

Polymorphism of BCL-2 (-938C > A) and associated with breast cancer susceptibility of Iraq population

Najwa Shihab Ahmed¹, Mahmood² Ahmed Muhi, Najlaa³ Qassim Muftin³

¹Biotechnology Research Center, Al-Nahrain University, Baghdad

²Biology Department, Al-Farabi university college, Baghdad-Iraq

³Chemistry Department, College of Science, Mustansiriyah University, Baghdad-Iraq.

Abstract

Background:

The B-cell leukemia/lymphoma 2 gene (BCL-2) family encodes pro- and anti-apoptotic proteins that play a significant role in a programmed cell death. It has been observed that (SNP; -938C > A) associated with breast cancer incidence. The aim of this study is to investigate the effect of polymorphism of BCL-2 gene (-938C > A) on breast cancer injury.

Methods:

This study involved the distribution of (-938C > A) among 60 patients and 30 controls by PCR-RFLP method. PCR products of forward strands of the gene BCL-2 were sent for sequencing in order to confirm the results of RFLP-PCR and these sequences were alignment with the standard sequence of BCL-2 gene found in NCBI through Bioedit program.

Results :

This study observed absent of TT genotype (0) 0% in patients and controls. The distribution of A allele was 0.48 versus 0.13 in patients and controls respectively while the distribution of C allele was 0.87 versus 0.52 in controls and patients respectively. Also this study revealed that subjects with heterozygous CT genotype of the BCL-2 gene have a 79 fold increased risk of breast cancer compared to those carrying the CC genotype (OR=79.8, CI=15.97-398.32, X²=50.1, and P=2.4x10⁻¹²). This study also included registration two sequences with GG,GT genotypes in Gene Bank NCBI.

Conclusion :

We concluded from this study that subjects with CT genotype of BCL-2 (-938C > A) are associated with breast cancer injury, we need more investigation to confirm this result.

Keywords: Breast cancer, BCL-2 gene, Polymorphism, Allele frequency

INTRODUCTION

Breast cancer has become the most common female malignancy around the world. Each year, there're over one million women diagnosed with this disease, With 400,000 deaths (1). As in other cancers, breast cancer happens depending on the interaction between genetic changes and the environment. It has been reported that an accumulation of genetic variants is involved in the process of breast carcinogenesis (2). There are a number of genetic variants that have a part in apoptosis or cell division proliferation, since the equalization between those two processes. The direction of the breast determines either the development of normal breast tissue or the direction of mastectomy of the mammary gland (2). Apoptosis is a process by which the cell dies in a programmed manner. It is excited through several pathological or physiological death signals to eliminate damaged and excessive cells for keeping of tissue homeostasis, therefore it plays a significant role in the growth and functionality of multicellular organisms. At natural tissue, there is accurate homeostasis between apoptosis and proliferation, it is organized by large assortment of the BCL2 family proteins, these proteins behave as either oncogenic or tumor suppressive. These conflicting of BCL2 family proteins appear to be tissue specific especially with respect to different cancers (3). The BCL2 gene, situated on chromosome 18q21.3, composed of three exons and two promoters (P1, P2) with various functions. The P1 promoter is responsible of the transcription process, where most of the BCL-2 transcription is initiated by P1, it

located on about 1.7 KB upstream of translation, this region is rich in GC box that can be incorporated with Sp-1, however it has no typical TATA box. The P1 promoter initiated transcription mainly from GC box near the beginning, this is very like with other housekeeping gene promoter. It has been observed from analysis of chromosome structure of P1 that it could be an essential promoter. (4,5) while the P2 promoter situated at 1400-bp upstream of the translation initiation site, it acts as a negative regulator of the P1 promoter (6). Compared with P1, the P2 promoter is fundamentally an inducible promoter, and a small part of Bcl-2 transcription is driven by the P2 (4).

The SNP (rs2279115) is located in the inhibitory P2 promoter of the BCL2 gene (7,8). The C allele in comparison to the A allele showed significantly increased inhibition of BCL2 promoter activity and binding of nuclear proteins (9). There is increasing evidence that Bcl-2 gene polymorphism may be associated with cancer susceptibility and prognosis (4). Several researchers show that cancer, neurodegenerative disorders, ischemia and autoimmune diseases are associated with Bcl-2 function abnormalities (10-12,13). High expression of Bcl-2 is associated with different cancer types, and has been reported in esophageal cancer, non-small cell lung cancer, endometrial cancer, breast cancer, prostate cancer, lung cancer, chronic lymphocytic leukemia, diffuse large B-cell lymphoma etc. [14-18]. Furthermore, -938C>A genotypes have an impact on risk [19, 20], outcome [21, 22], and drug response in different diseases [23, 24]. Therefore this

study were designed to identify the association between BCL2 (-938C>A) polymorphisms and breast cancer susceptibility.

