et al., 2004) have shown that these cells have a high degree of numerical and structural irregularities in their genome which causes fundamental changes in the expression of their genes. Clinical examination reveals a growing mass in the anterior area of the neck with dysphagia and changes in the voice sound. In addition, patients have systemic symptoms, such as anorexia, weight loss and respiratory depression (Kebebew et al., 2005). In the clinical examination of the elderly people, the observation of a large, tight, non-moving, high-growth mass usually draws the physician's attention to the ATC (Kuma et al., 2007). Treatment of patients with anaplastic thyroid carcinoma is very difficult, because the conventional therapeutical approaches such as chemotherapy and radiotherapy, only have relief effect on ATC patients and cannot be used for pregnant women. Also, thyroidectomy can only reduce the airway congestion, therefore, this malignancy is associated with death and the study of new compounds with natural origin and therapeutic properties is inevitable (Viola et al., 2016; Broecker-Preuss et al., 2016; Broecker-Preuss et al., 2015). Curcumin is a yellow compound obtained from the *Curcuma longa* L. It is a member of the curcuminoid family and has been used in traditional medicine for centuries, and so far, no toxicity has been reported (Hatcher et al., 2008). Circular groups in the curcumin structure create a hydrophobic environment. Its tautomeric structure also influences the hydrophobicity and polarity of the curcumin molecule. These factors make the curcumin able to react with a large variety of biomacromolecules (Srivastava et al., 2016). Specific structure of curcumin molecule is responsible for its functionality and multiple potentials for modulating biological and molecular activities. This suggests that curcumin has potential to be used for treatment

of diseases with molecular bases related to the loss of balance and setting of messenger molecules. One of these diseases can be cancer (Mullaicharam et al., 2012). The results of many studies suggest that curcumin regulates the growth of cancer cells and inhibits it by regulating the vital signaling pathways of cells such as; the effect on cell enzymes, antioxidant activity, effect on the transcription of the apoptosis genes such as *p53*, *PARP*, *p21* and *Bcl-2* (Ravindran et al., 2009; Gowda et al., 2008). The *Bcl-2* gene that encodes a protein with the molecular weight of 25 kDa and 239 amino acids in length, is located at chromosomal position of 21q18. The *bcl-2* protein regulates the activity of caspase enzymes. *Bcl-2* protein releases cytochrome C from mitochondria which leads to activation of Caspase 9 and Caspase 3 respectively. This protein can play a role in the survival and prevention of programmed cell death. Collaboration of *Bcl-2*, *Bcl-X* and *Mcl-1* proteins triggers an anti-apoptosis action (Fesik et al., 2001; Nagata, 2000). The *PARP* protein is encoded by the *PARP1* gene that is located on chromosome 1. This protein is responsible for single-stranded DNA repair by attaching to it. *PARP* is a substrate for special caspase proteins, including caspases 3 and 7, and shows its suppressing character through mediation of apoptosis pathway. Caspases are continually made in all cells as pro-enzyme and activated in a response to the pro-apoptotic stimulator (*PARP*). Activated caspases destroy key cellular compounds, such as structural proteins in the cellular skeleton and nuclear proteins, like DNA repairing enzymes, and eventually induce cell death (Leng et al., 2003; Mishra et al., 2016). The *p53* protein is encoded by *p53* tumor suppressor gene, located on chromosome 17. This protein has short half-life and is not naturally accumulated in the cell, but as soon as DNA damage occurs, in order to repair DNA or induce apoptosis, its level increases. At this time, the *p53* protein, by binding to the DNA, stimulates the *WAF1* gene, which produce *P21* protein. The *p21* protein from the *Cip / kip* family is located at the chromosomal position 6p21.2., It plays an important role in passing from G1 to S phase and in the control of the cell cycle by cyclin-dependent kinases. In addition, this protein plays an important role in DNA repair, cell growth and apoptosis. The activity of gene that encodes *p21* protein is to arrest or slow down the cell cycle until the cell's

energy level returns to normal. Once DNA damage occurs and *WAF1* gene is stimulated by p53 protein, p21 attaches to the CDK2 protein and prevents cell entry to the next phase and arrests the cell cycle to repair (Suryanarayana et al., 2005; Kuttan et al., 1987). Therefore, it seems that curcumin can be used to prevent cancer or if taken simultaneously with chemotherapy drugs to improve cancer treatment. Therefore, in this study, the anticancer ability of curcumin in the human anaplastic thyroid carcinoma cell line (SW-1736) was examined.

