Original Article

The Role of Functional Polymorphisms of Apoptotic *BAX* (-248G>A) and Anti-Apoptotic *BCL2* (-938C>A) Genes in the Development of Vitiligo: A Cross-sectional Study

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Abstract

Background: Vitiligo is an autoimmune skin disorder in which apoptosis plays an exceptionally vital role in its occurrence. Research has shown a strong association between the presence of polymorphisms and the occurrence of diseases. This study aimed to determine the association of *BAX*-248G>A and *BCL2*-938C>A polymorphisms with vitiligo.

Methods: This cross-sectional study utilized the tetra-primer amplification refractory mutation system polymerase chain reaction (ARMS PCR) in a cohort of vitiligo patients referred to the esteemed dermatology department of Shahid Faqihi Hospital in Shiraz between 2022 and 2023. The Chi square test and *t* test were used to analyze data, and logistic regression was employed to calculate odds ratios (ORs) and 95% confidence intervals (CIs) for vitiligo risk associated with *BAX*-248G>A, *BCL2*-938 C>A polymorphisms. P values<0.05 indicated statistical significance.

Results: From 112 healthy individuals and 107 vitiligo patients, our research uncovered a significant association between the C allele (*BCL2*-938C>A) and the chance of vitiligo, with the CC genotype increasing the risk of developing this disease (OR=2.59, 95% CI=1.66-4.05, P<0.001). We also found that in individuals with the GG genotype (*BAX*-248G>A), the risk of vitiligo is higher (OR=4.576, 95% CI=1.19-17.60, P=0.027). Parental kinship was strongly correlated with vitiligo (OR=1.83, 95% CI=1.00-3.33, P=0.048). Moreover, no significant association was observed between smoking and vitiligo.

Conclusion: The results showed that *BCL2*-938C>A and *BAX*-248G>A polymorphisms may play a role in the pathogenesis of vitiligo and can be used as prognosis markers. However, further studies in larger groups and different populations are needed.

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Keywords ● Polymorphism ● Apoptosis ● Bcl-2 associated X ● B-cell lymphoma-2 ● Vitiligo

What's Known

- Vitiligo may be associated with disruption of apoptosis regulatory molecules and altered expression of BCL2 and BAX.

What's New

- Identification of molecular markers that can be investigated by rapid methods, such as Polymerase chain reaction (PCR), can help provide warning information about the conditions of people at risk.
- Investigation of *BAX* 248G>A and *Bcl-2* 938C>A polymorphisms shows whether they increase the risk of vitiligo.

Introduction

Vitiligo, an acquired and progressive disorder, is characterized by the emergence of circumscribed white macules on the skin. This depigmenting condition results from the chronic and

progressive loss of functional melanocytes in the epidermis.[1] It seems that vitiligo is affected by various factors, including environmental and genetic factors, that can cause melanocyte dysfunction.[2]

In vitiligo disease, melanocytes seem to be destroyed through apoptosis.[3] Apoptosis in melanocytes is induced through active caspases and BAX/BCL2 systems.[4] Anti-apoptotic B-cell lymphoma-2 (BCL2) protein prevents cell apoptosis, contrary to pro-apoptotic Bcl-2-associated X (BAX) protein, which activates the apoptosis pathway.[5, 6] It was found that the expression level of BCL2 in the melanocytes around the lesion is lower than in control melanocytes, and the expression level of BAX protein is high, showing that the melanocytes of vitiligo patients are involved in the process of apoptosis.[7]