MATERIAL AND METHOD

Subjects

This study consists of 60 patients with breast cancer. The mean of age of these patients is 31-70 years and 30 females as a control group with mean age is (30-60) years. All these samples of patients are collected from Hospital in Baghdad- Iraq. Five mL of blood were collected into EDTA tube from both cases and controls. DNA was extracted using genomic DNA extraction kit (Quiagen). The products of DNA extraction were verified by horizontal electrophoresis in 1% agarose. DNA concentrations were determined using Nanodrop and DNA quality was determined by gel electrophoresis of extracted products.

PCR-restriction fragment length polymorphism (PCR-RFLP) method analysis of BCL2 polymorphism

SNP rs2279115 (-938C > A) Genotyping on promoter region. Primers were designed specific to this region that flanked SNP rs2279115 as follows: up strand primer 5'TTATCCAGCAGCTTTTCGG-3' and down strand primer 5'-GGCGGCAGATGAATTACAA-3'. The PCR conditions were set as follows: 94°C for 7 min, 40 cycles of 94°C for 30 s, 61°C for 30 s, and 72°C for 30 s and a final extension step of 72°C for 7 min. After the completion of PCR reaction, 10 µl product from each sample was digested by *BclI* Enzyme (NEB, Beijing, China) for 2 hours. After electrophoresis on 3% agarose gel, photographs were taken under ultraviolet light trans-illuminator. The amplified PCR fragments (252bp) were processed with Nucleotide Removal Kit (Quiagen, Catalogue # 28304) to remove excess dNTPs, MgCl₂, DNA, Primer, loading dye. Sequencing of gene having 50-100 ng/µl concentrations was performed by macrogen company / Korea online at (<https://dna.macrogen.com/eng/member/login.jsp>), machine is DNA sequencer using 23 ABI 3730XLs, Applied Biosystem, Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (ABI Prism, Catalogue # 4303152) was used For sequencing reactions. Cycle sequencing employs a thermo-stable DNA polymerase and di-deoxy-nucleotides are used in a polymerization reaction to create a nested set of DNA fragments with dideoxynucleotides at the 3' terminus of each fragment. As each of the four dideoxies labeled with a different color fluorescent dye, the reaction can be performed in single tube and the sequence can be determined easily. Electropherograms of each sample were analyzed and ambiguities in the sequence data are solved by comparison of the results of both forward and reverse primers as well as by comparison with the BCL2 sequences in the NCBI database. Allele of each sample were determined after sequence analysis. The compatibility of nucleotide bcl2 gene with gene bank was available at the National Center Biotechnology Information (NCBI) online at (<http://www.ncbi.nlm.nih.gov>) was conducted using Basic Local

Alignment Search Tool (BLAST) program and BioEdit program.

Submission of Nucleotide sequencing in NCBI

The DNA sequencing of B-cell leukemia/lymphoma 2 gene have been deposited in gene bank database under the accession number KY996478.1 that represent bcl2 gene were isolated from healthy Iraqi population (Homozygote GG) and KY996479.1 from breast cancer (Heterozygote TG).

Statistical analysis

The Pearson χ^2 criterion ($p < 0.05$), odds ratios (OR) and 95% confidence intervals (CI) tests were used to compare the frequencies of polymorphisms of BCL-2 between the two groups, using the SASS program.

RESULTS AND DISCUSSION

The current study investigates -938C > A polymorphism in promoter region of BCL-2 gene, the correlation between the polymorphism of this gene and risk of breast cancer were studied. A 252 bp fragment containing -938C>A polymorphism in promoter region of BCL-2 gene was amplified as band, Figure (1). Several samples from cancer group were chosen randomly to show the PCR-RFLP results, Undigested 252 bp products represent CC genotype, and totally digested 154 and 98 bp products represent AA genotype; CA heterozygous genotype showed both the undigested 252 bp band and digested 154 and 98bp bands, Figure (2).

