University of Sistan and Baluchestan

Journal of Epigenetics

Online ISSN: 2676 - 4350

Homepage: https://jep.usb.ac.ir

Iranian Genetics Society

# Transcriptional changes of BAX and BCL-2 genes under the effect of vitamin E in colon cancer cell line (HT29)

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**ARTICLE INFO**

**Article type**:
Research Article

**Article history**:
Received: 3 February 2024
Revised: 15 March 2024
Accepted: 17 April 2024

**Keywords:**
vitamin E,
cancer cell line HT29,
apoptosis,
*BAX*, *BCL*-2

**A B S T R A C T**

Despite significant advances in cancer diagnosis and treatment, cancer is still a fatal disease due to a lack of prevention, early detection, and effective drugs. Therefore, there is a need to discover anticancer treatments that are specific to cancer cells and are affordable, safe, and tolerable for patients. Vitamin E is a potential candidate because of its safety. A growing body of evidence on the anti-cancer power of vitamin E has shifted the focus away from chemical drugs and antioxidants. The purpose of this study is to investigate the effect of vitamin E on the survival of colorectal cancer cell lines and the effect of this vitamin on two important genes involved in apoptosis. MTT test was performed on this cell line treated with different concentrations of vitamin E (500 to 33350 µg/ml). After determining the IC50 of this vitamin, RNA was extracted from cells treated with vitamin E and untreated cells, and then cDNA synthesis was performed. Using the specific primers of *BAX* and *BCL2* genes and considering the *GAPDH* gene as the reference gene, Real-time PCR was performed and the results were analyzed. MTT test showed that vitamin E in low concentrations strengthens and increases the survival of the HT29 cell line, but at a concentration of 10000 µg/ml, it decreases the survival percentage of this cancer cell line. Analysis of gene expression also showed that vitamin E leads to increased transcription of both *BAX* and *BCL2* genes. However, the increase in *BCL2* transcription is several times higher than that of *BAX*. In general, the cytotoxicity and lethality of vitamin E in colorectal cancer cell line was proved and it was found that this toxicity is not through *Bax* and *Bcl*-2 genes and probably the apoptotic effect of this vitamin is through other ways such as reducing survivin expression.



## Introduction

Colorectal cancer is the fourth most common cancer in the world and the third most common cancer in Asia, according to a GLOBOCAN website report in 2022. Colorectal cancer is the second most common type of cancer that causes death in the United States (Siegel et al., 2023). With the occurrence of metastasis, which is very common in this type of cancer, this disease spreads

**DOI**: https://doi.org/ 10.22111/JEP.2024.47887.1068

**Publisher**: University of Sistan and Baluchestan

**How to Cite**: Diyarkojouri, M., Habibollahi, H., & Ahmad Sharbafi, M. (2024). Transcriptional changes of BAX and BCL-2 genes under the effect of vitamin E in colon cancer cell line (HT29). *Journal of Epigenetics*, 5(1), 41-47. https://doi.org/ 10.22111/JEP.2024.47887.1068

