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Original Article

Evaluation of *Bax* and *BCL 2* Genes Polymorphisms in Iraqi Women with Breast Cancer

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Abstract

The present study aimed to examine the polymorphism -938C > A *of BCL-2* gene and promoter -248G>A in the *Bax* gene, as well as their relationship with specific clinical-pathological characteristics, in patients with breast cancer. Blood samples were obtained from 70 patients who had been diagnosed with breast cancer and 34 healthy women as the control group. Polymorphic analysis was performed using the polymerase chain reaction-restriction fragment length polymorphism assay. Anthropometric data were assessed. Estrogen receptor (ER), human epidermal growth factor receptor 2 (Her-2), and progesterone receptor (PR) were measured by immunohistochemistry. The data of age and body mass index (BMI) demonstrated no significant variations between the two groups (*P*>0.05). The results of HER-2 revealed that 42.86% of breast cancer patients reflected positively for Her-2/neu expression, while 24.29% reflected negative results of Her-2/neu. Moreover, the results of ER revealed that 42.86% and 28.57% of subjects were positive and negative ER, respectively; moreover, the missing data was 28.57%. In addition, the results of PR indicated that 35.71% of patients (25/70) were positive for PR, while 28.57% reflected negative results, and the missing results were 35.71%. The genotype and allele frequencies of *BCL-2*(-938C>A) were not statistically significant in women with breast cancer and the control group (*P*=0.574, *P*=0.533) for heterozygous and recessive models, respectively. The genotype of *BCL-2*(-938C>A) in control and patients in codominant, dominant, recessive, and additive models demonstrated no significant variations of all genotypes in all groups. Genotypes and allele frequencies for *Bax* (-248G>A) in patients with breast cancer and control indicated that the frequencies of GG, AG, and AA genotypes in cases were 16.67%, 3.33%, and 80 %, while in controls, these values were 3.23 %, 58.06 %, and 3.23 %, respectively. The heterozygous genotype (AG) in the codominant model was OR=36.00 (95% CI: 4.5608 - 284.1608; *P*=0.0007). In comparison with the wild type (GG), there was a 36-fold increase in the risk of breast cancer. Furthermore, the findings of this study revealed a significant correlation between *Bax* (-248G>A) polymorphism and breast cancer risk under the dominant and overdominant (OR=6.33; 95% CI: 2.2604 -17.7452; *P*=0.0004, and OR=40.154; 95% CI: 5.1365 - 313.8949; *P*=0.0004, respectively. The recessive model revealed that there was a decreased risk of breast cancer (OR= 0.167; 95% CI: 0.0303 to 0.9168; *P*=0.039). Based on the results, it can be concluded that there were no significant variations in *BCL-2* (-938C>A) polymorphism of all genotypes models when breast cancer women are compared with healthy ones. In a similar vein, there was no significant association between the *BCL-2* (-938C>A) polymorphism and breast cancer risk under dominant, codominant, or recessive models.

Keywords: *Bax* gene, *BCL-2* gene, Breast cancer, 248G>A polymorphism, -938C > -97h526A polymorphism, Iraq

1. Introduction

Breast cancer has presently overpowered lung cancer as the leading cause of global cancer incidence in 2020, with an assessed 2.3 million new cases, accounting for 11.7% of overall cancer (1). Breast cancer is a heterogeneous malignancy that can be classified into several molecular subtypes concerning estrogen receptor (ER), progesterone receptor (PR), and human

epidermal growth factor 2 (HER2) (2). The HER2 receptor is a 185 kDa transmembrane glycoprotein which is encoded by the *HER2* gene found on the long arm of chromosome 17 (17q12–21.32) plays a fundamental function in cell growth control, differentiation, and survival (1).

