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Research Article

EVALUATION OF *BAX: BCL-2* EXPRESSION BY REAL-TIME PCR AND POSSIBLE SEQUENCE ALTERATIONS OF *BCL-2* AND *BAX* PROMOTERS IN IRANIAN BREAST CANCER PATIENTS

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ABSTRACT
The anti-apoptotic *BCL-2* and pro-apoptotic *BAX* genes are involved in the apoptosis mitochondrial pathway that can play a great role in carcinogenesis. In this research we evaluated the expression ratio of *BAX: BCL-2* genes in the tumor cells compared to border cells in Iranian breast cancer patients and also studied the probable polymorphisms related to the expression changes of these genes.
We evaluated *BAX:BCL*-2 expression by Real-Time PCR technique in tumor cells of 40 breast cancer patients. We analyzed the polymorphisms by traditional PCR technique. Data statistical analyze performed by descriptive statistics and the χ^2 and Fisher's Exact Test through the SPSS version18 (PASW) statistical software. Expression of *BAX: BCL-2* was associated with significant decrease in tumor cells (P=0.001) and also *BCL-2* expression displayed a significant increase in tumor cells (P=0.001). But there is no such significant relationship regarding the *BAX* expression (P=0.150). The results of the follow-up tests demonstrated that *BCL-2* polymorphism (-938 C>A) with frequency of 87.5% was observed in the patient with breast cancer and it has significant relationship with increasing expression value of *BCL-2* in tumor cells (P=0.041). We found that reduced *BAX: BCL-2* expression and increased *BCL-2* expression are significantly associated with tumor genesis in breast cancer. And also the *BCL-2* (-938C>A) polymorphism is considered as one of the factors that increase the expression of *BCL-2* gene. Although it seems that the *BCL-2* and *BAX: BCL-2* are effective prognosis marker of the breast cancer.

Keywords: *Breast Cancer, Apoptosis, BAX Gene, BCL-2 Gene, Border Cells, Real-Time PCR Technique*

INTRODUCTION
Breast cancer as the most common cancer in women is a complex genetic disease. Several factors and mechanisms involved in the onset and progression of the disease, one of these mechanisms is the internal pathway (mitochondrial pathway) of apoptosis and one of the most important regulators of this pathway are BCL-2 proteins (Vander and Vander, 1999) This family of proteins consists of two groups of anti-apoptotic BCL-2 proteins (inhibition of apoptosis) and pro-apoptotic BAX proteins (stimulate of apoptosis) (Adams and Cory, 2001). It has been shown that BAX and other members of this group, form heterodimer with BCL-2 and it seems that the ratio of BAX: BCL-2 is an important criterion to induce or inhibit apoptosis. In normal cells there is a balance between the expression of apoptotic proteins and anti-apoptotic family of BCL-2 (Reed, 1996). The collapse of this balance both the reduction or the increase of each one of the BCL-2 and BAX proteins leads to disorder in the mitochondrial pathway of apoptosis which is one of the features of malignant tumors (Wong, 2011; Zhang *et al.*, 2005). Researches have shown that BCL-2 is expressed in 80% of the cancer cells (Parton *et al.*, 2001). Since BCL-2 has an important role in inhibiting apoptosis thus it is expected that there is a close relationship between the over expression of BCL-2 and tumorgenesis of different tissues (Villar *et al.*, 2001). The BCL-*2* gene is located at Chromosome18q21 and includes three exons and 2 promoters with different functions (Zhang *et al.*, 2011). The second promoter (P2) activity down regulates the first promoter (P1) (Linjawi *et al.*, 2004). *BAX* (*BCL-2* Antagonist) gene is located at 19q13.3-q13.4 chromosome (Nuckel *et al.*, 2007) and the protein product of this gene's expression is a BCL-2 homologue structurally (Tsujimoto *et al.*, 1985). In many tumors, found mutation among the *BCL-2* family, one of the most common mutations is (-

938C>A) in promoter 2 (P2) of *BCL-2* gene (Park *et al.*, 2004). The aim of this study was to evaluated significant differences associated *BAX: BCL-2* expression in tumor cells of the Iranian patients with breast cancer and also analyzed distribution of *BCL-2*(-938C>A) polymorphism frequency in tumor cells of patients and evaluated significant relationship with the expression changes of *BAX:bcl-2* and *BCL-2* expression to identify a marker for the prediction of the breast cancer risk as well as finding appropriate strategies to cure and prevent breast cancer.