to other body parts and multiplies in them (Einolghozati, 2017) This type of disease occurs when the normal process of cell growth is disturbed and old, and damaged cells survive when they should die. The main reasons for the formation of this abnormality in the body are mainly improper diet, aging, lifestyle, and in rare cases genetic disorders. In mountainous areas, it is due to the high consumption of red meat, and in Europe, many reported cases are due to the high consumption of alcohol. In most cases, the occurrence of colon cancer first begins with the appearance of a benign tumor and turns into cancer over time. The treatment that has been proposed and used for this cancer so far is a series of drug treatments, radiation therapy, and immunotherapy, although this treatment combination is effective until metastasis occurs and cancer has not spread to all tissues of the body (McCracken et al., 2007). Tumor suppressor p53 participates in the control of cell survival and proliferation under various stresses, and in addition to its effect on apoptosis, autophagy, and cell cycle, p53 is also a regulator of ferroptosis through post-translational mechanisms (Kang et al., 2019). A large percentage of colorectal tumors are caused by mutations in the *p53* gene, suggesting that cell signaling pathways must be modified as much as possible in the body to control colon cancer (Kung and Murphy, 2016). In this regard, any alteration in the apoptosis pathway or programmed cell death that acts as a protector against tumor formation should be avoided. In cancer, the lack of regulation of apoptotic signaling, especially the activity of the anti-apoptotic system, causes cancer cells to escape from the apoptotic program and leads to uncontrolled cell proliferation and tumor formation (Mohammad et al., 2015). Colorectal cancer is caused by mutations of target oncogenes, tumor suppressor genes, and genes related to DNA repair mechanisms (Alzahrani et al., 2021). One of the methods to investigate the apoptosis pathway in the cell is to study the expression of apoptosis inhibitory genes, i.e. *BCL-2*, the mitochondrial pathway, and the *BAX* apoptosis gene (Czabotar et al., 2023). Studies show that substances and drugs such as alkaloids, polysaccharides, polyphenols, terpenoids, and fatty acids that can increase *BAX* gene expression have an anti-cancer effect. BAX protein is considered a key protein in apoptosis and acts by various factors in the internal pathway of apoptosis (Huang et al., 2019). Also, the Bcl-2 protein has an anti-apoptotic effect by preventing the release of cytochrome C from mitochondria in response to various apoptotic stimuli (Strasser and Vaux, 2018). Therefore, *BAX* and *BCL-2* are among the important genes involved in the mitochondrial pathway of apoptosis (Maes et al., 2017). Antioxidants such as Myricetin have anticancer effects against a range of cancers including hepatocarcinoma, pancreatic cancer, and colon cancer. It has been proven that the effectiveness of this antioxidant is through the pathway related to Bax and BCL2 (Kim et al., 2014). Many phytochemicals (phytochemicals) in the foods we eat act as antioxidants (Shahidi and Zhong, 2015). These nutrients work by inhibiting the formation of free radicals and may reduce the damage they cause in the body. This is thought to be at least part of the reason why a diet rich in vegetables and fruits is associated with a reduced risk of many diseases (Gülcin, 2012). Therefore, by identifying and fully mastering the causes of the occurrence and non-occurrence of cancer, it is possible to help increase the patient's survival rate. The patient's diet is one of the things that can be modified in this regard. Vitamin E is a fat-soluble vitamin with antioxidant properties that can be added to the diet (Niki and Traber, 2012). Various studies have proven that vitamin E acts as a scavenger of ROS and also inhibits lipid peroxidation chain reactions. This vitamin plays a role in the physiological function of embryos and embryo implantation (Blaner et al., 2021). Recent discoveries show that the simultaneous use of anticancer drugs with vitamin E leads to a significant inhibition of cancer cell growth (Natesan et al., 2014). In general, the prevalence of cancer in different areas of the digestive system, especially the large intestine, as well as the presence of many physical complications even after treatment, its impact on the economic structure of the individual and society, shows the importance of studying non-invasive methods for cancer prevention and treatment. In modern science, efforts have always been made to conduct research in the field of disease prevention and to find clinical treatment methods before the occurrence of diseases. Considering that currently in developed countries vitamin E is used as a therapeutic supplement to reduce side effects after chemotherapy and radiation therapy, in this research the effect of vitamin E alone and as a

separate drug that has high economic efficiency and It causes the least damage to the body. We hypothesize that vitamin E can be effective as an antioxidant in the signaling pathway leading to apoptosis in the HT29 cancer cell line.

## Materials and methods

### *Cell Culture*

A colon cancer cell line (HT29) with reference number IBRC C10097 was purchased from a reliable cell bank (Royan Research Institute). Cell culture was carried out in a flask containing DMEM (Dulbecco's Modified Eagle Medium) culture medium. After multiplying the number of cells to the required density, Cells were passaged in the complex culture medium containing 10% FBS (Fetal Bovine serum), 1% penstrep (Penicillin/Streptomycin), 1% glutamax and 1% non-essential amino acids.

