ORIGINAL ARTICLE

Study of the regulatory promoter polymorphism (−938C>A) of B-cell lymphoma 2 gene in breast cancer patients of Mazandaran province in Northern Iran

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Background: The incidence rate of breast cancer has been dramatically increasing since the last decade in Iran, and it is now one of the most common female malignant tumors. B-cell lymphoma 2 (BCL2) family is the most important regulator of apoptosis, and −938C>A single nucleotide polymorphism (SNP) of *BCL2* gene promoter has been demonstrated to influence breast cancer susceptibility. In this research, we study the effect of −938C>A allelic variants on breast cancer risk in Mazandaran province at the North of Iran. **Materials and Methods:** This analysis performed on 120 breast cancer patients who underwent surgery in some referenced hospitals at Mazandaran province along with 130 healthy individuals as a control. DNA extracted from peripheral blood samples was applied in polymerase chain reaction-single-strand conformation polymorphism analysis to determine −938C>A genotype. The association of the −938C>A genotype and breast cancer risk as well as clinicopathological characters were analyzed by logistic regression method. **Results:** Results showed that genotype frequency of AA, AC, and CC genotypes was 10%, 62%, and 28% for case and 28%, 50%, and 22% in control group, respectively. In the logistic regression model, *BCL2* – 938C/A variant genotype AA was associated with a decreased risk of breast cancer ($P = 0.041$) by 0.31-fold (odds ratio = 0.31, confidence interval = 0.091–0.909) compared to CC genotype. However, no significant association found between −938C>A genotype and clinicopathological characters. **Conclusion:** The study showed that AA genotype of BCL2 gene (−938C>A) is associated with decreased susceptibility to breast cancer. Hence, investigating the −938C>A SNP of BCL2 gene promoter could be an appropriate molecular marker to determine individual sensitivity to breast cancer.

Key words: −938C>A, B-cell lymphoma 2, breast cancer, polymerase chain reaction-single-strand conformation polymorphism, polymorphism

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INTRODUCTION

Breast cancer is the most prevalent malignancy and the causal agents of 19% of cancer-related death among women worldwide.[1-3] The incidence rate of breast cancer has been dramatically increasing since the last decade, and with the age-standardized incidence rate of 28.25% in 100,000 women, it is now the most frequent malignancies among Iranian women.[4] Reports show that Iranian breast cancer cases are approximately one decade younger than their Western counterparts.[5] Epidemiological studies defined various risk factors of breast cancer including genetic mutations, reproductive history, and environmental carcinogens in different population with ethnic and geographical variations.[6-8] The genotypic variants specially those play roles in apoptosis or cell proliferation have also been reported to be involved in breast carcinogenesis. Apoptosis or programed cell death occurs normally during development as a homeostatic mechanism to maintain

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cell populations in tissues.[9] Inappropriate apoptosis either too little or too much has the effective role in cancer progress and metastasis.[10,11] Apoptosis can be achieved through two different pathways including death receptor and mitochondrial pathway.[12] The B-cell lymphoma 2 (BCL2) family proteins as the most important regulator of mitochondrial pathway include both proapoptotic members such as BCL2-associated X protein (Bax) and BAX-like death factor (Bak) and antiapoptotic/antiproliferative members such as BCL2 itself and BCL-xL.[13] *BCL2* gene which is placed on 18q21.3 consists of three exons and two introns coding for a 25 KDa protein.[14] Despite the antiapoptotic property of *BCL2* gene, conflicting results have been acquired regarding to the association of its expression level and patient's survival and seems to be tissue specific.[15-17] *BCL2* gene expression is regulated through alternative promoters including P1 and P2 having distinct regulatory roles. The P1 promoter has positive regulatory effect on *BCL2* gene expression, whereas P2 promoter located 1400 bp upstream of the translation initiation site modulates negative regulatory effect of *BCL2* gene expression which is exerted through P1 promoter.[18] Single nucleotide polymorphisms (SNPs) of *BCL2* genes have been reported to be involved in several cancer types including chronic lymphocytic leukemia,[19,20] prostate cancer,[21] ovarian cancer,[22] breast cancer,[23] renal cancer,[24] oropharyngeal squamous cell carcinoma,[25] and sporadic medullary thyroid carcinoma.[26] The −938C>A SNP in P2 promoter of *BCL2* gene was initially reported by Park *et al*., through sequencing of this region in 24 DNA samples of Korean females.[27] The association between −938C>A genetic variants and cancer progression was subsequently studied through investigation of several cancers in different population. Nückel *et al*. showed that − 938C allele is significantly associated with an increase in P2 promoter activity which inversely decreases *BCL2* gene expression in B-cells derived from chronic lymphocyte leukemia (CLL) patients.[28] Zhang *et al*. showed that AA genotype of −938C>A is positively associated with increased breast cancer susceptibility in Chinese population.[29,30] However, some reports indicated no significant association between *BCL2* gene expression and −938C>A allelic variants.[31] In this report, we studied the association between −938C>A allelic variants and breast cancer risk of women in Mazandaran province at Northern Iran.