Material and methods

The experimental study was carried out in October, 2016 at the Cellular and Developmental Research Center of Islamic Azad University, Shahrekord Branch.

Cell culture

SW-1736 cell line (IBRC C10311) was purchased from Iranian Biological Resource Center. SW-1736 cells with RPMI culture medium (Roswell Park Memorial Institute) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA) were cultured and incubated at 37°C in an environment with 5% CO₂ and 90% humidity.

Preparation of curcumin solution

To prepare curcumin solution for cell culture treatment, 10 mg of curcumin powder was added to 1 ml ethanol in the laminar flow hood and filtered through a 0.2 µm filter. The 23.15, 46.3, 69.45, 92.6 µl values of filtered extract respectively were used to prepare final concentrations (2.5, 5, 7.5 and 10 µM solutions). (Microliter amounts of curcumin was calculated using $M1V1 = M2V2$ formula based on the molecular weight of curcumin (368.38 g / mol)).

Cytotoxicity analysis

Cells were seeded in 96-well plates (5×10^3 cells/well). After 24 h, 2.5, 5, 7.5 and 10 µM of curcumin dilutions were added to the wells. Control group was exposed to culture medium without

curcumin. Following the indicated incubation period (24 and 48h), cell viability was evaluated using the MTS assay according to the manufacturer's instructions. Briefly, the medium was replaced with 100 µL of fresh medium and 20 µL of MTS reagent (CellTiter 96 Aqueous Solution) in each well, and the plate was returned to the incubator for 4 h. The optical density measurements were obtained at 492 nm using a microplate reader.

Apoptosis analysis

SW-1736 cells were seeded in 6-well tissue culture plates (5×10^5 cells/well). After 24 h, the medium was changed and cells were treated with 2.5, 5, 7.5 and 10 µM of curcumin preparations. After treatment (for 24, 48 and 72h), the adherent cells were detached with 0.025% trypsin. The cells were stained with Annexin V-FITC and PI according to the manufacturer's instructions. Untreated cells were used as control for the double staining. The cells were analyzed immediately after staining using a FACScan flow cytometer. For each measurement, at least 10,000 cells were counted.

Gene expression analysis

In order to determine the expression of *p53*, *PARP*, *p21* and *Bcl-2* genes, 3×10^5 cells were cultured in 6-well tissue culture plates, then were treated with 2.5, 5 and 10 µM of curcumin dilutions and incubated for 24, 48 and 72 hours. After 24, 48 and 72 hours, cells from three experimental and control groups were picked up and after washing with PBS buffer, total RNA, using the Biozol reagent (Biozol Total RNA Extraction Reagent) (BSC51M1) (BioFlux, China) was extracted. To perform the Heat block stage, samples were placed in thermal cycler at 60°C for 20 minutes. Subsequently, all RNA sequences were converted to cDNA using cDNA synthesis kit (RevertAID First Standard cDNA Syn Kit), (Termo Scientific, Lithuania) and transferred to -20°C. Expression of *p53*, *PARP*, *p21* and *Bcl-2* genes were analyzed by Real Time PCR (Rotor gene 3000 corbett, Australia). To carry out the reaction, reagent containing 1/5µl cDNA, 0.3µl of each primer (forward and reverse) and 4.5µl of RNase-free water

were prepared. Sequences of forward and reverse primers of *GAPDH*, *p53*, *PARP*, *p21* and *Bcl-2* genes were designed by Oligo6 software and then blasted with NCBI to ensure their integrity and were finally synthesized by Macrogen Inc (Table 1).