### *MTT Test*

The cytotoxicity of vitamin E for the colon cancer cell line was measured by the MTT test. An amount of the cells with an approximate density of 10,000 cells per well was prepared in a 96-well plate. The wells of the plate were treated with a concentration gradient of vitamin E (500µg/ml, 1000 µg/ml, 2500 µg/ml, 4000 µg/ml, 5000 µg/ml, 10000 µg/ml, 15000 µg/ml, 30000 µg/ml, 33350 µg/ml). After incubation at 37°C for 24 hours, 50 µl of 5 mg/ml MTT solution was added to each well. The 96-well plate was incubated for 4 hours at 37°C and 5% $CO_2$. After discarding the MTT solution, to dissolve the formazan crystals, 100 µl of DMSO (Dimethylsulfoxide) was added to each well and the plate was shaken for 15 minutes, and finally, the optical absorption of the samples was determined with an ELISA reader at a wavelength of 570 nm. After discarding the MTT solution, to dissolve the formazan crystals, 100 µl of DMSO was added to each well and the plate was shaken for 15 minutes, finally, the optical absorption of the samples was determined by an ELISA reader at a wavelength of 570 nm. The percentage of cell survival was calculated according to the following formula:

$$\frac{\text{The amount of optical absorption of the treated group}}{\text{amount of optical absorption of the control group}} \times 100$$

A concentration of vitamin E in which only 50% of cells survived was considered as $IC_{50}$.

### *RNA extraction and cDNA synthesis*

RNA extraction was performed from the colon cancer cell line (HT29) treated with $IC_{50}$ concentration of vitamin E as well as the untreated cell line using the Sina Colon Extraction Kit. The quality of the extracted RNA was evaluated by electrophoresis. The extracted RNAs were used for cDNA synthesis using the Sina clone kit and according to the instructions of this company.

### *Real-Time PCR*

To investigate the changes in the transcription level of *BAX* and *BCL-2* genes, Real-time PCR was performed using specific primers of these two genes and the GAPDH reference gene as well as synthesized cDNA. The sequence of primers used is shown in Table 1. (Kandhavelu et al, 2024).

**Table 1-** Sequence of primers used in real-time PCR

| Primers | sequences | Number of nucleotides | Size |
|---|---|---|---|
| *BAX*_F | 5'-TTCATCTCAGTCCCCTGCCC-3' | 20 | ۴۸bp ۷ |
| *BAX*_R | 3'-GGAGACAGGGACATCAGTCG-5' | 20 | |
| *BCL*-2_F | 5'-CCTGTGGATGACTGAGTACC-3' | 20 | ۱۲bp ۷ |
| *BCL*-2_R | 3'-GAGACAGCCAGGAGAAATCA-5' | 20 | |
| *GAPDH*_F | 5'-TGCGTCCTGCACCACCAACT-3' | 20 | ۳۴bp ۹ |
| *GAPDH*_R | 3'-CGCCTGCTTCACCACCTTC-5' | 19 | |

The temperature programs used include 94°C for 10 minutes, 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds in 40 cycles. The results of real-time PCR were analyzed using Genex software and the $2^{-\Delta\Delta CT}$ method.

### *Statistical analysis*

The statistical analysis of the data was done with the statistical software SPSS version 16.0. One-way ANOVA was used for statistical comparisons between different groups. All tests were repeated three times. Differences were considered statistically significant when the P-value was less than 0.05.

## Results

After 48 hours, the colon cancer cell line (HT29) reached 80% confluence in the DMEM medium (Fig.1)

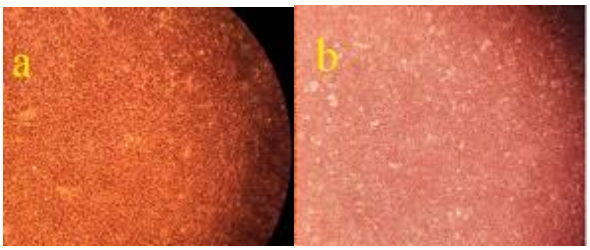


**Fig.1-** Cell density during primary cell subculture (Before treatment).

a: 40 X magnification; b: 10 X magnification

MTT assay, which was performed in a vitamin E concentration gradient and repeated 6 times, showed that vitamin E at low concentrations (from 500 to 2500 µg/ml) not only did not cause the death of HT29 cells but also increased the percentage of survival (compared to the control group). But from the concentration of 4000 µg/ml or more of vitamin E, the lethal effect increases gradually. So vitamin E in concentrations of 10000 µg/ml and above causes a 50-60% decrease in cell survival. (Fig.2)