MATERIALS AND METHODS

This research is a causal-comparative descriptive research and it is classified as cross-sectional study based on time. This study involved 40 Iranian patients with breast cancer who were not exposed to radiation and chemotherapy. The tumor tissue samples and border cells of patients were selected randomly among the samples of the tumor specimens in Imam Khomeini Cancer Research Center and Tumor Bank during 2011-2013. All of the samples examined and approved by pathology specialty. Expression levels of *BAX:BCL-2* in tumor cells compared to border cells were analyzed by the quantitative Real-Time PCR technique and the expression levels of these genes was determined relative to *ACTB* (housekeeping gene) as the internal control based on the following process.

First the RNA of the samples was extracted using High Pure miRNA Isolation Kit (Version 05) made by Roche Company, Germany (www.roche-applied-science.com) and Using Nanodrop and gel electrophoresis for evaluation quantitatively and qualitatively. Then the genomic DNA was eliminated using DNase Fermentas Kit and then synthesized cDNA from mRNA with Fermentas Kit (Thermo Scientific, RevertAid First Strand cDNA Synthesis Kit,K#1621) and the samples were prepared as cDNA to perform Real-Time PCR.

The Primers for the Real-Time PCR were designed according to sequence of *BAX, BCL-2* and *ACTB* with Gene Runner software that listed in table 1.

Real-Time PCR reaction was performed for the respective genes of patient's tumor cells and border cells with using TaKaRa SYBR® Premix Ex TaqTM II(Tli RNaseH Plus) cat.# RR820Q kit in Real-Time rotary analyzer Corbet ™ (Rotor-Gene 6000). The cycling conditions were: 95^{oC} for 10 min and 40 cycles at 95^{oC} for 10 sec, and 60 ^{oC} for 20 sec, and 72 ^{oC} for 15 sec. In this study SYBR® Green dye (a dsDNA binding dye) was used to detect proliferation of PCR products as it accumulates during PCR in Real-Time PCR. For determination of *BCL-2* genotypes at the first, genomic DNA was extracted following the manufacturer's instruction of QIAamp DNA Mini Kit from both of the tumor and border cells of each patient and after that evaluated extracted DNAs with Nanodrop and Gel Electrophoresis. *BCL-2* primer was designed by Gene Runner software. The following primers were used: forward primer, 5′-GCATTTGCTGTTCGGAGT-3′ and the reverse primer, 5′-CTCTGCGACAGCTTATAATG-3′. The PCR was performed using BioRad® Thermocycler set by 35 cycles with 50 seconds denaturation at 95^{oC},50 seconds annealing at 61^{oC} , and 1 minute extention at 72^{oC}. The PCR products were sequenced after the quality evaluation of them. Distribution of *BCL-2 (-938C>A)* genotype was studied with comparison between tumor cells and border cells of patients. All statistical analysis in this study were carried out SPSS Statistics software version18 (PASW). χ^2 test or Fisher's exact test were employed to indicate the significant differences in expression and genotype between tumor cells and border cells in patients. Differences were considered significant at P-values be less than 0.05.

RESULTS AND DISCUSSION

Results

The Real-Time PCR data demonstrated decreased expression of *BAX: BCL-2* ratio in tumor cells compare with the border cells in the most of patients (62.5%) (Figure 1). According to Chi-square test results were indicated significant difference between the expression level of *BAX: BCL-2* in tumor cells compare with the border cells of patients (P=0.001). So followed this result we expected increased expression of *BCL-2* and reduced expression of *BAX* in tumor cells. Thus it was assumed that there is a significant relationship between the changes in expression level of *BAX* and *BCL-2* in tumor and Border cells and these genes

have regulative effect on each other and the increase in expression level of *BCL-2* in tumor cells is followed by the reduction of *BAX* in these cells. The results of Fisher's exact test demonstrated significant relationship between the expressions of *BAX* and *BCL-2* (P=0.001). Reviewing the data it was observed that there is a direct relationship between *BAX* and *BCL-2* expression levels and the value of this relationship based on the Cramer's V is 0.50. According to this direct relationship between *BAX* and *BCL-2*, and the reduced expression of *BAX: BCL-2* ratio in tumor cells compared to the border cells, this question is raised that how the direct relationship of these two genes may change the expression ratio of *BAX: BCL-2*? Therefore we decided to analyze the both of *BAX* and *BCL-2* genes expression levels in tumor and border cells separately.