Progesterone receptor (PR) intercedes the effects of progesterone in mammary tissues and plays a significant role in normal breast development and breast cancer. Although PR is present as two isoforms, progesterone receptor alpha (PRA) and progesterone receptor beta (PRB), with varying capabilities to activate target genes, it is not clear whether PRA and/or PRB mediate progesterone action in normal and cancerous breast tissues (3). Apart from its involvement in normal breast development and function, PR has been correlated with breast cancer occurrence.

The PR-positive primary breast cancers are more likely to be smaller, less proliferative, and more differentiated (3, 4). Both human epithelial growth factor receptor 2 (HER2) and the estrogen receptor are involved in the development and prognosis of breast cancer tissues (5). The estrogen receptor, progesterone receptor, and HER2 profile of a primary breast carcinoma all have a role to play in patient care and treatment. Surgically-resected carcinomas often exhibit a substantial treatment impact as a result of the increased use of neoadjuvant chemotherapy or hormone therapy (6).

The development of diseases is influenced by a number of factors. While heredity performs a key function in the development of cancer, the likelihood of the disease is also increased by environmental influences, nutrition habits, and genetic variants (7). The physiological mechanism of cell death that relies on proteins that already exist and de novo protein synthesis is known as apoptosis, and the apoptotic process is necessary for the development of the normal mammary gland and many diseases, including breast cancer (8). Different factors are generally linked to apoptosis, including bacteria, environment, age, gender, genetic factors, tumor suppressor gene mutations, as well as the genes involved in the apoptotic process, such as tumor necrosis factor-alpha (TNF-α) (9, 10).

One of the main problems in breast cancer, as in all other malignancies, is the altered regulation of apoptosis. Apoptosis has two diverse mechanisms in breast cancer: the first pathway (extrinsic) is activated by the death receptors, a subgroup of the tumor necrosis factor (TNF) receptor family (7), and the intrinsic pathway which focuses on mitochondria. The Bcl-2 family proteins regulate the mitochondrial release of death factors, regulating this pathway (11).

The *BCL2* gene contains six exons and two distinct functional promoters localized on chromosome 18q21.33; moreover, it is the first promoter affected by negative regulation of the second promoter (12, 13). The anti-apoptotic B-cell lymphoma 2 (BCL2) and the pro-apoptotic B-cell lymphoma 2-associated X protein (BAX) are both good apoptosis regulators (14-16). The BCL-2 is a protein group with three distinct classifications depending on the existence of BCL-2 homology domains (BH1-4 domains): anti-apoptotic BCL-2 proteins (e.g., BCL-2), the effectors pro-apoptotic members (e.g., Bax), and the BH3-only proteins (17).

The *Bax* gene, which has six exons and is found on human chromosome 19q13.3, is the first known apoptotic gene that is directly activated by p53 (18). Although altered *Bax* gene expression appears to be more correlated with the process of carcinogenesis, mutations that cause this dysregulation and their associations with various cancers are a hot topic among researchers. *Bax* gene promoter region includes p53 response elements, which influence gene expression (18). Despite the importance of BCL-2, BAX, and HER2/neu in the progression of breast cancer, the precise molecular mechanism is still unclear.

In light of the aforementioned issues, the current study aimed to evaluate the polymorphism -938C > A *of BCL-2* gene and promoter -248G>A in *Bax* gene, as well as their relationship with specific clinical-pathological characteristics, in patients with breast cancer.

2. Materials and Methods

2.1 Study Subjects

This case-control study was conducted on 70 women with malignant breast tumors who attended the Middle Euphrates Cancer Center in Al-Najaf, Iraq. The 70 patients with breast cancer were within the age range of 28-71 years (49.814±9.2806), and 34 healthy women were in the age group of 25-65 years (47.0542+10.412). The related clinical history of all of the studied cases was collected, and the clinical history was used to identify suitable cases based on the exclusion/ inclusion criteria.

