Transcriptomic Study of B-cell associated X (BAX) and B-cell lymphoma (BCL2) Protein in Breast Cancer

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Abstract

Background: Cancer is one of the major human problems and breast cancer is a significant health problem. In spite of the developed treatment methods, breast cancer cannot be treated very effectively. Therefore, the identification of novel genes that will have played a role in the treatment and diagnosis of breast cancer and the high interest of B-cell lymphoma (BCL2) family proteins are critical regulators of apoptosis).

Objective: The current study aimed to investigate the relationship between the levels of BCL2, BAX, and B-cell integrate medium (BIM) proteins which the prognosis and apoptosis processes of the breast cell line.

Patients and Methods: Collected tissue samples from normal and cancer tissue type, RNA isolated from the cell, then quantitation done, cDNA synthesized, cDNA quantitation and Real-time PCR was performed to see the level between two kinds of genes.

Results: BCL2 protein level was significantly lessened in regulator molecule tissues of a cell line with breast cancer. In contrast to Bax protein, no significant difference was detected in the BIM expression level. Our result also showed that low BCL2 expression level is associated with significant clinical characteristics of the patient such as tumor grade, stage and breast cancer type.

Conclusion: Findings of our study showed that BCL2 has a role in breast cancer formation and might be a novel biomarker for the diagnosis and treatment of breast cancer.

Keywords: Apoptosis, BCL2, Breast Cancer, gene expression, BAX, BIM.

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Introduction

Breast cancer is a major health problem in all countries and predominantly affects women population.1It is the most common type of cancer in women and responsible for the most cancer-related death among women population[2].In the United States, it is estimated that 255,180 new cases of breast cancer and 41,070 breast cancer-related deaths will occur3. Also, according to 2014 cancer statistics in Turkey, breast cancer (24.9%) is the most common cancer of women in all age groups[1].

Apoptotic regulatory molecules triggered by p53, such as Bax, FAS/pol, and BCL2, provide the tumor suppressor property of p53.4 Myc and p53 direct cells to apoptosis through Max pathway and DNA damage, respectively[5].Also, Bcl2 proto-oncogene maintains cell viability by inhibiting apoptosis. Presence of Bcl-2 is essential for maintaining cell viability. While some of the members of the BCL family proteins such as Bcl-2, Bcl-XL, Bcl-W and Mcl-1interferes with the induction of apoptosis and some of them such as Bax, Bak, Bad, Bik, Bid and BclxS induce apoptosis [6].

In many types of cancer, tumor cells gain resistance to apoptotic death by inducing the expression of anti-apoptotic Bcl-2 proteins and inhibiting the expression of pro-apoptotic proteins such as Bax, leading to disruption of the p53 mechanism. 7 Bax proteins are one of the key molecules that lead to multidrug resistance to the cell, especially in cancer patients. 8 The excess level of Bcl-2 inhibits cell death whereas low levels of Bcl-2 increase the susceptibility of the cell to various toxins.9 The ratio of Bax to Bcl-2 is an essential indicator of the susceptibility of the cell to apoptosis 10. Therefore, the Bax / Bcl-2 ratio can be used as criteria in assessing the course of the disease in various carcinomas[11].Caspase-3 regulates the Bax / Bcl-2 ratio and plays a crucial role in apoptosis. Bcl-2 is also a death mediator for caspases and accelerates cell death by inactivating the function of Bcl-2 against apoptosis[12].

The Bcl2 is a major protein which plays an important role to regulate cell death. These contact between two proteins can form a complex between four protein each other called homologous regions termed Bcl homology (BH) domains; It has been found that Bcl2 play an essential role in cancer cell for treatment and radiation therapy and chemotherapy. Bcl2 can promote the expansion level tumor cell by preventing normal cell turnover caused by physiotherapy cell death mechanisms[13,14].Bcl2 in different human cancer cell cause to a high level in expression range of Bcl2 effect on the powerful of cancers to a wide range of chemotherapeutic drugs and γ-irradiation which active of death cell in a tumor cell by inhibition of Bcl2[15].