Data analysis of 40 patients demonstrated reduced *BAX* expression in 8 patients (20%), and increased *BAX* expression in 18 (45%) patients and equal expression of *BAX* in tumor cells and order cells in 14 patients (35%) (fig.1). Chi-square test results indicate that there is no significant relationship between the expression level of *BAX* in the tumor and border cells (P=0.150). While the expression level of *BCL-2* in tumor cells of the most patients (67.5%) is increased compared to the Border cells (fig.1) and Chi-square test indicated that there is a significant relationship between the expression level of *BCL-2* in tumor and the border cells (P=0.001).

Followed the results of Real-Time PCR that demonstrated the expression level of *BCL-2* and *BAX: BCL-2* in tumor cells therefore we decided to analyze the polymorphism *BCL-2*(-938C>A).

After the performing traditional PCR we sequenced the promoter 2 of *BCL-2* and investigated distribution of *BCL-2* (-938C>A) polymorphism in 40 patients that was not evenly distribution among patients. We found 87.5% of patients displayed *BCL-2* (-938C>A) polymorphism which was no observed in the Border cells of the same patient, 45% homozygous (AA) and 42.5% heterozygous (AC), while only 12.5% of patients didn't display this polymorphism and so were normal (Figure 2). According to the Chi-square test this polymorphism is significantly correlated with tumor genesis (P=0.020).

Based on the Fisher's exact test, *BCL-2*(-938C>A) polymorphism has significantly related with the expression level of *BCL-2* (P=0.045). Reviewing the data showed that the mentioned polymorphism existed in 88.88% of the patients who their tumor cells indicated the increased expression level of *BCL-2* that 66.66% of them were Homozygous and 33.33% were Heterozygous in this mutation. Therefore, it seems that *BCL-2*(-938C>A) plays an important role in increasing expression level of *BCL-2* and tumor genesis.

The result of Fisher's exact test showed that there is no significant relationship between polymorphism *BCL-2*(-938C>A) with the changes of expression level of *BAX* (P=0.984) which seems reasonable. The analysis of the relationship between the changes of expression level of *BAX: BCL-2* and *BCL-2*(-938C>A) polymorphism with review of the data indicated that in 47% of Heterozygous and 83% of Homozygous patients the expression level of *BAX: BCL-2* is reduced (Figure 3) and in 47% of Heterozygous and 89% of Homozygous patients the expression level of *BCL-2* is increased (Figure 4).

Based on the statistical results of the Fisher's exact test the relationship of the expression level of *BAX: BCL-2* with *BCL-2*(-938C>A) polymorphism is significant (P=0.043). The data analysis shows that 92% of the patients who showed the reduction in the expression of *BAX: BCL-2* in tumor cells have the polymorphism *BCL-2*(-938C>A) and 65% of them are Homozygous and 35% are Heterozygous.

Thus it seems that the polymorphism *BCL-2*(-938C>A) play an important role in the reduction of the expression level of *BAX: BCL-2*. The correlation rate of this polymorphism with the expression level of *BAX: BCL-2* based on the Cramer's V is 0.36 and with the expression level of *BCL-2* is 0.35 which indicate that polymorphism *BCL-2* (-938C>A) is not the only factor that plays a role in the expression level changes of *BAX: BCL-2* and *BCL-2*, but it can be considered as one of the effective factors in changes of expression level.

Table 1: The primers used for amplification of the mentioned genes with Real-Time PCR

Gene	Forward Primer	Reverse Primer
BCL-2	5′-GGTGGGGTCATGTGTGTGG-3′	5′-CGGTTCAGGTACTCAGTCATCC-3
BAX	5′-CCCGAGAGGTCTTTTTCCGAG-3′	5′-CCAGCCCATGATGGTTCTGAT-3′
ACTB	5′- AACGGTGAAGGTGACAGCAGTCG -3′	5′-GGCAAGGGACTTCCTGTAACAACG-3′

