Study of a Nano-Oleuropein's Effect on the TCA Cycle`s Protein Expression in the Breast Cancer Cell Line Using Proteomics

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Abstract: Breast cancer is the most common cancer and a common cause of death, which occurs due to cancer among women in the world. Cancer cells need a lot of energy to their uncontrolled growth, so it seems that the expression of the enzyme in the Krebs cycle is changing. There are some reports about mutations and altered expression of *succinate dehydrogenase*, *fumarate Hydratase*, and *isocitrate dehydrogenase* in human cancers.

This research aimed to investigate the role of magnetite nanoparticle Oleuropein on the Krebs cycle proteins expression on the breast cancer cell line. Oleuropein is one of the polyphenolic components in olive trees and has some benefits in some diseases, including cancer. In addition to testing the viability test MTT (3- 4,5 Dimethylthiazol-2-yl -2,5-diphenyltetrazolium bromide) assay, in three levels of Oleuropein 0ppm, 300ppm, 600ppm proteomics analysis was also performed in cell line MCF7 in this study. The results of differential protein spots identification into two-dimensional electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MS-MALDI-TOF-TOF), showed that *fumarylacetoacetate hydrolase, succinate-coenzyme A ligase and isocitrate dehydrogenase1* are differential proteins upregulated after treated with 300ppm and 600ppm of oleuropein. It seems that Nano Oleuropein is a booster of Krebs cycle with upregulation of *Fumarylacetoacetase, succinate-CoA ligase, and isocitrate dehydrogenase1*. Uncoordinated Overexpression of some Krebs cycle protein can be one of the inhibition mechanisms on the breast cancer cell line under Oleuropein treatment.

Keywords: Breast cancer, Oleuropein, Krebs cycle, Proteomics.

1. INTRODUCTION

Breast cancer is the most prevalent cancer and second mortality agent between women. There are one million new cases of this disease, annual. Investigation of the statistics shows that breast cancer is increased in the world [1].

During the long years, human has known the different effect of organic extracts like olive extract. Absence of side effects and range spread spectrum are the advantages of these extracts. Oleuropein is ingrained in the daily lives of people [2].

The olive tree is essential in medicine because of its polyphenolic components [3]. Oleuropein decreases cell viability in breast cancer cells [4], acts as anti-metastatic agent [3], induces apoptosis [5-7] and inhibits cell proliferation via delaying the cell cycle in S phase and up-regulating cyclin-dependent inhibitor *p21* [6].

It is determined that cancer cells have metabolic deferent toward healthy cells. For example, most of the cancer cells show high levels of glycolysis disclaimer irrespective of available oxygen levels for oxidative phosphorylation. This is known as aerobic glycolysis or Warburg effect [8]. These observations demonstrated that cancer cells have faulty or disturbed mitochondrial metabolism. However, recent evidence determined that most of the cancer cells don't have mitochondrial function disorder, but they have a high level of ATP-dependent Oxygen consumption, toward normal cells [9]. Furthermore, cancer cells are resistance against standard therapeutic regimens, have increased mitochondrial capacity [8]. There are reports about the altered expression of the Krebs cycle enzymes in these cells, include *Fumarate hydratase, succinate dehydrogenase* [10], and *Isocitrate dehydrogenase* [11].

Damaged proteins are the main factor of cancers; therefore, they are the actual target for diagnosis and therapeutics in cancer. Also, proteins are the main aim of drugs, and they are base for designing drugs. So that proteomics consideration is beneficial for understanding the role of proteins in the creation and controlling cancer [12-14].

To determine the effects of Oleuropein on the Krebs cycle enzymes in breast cancer cell line, we designed this study.

2. METHOD

This study was into observational and was a cross-sectional analytic study. MCF-7 cell line was the

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population used that was prepared from Pastor Institute. The cell line was grown in RPMI 1640 medium containing 10% FBS and 1% penicillin-streptomycin. Cells were cultured in 5% CO_2, at 37 °C and 90% humidity incubator, after defreezing. Next, cells were treated with Nano Oleuropein in 5 levels and one control sample. Synthesis of Nano Oleuropein was done through the four-stage co-precipitation method [15].

