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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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 μM to examine bioavailability, and for 24, 48 and 72 hours with concentrations of 2.5, 5, 7.5, 10 μM and 2.5, 5 and 10 μM 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 μM 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.

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; Talroth 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 \textit{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.

\section*{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.

\subsection*{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\(^\circ\)C in an environment with 5\% CO\(_2\) and 90\% humidity.

\subsection*{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 \(\mu\)m filter. The 23.15, 46.3, 69.45, 92.6 \(\mu\)l values of filtered extract respectively were used to prepare final concentrations (2.5, 5, 7.5 and 10 \(\mu\)M solutions). (Microliter amounts of curcumin was calculated using \(M_1V_1 = M_2V_2\) formula based on the molecular weight of curcumin (368.38 g / mol)).

\subsection*{Cytotoxicity analysis}

Cells were seeded in 96-well plates (5 \(\times\) 10\(^3\) cells/well). After 24 h, 2.5, 5, 7.5 and 10 \(\mu\)M 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 \(\mu\)L of fresh medium and 20 \(\mu\)L 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.

\subsection*{Apoptosis analysis}

SW-1736 cells were seeded in 6-well tissue culture plates (5 \(\times\) 10\(^5\) cells/well). After 24 h, the medium was changed and cells were treated with 2.5, 5, 7.5 and 10 \(\mu\)M 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 FACSscan flow cytometer. For each measurement, at least 10,000 cells were counted.

\subsection*{Gene expression analysis}

In order to determine the expression of \textit{p53}, \textit{PARP}, \textit{p21} and \textit{Bcl-2} genes, 3 \(\times\) 10\(^5\) cells were cultured in 6-well tissue culture plates, then were treated with 2.5, 5 and 10 \(\mu\)M 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\(^\circ\)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\(^\circ\)C. Expression of \textit{p53}, \textit{PARP}, \textit{p21} and \textit{Bcl-2} genes were analyzed by Real Time PCR (Rotor gene 3000 corbett, Australia). To carry out the reaction, reagent containing 1/5\(\mu\)l cDNA, 0.3\(\mu\)l of each primer (forward and reverse) and 4.5\(\mu\)l 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 NBCI to ensure their integrity and were finally synthesized by Macrogen Inc (Table 1).

**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 ACATAGTGTTGTTGTTGCCCT 20 R ACCTCAAGCTGTCCGTCC 20 |
| 2 PARP               | NM-001618             | F GGCCTACGCTCTGCTTAC 20 R TTCGCCACTTCATCACCTCC 20 |
| 3 GAPDH              | NM-001256799.2        | F CACATGGCGCTCCAAGGATAG 22 R AGGGAGATTCTAGTGTTG 20 |
| 4 p21                | NM-001291549.1        | F CATGTCAGCTGACTTCATTT 22 R CTGGTCTGCCCGCCCGTTTTC 19 |
| 5 Bcl2               | XM-01702591701        | F GGGAGGATTGGCCGCTCCTTTC 21 R GGTCAGGTACTCAGTCATCA 22 |

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

| Time-temperature condition | Time PCR |
|---------------------------|----------|
| 1 First denaturation       | 95ºC     |
| 2 Anneling p53 gene       | 62ºC     |
| 3 Anneling p21 gene       | 62ºC     |
| 4 Anneling PARP gene      | 61ºC     |
| 5 Anneling Bcl-2 gene     | 61ºC     |
| 6 Anneling GAPDH gene     | 61ºC     |
| 40cycle                   | Extention |
| 8 Extention               | 72ºC     |

**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 7.5μM was significantly reduced compared to the control group and other treatments (Figure 1).

**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.
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 ± SD. Sign (*) denote significant differences (P<0.05).](image)

Changes in gene expression by curcumin

Analysis of Real Time PCR Ct values by using of 2^{-\Delta\Delta\text{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).

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-κB 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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