Five milliliters of whole blood were withdrawn from each patient and healthy individual and deposited into Ethylenediaminetetraacetic acid (EDTA)-tubes. Thereafter, they were transported to the laboratory under cooling circumstances at the earliest opportunity. For histological analysis, tissue biopsy specimens from the patients were fixed in 10% formaldehyde; subsequently, the tissues were embedded in paraffin, sectioned, and stained with Hematoxylin and Eosin. Following a breast cancer diagnosis, the availability of hormone receptors ER, PR, and HER2 was investigated using all blocks that have been formalin-fixed and embedded in paraffin.

2.2. Measurements

Anthropometric measures, such as body mass index (BMI) and age, were calculated. The primary tumors were examined for the presence of HER2, ER, and PR by immunohistochemistry staining of the blocks that have been formalin-fixed and embedded in paraffin using Dako kits (Denmark) which included particular monoclonal antibodies (19).

2.3. Genotyping of Studied Genes

2.3.1. DNA Isolation

The manufacturer's protocol was followed to extract genomic DNA from whole blood using the G-spinTm complete DNA extraction mini Kit (Intron/Korea). The purity and concentration of DNA were determined using BioDrop spectorophotometer (UK). Following that, the extracted genomic DNA quality was evaluated by horizontal electrophoresis with Safe-Green stain. The DNA specimens were kept at -20°C for polymerase chain technique.

2.3.2. Amplification of Studied Genes

The intended segment of genomic DNA was amplified with specific sets of primers to evaluate *BCL2* and *Bax* polymorphisms of all samples. Genotyping for the studied SNPs was carried out using restriction fragmented length polymorphism polymerase chain reaction (RFLP-PCR) figures 1 and 2.

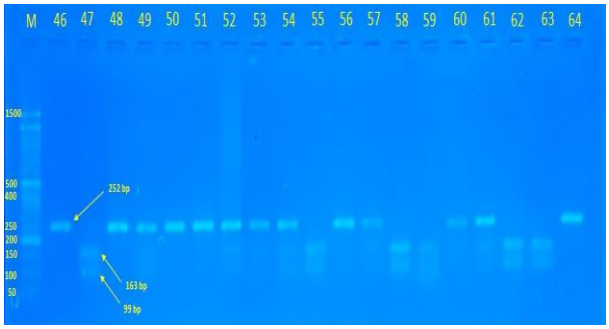

Figure 1. PCR-RFLP bands of SNP rs2279115 (-٩٣٧C>A) of the human gene, *BCL2*

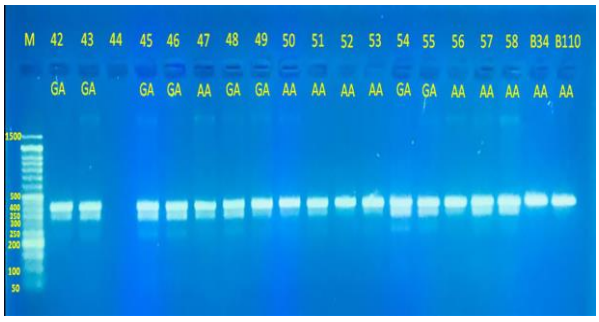

Figure 2. RFLP-PCR product bands of SNP (-248G>A) of human gene, *Bax*

GoTaq® G2 Green Master Mix was mixed with dye comprising an inherited green dye and a stabilizer that allows direct loading of the final products onto a gel for analysis. Each PCR amplification was carried out in a 50 µl reaction mixture consisting of 25 µl of GoTaq® G2 Green Master Mix (1X), 4 µl of each primer of (10 pmol), 13 µl of ddH_2O, and 4 µl (40 ng) of genomic DNA templates. The PCR program was performed in

an Optimus 96G thermal cycler (QLS, UK).

The sequences of forward primer of *BCL-2* gene was 5'-TTATCCAGC AGCTTTTCGG-3' and the reverse was 5'-GGCGGCAGATGAATTAC A-3' (20). The PCR protocols consisted of an initial 94°C for 3 min and temperature cycling was set at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 1 min for 35 cycles, followed by a final extension at 72°C for 5 min to ensure polymerization of all unreacted nucleotides. Upon the completion of the PCR reaction, 10 µl volume of PCR product reaction of each sample was incubated for 1.5 h with BccI restricted enzyme (NEB, England) for digestion (18).