MTT assay: The MTT substrate is prepared in a physiologically balanced solution, added to cells in culture, at a concentration of 0.2 mg/ml, and incubated for 2 to 4 hours. The quantity of formazan (presumably directly proportional to the number of viable cells) is measured by recording changes in absorbance at 570 nm using a plate reading spectrophotometer. Viable cells with active metabolism convert MTT into a purple colored formazan product with an absorbance maximum near 570 nm. When cells die, they lose the ability to convert MTT into formazan; thus color formation serves as a useful and convenient marker of only the viable cells.

2.1. Protein Extraction

Protein was extracted using standard techniques, according to Wang *et al.* [16]. In this way, we added 750 µl of buffer for each 1 million cells. So we set the cells in 4°C ultrasound for 25-60 minutes, after homogenization of cells in the buffer. Afterward, we exported microtube containing the cells and protein extraction buffer from the ultrasound. The centrifuge was done in 10000 rpm and 4°C. At the end of Centrifuge, we transferred containing fluid covering proteins to new microtube.

Two Dimensional electrophoreses: 2D electrophoresis was done after protein extraction. For 2-DE, the extracted proteins were re-suspended in the rehydration buffer (350 µl). Eighteen cm IPG strips with PH range between 3 to 10 were loaded in the rehydration buffer either with 1000µgprotein.

Afterward, strips were put on the SDS PAGE gel, flux was established in constant voltage. So, it was 100V in the first 30 minute and 70V in continuation. Then, gel stained with Coomassie blue. All of the electrophoresis stages were done in the laboratory of the Iran Genetic engineering and Biotechnology national center.

MALDI-TOF-TOF: The spots were identified using Proteomics. After cutting the spots, they were sent to the Wave University-England by Sina Colon Company. The molecular weight of pieces was measured with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-TOF). Type of proteins and score of them recognized with Mascot database.

3. RESULTS

MTT results: We can account the number of cells by recording the absorbance in 570 nm and through the standard curve. The results of MTT show 99.5%, 99.2%, and 99.7% cells relative inhibition after adjacency with Nano Oleuropein for 24h, 48h, and 72h, respectively.

2D-electrophoresis results: investigate of the 2D-electrophoresis was done at three levels of Oleuropein concentration, after investigating the quality of extraction proteins with horizontal electrophoresis (1D electrophoresis). The results of 2D-electrophoresis for 0 ppm, 300 ppm, and 600 ppm Oleuropeinare in Figures **1**, **2,** and **3**. Differential spots areas measured by Melani software in three replication. And three spots identified according to PI and MW and working by MALDI-TOF-TOF.

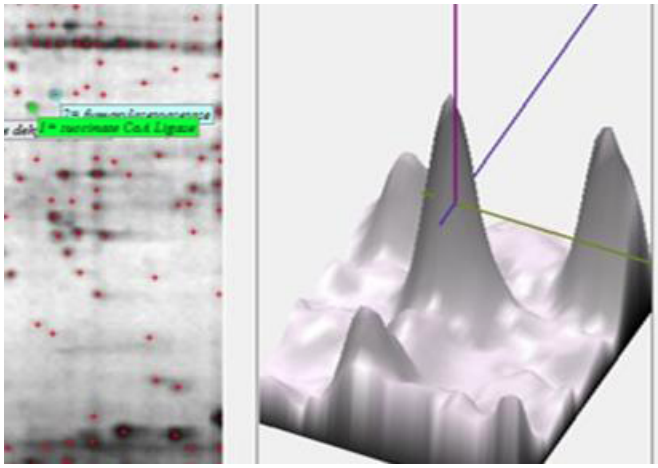


Figure 1: Protein expression of succinate-CoA ligase [ADP-forming] in 2D Gel electrophoresis under Oleuropein treatment.