BCL2-938C>A and *BAX*-248G>A polymorphisms are in the promoter region and can bind to various transcription factors and regulatory proteins.[8] Therefore, polymorphisms located in the promoter of *BCL2* and *BAX* genes can alter gene expression or protein function, which may affect apoptosis regulatory mechanisms.[9] Polymorphisms are considered to be one of the most influential factors in people's sensitivity to diseases and drugs.[10] The study of polymorphisms and gene expression is necessary to discover the diseases' molecular mechanisms, determine the disease's genetic risk, and use it in personalized medicine in the future.[11] In this regard, the pathogenesis of vitiligo and the association of polymorphisms with this disease are not fully understood.[12] Considering the role of apoptosis in vitiligo disease and the effectiveness of the BAX/BCL2 expression ratio in the process of apoptosis, the study of polymorphisms of *BCL2* and *BAX* genes and their association with vitiligo is particularly important**.** Apoptosis plays an exceptionally vital part in causing immune system reactions and the devastation of melanocytes in vitiligo. Apoptosis is mediated by BCL2 and BAX proteins in the intrinsic apoptosis pathway. Vitiligo may be associated with disruption of apoptosis regulatory molecules and altered expression of BCL2 and BAX.[13, 14] *BCL2*-938C>A and *BAX*-248G>A polymorphisms are in the promoter region of *BAX* and *BCL2* genes.[15] These polymorphisms can cause changes in the binding sites of transcription factors and have significant functional effects in regulating gene expression, ultimately affecting disease susceptibility. Therefore, single-nucleotide polymorphisms (SNPs) can act as biomarkers for the diagnosis and prognosis of diseases.[16]

Therefore, this cross-sectional study aimed to investigate the association of *BAX-248G>A* (rs4645878) and *BCL2-938C>A* (rs2279115) polymorphisms with vitiligo disease in Shirazi patients in the Iranian population. The studied polymorphisms had not previously been investigated in vitiligo patients.

Patients and Methods

This cross-sectional study was approved by the Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran (ID number IR.SUMS.REC.1400.095). The inclusion criteria of this case-control study were age (18 years old or older) and clinical diagnosis of vitiligo. The case group consisted of vitiligo patients, while the control group included healthy individuals who were matched for age and sex across both groups. The sample size was confirmed by the *post hoc* power analysis that uses the observed sample size and estimated effect, along with the assumed type I error, to calculate the observed power.[17] The vitiligo evaluations were clinically performed by dermatologists, with the skin lesions examined under a Wood's lamp. We designed the sample size based on the average number of vitiligo patients visiting the dermatology clinic.

For all volunteers referred to dermatology clinics at Shiraz University of Medical Sciences, a clinical history form and general information (age, sex, smoking, parental consanguinity, age of onset, duration of vitiligo) were completed by the researcher. Next, whole blood samples were collected in Ethylenediaminetetraacetic acid (EDTA) tubes (Easy, Iran) for genetic analysis. In this study, all ethical principles were reviewed and approved by the ethics committee of Shiraz University of Medical Sciences.

The genotypes of *BAX*-248G>A (rs4645878) and *BCL2*-938C>A (rs2279115) gene polymorphisms were investigated in all subjects enrolled in this study using the tetra-primer amplification refractory mutation system polymerase chain reaction (ARMS PCR) technique.[18]

DNA Extraction and Genotyping of Samples

Sampling Procedure

Whole blood samples collected from the case and control groups were transferred into EDTA tubes and stored. Then, the genomic Deoxyribonucleic acid (DNA) was extracted from the blood samples using a FavorPrep™ Blood/Cultured Cell Genomic DNA Extraction Mini Kit (Favorgen Biotech Corp., Taiwan), following the manufacturer's instructions.

Table 1: Primers for *BAX*-248G>A and *BCL2*-938C>A polymorphism

Primer	Sequence	Amplicon (bp)	T_m (°C)
BAX-248G>A			
Forward outer primer	5′- CCTGGAAGCATGCTATTTTGGGCCT -3′	323	65
Reverse outer primer	5′- ACGTGAGAGCCCCGCTGAACGT -3′		
Forward inner primer (G allele)	5′- GGCATTAGAGCTGCGATTGGACTGG -3′	209	
Reverse inner primer (A allele)	5′- AGTGGCGCCGTCCAACAGCAGT -3′	160	
BCL2-938 C>A			
Forward outer primer	5′- CCGGCTCCTTCATCGTCTCC -3′	300	58
Reverse outer primer	5′- CCCAGGAGAGAGACAGGGGAAAT -3′		
Forward inner primer (A allele)	5′- AATAAAACCCTCCCCCACCACCT -3′	220	
Reverse inner primer (C allele)	5′- CCCTTCTCGGCAATTTACACGC -3′	121	