The primers used to identify the polymorphism in the *Bax* gene were 5'-CGG GGTTATCTCTTGGGC-3' forward and 5'-GTGAGAGCCCCGCTGA AC-3' revers (21). The PCR protocols consisted of an initial 94°C for 3 min and temperature cycling was adjusted at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 1 min for 35 cycles, followed by a final extension at 72°C for 5 min to ensure the polymerization of all unreacted nucleotides. After the completion of the PCR reaction, Aci I was used to digest the PCR products. (NEB, England) at 37°C for 1.5 h.

The digested and undigested products were visualized using gel electrophoresis with 2% agarose, and images were then taken using an ultraviolet light transilluminator. Several PCR products were randomly selected for sequencing to verify the genotyping. Sequencing of *Bax* genes was performed by macrogen company/Korea as presented in figure 3.

2.4. Bio Statistical Analysis

The results were analyzed in SPSS software (version 19) (SPSS Inc, Chicago, USA). The student's t-test was applied to examine numerical quantities. To evaluate the continuous allele from generation to other, Hardy Weinberg Equilibrium was utilized for both groups (patients and control) in studied SNPs and considered suitable when P>0.05. Moreover, the chi-square test was performed to examine variations in features between patients with breast cancer and controls, as well as variations in genotype and allele frequencies. P-values less than 0.05 were considered statistically significant.

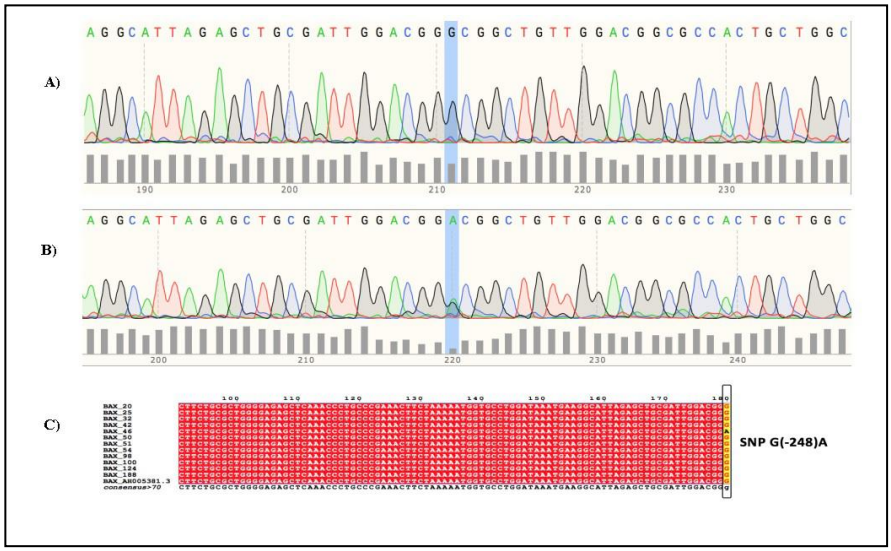

Figure 3. Chromatography of partial *BAX* gene sequence of sample 25 against a reference *BAX* gene sequence of Homo sapiens for detection of G(-248)A SNP, **A)** represent G/G homozygous wild type, **B)** represent heterozygous A/G model, **C)** represent the aliment of samples relative to Homo sapiens in the NCBI (Accession number: AH005381.3) for the detection of G (-248)A SNP (highlighted in the black box)

3. Results

3.1. Anthropometric Data

The 70 patients with breast cancer were within the age range of 28-71 years (49.814±9.2806), and 34 healthy women were in the age group of 25-65 years (47.0542+10.412). The mean BMI scores were reported as 28.9313±2.5 and 28.301±4.9 for breast cancer patients and healthy women, respectively. The data of age and BMI showed that there were no significant variations between the two groups (P>0.05).