MATERIALS AND METHODS

Cases and controls

This case–control study involved 120 patients who underwent surgery in some referenced hospitals in Mazandaran province from September 2012 to December 2014. Patients' age ranged from 29 to 72 years. Demographic and clinicopathological data were extracted from patients' record available in hospitals. A group of 130 healthy females ranging from 26 to 79 years were also included in this study to investigate whether certain −938C>A genotype is a susceptible marker. Five milliliters peripheral blood was collected from both patients and control group and stored at −80°C.

DNA extraction and polymerase chain reaction amplification

Blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes, and genomic DNA was extracted from blood lymphocytes by proteinase K/SDS digestion and phenol–chloroform extraction method.[20] DNA concentration was measured spectrophotometrically, and its purity was checked through agarose gel electrophoresis. A 253 bp fragment containing − 938C/A allelic variant was amplified using forward: 5-TTATCCAGCAGCTTTTCGG-3 and reverse: 5-GGCGGCAGATGAATTACAA-3 primers. Polymerase chain reaction (PCR) amplification performed in 25 μl reaction containing 1X PCR buffer, 100 ng genomic DNA, 1.5 mM MgCl2, 0.3 mM each forward and reverse primers, 0.2 μM dNTPs, and 2.5 U taq DNA polymerase (10 U/μl). The cycling conditions including an initial denaturation at 94°C for 4 min, 32 cycles of denaturation at 94°C for 40 s, annealing at 52°C for 35 s, and extension at 72°C for 37 s and a final extension at 72°C for 7 min. Products were analyzed by electrophoresis at 1.5% agarose gel and visualized by ethidium bromide staining.

–938C>A genotyping

The −938C>A genotype of *BCL2* P2 promoter was determined using single-strand conformation polymorphism (SSCP) analysis. The amplified fragments were purified using GF-1 Gel DNA Recovery (extraction) Kit (Vivantis, Malaysia) according to manufacturer's instruction and their concentration determined by spectrophotometer. Equal amount of each sample (approximately 1 μl) was added to 9 μl of denaturing solution (95% formamide, 20 mM EDTA pH 8.0, 0.05% bromophenol blue, and 0.05% xylene cyanol), boiled for 10 min, and immediately chilled on ice. Electrophoresis performed in a 12% nondenaturing acrylamide gel at 350 volts for 3 h. The gel was visualized after silver staining, and the genotypes were determined based on resulting banding pattern.[32] Two samples of each SSCP profiles were gel-purified and sequenced in Bioneer company (South Korea) to verify the results of genotyping.

Statistical and sequence analysis

The genotype and allele frequency of −938C>A genotype were tested for Hardy–Weinberg equilibrium (HWE) for both patient and control group using Chi-square test. Odds ratio (OR), confidence intervals (CIs), and *P* values were calculated using unconditional logistic regression and adjusted for age to estimate the association between genotypes and the risk of breast cancer or some demographic and

clinicopathological data. Data analysis carried out by SAS 9.1 statistics software (The SAS Institute, NC, USA) and $P < 0.05$ was considered statistically significant.[33] Nucleotide sequence analysis performed by BioEdit 7.9.1 program. The acquired sequences were compared with those available in databases using basic local alignment search tool (BLAST) program and alignment done by ClustalW method.[34]

