

Oleuropein induces apoptosis via p53 pathway on breast cancer cells

Zeinab Korany Hassan^{1,2*}, Maha H Elamin¹, Sawsan Omer¹, Maha Daghestani¹, Ebtesam Al-Olayan¹, Mai Elobeid¹, Promy Virk¹

¹Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia, ²Cancer Biology Department, National Cancer Institute, Cairo University, Cairo, Egypt

*For correspondence: hildahafez@hotmail.com

Abstract:

Breast cancer is a major health problem worldwide. Olive oil induces apoptosis in some cancer cells due to its phenolic compounds e.g. oleuropein. Although oleuropein has an anticancer activity, the underlying action mechanism remains unknown. We aim to study the mechanism of oleuropein-induced breast cancer cell apoptosis. P53, Bcl-2 and Bax genes expression were evaluated by Semi-Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) in the luminal MCF-7 cells. Oleuropein-induced apoptosis was accompanied by an up-regulation of both p53 and Bax genes expression levels and down-regulation in Bcl2 gene expression level. In conclusion oleuropein induced apoptosis in tumor cells via a p53-dependent pathway mediated by Bax and Bcl2 genes. Therefore, oleuropein may have therapeutic potential in breast cancer patients by inducing apoptosis via activation of p53 pathway.

Keywords: Oleuropein, Breast cancer, apoptotic pathway, p53, Bcl2 and Bax genes

Introduction:

Breast cancer is a leading cause of deaths with increasing rate in developing countries (Saad 2011). Therapeutic Bioactive nutritional agents come into view to have importance in preventing and treating diseases. Olive is important in medicine due to its phenolic content (Visioli et al., 2002) e.g. oleuropein. In breast cancer cells, oleuropein decreases cell viability (Menendez et al., 2007), acts as anti-metastatic (Hassan *et al.* 2012), and induces cell apoptosis (Campolo *et al.* 2013; Elamin *et al.* 2013; Oi-Kano *et al.* 2013; Pasban-Aliabadi *et al.* 2013). Oleuropein inhibits cell proliferation via delaying the cell cycle at S phase and up-regulating cyclin-dependent inhibitor p21 (Elamin *et al.* 2013).

Apoptosis is a cell death to remove aberrant cells. The milestone in considering the effectiveness of chemopreventive agent is the ability to induce apoptosis as a mechanism for tumor

suppression (Choudhuri *et al.* 2002). The mitochondrial apoptotic pathway is initiated within the cell in which pro-apoptotic proteins are released from the mitochondria to activate caspase proteases triggering apoptosis (Lessene *et al.* 2008) .

The P53, tumor suppressor gene, is aberrant in most cancers (Hollstein *et al.* 1991) and mediates apoptosis in response to DNA damage (Das *et al.* 1999). In normal cells, p53 arrests cells, reduces genetic instability and allows the cells repair before progressing through the cell cycle (Taylor *et al.* 1999), but apoptosis induced by p53 is necessary for eliminating aberrant cells. The Bax, pro-apoptotic gene, is up-regulated in some systems during p53-mediated apoptosis. The up-regulation of Bax gene and down-regulation of Bcl-2 expression are established during apoptosis (Moon *et al.* 2007). P53 signaling pathway is implicated in the transcriptional activation of Bax in apoptosis (Li-Weber 2009). The pro- and anti-apoptotic genes are important in the fate of a cell. As the effect of oleuropein on apoptotic genes is not studied, this study aimed to investigate the mechanism of oleuropein-induced human breast cancer cell apoptosis.