B-cell CLL/lymphoma 2 (BCL2) encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death of some cells such as lymphocytes, and it is located on chromosome 18q21.3 and has six exons[16]. The BCL2 protein, encoded by the BCL2 gene, is the founding member of the BCL2 family of regulatory proteins that regulate cell death (apoptosis), by either inducing (proapoptotic) or inhibiting (antiapoptotic) apoptosis. BCL2 is the essential antiapoptotic protein, and thus its gene is classified as an oncogene in general[17].

Although BCL2 was usually present in human follicular B cell lymphoma, carry genetic information of translocation,18 and the scientist explain that this protein has an essential role in cancer cell line especially in

leukemia, cancer, prostate cancer, gastric cancer, and pancreatic cancer, among others[19].Callagy [20] reported that BCL2 is an independent predictor of favorable outcomes in breast cancer, particularly in the first five years after diagnosis. Berardo[21] Reported that high BCL2 expression is associated with some good prognostic factors and independently with a better clinical outcome for patients of lymph node-positive breast carcinoma[22].

Bcl-2 family proteins are classified into three subfamilies. These subfamilies are apoptosis inhibitors (anti-apoptotic), apoptosis promoters (pro-apoptotic) multidomain proteins and BH3-only proteins[23].

The aim and scope of this study were the BCL2, and BAX is high interest in cancer treatment, even though the biological significance remains still under scientific investigation with major key questions yet to be elucidated.

Patients and Methods

Participants

Collection of the sample from normal and cancer tissue type in cell line RNA isolation from the cell, RNA quantitation, cDNA synthesis, cDNA quantitation, and Real-Time- PCR analysis.

RNA isolation from cell line

After cells reach 80-90% density, they are removed for RNA isolation under appropriate conditions. RNA was extracted from cell culture using High Pure RNA Isolation (Roche, Mannheim, Germany) kit. The RNA isolation protocol is as follows; The cells with appropriate density are removed with Trypsin, and DMEM containing FCS is added to stop the effect of trypsin Cells are centrifuged at 3500 rpm for 5 min. The supernatant is removed without touching the pellet. The remaining pellet is resuspended in 200 µl PBS. Add 400 µl of Lysis Buffer to this mixture and vortex for 15 seconds. The whole mixture is transferred to filter tubes, centrifuged for 30 seconds at 9200 rpm. The lower part is discarded. Add 100 µl (10 µl DNAse and 90 µl DNAse incubation buffer) to the filtered tubes and wait at room temperature for 45 minutes. Add 500 µl Wash Buffer I and centrifuge at 9200 rpm for 30 seconds. The lower part is discarded. Then add 500 µl Wash Buffer II and centrifuge at 9200 rpm for 30 seconds. The bottom tube is replaced with the new one. Add 200 µl of Wash Buffer II and centrifuge for 2 minutes at 11800 rpm. The lower tube is discarded, and a new tube is inserted. After this, add 50 µl of Elution Buffer and wait for 1 minute at room temperature. Centrifuge at 9200 rpm for 1 minute. The filtered tube is discarded. Measurements were made on the NanoDrop 1000 to determine the amount of RNA. RNAs are stored at -80 ºC until the working period.

RNA Quantitation

Determination of quantity and quality of obtained RNA samples were done by detecting A260/A280 ratio using Nanodrop spectrophotometer. For PCR reactions, RNA was diluted according to their density.

cDNA components and their amounts

Single-stranded cDNA synthesis from RNA-

isolated samples and tissue RNAs was performed with Maxima H Minus First Strand cDNA Synthesis Kit # K1652 (Thermo Scientific). From the cell culture samples, cDNA synthesis was performed by adding RNA at a final concentration of 2 µg / µl RNA and tissue RNA at a final concentration of 1 µg / µl RNA.