3.1. Spot 1

According to obtained peak, pieces with molecular weigh 1032.4521, 2678.3637, 1063.5186, 1018.5335, 919.4797, 3020.5455, 146.1055, and 972.5855 had much abundance in MALDI-TOF-TOF system. In align mascot pieces with above MW, proteins with 348 score and probability P>0.5 identified (Figure **1**). This protein was *succinate-CoA ligase [ADP-forming] subunit beta*,

mitochondrial. It is a human protein that its PI and Mw are 7.5 and 50627, respectively. Its Sequence coverage was 75% succinate-CoA ligase had up-regulation in treated with Oleuropein. Therefore, it seems that Oleuropein causes the up-regulation of this protein.

3.2. Spot 2

According to obtained peak, pieces with molecular weigh 2838.3687, 1666.9869, 747.3399, 1937.9379, and 808.5058 had much abundance in MALDI-TOF-TOF system. In align mascot pieces with above MW, proteins with 193 score and probability P>0.5 identified (Figure **2**). This protein was *Fumarylacetoacetase*. It is a human protein that its PI and Mw are 6.46 and 46743, respectively. Its Sequence coverage was 61%. *Fumarylacetoacetase* had up-regulation in treated with Oleuropein. Therefore, it seems that Oleuropein causes the up-regulation of this protein.

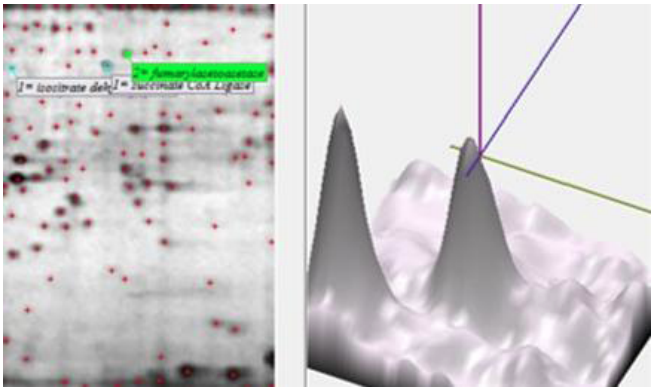


Figure 2: Protein expression of *Fumarylacetoacetase* with Oleuropein treatment in 2D Gel Electrophoresis.

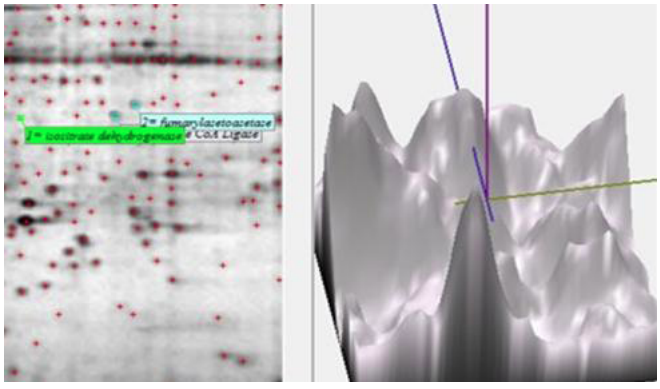


Figure 3: Isocitrate dehydrogenase expression in consumption of Oleuropein treatment in 2D Gel Electrophoresis.