The Demographics and case line clinical-pathological features of patients are demonstrated in table 1. The assay of HER-2 has just two possible outcomes: positive, showing that the *HER-2/neu* gene is expressed, or negative, signifying that the gene is not produced excessively. The results of HER-2 in table 1 revealed that 42.86% of cases (30/70) reflected positively for Her-2/neu expression. Out of 70 patients, 17 (24.29%) subjects reflected negative results of Her-2/neu. In addition, the results of ER revealed that there was 42.86% (30/70) with positive ER and 28.57% (20/70) with negative ER, while the missing data was 28.57% (20/70). Furthermore, the results of PR indicated that 35.71% of patients (25/70) reflected positive results, 28.57% reflected negative results, and the missing results were 35.71% (25/70).

3.2. Association between *BCL-2* (-938C>A) and breast cancer

In this study, the *BCL-2* (-938C>A) was determined in 70 cases with breast cancer and 34 healthy controls. Genotypes and allele frequencies for *BCL-2* (-938C>A) polymorphism in patients with breast cancer and control groups are displayed in table 2. The genotype frequency of *BCL-2* (-938C>A) in cases revealed that 77.14%, 12.86%, and 10% of cases were CC wild-type, AC heterozygous, and AA recessive, respectively. Moreover, an allelic frequency was 81.25%, 18.75%, and 6.25% CC, AC, AA, respectively, in controls. The genotype frequency of the described SNP was tested for Hardy-Weinberg equilibrium (HWE). The *BCL-2* SNP was clear to be harmonious with HWE in the control group, indicating that the distribution of genotypes in our population is constant from generation to generation. Therefore, the distributions of genotype changes in the breast cancer group could be appropriately interpreted. It is reasonable to attribute these changes to the disease occurrence.

The genotype and allele frequencies of *BCL-2*(-938C>A) were not statistically significant in women with breast cancer and the control group (P=0.574 and P=0.533 for heterozygous and recessive model, respectively). The genotype of *BCL-2*(-938C>A) in controls and breast cancer patients in codominant, dominant, recessive, and additive models were examined by multinomial logistic regression analysis (Table 2), and no significant variations were observed in all genotypes in both groups. In a similar vein, there was no significant association between the *BCL-2* (-938C>A) polymorphism and breast cancer risk under dominant, codominant, or recessive models.

Table 1. Patient demographics and baseline clinical pathologic features

Clinical features	N	(%)
Age	49.814±9.2806	
BMI	28.301±4.946	
T stage		
T_1	14	20%
T_2	41	58.57%
T_3	10	14.29%
T_4	5	7.14%
L.N		
N_0	21	30%
N_1	23	32.86%
N_2	20	28.57%
N_3	6	8.57%
M		
M_0	38	54.29%
M_1	32	45.71%
Her-2		
Positive	30	42.86%
Negative	17	24.29%
Missing	23	32.86%
ER		
Positive	30	42.86%
Negative	20	28.57%
Missing	20	28.57%
PR		
Positive	25	35.71%
Negative	20	28.57%
Missing	25	35.71%

T: Tumor stage, LN: lymph node stage, M: metastasis stage

Table 2. Genotypes and allele frequencies for *BCL-2* (-938C>A) in breast patients and controls

SNP	Control 34	Patients 70	OR (CI 95%)	*P* value
Codominant				
CC (Wild type)	26 (81.25%)	54	-	
CA	6 (18.75)	9	0.72 0.2324 to 2.2448	0.574
AA	2 (6.25%)	7	1.685 0.3270 to 8.6845	0.533
Dominant				
CA+AA	8	16	0.96 0.3654 to 2.5380	0.94
Over dominant				
CC+AA	28	61	-	
AC	6	9	0.688 0.2234 to 2.1224	0.52
Recessive				
CC+CA (Wild type)	32	63	-	
AA	2	7	1.778 0.3490 to 9.0561	0.488
Additive				
2CC+CA	58	117	-	
2AA+CA	10	23	1.14 0.5091 to 2.5537	0.75