BAX: Bcl-2 associated X; BCL2: B-cell lymphoma-2

The Quality Control of the Extracted DNA

The DNA optical absorbance was determined using a nano-drop device, and the samples with Optical Density (OD) values between 1.8 and 2 were confirmed. In addition, the electrophoresis technique was used to validate the quality of DNA extraction. DNA samples were loaded and run on an agarose gel (Sigma-Aldrich, US). The DNA bands were visualized using the gel documentation imaging system, and molecular markers confirmed the band's validity on the gel.

Tetra-primer ARMS PCR

The tetra-primer ARMS PCR reaction was utilized to amplify the polymorphisms of the target gene. In this technique, four primers are needed, including an outer primer pair and an internal primer pair that contain mismatches at the 3′-end so that two bands for a homozygous individual and three bands for a heterozygous individual will appear in the agarose gel.

Table 1 presents the primers used in this study. The genotype determination was investigated in a single tube with a total volume of 20 µL: master mix (10 µL), 100 ng of template DNA, and one µL each primer (10 pmol).

The PCR condition was set as follows: 5 min of denaturation at 95 °C, then 35 cycles of 30 sec for denaturation at 95 °C, 30 sec for primer annealing at 59 °C, and 20 sec for the extension at 72 °C, and 5 min at 72 °C as a final extension.

Finally, the PCR products of different samples were run on a 2% electrophoresis gel, and the bands related to alleles were identified according to figure 1.

Statistical Analysis

Statistical analysis was done using an IBM personal computer with Statistical Package of Social Sciences (SPSS) version 22 (SPSS, Inc., Chicago, Illinois, USA). Two types of statistics were used: (a) descriptive statistics, where quantitative data were presented in the form of mean, SD, and range, and qualitative data were presented in the form of numbers and percentages; and (b) analytical statistics, including the Hardy-Weinberg equilibrium (HWE) for each single nucleotide polymorphism (SNP)

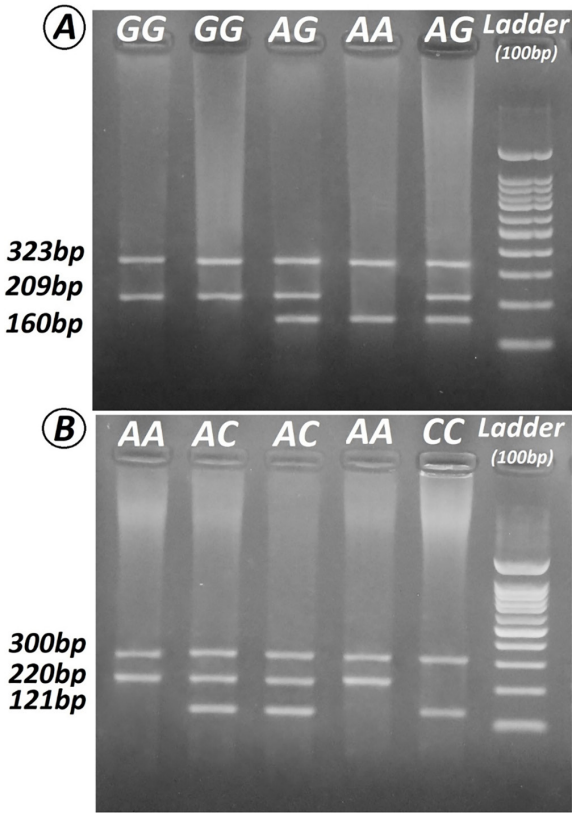

Figure 1: *BAX*-248G>A (rs4645878) and *BCL2*-938 C>A (rs2279115) polymorphisms are shown on 1.5% agarose gel electrophoresis; A) *BAX*-248G>A; the individuals homozygous for the G allele (GG genotype) were identified by the presence of products of 323 bp and 209 bp. The individuals homozygous for the A allele (AA genotype) were identified by the presence of products of 323 bp and 160 bp. The heterozygous individuals (AG genotype) were identified by the presence of all products of 323 bp, 209 bp, and 160 bp. B) *BCL2*-938 C>A; the individuals homozygous for the A allele (AA genotype) were identified by the presence of products of 300 bp and 220 bp. The individuals homozygous for the C allele (CC genotype) were identified by the presence of products of 300 bp and 121 bp. The heterozygous individuals (AC genotype) were identified by the presence of 300 bp, 220 bp, and 121 bp products. DNA ladder; 100 bp DNA size marker.