Materials and methods:

MCF-7 cells were obtained from ATCC and were cultured following the instructions of the company. MCF-7 was maintained in RPMI-1640 (GIBCO, USA), L-glutamin 1%, 10% fetal bovine serum (FBS), 1% antibiotic/anti-myotic (penicillin/streptomycin) (Sigma Aldrich, USA). Cells were maintained at 37 °C in humidified incubator with 5% CO₂. Cells were treated with 100 µM or 200 µM concentration of Oleuropein. The incubation period and oleuropein concentrations were determined according to our previous study (Elamin *et al.* 2013). The untreated cells were used as control. All treated and untreated cells were incubated for 72 hrs and then collected for the gene expression studies

Gene Expression by Semi-Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR):

RNA extraction: Total RNA was extracted from the Oleuropein -treated and control cell line with TRIzol (Gibco BRL), in accordance with the manufacturer's instructions. Concentrations and purity of RNA were quantified spectro-photometrically by measuring A₂₆₀ and A₂₈₀; the ratio A₂₆₀/A₂₈₀ of pure RNA is approximately 1.8.

Semi-Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR): Total RNA isolated from treated and untreated cells was analyzed for anti-apoptotic Bcl-2 gene and pro-apoptotic Bax and p53 genes by semi-quantitative reverse transcriptase-PCR. cDNA was prepared from total RNA as described in manufacturer's protocol (Invitrogen, USA). Reverse transcription using oligo-dT primers in a 20 μ l total volume reaction mixture using a superscript system (Invitrogen, USA) and PCR were performed as previously (Huang *et al.* 2012), with an endogenous control gene (GAPDH).

The primer sequences for PCR were as follows: 5'-TG TGTGTGGAGAGCGTCAACC-3' and 5'-TTCAGAGACAGCCAGGAGAAATC-3' used for Bcl-2; 5'-TCAGGATGCGTCCACCAAGAA-3' and 5'-TCCCGGAGGAAGTCCAATGTC-3' for Bax; 5'-AGCG ATGGTCTGGCC CCTCC-3' and 5'-GCGCCGGTCTCTCCAGGA-3' for p53; and 5'-ATTCAACGGCACAGTCAAGG-3' and 5'-GCAGAAGGGGCGGAGATGA-3' for GAPDH internal control. Amplification was done at the temperature of 94 °C for 5 min, 25 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s, and 72 °C for 10 min (Huang *et al.* 2012). Experiments were performed in triplicate. Prior to amplification of each gene, normalization was carried out with endogenous control gene GAPDH. Aliquots of the PCR reaction were subjected to electrophoresis on 2% agarose gels and PCR fragments were visualized by ethidium bromide staining and photographed on gel documentation system. mRNA gene expression of the housekeeping gene was used as a quality control for the samples showing equal cDNA in all samples.

Statistical Analysis:

All experiments were performed in triplicate and analyzed by one way ANOVA (Graphpad prism 5) for significant differences. P values of <0.05 were considered statistically significant. The data are presented as the mean \pm SD.

Results:

P53 is targeted by a broad range of agents in apoptosis. The Bcl-2 families have a regulatory effect on the anti- and pro-apoptotic genes to determine the cells fate. To determine the mechanisms of oleuropein-induced apoptosis, we investigated the gene expression levels of the anti-apoptotic Bcl-2 and pro-apoptotic Bax and p53 in MCF-7 breast cancer cells.

The effect of Oleuropein on p53 gene expression level

Oleuropein significantly increases the gene expression level of p53 by 2.5 folds in the MCF-7 cells treated with 100 μ M oleuropein, while in the cells treated with 200 μ M oleuropein, the p53 gene expression level was increased significantly by 3.5 folds compared to the untreated cells. On the other hand, the up-regulation of p53 gene expression by 200 μ M oleuropein was not significant compared to the 100 μ M oleuropein concentration as shown in Figure 1. Our observation showed that oleuropein induces MCF-7 cell apoptosis in a p53-dependent pathway.

The effect of Oleuropein on Bcl-2 and Bax genes expression level

In the MCF-7 cells treated with 100 μ M oleuropein no significant differences were observed in the Bcl-2 and Bax genes expression levels compared to the untreated cells. However, at 200 μ M of oleuropein, the Bax gene expression was up-regulated significantly compared to both the untreated control cells and the others treated with 100 μ M oleuropein as shown in figure 2. Whereas Bcl-2 gene expression level was down-regulated significantly in the cells treated with 200 μ M oleuropein by 0.6 folds compared to both the untreated cells and the others treated with 100 μ M oleuropein as shown in figure 3. In the cells treated with 200 μ M oleuropein the Bax/Bcl-2 ratio was higher than that of both the cells treated with 100 μ M oleuropein and the untreated cells.