Real-Time PCR (qPCR) Analyzes

QPCR was performed to determine expression levels of target genes more effectively in tissues and cell lines. For this experiment, a Rotor-Gene Q (QIAGEN,) Real-Time PCR instrument was used. qPCR experiments were performed in the direction of the manufacturer's firm using Maxima SYBR Green / ROX qPCR Master Mix (# K0251). For this study, appropriate synthetic primers were designed for exon regions of Bcl2 and Bax and bim genes by using the NCBI/Primer Blast database. Forward and reverse BCL2 gene are: 5-GTTGCATCAGCTGTCCTCCT -3 ,3 AAAAAGGGGTTGGGGTAGG5. Forward and reverse Bax gene are 5-ACCAGACCTACTCTTCCGCT-3, 3-GGGAAGAGCCAAGTCAGAC-5. Forward and reverse Bim gene are: -5 ATCTGGTGAGCCAGGTAGGA -3 , -3GGGAAGAGCCAAGTCAGAC -5.

Statistical analysis

The evaluation was performed by using GraphPad Prism 7 for evaluation of the invasive ductal + lobular subtype.

Results

The characteristics of the cell line who diagnosed with breast cancer were listed in Table (1). The mean age of the patients included in the study was 51.91 ± 13.05. Of these, 32.8% are under 45 years of age, 51.7% are between 45-65 years of age, and 15.5% are over 65 years of age. According to breast cancer subtypes, 79.3% of these patients were invasive ductal carcinoma, 19% were invasive lobular carcinoma, and 1.7% were the invasive ductal + lobular type.

Figure (1) illustrates the expression levels of Bcl2 protein was found to be significantly diminished in the tumoral tissues of breast cancer patients as compared to normal tissues (p=0.0078).

In Table (1) BCL2 expression levels of breast cancer patient were shown according to age (<45, 45-65, >65). BCL2 protein levels compared with the age distribution, it was found to be more prevalent in 65 year age tumor samples . Also, the expression level of bcl2 protein was found to be higher in patients over 65 years of age than in any other age groups.

Moreover, the expression level of Bax protein was shown according to the invasive ductal and lobular subtypes of breast cancer. Particularly, expression levels of Bax protein were found to be significantly decreased in breast cancer patients with invasive ductal carcinoma. Also, Bax protein expression levels were also found to be diminished in invasive lobular carcinoma patients, yet this change was statistically insignificant (p=0.0271) Figure (2). Statistical evaluation was performed for the invasive ductal + lobular subtype due to the presence of only one patient.

According to the histopathological staging of the patients, 8.6% were stage 1, 36.2% were stage 2, and 34.5% were stage 3. Also, the histopathologic stage of 20.7% of these patients is unknown. TNM is very important in the detection of breast cancer stages for the determination of appropriate diagnosis and treatment method. The frequency of patients was determined according to each parameter (T, N, and M) in the TNM classification. Detailed information about the patients is shown in Table (2).

Expression levels of Bax protein were found to be significantly diminished in the tumoral tissues of breast cancer patients as compared to normal tissues (p=0.0078) Figure(1).

There was no significant difference in BIM protein expression between normal and tumor tissues in breast cancer patients (p>0.05) Figure(3). Expression levels of Bax were found to be significantly increased in the tumoral tissues of breast cancer patients with invasive ductal carcinoma (p=0.0001) Figure (5). However, in invasive lobular carcinoma, Bax protein was not detected in normal and tumorous breast tissues. Figure 4 show the expression levels of Bax protein according to different age groups.

Table (1): Clinical characteristics of the cell line included in the study.

Demographic characteristics			n=58	% n
Age		<45 (38,47 ± 4,40)	19	32,8
		45-65 (53,66 ± 5,52)	30	51,7
		>65 (74,44 ± 6,44)	9	15,5
Diagnosis		Invasive ductal	46	79,3
		Invasive lobular	11	19,0
		Invasive ductal + lobular	1	1,7
Grade		G1	5	8,6
		G2	21	36,2
		G3	20	34,5
		Unknown*	12	20,7
TNM	T	T1	11	19,0
		T2	33	56,9
		T3	12	20,7
		T4	1	1,7
	N	N0	20	34,5
		N1	13	22,4
		N2	13	22,4
		N3	11	19,0
	M	Mx	57	100,0

*T category describes the primary tumor site, N category describes the regional lymph node involvement, M category describes the presence or otherwise of distant metastatic spread

Table (2): Representation of the Bax protein expression levels according to the patient characteristics.