were used, which was calculated among controls. The Chi square test assessed the distribution of genotypes between groups. A *t* test was also used to analyze data using quantitative variables and continuous values. Odds ratios (ORs) and 95% confidence intervals (CIs) were used to investigate the association of polymorphisms with vitiligo risk in two genetic models.

Results

Profile of the Participants in the Study

This study had 219 participants, 112 of whom were in the control group and 107 were in the patient group. Based on *post hoc* power analysis, this sample volume obtained a power equal to 94.4% in detecting differences, which is acceptable. In terms of gender, the two groups were homogenous (The sex distribution was compared between the two groups using Pearson's Chi square test). The mean age was 41.39 years in the patient group and 41.08 years in the control group. There was no significant difference between the means (age was compared between the two groups using the Independent Samples *t* test, depending on the result of Levene's test of the homogeneity of the variances) (table 2).

The results are presented as frequency and percentages or mean±SD. The Chi square test and the independent samples *t* test was used; P<0.05 was considered statistically significant.

Investigation of Hardy-Weinberg Equilibrium in the Population

To check for balance in the population, the allelic and genotypic frequencies in the healthy population are given below for *BAX*-248G>A (rs4645878) and *BCL2*-938C>A (rs2279115) polymorphisms.

The results of our investigation showed that the genotypic frequencies of the *BAX*-248G>A gene polymorphism (rs4645878) in the healthy group (χ^2=0.035, P=0.987) followed the Hardy-Weinberg equilibrium. Additionally, as to the *BCL2* gene, the research results showed that the genotypic frequencies of polymorphism 938C>A (rs2279115) in the healthy group (χ^2=0.98, P=0.611) followed the Hardy-Weinberg equilibrium.

Assessment of Genotypic and Allelic Frequencies between the Control and Patient Groups

Data analysis showed that the GG genotype of the *BAX*-248G>A polymorphism significantly increased the probability of vitiligo. Compared to the A allele of this polymorphism, the G allele increased the risk of vitiligo disease. In other words, the results indicated that the A allele in genotypes could decrease the probability of vitiligo (table 3).

Regarding the *BCL2* gene, data analysis showed a significant relationship between the CC genotype of the *BCL2*-938C>A polymorphism and the risk of vitiligo disease. The C allele

Table 2: Demographic data of the vitiligo and control groups

Demographic data		Patients group	Control group	P value
Number of samples		107	112	
Sex	Men	41 (38.3)	56 (50)	0.082
	Women	66 (61.7)	56 (50)	
Age (years)		41.39±11.85	41.08±13.75	0.121

Table 3: Distributions of *BAX*-248G>A (rs4645878) and *BCL2*-938 C>A (rs2279115) polymorphism in the cases and controls and risk of vitiligo

Genotype		Patients (%)	Controls (%)	OR (95% CI)	P value
BAX-248G>A	AA	3 (2.8)	9 (8.1)	1	
	AG	14 (13.0)	44 (39.2)	0.95 (0.22-4.02)	0.949
	GG	90 (84.2)	59 (52.7)	4.57 (1.19-17.6)	0.027
	AA+AG vs. GG	17	53	0.21 (0.11-0.39)	<0.001
	A allele	20 (9.3)	62 (27.7)	1	
	G allele	194 (90.7)	162 (72.3)	3.71 (2.15-6.40)	<0.001
BCL2-938C>A	AA	1 (0.9)	7 (6.3)	1	
	AC	34 (31.8)	50 (44.6)	4.76 (0.56-40.46)	0.153
	CC	72 (67.3)	55 (49.1)	9.16 (1.09-76.68)	0.041
	AA+AC vs CC	35	57	0.46 (0.27-0.81)	0.007
	A allele	36 (16.8)	84 (28.5)	1	
	C allele	178 (83.2)	160 (71.5)	2.59 (1.66-4.05)	<0.001