Discussion:

In spite of early diagnosis, radiation and chemotherapy, breast cancer is still a leading cause of cancer death (Jemal *et al.* 2011). Some studies have shown that certain cancers, are relatively low in the Mediterranean basin compared to other countries. Nutritional agents come into view to have importance in cancer treatment. Therefore, there is need to explore into new anti-cancer drugs. Some epidemiological studies showed correlation between olive products consumption and incidence of breast cancer. Due to its little side effects, oleuropein is one of the phenolic compounds in olive with a powerful effect on inhibiting the proliferation and migration of cancer cells (Sirianni *et al.* 2010; Santiago-Mora *et al.* 2011). Oleuropein can also target steps in cancer progression (Abe *et al.* 2011). Nowadays, oleuropein has been focused due to its ability to inhibit proliferation, metastasis and promote apoptosis in several tumor cell lines (Hassan *et al.* 2012; Zare *et al.* 2012; Elamin *et al.* 2013; Kostomoiri *et al.* 2013; Oi-Kano *et al.* 2013; Parzonko *et al.*

2013). In our previous report, oleuropein shows cytotoxic effect on the luminal MCF-7 cells via the induction of the mitochondrial apoptotic pathway (Elamin *et al.* 2013). However, the effect of oleuropein on the pro- and anti-apoptotic genes is not studied. Therefore this study aimed to determine the mechanisms of oleuropein-induced breast cancer cell apoptosis.

The proapoptotic activity of p53 is due to its transactivation capabilities through the transactivation of target genes correlated to apoptosis, or can directly activate the transcription of genes known to promote apoptosis (Fridman & Lowe 2003). P53 is a transcription factor that mediates cell cycle arrest and apoptosis (Struckhoff *et al.* 2010). In the current study, oleuropein treatment significantly increases p53 gene expression level in MCF-7 cells treated with 100 μ M oleuropein compared to untreated cells. However, in the cells treated with 200 μ M oleuropein the expression level was further increased significantly from the untreated cells. The current observation showed that oleuropein induces MCF-7 cell apoptosis in a p53-dependent pathway. The tumor suppressor p53 is mutated in 30% of breast cancers (Borresen-Dale 2003).

There are two major apoptosis signaling pathways, mitochondrial and death receptor pathway. The p53 gene is mediated apoptosis via the ability to control transcription of proapoptotic members of the Bcl-2 family. These include the 'multidomain' Bcl-2 family member Bax (Fridman & Lowe 2003). Bcl-2 is an apoptosis inhibitor and Bax is an apoptotic protein. The anti-apoptotic protein Bcl-2 mainly inhibits the mitochondrial pathways (Huang *et al.* 2012). In the current study, the MCF-7 cells treated with 100 μ M oleuropein showed no significant difference in the Bcl-2 and Bax genes expression compared to the untreated cells. With increasing the oleuropein concentration, the level of Bax gene expression was up-regulated significantly from both the untreated cells and the others treated with oleuropein 100 μ M. Whereas Bcl-2 gene expression level was down-regulated significantly in the MCF-7 cells treated with oleuropein 200 μ M. The change in the ratio of Bax/Bcl-2 is recognized to initiate caspase signaling (Kirkin *et al.* 2004). In the current study, the cells treated with 200 μ M oleuropein showed Bax/Bcl-2 ratio higher than that of both the cells treated with oleuropein 100 μ M and the untreated cells. Similarly in previous study, the up-regulated P53 directly promotes Bax expression that destroys the integrity of the mitochondria to induce apoptosis (Chipuk *et al.* 2004).

In conclusion oleuropein induced apoptosis in tumor cells via a p53-dependent pathway mediated by Bax and Bcl2 genes. Therefore, oleuropein may have possible therapeutic potential in breast cancer patients by inducing apoptosis in cells via activation of p53 pathway.

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