Characteristic			Bcl2-normal	Bcl2-tumor	P value
Age	<45		4,36 ± 2,33	3,73 ± 1,82	0,175
	45-65		4,16 ± 2,03	3,96 ± 2,12	0,415
	>65		5,4 ± 2,06	4 ± 1	0,062
Diagnosis	Invasive ductal		4,3 ± 2,02	3,69 ± 1,54	0,0271
	Invasive lobular		5 ± 2,75	4,72 ± 2,8	0,500
	Invasive ductal + lobular		4	4	1
Grade	G1		4,6 ± 2,4	4 ± 2,34	0,5
	G2		4,23 ± 1,57	3,19 ± 1,12	0,002
	G3		4,3 ± 2,4	4,15 ± 1,63	0,769
TNM	T	T1	4,63 ± 2,06	2,72 ± 1,55	0,001
		T2	4,51 ± 2,16	4,24 ± 1,83	0,403
		T3	3,83 ± 2,16	3,75 ± 1,65	0,999
		T4	3	3	1
	N	N0	4,4 ± 1,87	3,95 ± 1,60	0,300
		N1	5,23 ± 2,62	4,3 ± 2,59	0,031
		N2	4,23 ± 2,04	3,92 ± 1,49	0,562
		N3	3,45 ± 1,80	2,9 ± 1,13	0,25
	M	Mx	4,43 ± 2,15	3,89 ± 1,87	0,014

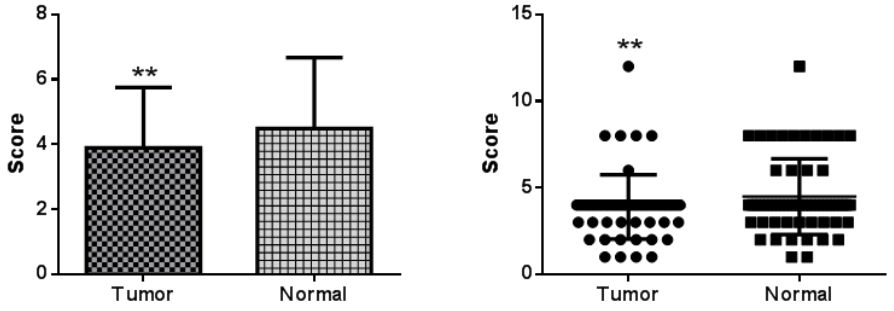
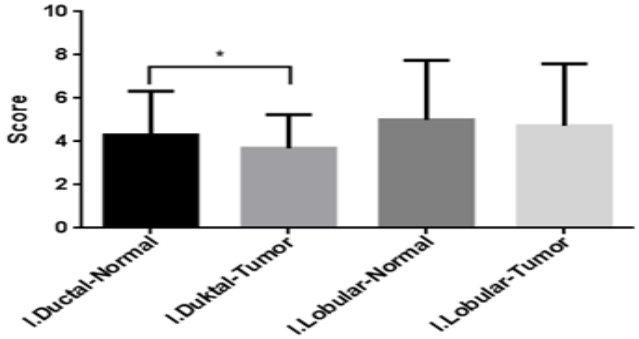


Figure (1): The expression level of Bcl2 protein in normal and tumor tissues of breast cancer patients ** p=0.0078.



Figure(2): Bcl2 protein expression levels according to breast cancer diagnosis,* p=0.0271.

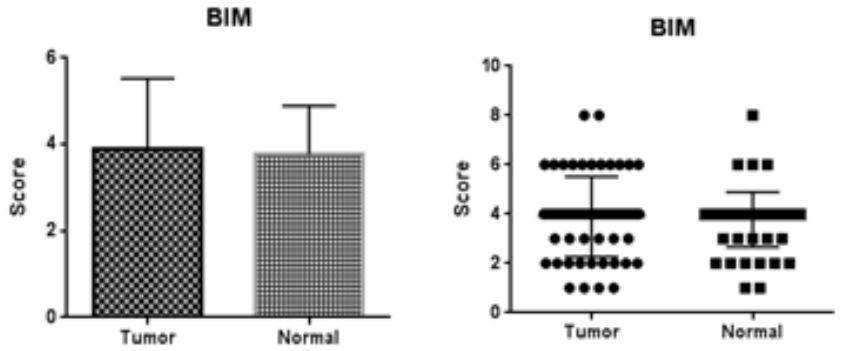


Figure (3): Expression levels of BIM protein in normal and tumor tissues of breast cancer patients.