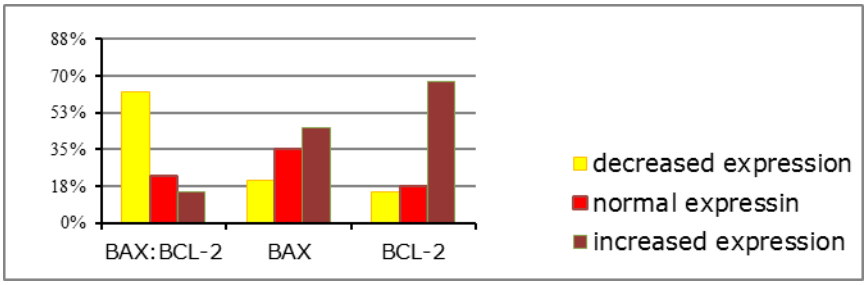

Figure 1: Frequency Column Chart of patients with breast cancer according to the expression of BAX: Bcl-2, BAX and BCL-2 in tumor tissue compared to the Border

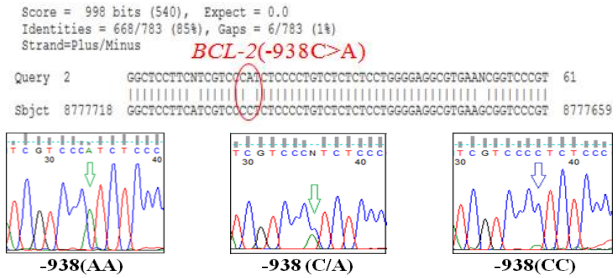

Figure 2: The position of mutation -938 in promoter 2 sequence of the BCL-2gene

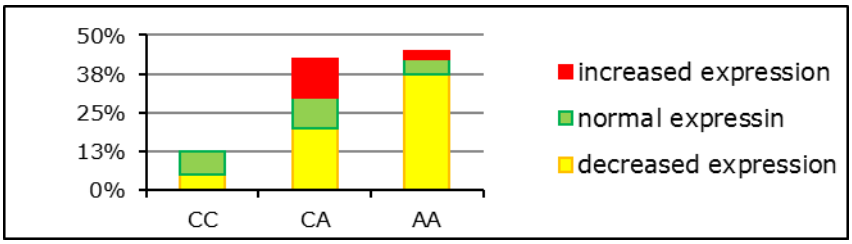

Figure 3: The relationship between the *BAX: BCL-2* expression and polymorphism *BCL-2*(-938C>A)

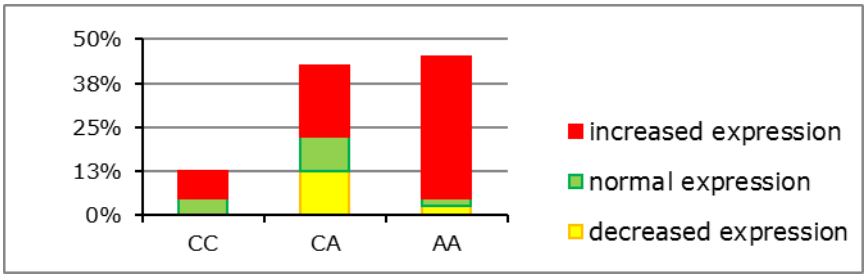

Figure 4: The relationship between the *BCL-2* expression and polymorphism *BCL-2*(-938C>A)