GAPDH gene and SYBR Green were used as an internal control and reporter, respectively. Then, the reaction mixture with a final volume of 15 μ l was prepared on ice and placed under time-temperature conditions (Table 2)

Table 1. Forward and reverse primer sequences for RT- Real Time PCR

Official Name (gene)	mRNA accession number		Primers	
1 p53	NM_00546.5	F	ACATAGTGTGGTGGTGCCCT	20
		R	ACCTCAAAGCTGTTCCGTCC	20
2 PARP	NM-001618.	F	GGCTTCAGCCTCCTTGCTAC	20
		R	TTCGCCACTTCATCCACTCC	20
3 GAPDH	NM-001256799.2	F	CACATGGCCTCCAAGGAGTAG	22
		R	AGGGGAGATTCAAGTGTGGTG	20
4 p21	NM-001291549.1	F	CATGTGGACCTGTCACTGTCTT	22
		R	CTGGTCTGCCGCCGTTTTC	19
5 Bcl2	XM-01702591701	F	GGGAGGATTGTGGCCTTCTTT	21
		R	GGTTCAGGTACTCAGTCATCCA	22

Table 2. Time-temperature condition of RT- Real Time PCR

1	First denaturation	95 ⁰ c	10 Min
1cycle			
2	denaturation	95 ⁰ c	15 sec
3	Anneling <i>p53</i> gene	62 ⁰ c	60 sec
4	Anneling <i>p21</i> gene	62 ⁰ c	60 sec
5	Anneling <i>PARP</i> gene	61 ⁰ c	60 sec
6	Anneling <i>Bcl-2</i> gene	61 ⁰ c	60 sec
7	Anneling <i>GAPDH</i> gene	61 ⁰ c	60 sec
40cycle			
8	Extention	72 ⁰ c	1 Min

Statistical analysis

Statistical analysis was performed using ANOVA test, SPSS 16 software, as well as the Excel program. The level of statistical significance was set at $p < 0.05$.

Results and Discussion

Cytotoxicity effect of curcumin on anaplastic thyroid cells

The results of the MTS indicated that anaplastic thyroid cells at both incubation times (24 and 48h) when treated with curcumin at concentration of 7.5 μ M was significantly reduced compared to the control group and other treatments (Figure 1).

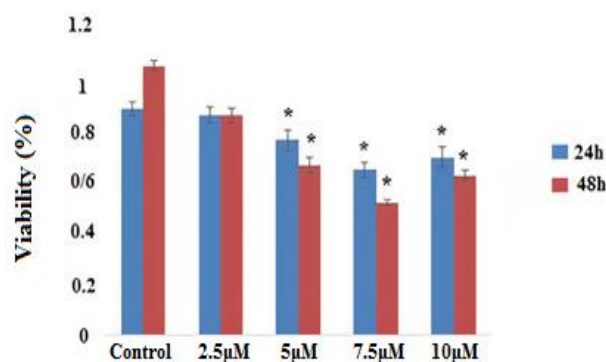


Figure 1. Inhibition of SW-1736 cell line viability by curcumin. Data were presented as mean \pm SD. Sign (*) denote significant differences ($P < 0.05$).

Curcumin-induced apoptotic cell death in SW-1736 cells

The Annexin V-FITC results showed an increase in the percentage of cell death in the treated groups. Thus, by increasing the dose and treatment time, the percentage of viable cells in the treated groups was reduced compared to the control group.

As shown in Figure 2 in each incubation time the percentage of viable cells in all groups is lower and statistically significant compared to the control group ($P = .032$). Also, with increasing curcumin concentration and incubation time, the percentages of cells that are in significantly increased in comparison with the control group at all three incubation times (Figure 2).