The PCR product of samples was digested using the restriction enzyme (BccI) that cuts the PCR product (252 bp) into two pieces (163 bp and 99 bp) in the presence of the SNP rs2279115 (-938C>A). The wild-type genotype (GG) would have only one band (252 bp). The genotype (GA) is confirmed once three bands of (252 bp, 163 bp, and 99 bp) are demonstrated in the gel. Finally, the mutant type (AA) genotype is detected once the gel expresses only two bands (163 bp and 99 bp). The gel was 2% and the DNA dye is RedSafe (Intron, Korea). V: 95, Time: 45 minutes. M: DNA ladder

3.3. Association between *Bax* (-248G>A) and Breast Cancer

The current study examined the *Bax* (-248G>A) in the promotor region of the *Bax* gene, as well as the association between this polymorphism and breast cancer risk. *Bax* gene (-248G>A) polymorphism was determined in 62 patients with breast cancer and 30 healthy controls since some samples did not give results due to damaged DNA. Genotype distributions for *Bax* (-248G>A) polymorphism in both breast cancer and control groups were in disagreement with the Hardy-Weinberg equilibrium ($P<0.05$).

Genotypes and allele frequencies for *Bax* (-248G>A) polymorphism in breast cancer patients and control are presented in table 3. In breast cancer cases, the frequencies of AA, AG, GG, and genotypes were 80%, 3.33%, and 16.67%, respectively, while in controls, the frequencies were 3.23%, 58.06%, and 3.23%, respectively

Table 3. Genotypes and allele frequencies for *Bax* (-248G>A) in breast patients and controls

SNP	Control 30	Patients 62	OR (CI 95%)	*P* value
Codominant				
AA (Wild type)	24 (80 %)	24 (38.71%)	-	
AG	1 (3.33%)	36 (58.06 %)	36.00 4.5608 to 284.1608	0.0007
GG	5 (16.67%)	2 (3.23 %)	0.40 0.0706 to 2.2669	0.301
Dominant				
AG+GG	6	38	6.33 2.2604 to 17.7452	0.0004
Over dominant				
AA+GG	29	26	-	
AG	1	36	40.154 5.1365 to 313.8949	0.0004
Recessive				
AA+AG(Wild type)	25	60	-	
GG	5	2	0.167 0.0303 to 0.9168	0.039
Additive				
2GG+AG	11	40	3.63 1.5160 to 8.7221	0.004

The heterozygous genotype (AG) in the codominant model was OR=36.00 (95% CI: 4.5608 - 284.1608; *P*=0.0007). In comparison with the wild type (GG), there was a 36-fold increase in the risk of breast cancer. In addition, the findings of the present study revealed a significant correlation between *Bax* (-248G>A) polymorphism and breast cancer risk under the dominant and overdominant (OR = 6.33; 95% CI: 2.2604 -17.7452; *P*=0.0004, and OR=40.154; 95% CI: 5.1365 - 313.8949; *P*=0.0004 respectively. The recessive model demonstrated that there was a decreased risk of breast cancer (OR= 0.167; 95% CI: 0.0303 to 0.9168; *P*=0.039).

The PCR product of these samples was digested using the restriction enzyme (AciI) that cuts the PCR products into two pieces (256 bp and 183 bp) in the presence of SNP (-248G>A). The mutant type genotype (AA) would have only one band (438bp). The genotype (GC) is confirmed once three bands of (438 bp, 256 bp, and 183 bp) are shown in the gel. Finally, the wild type (WT) genotype (GG) is detected once the gel expressed only two bands (256 bp and 183 bp). The gel was 2% and the DNA dye is RedSafe (Intron, Korea). V: 95, Time: 45 minutes. M: DNA ladder.