Discussion

Data analysis caused to confirm that the significant decreased *BAX: BCL-2* expression and significant increased *BCL-2* expression in tumor cells of patients. Also through the synchronous analysis of the inferential and descriptive statistics demonstrated that *BCL-2(-938C>A)* polymorphism associated with the increased *BCL-2* expression. *BCL-2(-938A)* mutant allele observed in 89% of the patients with increased of *BCL-2* expression, and also 92% of them with decreased expression of *BAX: BCL-2* ratio in their tumor cells. This caused to increased importance of mutant allele "A" in locus -938 of *BCL-2* gene. No studies have been conducted in Iran on the evaluation of the expression level of *BAX: BCL-2* in breast cancer with Real-Time PCR technique. Although, studies conducted by different techniques such as immunohistochemistry in the other countries that presented different idea about the expression of *BAX* and *BCL-2* genes but the majority of them were suggested the increased expression *BCL-2* in tumor cells. Studies on prostate cells investigated increased *BAX: BCL-2* expression was associated with stimulate of apoptosis in Epithelial cells of prostate (Perlman *et al.*, 1999). Melanoma cells with reduced *BAX: BCL-2* expression were more apoptotic resistant and the over expression of *BCL-2* disturbed the apoptosis stimuli and defined the critical role of *BAX: BCL-2* as an apoptosis sensitivity regulator in the Melanoma cells (Raisova *et al.*, 2001). Evaluation the *BAX: BCL-2* expression in 46 patients with Myasthenia gravis introduced the increased *BAX: BCL-2* expression as apoptotic-stimulating factor in patients' Thymus (Salakou *et al.*, 2007). This study which is conducted on Iranian breast cancer patients confirmed the reduced *BAX: BCL-2* expression in the breast tumor cells. Therefore, based on the convergence of the results of this research with the previous studies it seems that the expression ratio of *BAX: BCL-2* can be an effective factor in the prognosis of breast cancer. In fact the reduction in the expression ratio of *BAX: BCL-2* can be accompanied with the increase in *BCL-2* or the reduction of *BAX* which has led to the reduction of this ratio. Evaluated the *BCL-2*: *BAX* expression through cytometry technique in invasive breast carcinoma indicated that the ratio of *BCL-2: BAX* >1 is not significantly correlated with Overall Survival (OS) or the Disease-Free Survival (DFS). But the increase in overall survival and the disease-free survival (OS and DFS) is significantly correlated with the positive expression of *BCL-2* with the univariate analysis (Schiller *et al.*, 2002). In the bladder cancer with low malignancy it was concluded that the patients that have higher *BCL-2* expression level than *BAX* expression level in their tumor cells the early recurrence of the disease is much higher, also in this study the expression ratio of *BAX:BCL-2* is introduced as the diagnosis factor (Gazzaniga *et al.*, 1996). In the studies conducted on the Rectum cancer it was observed that the increase in expression level of *BCL-2: BAX* in patients leads to the higher resistance of the tumor cells against radiotherapy. Also this ratio is introduced as a marker to prognosis the tumor existence (Scopa *et al.*, 2001). In patients suffering from CLL was observed the increased of *BCL-2:BAX* expression (Molika *et al.*, 1998), on the other hand, the expression level of *BCL-2:BAX* in invasive breast carcinoma did not show significant correlation with survival while this ratio is increases in the tumor cells of the patient under this study (Schiller *et al.*, 2002). the studies of the CLL and invasive breast carcinoma was performed by Cytometry (Molika *et al.*, 1998) but in this study Real-Time PCR technique was used, so it seems that one of the differences in results is the method of the study. On the other hand it is possible that the rate of the changes in *BAX: BCL-2* expression be tissue-specific because the expression is different in various tissues. Some articles consider *BCL-2* as the *BAX* regulator and concluded that the reduced levels of *BAX* are associated with the increased levels of *BCL-2* and also the expression of *BAX: BCL-2* ratio which is critical in natural evolution of the breast cells (Reed, 1996). In one of the studies mentioned to importance of the *BCL-2: BAX* expression effect on the apoptosis sensitivity compare to the absolute evaluation of these genes separately. They pointed to the decreased *BAX* expression and removing *BCL-2* in tumor cells of the breast tissue (Reed, 1996). The studies that have been previously conducted around the relationship between the expression level of *BAX* and the breast cancer showed that there is a significant relationship between the *BAX* expression and the breast cancer. Increased *BAX* expression indicates the malignancy and the worst prognosis for the breast cancer (Binder *et al.*, 1996). Against the previously studies, in our study no significant changes in *BAX* expression observed in tumor cells of the patients. The reason of this non-compliance may be due to the fact that many factor including hormone changes, the stage, degree and type of tumor, the mutations of

the genes, and many environmental and within the intra cell factors affect the expression level of this gene also ethnical differences can also be one of the factors that caused to this study is non-convergent with the previous studies.

The present study shows the increased expression of *BCL-2* in tumor cells compared to the Border cells. In a study that is conducted by a research team on the 50 samples of invasive breast cancer patients the increased expression level of *BCL-2* was shown in tumor cells and it is introduced as the reason of the tumor cells' survival and an effective maker in prognosis of the breast cancer (Abdel *et al.*, 2006). Another study conducted on invasive breast carcinoma demonstrated direct associated *BCL-2* expression with clinic pathological prognostic factors.

They using the Immunohistochemistry technique and concluded that the expression of *BCL-2* was recognized in 55% of the invasive breast carcinomas (Dema *et al.*, 2008). In a study conducted by Villar *et al.*, (2001) it was concluded that the expression level of *BCL-2* gene and the rate of apoptosis are the factors that can provide useful information regarding the prognosis of the breast cancer (Villar *et al.*, 2001). However, some experiments indicated that over expression of *BCL-2* in breast cancer indicates reduced malignancy and relative better prognosis (Yang *et al.*, 2003). But based on the present study since the overexpressed BCL-*2* in most cancer cells are seen, it seems that *BCL-2* is considered as an apoptosis inhibitor and preventing the cell death, so stimulate irregular cell proliferation and tumor induction and finally the cancerous breast tissue. The increase in the expression of *BCL-2* for the cell survival and tumor genesis induction is more important than the changes in the expression level of *BAX*. Thus it can be concluded that the increase in the expression level of *BCL-2* can be reduced the expression ratio of *BAX: BCL-2* in tumor cells. These changes can be a prognosis marker of the breast cancer.