Data are presented as numbers (%). Genotypes and alleles were compared between groups using the Chi Squared test. Binary logistic regression analysis was used. P<0.05 was considered statistically significant. OR: Odds ratio; CI: Confidence interval; BAX: Bcl-2 associated X; BCL2: B-cell lymphoma-2

Table 4: The effect combination of the genetic polymorphism of the two genes *BAX* and *BCL2* with vitiligo

BAX	BCL2	Case (%)	Controls (%)	OR (95% CI)	P value
AA+AG	CC	14 (13.1)	20 (17.8)	1	-
AA+AG	AA+AC	3 (2.8)	33 (29.5)	0.13 (0.03-0.50)	0.003
GG	CC	58 (54.2)	35 (31.3)	2.36 (1.06-5.27)	0.035
GG	AA+AC	32 (29.9)	24 (21.4)	1.90 (0.80-4.51)	0.144

Data are presented as numbers (%). Genotypes were compared between groups using the Chi square test. Binary logistic regression analysis was used to calculate. P<0.05 was considered statistically significant. BAX: Bcl-2 associated X; BCL2: B-cell lymphoma-2; OR: Odds ratio; CI: Confidence interval

Table 5: Association between parental consanguinity, smoking, and vitiligo

Variables		Patients	Controls	OR (95% CI)	P value
Family Relationships	Yes	37	24	1	
	No	74	88	1.83 (1.00-3.33)	0.048
Smoking	Yes	19	19	1	
	No	88	93	0.94 (0.47-1.90)	0.877

Data are presented as numbers. Genotypes were compared between groups using the Chi square test. Binary logistic regression analysis was used to calculate. OR: Odds ratio; CI: Confidence interval. P<0.05 was considered statistically significant.

was also associated with the vitiligo risk, and it increased the disease risk. In other words, the results revealed that the presence of the A allele in genotypes could reduce the probability of vitiligo (table 3).

Association of Vitiligo with the Concurrent Effect of BAX and BCL2 Gene Polymorphisms

Another investigation showed the effect of *BAX* and *BCL2* gene polymorphisms simultaneously with the risk of vitiligo. The results of this study are given in table 4.

The results in table 4 show that the combination of AA+AG genotypes of *BAX*-248G>A polymorphism, along with AA+AC genotypes of *BCL2*-938C>A polymorphism, has a significant association with reducing the risk of vitiligo. The combination of GG genotypes of *BAX*-248G>A polymorphism and CC genotypes of *BCL2*-938C>A polymorphism has a significant association with an increased risk of vitiligo.

Association between Parental Consanguinity, Smoking, and Vitiligo

The data analysis of this study showed a significant association between parental consanguinity, which indicates the genetic background of vitiligo patients. Consequently, parental consanguinity is a risk factor for vitiligo. Moreover, other results showed that there was no significant association between smoking and vitiligo (table 5).

Discussion

In the present study, data analysis showed that the CC genotype of the *BCL2*-938C>A polymorphism significantly increased the probability of vitiligo. Considering the role of the C allele as mentioned, it can be concluded that the CC genotype is associated with a decrease in the *BCL2* expression level. The increase in the frequency of the CC genotype in vitiligo compared to the control group shows that the increase in apoptosis may destroy more melanocytes and, as a result, the appearance of vitiligo symptoms. Until now, no study has been conducted on the association between *BCL2*-938C>A polymorphism and vitiligo disease. However, since the process of apoptosis in melanocytes causes the appearance of vitiligo symptoms, studies were conducted that showed the association between *BCL2*-938C>A polymorphism and the expression level of this gene.