RESULTS

Demographic and clinicopathological data

This study performed on 120 breast cancer patients and 130 healthy controls with known demographic and clinicopathological data [Table 1] that belong to Mazandaran province in Northern Iran. The mean age of patient and healthy individuals was 47.13 ± 8.4 years and 46.5 ± 7.2 years, respectively, and Student's *t*-test showed no significant relationships between two groups ($P = 0.312$). Results of Chi-square test showed that the genotype frequency of case and control groups did not significantly diverge from HWE (both $P > 0.05$). Demographic data analysis indicated that only 10% of patients had a positive familial history and just 7% of them belonged to smoker group. Pathologic information demonstrated that 93.7% of patients were diagnosed at Grade II and III and 58% of them were at advanced stage when the disease detected for the first time.

The −938C>A genotype distribution and its association to known clinicopathological data

A single DNA band was appeared in 1% agarose gel following PCR amplification [Figure 1]. SSCP analysis showed that three distinct profiles were appeared in acrylamide gel [Figure 2]. Results of nucleotide sequencing and sequence database searches by the on-line BLAST tool confirmed them as belong to *BCL2* gene P2 promoter. Nucleotide sequence analysis of the amplified fragments each having unique SSCP profile with BioEdit program determined the presence of variant alleles at nucleotide position −938C>A [Figure 3]. The −938C>A genotype of all samples was subsequently determined according to known SSCP profiles and resulting genotype distribution was presented in Table 2. Results showed that the allelic frequency of AA, AC, and CC genotypes was 10%, 62%,

Table 1: The demographic and clinicopathological characteristics of patients[a]

Clinicopathological variables	Number of patient (%)
Age	
≤45	45 (46)
>45	53 (54)
Menopause	
Positive	43 (44)
Negative	55 (56)
Grade	
I	6 (6.2)
II	56 (58.3)
III	34 (35.4)
TNM staging	
I-II	32 (42)
III-IV	44 (58)
Family history	
Positive	11 (10)
Negative	99 (90)
Smoking	
Positive	8 (7)
Negative	106 (93)

[a]Numbers and percentages of patients who their characteristics were available. TNM=Tumor node metastasis

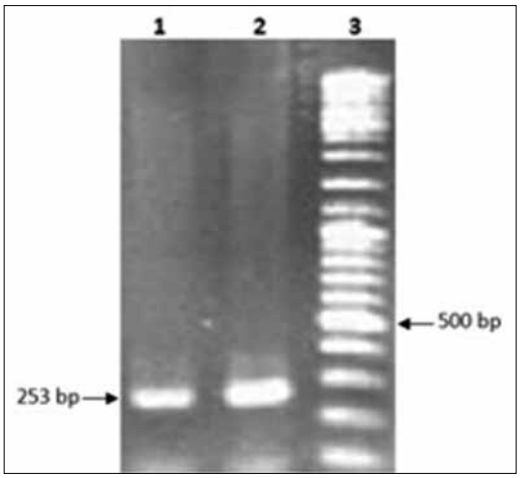

Figure 1: Electrophoresis of the amplified fragment of B-cell lymphoma 2 P2 promoter in 2% agarose gel. 1, 2: 253 bp fragment; 3: 100 bp DNA ladder (fermentas)

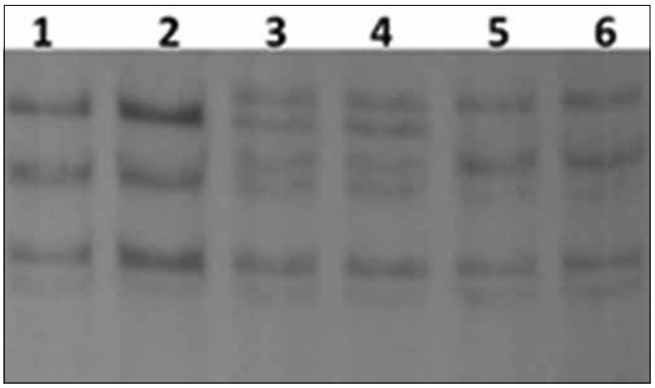

Figure 2: Single-strand conformation polymorphism profile of the amplified 253 bp fragment of B-cell lymphoma 2 P2 promoter in 12% nondenaturing acrylamide gel. 1, 2: CC genotype; 3, 4: AC genotype; 5, 6: AA genotype