3.3. Spot 3

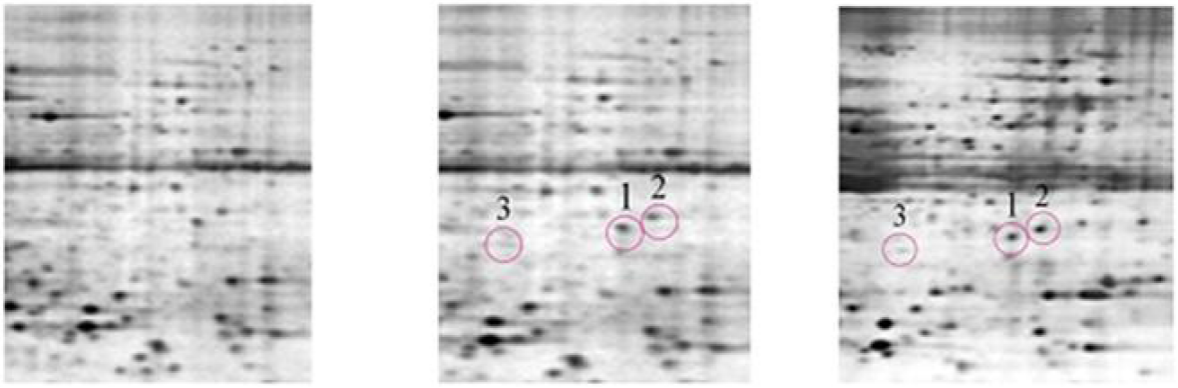
According to obtained peak, pieces with molecular weigh 1649.7604, 2405.2267, 975.5502, 1086.5710, 902.4399, 1877.9272, 672.3806, and 131.0946 had much abundance in MALDI-TOF-TOF system. In align mascot pieces with above MW, proteins with 405 scores and probability P>0.5 identified (Figure **3**). This protein was *isocitrate dehydrogenase* [NADP] cytoplasmic. It is a human protein that its PI and Mw are 6.46 and 46743, respectively. Its Sequence coverage was 87% *isocitrate dehydrogenase* had up-regulation in treated with Oleuropein. Therefore, it seems that Oleuropeincauses the up-regulation of this protein.

4. DISCUSSION

Succinate-CoA ligase (SUCLA): this protein catalyzes the ATP depending reaction of succinate and CoA to succinyl CoA. It involves in the first stage of subsidiary pathway synthesis succinate from succinyl CoA. This subsidiary pathway is a part of Three Carboxylic Acid cycle that is a part of carbohydrate metabolism. This enzyme is associated with kidney cancer and colon cancers in mice. The results of the experiment showed that *SUCLA* upregulated in consumption of 300 ppm and 600 ppm Oleuropein (Figure **4**).

Due to the *SUCLA* function (Succinate to fumarate conversion), this result was expected. According to the Warburg effect, cancer cells get their basic energy from glycolysis. Pyruvate converts to lactate, rather than be metabolized in the mitochondria. However, it is rare, but the development of tumor cells destroys the mitochondrial function. So it is expected that consumption of Oleuropein Increases the function of the Krebs cycle. One of the stimulating factors for Krebs cycle is the low rate of ATP. Because the *SUCLA* activity is dependent on ATP, and it advances the Krebs cycle, so this result was expected (Figure **4**).

At a study entitled "coordinated upregulation of oxidative pathways and downregulation of lipid biosynthesis underline obesity resistance in perilipin knockout Mice a microarray gene expression profile" by Fernando Castro-Chavez *et al.* [17] was conducted in 2003 in America, such an outcome was achieved. This study was done on 12 mice in two groups of 6- to 10-week-old male mice were used in this study with six perilipin knockout (*plin-/-*) mice in one group and six littermate wild-type (*plin-/-*) mice in the other. Five different tissues—WAT, liver, heart, skeletal muscle, and kidney—were isolated from each of the 12 mice and were tested. The analysis of gene expression showed that beta subunit of *succinate-CoA ligase, ADP*



(**A**) (**B**) (**C**)

Figure 4: The result of 2-D electrophoresis in 0 ppm of Oleuropein/ control case (**A**), 300 ppm of Oleuropein treatment (**B**), and 600 ppm of Oleuropein treatment (**C**).

forming in skeletal muscle cells from *plin-/-*mices had downregulation 1.56 times toward the natural kinds.

Also, Bertrand Perroud *et al.* [18] reported a similar result about alpha subunit of *succinate-CoA ligase, GDP forming*. At their study entitled "Pathway analysis of kidney cancer using proteomics and metabolic profiling" that done in 2006 in California it has been shown that expression of the alpha subunit of *succinate-CoA ligase, GDP forming* is different between Healthy and cancerous tissues.

Daneida Lizarraga *et al.* [19] in their article entitled "A Lyophilized Red Grape Pomace Containing Proanthocyanidin-Rich Dietary Fiber InducesGenetic and Metabolic Alterations in Colon Mucosa of Female C57BL/6J Mice" treated Female C57BL/6J mice with colon cancer by grape antioxidant dietary fiber (GADF) for two weeks. So that they investigated the gene expression with cDNA microarrays, after sampling from colon mucosa. Results showed that beta subunit of *succinate-CoA ligase, GDP forming* downregulated 1.60 times toward untreated samples.