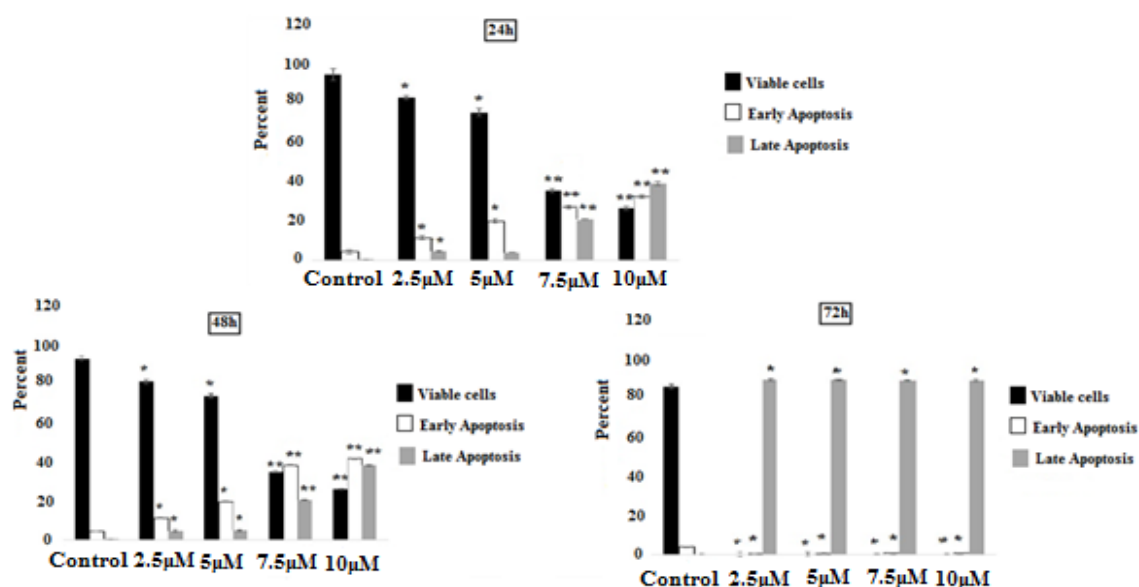


Figure 2. Effects of curcumin on SW-1736 cell apoptosis for 24, 48 and 72 hours treatments. Percentage of viable, early and late apoptotic cells. Data were presented as mean \pm SD. Sign (*) denote significant differences ($P < 0.05$).

Changes in gene expression by curcumin

Analysis of Real Time PCR Ct values by using of $2^{-\Delta\Delta Ct}$ formula revealed a significant decrease in the expression of Bcl-2 gene and a significant increase in the expression of p53, PARP, p21 genes at some concentrations compared to the reference gene (Figure 3).

Curcumin is the active ingredient and the main component of *Curcuma longa* that due to its specific structure and pharmacological effects such as; antimicrobial, antioxidant, inflammatory factors inhibitor, cell death induction and anti-carcinogen. In traditional medicine of some countries, such as Iran and India, it has been used to treat various diseases, especially respiratory, liver, gastrointestinal and for pain relief (Goel et al., 2010; Fallah et al., 2010; Moreillon et al., 2013; Anand et al., 2008). This compound by decreasing the expression of anti-apoptotic genes leads to increased

expression of pro apoptotic proteins and targets the apoptotic pathway. These proteins facilitate the induction of cell cycle arrest, activation of proteins involved in DNA repair and induction of apoptosis in cancerous cells due to its multiple functional mechanisms (Broecker-Preuss et al., 2016). In the present study, the ability of curcumin to inhibit growth of the SW-1736 cell line was investigated. Results showed that curcumin significantly inhibited the growth of SW-1736 cell line. In the same way: in 2006, the effects of curcumin on growth and apoptosis in the human ovarian cancer cell line (Ho-8910) was investigated. Results showed that curcumin could significantly inhibit the growth and induce apoptosis in Ho-8910 cells (Shi et al., 2006). In 2012, the ability of cyclohexanone analogues of curcumin was investigated for their effects on growth and apoptosis in PC-3 human prostate cancer cells. Results showed that curcumin analogues (cyclohexanone) had stronger inhibitory effects

compared to curcumin on the growth of cultured PC-3 cells (Wei et al., 2012). In 2018, the cytotoxicity effects of combination Berberine and Nano-curcumin in breast cancer cell line was evaluated.

Results of this study showed that co-treatment of Berberine and Nano-curcumin significantly inhibited the growth of MCF-7 breast cancer cell line (ZiaSarabi et al., 2008).