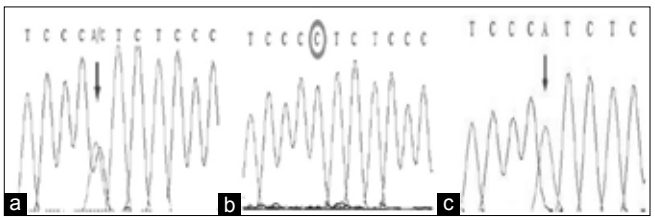

Figure 3: Sequencing profile of three allelic variants of the position −938C>A of *BCL2* gene. (a) AC genotype; (b) CC genotype; (c) AA genotype

and 28% for case and 28%, 50%, and 22% in control group, respectively. As shown in Table 2, in logistic regression model, *BCL2* −938C/A variant genotype AA was significantly associated (P = 0.041) with a decreased risk of breast cancer by 0.31-fold (OR = 0.31, CI = 0.091–0.909) compared to CC genotype. The variant genotypes AA and AC + CC were not significantly correlated with demographic and clinicopathological characteristics including age at diagnosis (P = 0.69), menopause (P = 0.357), grade (P = 0.89), smoking (P = 0.777), and family history (P = 0.357) [Table 3].

DISCUSSION

The incidence rate of breast cancer has dramatically increased since last decade in Iran and it is now one of the most common female malignant tumors.[4] Antiapoptotic BCL2 protein involves in mitochondrial pathway of apoptosis through cell cycle arrest which is commonly led to patients survival. Nevertheless, its increased expression was denoted to be associated with unfavorable outcome in some cancer types.[15-17] The *BCL2* gene −938C>A SNP initially identified by Park *et al.* have also been reported to be controversially involved in CLL development. However, consistent results were reported regarding to its association with solid tumors development such as breast cancer.[22-25] We conducted a case–control population-based study to determine the *BCL2* gene −938C>A allelic variants and its association with breast cancer risk or clinicopathological features in Mazandaran province at Northern Iran. The mean age of breast cancer patients in this study was 47.13 ± 8.4 years which is consistent with the results of

Table 2: Distribution of−938C>A polymorphisms of B-cell lymphoma 2 P2 promoter and its association to breast cancer risk

Genotype	Number of subjects (%)		Nonadjusted[a]		Adjusted[b]		
	Case	Control	*P*	OR	*P*	OR	CI
AA	12 (10)	36 (28)	-	1			
AC	74 (62)	65 (50)	0.0337	0.288	0.041	0.31	0.091-0.909
CC	34 (28)	29 (22)	0.0537	0.281	0.063	0.3	0.077-1.02
AC+CC	108 (90)	94 (72)	-	1			
AA	12 (10)	36 (28)	0.0559	0.345	0.065	0.365	0.116-1.027
AC+AA	86 (72)	101 (78)	-	1			
CC	34 (28)	29 (22)	0.4893	1.379	0.51	1.35	0.555-3.427
AA+CC	46 (38)	65 (50)	-	1			
AC	74 (62)	65 (50)	0.228	1.632	0.32	1.58	0.736-3.616
Total	120	130					

[a]Logistic regression model, nonadjusted, [b]Logistic regression model, adjusted for diagnostic age. OR=Odds ratio, CI=Confidence interval

Table 3: Relationship between−938 C>A allelic variants of B-cell lymphoma 2 P2 promoter and known clinicopathological variables