Considering the role of the C allele, it can be concluded that the CC genotype is associated with a decrease in the *BCL2* expression level.[15] Hirata and others observed that kidney cancer tissues carrying the CC genotype had a significantly lower expression of BCL2 than those carrying the CA+AA genotype.[19] Our study shows similarities with previous reports on the association of the CC genotype with increased apoptosis. Considering the role of *BAX* in apoptosis, G (-248) A polymorphism can cause changes in BAX expression and disrupt the apoptosis process.[8, 20]

Previous studies have shown that the GG genotype is associated with decreased BAX expression, which is inconsistent with our study.[21] Ma and others found that the GG genotype might lead to overexpression of BAX and cause abnormal cell apoptosis.[22] These results are consistent with our study. Additionally, the results of our research showed that the GG genotype of the *BAX*-248G>A polymorphism significantly increased the probability of vitiligo,

and considering the role of apoptosis in vitiligo, it can be concluded that the GG genotype is associated with a higher expression of BAX, which leads to clinical manifestations of vitiligo.

Wang and others demonstrated that the 938AA genotype of the *BCL2* gene might be a susceptible genotype for non-Hodgkin lymphoma (NHL), and this genotype is associated with a larger tumor size in NHL.[23] Yao and others showed a statistically significant association between rs2279115 and cancer susceptibility. Subgroup analysis indicated that rs2279115 was associated with a significantly higher risk of cancer susceptibility in Asia but not in Caucasians. Furthermore, rs2279115 was associated with a considerably higher risk of digestive system cancer and endocrine system cancer, but not breast, respiratory, and hematopoietic cancers.[24]

Cingeetham and others found that the *BCL2*-938CA and *BAX*-248GG genotypes are significantly associated with an increased risk of acute myeloid leukemia (AML). The *BAX*-248A allele showed a lower risk of AML. They concluded that the gene polymorphisms *BCL2*-938C>A and *BAX*-248G>A might contribute to the development of AML. In Addition, the GG genotype and G allele frequencies were significantly higher. Conversely, the A allele was linked to a reduced risk of AML.[25]

Moreover, in previous chronic lymphocytic leukemia (CLL) studies, the GG genotype and G allele of the polymorphic locus 248 G>A were associated with an elevated risk of CLL.[26] However, the rs4645878 variant (G125A) of the *BAX* promoter showed an association with decreased CLL risk.[27] Fernandes and others have provided evidence that G allele carriers in the promoter regions of *BAX*-248G>A are related to the risk of squamous intraepithelial neoplasia development but not the disease severity.[28] In contrast, Al-Zubaidy and others concluded that there was no significant difference in the *BCL2*-938C>A polymorphism of all genotype models compared to healthy models in Iraqi women with breast cancer.[29]

Results from previous studies and our research show that polymorphisms in the promoter of *BCL2* and *BAX* genes can significantly alter protein function or expression, thereby disrupting the regulation of apoptosis. This disruption can lead to a disturbance in cellular homeostasis and potentially contribute to malignant transformation.

In general, the results of different studies related to the association between *BAX*-248G>A and *BCL2*-938C>A polymorphisms and BAX and BCL2 expression levels are dissimilar. However, further confirmation in more extensive studies is needed to clarify the role of these polymorphisms in vitiligo.

One limitation of this study was not using the matching process, which is recommended to be used in future studies. Moreover, contrary to previous studies, we found no significant association between gender and smoking and the risk of vitiligo in men and women.[30, 31] One of the key findings of the present study was a significant association between parental kinship and the risk of vitiligo, suggesting that inherited risk factors from parents may contribute to the development of the condition. These results are in line with previous studies that have shown that individuals with consanguineous parents have an increased risk of developing vitiligo.[32, 33]

Conclusion

Our data support the role of apoptosis-related *BAX*-248G>A (rs4645878) and *BCL2*-938C>A (rs2279115) polymorphisms in vitiligo susceptibility. Therefore, these polymorphisms can be utilized as biomarkers for the early diagnosis of vitiligo.

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Authors' Contribution

A.D: Data gathering, data analysis, drafting, and critical reviewing; N.H: Data gathering, data analysis, and critical reviewing; F.H: Data gathering and critical reviewing; I.J: Study design, data gathering, data analysis, and critical reviewing. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work to ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflicts of Interest: None declared.

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