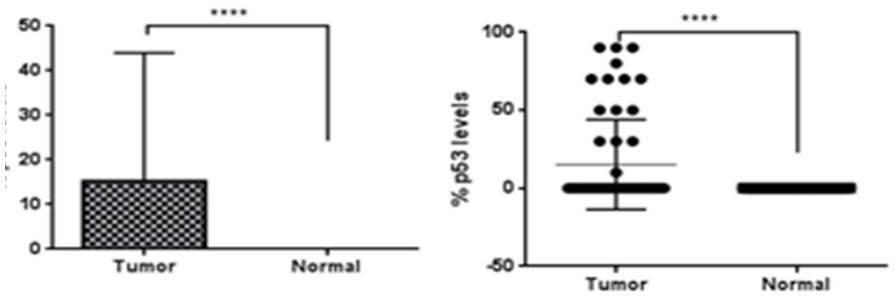


Figure (4): Expression levels of Bax protein in normal and tumor tissues of breast.

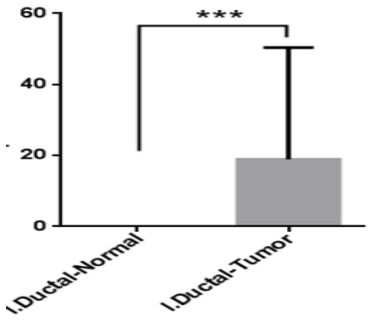


Figure (5): Expression levels of Bax protein in invasive ductal carcinoma breast cancer subtype.
***** p=0.0001.**

Discussion

Breast cancer is the cause of death in female all over the world, with about 1.3 million cases affected yearly and approximately 500,000 deaths[21].

For therapeutic and prognostic reasons, breast cancer of female is treated on the expression of the estrogen receptor (ER), progesterone receptor (PR), and the epidermal growth factor receptor (EGFR)

subtype HER2.25Another more differentiated methodology used in the characterization of breast cancers is tumor grading.26 For this reason, oncologists classify breast cancer subtypes as HER2-overexpressing breast cancer causing to the luminal B (higher grade, ER-positive), luminal A (low grade, ER-positive) , TNBC (triple-negative breast cancers lacking ER, PR, HER2), normal breast-like tumors, and claudin-low (triple-negative invasive ductal carcinomas with a low expression of cell adhesion molecules). ER-positive breast cancers, which represents approximately 70% of all breast cancers 27, are estrogen dependent and thus responds to treatment with anti-estrogens, such as ER antagonists 4-hydroxytamoxifen and raloxifene 28. ER-, PR-, and HER2-negative (triple negative) breast cancers still cannot be effectively treated. Therefore, to develop effective treatment approaches specific to breast cancer subtypes, expression profiles need to be studied in detail[29].

In normal cells, apoptosis is considered as a natural defensive mechanism against cancer development and the resistance of cancer cells to apoptosis is listed as one of the most important hallmarks of cancer.30 Thus, the basis of the currently used cancer treatment methods is to trigger apoptosis in cancer cells. At the molecular level. Cancer is caused by a series of mutations and or disorders that occur in genes that play a role in the execution and regulation of vital cellular activities such as cell proliferation, death, and differentiation[31].The genes contain in the formation and progression of cancers are generally known as tumor suppressor genes and oncogenes[32].

In this study, the range of expression of the Bcl2 gene was determined in a total number of 58 patients who diagnosed with breast cancer Table(1). The expression level of bcl2 average in 65 years has increase than other age groups. The expression level of Bax can be increased in ductal tissue if when compared with the normal tissue depend on a grade of the tumor. The expression level of BIM was the same in normal and tumor cells that it means there is no any significant difference to Figure [3].

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