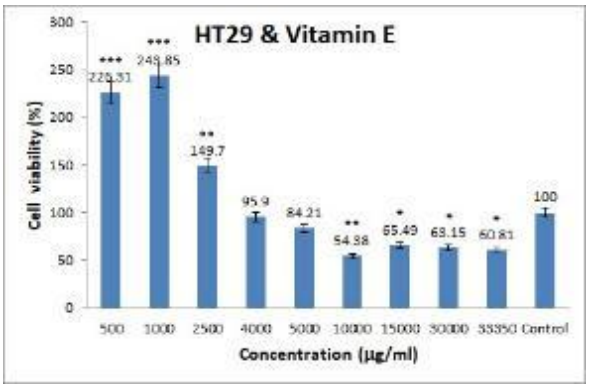


**Fig.2-**Results of MTT assay in HT29 cell line treated with different concentrations of vitamin E

The RNA extracted from the cells treated with $IC_{50}$ concentration of vitamin E (10000 µg/ml) as well as the untreated cells (control) had a good quality (Fig.3).

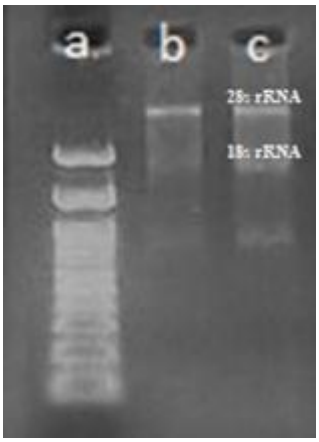


**Fig.3-** The quality of extracted RNA in 1% agarose gel (a: Ladder, b: Treated with vitamin E, c: Control)

The results of Real-Time PCR using cDNA obtained from the cell line treated with $IC_{50}$ concentration of vitamin E (10000 µg/ml) as well as the cell line without treatment (as control) showed that the transcription level of two important genes in apoptosis (*BAX* and *BCL*-2) increases in treated cells. Of course, the increase in the expression of the *BCL*-2 gene was very significant and was determined to be about 5.8 times the expression of the *BAX* gene (Fig.4).

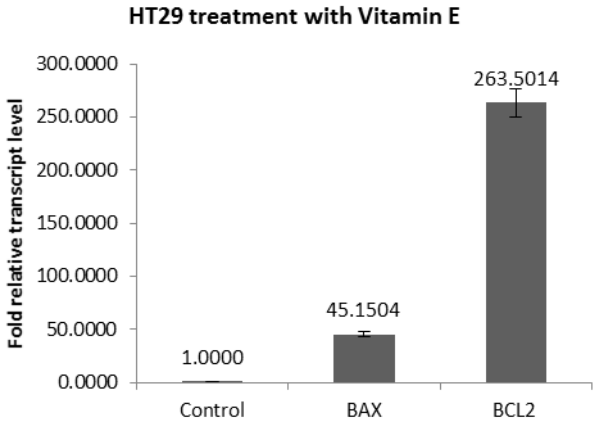


**Fig.4-**Changes in the transcription level of *BAX* and *BCL*-2 genes in the HT29 cell line treated with vitamin E (10000 µg/ml) compared to untreated cells.