Clinicopathological variables	All	Genotype (%)		Adjusted[a]		
		AA	AC+CC	*P*	OR	CI
Age						
≤45	45 (46)	4 (8.9)	41 (91.1)	-	1	-
>45	53 (54)	6 (11.3)	47 (88.7)	0.69	1.3	0.345–4.96
Menopause						
Negative	43 (44)	3 (7)	40 (93)	-	1	-
Positive	55 (56)	7 (12.7)	48 (87.3)	0.357	1.94	0.472–8.01
Grade						
I-II	62 (64.6)	5 (8.1)	57 (91.9)	-	1	-
III	34 (35.4)	3 (8.8)	31 (91.2)	0.89	1.1	0.247–4.928
TNM staging						
I–II	32 (42)	3 (9.4)	29 (90.4)	-	1	-
III–IV	44 (58)	4 (100)	40 (90.9)	0.966	0.967	0.201–4.653
Family history						
Negative	11 (10)	2 (18.2)	9 (81.8)	-	1	-
Positive	99 (90)	9 (9.1)	90 (90.1)	0.357	0.455	0.085–2.438
Smoking						
Negative	8 (7)	1 (12.3)	7 (87.5)	-	1	-
Positive	106 (93)	10 (9.4)	96 (90.6)	0.777	0.729	0.081–6.536

[a]Logistic regression model adjusted for diagnostic age. All statistical tests were two-sided with a significance of P<0.05. OR=Odds ratiop; CI=Confidence interval; TNM=Tumor node metastasis

other researches in Iran and verifying again the younger age of breast cancer development for Iranian women.[35,36] Numerous reports have highlighted positive association between familial disease history or smoking habit and cancer incidence. However, in this study, only 10% of cases appeared to have positive disease history and just 7% of them belonged to smoker group. Most patients were identified to be at Grades II (58.3%) and III (35.4%) and Stages III and IV (58%) when disease was firstly diagnosed. Similar results were also reported in a research conducted on a group of 3085 women with breast cancer in Iran.[36] These reports highlight that cultural and social issues, lifestyle change, and reproductive behaviors are among key factors for developing different clinicopathological features in Iranian women. Breast self-examination and regular evaluations may be helpful for screening of the disease at primary stage.[37] Genotype frequency of *BCL2* gene −938C>A SNP studied using PCR-SSCP analysis confirmed the presence of three AA, AC, and CC genotypes in both cases and healthy controls. Statistical analysis by logistic regression model showed that the variant genotype AA was significantly associated ($P = 0.041$) with a decreased risk of breast cancer by 0.31-fold (OR = 0.31, CI = 0.091–0.909). Previous researches conducted on the association between −938C>A polymorphism and cancer susceptibility including CLL and breast cancer reported positive association between AA genotype and favorite outcome for patients survival.[23,28,38] However, study of −938C>A polymorphism in breast cancer patients of Chinese Mainland showed that the homozygous AA genotype is associated with an increased risk of breast cancer development by 2.37-fold. Overall, these results emphasize that the *BCL2* (−938C>A) AA genotype can serve as a susceptible factor in breast cancer. However, its association with disease incidence either positive or negative remained to be elucidated and complementary results are needed to confirm whether *BCL2* (−938C>A) allelic variants can serve as a prognostic biomarker for breast cancer patients. The study of the relationship between *BCL2* (−938C>A) genotype and clinicopathological characteristics such as age, menopause, smoking, family history, and stage of cancer in this study demonstrated no significant correlation. The study of the association between *BCL2* (−938C>A) allelic variants and clinicopathological features did not create consistent results.[21,30,37] The discrepancy between the results of the clinicopathological properties and the association between SNP and breast cancer risk may be due to several factors including difference in ethnicity, diet, geographical variation, and environmental exposures.[31,39] Particularly, the effects of pesticides on disease incidence should be studied with more priority as it is massively applied in Northern Iran.[8] Further researches, conducted on a larger group, are needed to clarify these points.

CONCLUSIONS

This population-based case–control study performed on 120 breast cancer patients and 130 healthy women as control to determine −938C>A allelic variants of *BCL2* P2 promoter in Mazandaran province at Northern Iran. Results showed that AA genotype of *BCL2* gene (−938C>A) is associated with decreased susceptibility to breast cancer. No significant correlation found between −938C>A genotype and clinicopathological data. Hence, investigating the −938C>A SNP of BCL2 gene promoter could be an appropriate molecular marker to determine individual sensitivity to breast cancer.

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Conflicts of interest

There are no conflicts of interest.

AUTHORS' CONTRIBUTION

AB and SEM contributed in the conception of the work, revising the draft, approval of the final version of the manuscript, and agreed for all aspects of the work. NN performed surgery and provided samples.

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