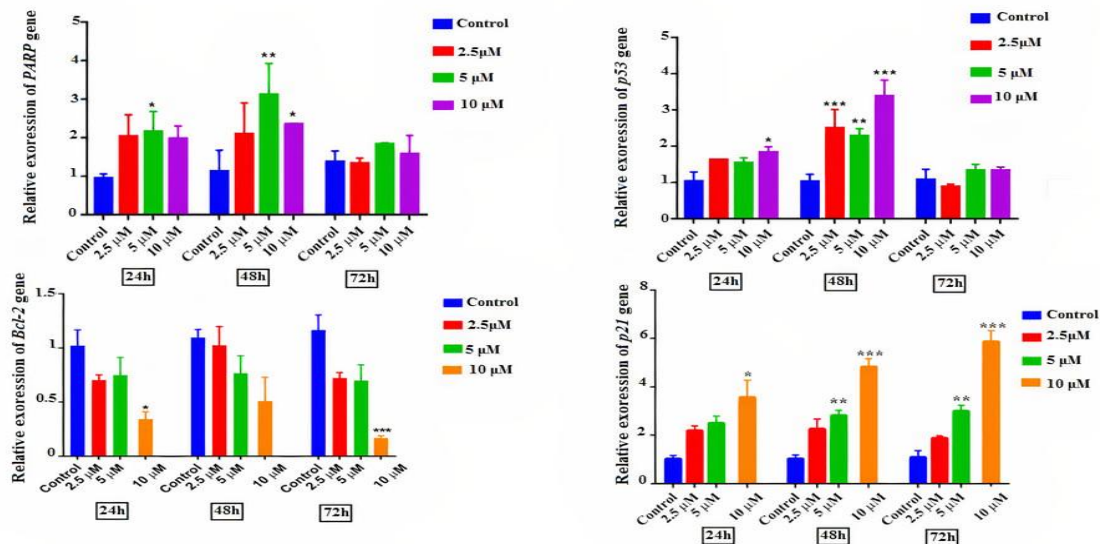


Figure 3. *PARP*, *P53*, *Bcl-2* and *P21* gene expression in SW-1736 cells by real time PCR after 24, 48 and 72h incubated by 2.5, 5 and 10 μM concentration of curcumin. Data were presented as mean ± SD. Sign (*) denote significant differences ($P < 0.05$).

The ability of curcumin to induce apoptosis in the SW-1736 cell line was also investigated by gene expression analysis of genes involved in the cell death pathway. The results showed that curcumin can alter the expression of, such as *Bcl-2*, *Bcl-X*, *p53*, *p21* and *PARP*, involved in the apoptotic process, and subsequently inhibit proliferation and invasion of human anaplastic thyroid cancer cells (SW-1736). Apoptotic and gene regulatory effects of both turmeric and curcumin were investigated in the MCF-7 human breast cancer carcinoma cell line. Results showed that curcumin alters gene expression up to 14-fold levels and induces apoptosis in breast cancer cells by regulation of multiple signaling pathways (Ramachandran et al., 2005). In 2014, the effects of curcumin on the cell viability, apoptosis, migration and invasion of human thyroid cancer cell lines FTC133 were investigated. Results showed that curcumin suppresses FTC133 cell invasion and migration by inhibiting PI3K and Akt signaling pathways (Xu et al., 2014). Treatment of BCPAP cells with curcumin showed that curcumin inhibits their adhesion, spreading and migration. Also,

curcumin inhibits TGF- β /Smad2/3 signaling pathway in BCPAP cells (Zhang et al., 2016). Also, human thyroid cancer cell lines TPC-1 (papillary), FTC-133 (follicular), and BHT-101 (anaplastic) were treated with curcumin. Results showed that curcumin increases the expression of redifferentiation markers and induces G2/M arrest, apoptosis, and downregulation of NF- κ B activity in thyroid carcinoma cells (Schwertheim et al., 2017).

Conclusion

The results of the present study indicate the ability of curcumin to decrease the growth of anaplastic thyroid carcinoma cells through affecting the pro-apoptotic genes and likely other mechanisms and its capacity to treat ATC malignancy.

Conflict of interest

Authors declare no conflict of interest.

Ethical Considerations

This study with research ethics code IR.IAU.SHK.REC.1397.028 has been approved by

Research Ethics Committee of Islamic Azad University of Shahrekord.

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