According to Krebs cycle, downregulation of *succinate-CoA ligase* causes to succinate aggregation; a metabolite which communicates with consolidation and activating the alpha subunit of hypoxia inducing factor 1. This factor activates the translation of genes involved in cancer biology.

Fumarylacetoacetase or Fumarylacetoacetate hydrolase (FAH): *Fumarylacetoacetase* has a role in decomposition reaction of L-phenylalanine and involved in level 6 of synthesis of acetoacetate and fumarate from L- phenylalanine. This pathway is a part of the breakdown of L-alanine. Ca^{2+} and Mg^{2+} are cofactors for *FAH.*

The function of *Fumarylacetoacetase* impaired in hereditary tyrosinemia type 1. Also, the relationship between *FAH* and liver cancer in mice and breast cancer was shown.

The results of the experiment showed that *FAH* upregulated in consumption of 300 ppm and 600 ppm Oleuropein (Figure **4**).

According to the Warburg effect, cancer cells get their basic energy from glycolysis. Pyruvate converts to lactate, rather than be metabolized in the mitochondria. However, it is rare, but the development of tumor cells destroys the mitochondrial function. So it is expected that consumption of Oleuropein increases the role of the Krebs cycle. According to the purpose of *FAH* (converting 4-fumarylacetoacetate to fumarate and acetate), this result was expected. Because the enzyme gene expression increased with the use of Oleuropein and its fumarate enter the Krebs cycle. Overexpression of this protein can be one of the mechanisms that increased Krebs cycle, so this result was expected. Therefore Oleuropein causes the Induction of Krebs cycle instead of glycolysis that it can cause to change direction cancerous cells to healthy cells.

A result similar to the result of the present reported in the Yanting Qi *et al.* [20] article entitled "Two-dimensional differential gel electrophoresis/analysis of diethylnitrosamine-induced rat hepatocellular carcinoma". In this study that done in China in 2008, Hepatocellular carcinoma cell samples of Mice exposed to DEN carcinogen and healthy mice were tasted. After analysis of the electrophoresis and Western blot, *FAH* was introduced as one of the

proteins that downregulated in liver cancer cells. This down regulation shows that hydrolase activity in the liver has a low necessity.

Gabriela A. Balogh *et al.* [21] achieve a similar result in breast cancer cells. They demonstrated that *FAH* gene expression was overexpressed 1.29 times in healthy women than women with breast cancer.

Isocitrate dehydrogenase1(IDH): *IDH1* and*IDH2* mutation occurs in 2-4 gliomas gradations (World Health Organization) and Acute myeloid leukemia with normal karyotype. Mutations in Arg 132 in *isocitrate dehydrogenase1* recognise in genome analysis of 22 patients with grade 4 glioma.

The results of the experiment showed that *isocitrate dehydrogenase1* upregulated in consumption of 300 ppm and 600 ppm Oleuropein.

According to the Warburg effect, cancer cells get their basic energy from glycolysis. Pyruvate converts to lactate, rather than be metabolized in the mitochondria. However, it is rare, but the development of tumor cells destroys the mitochondrial function. So it is expected that consumption of Oleuropein increase the function of the Krebs cycle. According to the function of *IDH* (converting *isocitrate* to alpha-ketoglutarate), this result was expected. Because the *IDH* gene expression increased with the use of Oleuropein. Overexpression of this protein can be one of the mechanisms that increased the Krebs cycle, so this result was expected.

5. LIMITATION OF THE STUDY

In this research, we didn't have healthy breast cells to compare with cancer cell line.

6. CONCLUSION

It seems that Nano Oleuropein is a booster of Krebs cycle with upregulation of *Fumarylacetoacetase, succinate-CoA ligase, and isocitrate dehydrogenase1. Oleuropein* works by inhibiting glycolysis in cancer cells and changing it to the TCA cycle and can be a sign of developing the cancer cells to healthy cells.

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