Considering the importance of *BCL-2* expression changes in tumor genesis we analyzed the *BCL-2* (-938C>A) polymorphism. We found that *BCL-2* (-938C>A) was associated significantly with the decreased expression of *BAX: BCL-2* and increased expression level of *BCL-2* in tumor cells of the patients. Most studies point to the SNP (-938C>A) in the promoter 2 of *BCL-2* gene (Bachmann *et al.*, 2007). In one of these studies which were conducted on 274 patients with invasive independent of lymph breast cancer which used the Immunohistochemistry method it was observed that both high expression of *BCL-2* and -938A allele resulted from the mentioned polymorphism led to increased survival of these patients. They are considered that increased expression of BCL-2 is a favorable prognosis marker of the breast cancer (Bachmann *et al.*, 2007).

Another study on breast cancer against the previous study, concluded the (-938A) allele caused to the decreased *BCL-2* expression and the existence of the genotype (-938AA) increased the risk of the breast cancer associated with lymph. In this study as well as the previous study the increased expression level of *BCL-2* is introduced as a marker in favorable prognosis of the breast cancer (Zhang *et al.*, 2011). Our study confirmed the result of the study regarding that -938A allele lead to the increase in the expression of *BCL-2* but it seems that the genotype -938AA increases the risk of the breast cancer through the increase of the expression of *BCL-2*. This result is against of another study conducted on the breast cancer is associated with lymph and considered (-938A) allele as the factor of reducing the expression of *BCL-2* but it confirms the study regarding the fact that the genotype (-938AA) increases the risk of the breast cancer. Another study is conducted on the European-Americans with prostate cancer in which they observed a 70% decreased risk for prostate cancer among the people who had the homozygous *BCL-2*(-938AA) genotype. This team introduced -938A allele as the factor that decreased risk of developing prostate cancer (Kidd *et al.*, 2006).Unlike the study on the prostate cancer that considered the (-938A) allele as the factor of decreasing the risk of the cancer, our study has introduced the *BCL-2* (-938C>A) polymorphism as an effective factor in the onset of the breast cancer and it is indicated that the -938A allele is the factor that increases the expression of *BCL-2*. This contrast may be due to the fact that the mutation effect varies in different tissues and the mutation in the prostate tissue cells that decreases the risk of prostate cancer may lead to the increased risk of the breast cancer, so it can be concluded that maybe this mutation effects are altered under the effect of hormones.

Since the promoter 2 is down regulates promoter 1 of the *BCL-2* gene the occurrence of the mutation *BCL-2*(-938C>A) located in promoter 2 may lead to the reduction of its inhibition effect and increase in the activity of promoter 1 and finally the increase in the expression level of *BCL-2*.

Conclusion

This study suggested that *BAX: BCL-2* expression is decreased and *BCL-2* expression is increased in tumor cells of the breast tissue compared to the Border cells. So we can be introduced these two factors with other factors that lead to the tumor genesis as a prognosis marker of the breast cancer. These factors caused to cancer trough the stimulation of the cells to escape from the apoptosis pathway and cell proliferation. Also the effect of the polymorphism *BCL-2*(-938C>A) in the expression changes of these genes led to the fact that the mutation of *BCL-2* (-938C>A) is effective in carcinogenesis and the -938A allele is considered as one of the factors that increase the expression of this gene and the genotype -938AA leads to the decreased expression ratio of *BAX: BCL-2* and increased risk of breast cancer. Also if *BCL-2*(-938C>A) polymorphism was observed in the case, it is possible to estimate the cancer risk. Although it seems that the *BCL-2* and *BAX: BCL-2* factors are effective in prognosis of the breast cancer but recommended in order to obtain more accurate results, other markers of prognosis breast cancer to be studied simultaneously.

Hope the present study would open new horizons to the researchers to prevention and treatment of the breast cancer and the aggressive treatments would be replaced by molecular treatments.

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