4. Discussion

Immunohistochemical examination of ER, PR, and HER2 markers is routinely used, in conjunction with histopathological grading and staging, to decide on a suitable therapeutic approach for patients with breast cancer (22). Breast cancer patients with ER^+ and/or PR^+ have a decreased risk of death after detection, as compared to women with ER^- and/or PR^- (23). The

expression of HER-2/neu is a diagnostic and predictive biomarker that correlates with a poor clinical outcome in breast cancer and other malignancies. As a result, anti-HER-2/neu therapy improved the clinical outcome (24, 25). In their study, Hatem, jabbar Alyaqubi (26) pointed out that about 26.7% of 30 Iraqi breast cancer cases were HER-2/neu positive. Several studies revealed that cancer, neurodegenerative disorders, ischemia, and autoimmune diseases are linked to abnormalities of Bcl-2 function (27, 28). The AA genotype of BCL-2 (- 938C >A) was reported to be correlated with breast cancer susceptibility by Zhang, Li (29); nonetheless, this genotype is just linked to nodal status and pathological diagnosis. The results of the BCL-2 (-938C>A) polymorphism were shown to be unrelated to the risk of breast cancer in this study. These results are consistent with those obtained by Bhatt, Verma (30) who reported that there was no significant variation in both *Bax* (-248G>A) allelic and genotype frequency, as well as *BCL-2* (-938C>A) polymorphisms, between breast cancer patients and controls. The findings of the present research on *BCL-2* (-938C > A) differed from those of a study conducted by Bhushann Meka, Jarjapu (31) who discovered that there was the coloration of AA genotype with an elevated risk (AA vs AC+CC) of breast cancer by 2.86 fold (*P=0.07*), and the frequency of A allele was also elevated. In comparison with the A allele, the -938C allele showed considerably higher suppression of *BCL-2* promoter activity and nuclear protein binding (32). Recently it was discovered that *Bax* plays a role in tumor suppression and carrying mutations in some cancers types; moreover, it can accelerate cell cycle entry in T-cells. *Bax* gene is able to create ion-conducting channels in planar lipid bilayers, which may be the biochemical mechanism whereby it performs its multiple effects. The findings of the present study regarding *Bax* (-248G>A) were not in line with those of the study by Yildiz, Yaylim (20) who found no significant differences in *Bax* (-248G>A) genotype and allele frequencies in patients with breast cancer and controls in Turkish women and a meta-analysis study by Sahu and Choudhuri (33) who reported that *Bax* (-248G>A) genotype and allele frequency were not correlated with cancer risk under various genetic models.

5. Conclusion

As evidenced by the results of the current study, it can be concluded that there were no significant variations in *BCL-2* (-938C>A) polymorphism of all genotypes models when breast cancer women are compared with healthy ones. In a similar vein, there was no significant association between the *BCL-2* (-938C>A) polymorphism and breast cancer risk under dominant, codominant, or recessive models.

On the contrary, the findings of this study pointed to a significant correlation between *Bax* (-248G>A) and breast cancer risk under the dominant and over dominant models (OR = 6.33; 95% CI: 2.2604 - 17.7452; *P*=0.0004, and OR=40.154; 95% CI: 5.1365 - 313.8949; *P*= 0.0004 respectively. The recessive model revealed that there was a decreased risk of breast cancer (OR= 0.167; 95% CI: 0.0303 to 0.9168; *P*=0.039).

Authors' Contribution

Study concept and design: H. F. S. A.
Acquisition of data: S. R. M.
Analysis and interpretation of data: H. F. S. A.
Drafting of the manuscript: S. R. M.
Critical revision of the manuscript for important intellectual content: S. R. M.
Statistical analysis: D. A. F. A.
Administrative, technical, and material support: S. R. M.

Ethics

All study protocols have been approved by the ethics committee of University of Kufa, Kufa, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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