## Discussion

The relationship between vitamin E and cancer risk has been studied by many researchers. According to Junyi Xin et al.'s findings 2022, vitamin E reduces the risk of breast cancer (Junyi Xin et al., 2022). In 2020, Bryson W. Katona and Jennifer M. Weiss pointed out the role of antioxidants such as vitamin E in preventing colorectal cancer (Katona and Weiss, 2020). Many factors such as diet and

vitamins change the epigenetic pattern and lead to changes in gene expression. However epigenetic irregularities can lead to an increased risk of cancer. Hatim Boughanem and his colleagues stated in 2023 that the simultaneous use of vitamin E with DNA methyltransferase inhibitors can help in the prevention and treatment of colorectal cancer (Boughanem et al., 2023). In 2012, Patacsil and her colleagues studied the antiproliferative effect of vitamin E succinate and its effect on the inhibition of apoptotic proteins in pancreatic cancer cells. The results of their research showed that vitamin E succinate inhibits cell proliferation and induces apoptosis in pancreatic cancer cells. This research proved that vitamin E succinate decreases survivin protein expression (Patacsil et al., 2012). Yasin Şenol and his colleagues evaluated the effect of albumin nanoparticles containing vitamin E on HepG2 cancer cells, in this study it was shown that HSA nanoparticles containing vitamin E have favorable properties in vitro for a drug delivery system intended to achieve are to the liver and have a positive effect on inducing apoptosis (Şenol et al., 2023). Victor Alves de Oliveira and his colleagues had a review article in the field of the effect of vitamin E on cancer, and again in 2023, during a systematic review article, they examined the effects of vitamin E in the treatment and reduction of side effects of cancer and found favorable results in this field (de Oliveira et al., 2023).

In the present study, two hypotheses were followed. The first hypothesis was related to the cytotoxic effect of vitamin E on the HT29 cell line. The results of the MTT test showed that with the change in vitamin E concentration, there is a significant difference between the survival of the control group and the treatment group. In other words, the average survival of the cells in the dose of 10000 µg/ml of vitamin E is much lower than the dose of 4000 µg/ml. This difference in lethality means the direct effect of vitamin E on the HT29 cell line and confirms the first hypothesis. Of course, it should be noted that low doses of vitamin E have helped the survival of this cell line, so the percentage of cell survival in doses of 500, 1000, and 2500 µg/ml is significantly higher than in the control group.

The second hypothesis of this research was related to the effect of vitamin E on the expression of two apoptotic genes. The results of real-time PCR in this study show that the expression of the BCL-2 gene has increased greatly under the influence of vitamin E. Although the expression of *BAX* also increased under the treatment of this vitamin, it was lower compared to the expression of *BCL-2*. Considering that BCL-2 protein is anti-apoptotic, increased expression of this protein compared to BAX protein can inhibit apoptosis in colorectal cancer cell lines. Research by researchers such as Patacsil in 2012 proved that vitamin E leads to apoptosis induction by reducing survivin protein expression. Survivin protein is one of the inhibitors of apoptosis that is produced in a large amount of cancer cells. Therefore, although it was found in the present study that vitamin E leads to an increase in the anti-apoptotic protein Bcl-2, this vitamin can still show its apoptosis-inducing effect by inhibiting the production of survivin protein.

## Conclusion

In explaining the findings of this research, after conducting experimental tests in a laboratory environment, it can be concluded that vitamin E can have a cytotoxic effect in high doses on the HT29 cell line and reduce the survival rate of this type of colorectal cancer cell. However low doses of this vitamin strengthen this cell line. Examining the changes in the transcription level of two genes involved in apoptosis (*BAX*, *BCL-2*) showed that, contrary to expectations, vitamin E increases the transcription of the antiapoptotic gene *BCL-2*. No reports were found about the effect of vitamin E on the expression of *BAX* and *BCL-2* genes, and no comparison can be made in this case. However according to the reports about the effect of vitamin E on reducing the expression of survivin protein, which is an anti-apoptotic factor in cancer cells, vitamin E in high doses may cause cancer cell death by affecting other genes involved in apoptosis or by affecting other signaling pathways, which requires more research in this field.

## Acknowledgments

The authors of this article would like to thank the Islamic Azad University, Rasht Branch, as well as the Vira Armanian Biotechnology Research Laboratory (Vab Lab) for their help and support.

## Conflict of interest

The authors declare that they have no conflict of interest.

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