In this paper, DNA sequencing was used to confirm our result of genotyping. The sequencing of amplified product of Bcl-2 gene from 90 sample, 24 of them appeared as 100% compatibility with standard gene, from 13 to 222 number of nucleotide from gene of Gene Bank results as shown in Figure 3, Sequence ID: ref[XM_011526135.1], and have number score (389) bits whereas 66 sequencing of amplified product of Bcl-2 appeared as 99% compatibility to standard gene in Gene Bank from 12 to 222 with score number (370) bits as shown in Figure 4. Frequency readings sequence of nucleotides of Bcl-2 gene shown in figure 3 and figure 4 by using BioEdit program, which was performed by National Instrumentation Center for Environmental Management (nicem) online at (http://nicem.snu.ac.kr/main/?en_skin=index.html), biotechnology lab, machine is DNA sequencer 3730XL, Applied Biosystem, Homology search was conducted using Basic Local Alignment Search Tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at (<http://www.ncbi.nlm.nih.gov>).

By the use of sequence of nucleotides and nitrogenous bases of BCL2 gene technology by the way mentioned in chapter "materials and methods", Initial genotyping data using the curve method revealed 11(73.3%) clear G/G homozygotes, 4(26.7%) clear G/T heterozygotes, as showed in table 2, figure 5 shows sample curves that represent each of the two genotypes.

The results of allele distribution in table (1) showed each allele and their related frequencies in each different population. The first SS number (ss1361473805) showed the higher frequency of C allele in Africa (AFR) population and the higher A frequency appeared in East Europe (EUR). While the SS137429571 showed only A allele and the both ss168385600 and ss203388312 showed equal frequencies for A and G alleles . the highest frequencies of G allele clearly noticeable in both ss227920630 and ss76894403. While the other accession numbers (ss237511604, ss243751036, ss3216615 and ss6904557) showed nearly a similar frequencies with the dominance of G allele.

After analysis of -938C > A polymorphism for females patients with breast cancer and females controls in this study, it was reached to absent of AA genotype (0) 0% in the two groups.

In control group ,the results showed that there was non-significant increase in CC genotype 22(73.3)compared to

AC genotype 8(26.7) ($X^2=0.710,P=0.3994$),whereas in patients group the results revealed that there was significant increase in CT genotype 58(96.66) compared to CC genotype 2 (3.33) ($X^2=52.51,P=0.0000$)as shown in Table(2). The distribution of A allele in patients was 0.48 versus 0.13 in control while the distribution of C allele was 0.87 versus 0.52 in control and patients, respectively. Our study showed that carriers of the heterozygous CT genotype at rs2279115 polymorphism of the BCL-2 gene have a 79 fold increased risk of breast cancer compared to those carrying the CC genotype($OR=79.8$, $CI=15.97-398.32$, $X^2=50.1$, and $P=2.4 \times 10^{-12}$),(Table 2). This observation was confirmed in the analysis of Meka *et al.*,2015(3)who also found that, AA genotype of BCL2-938C>A polymorphism might greatly influence BCL2 gene expression there by associated with higher risk for breast cancer development and progression.

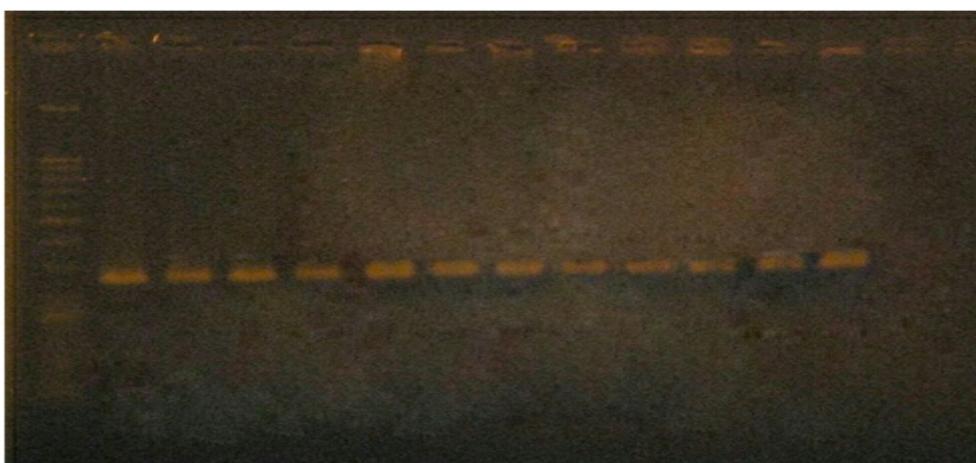


Figure 1:Agarose gel electrophoresis for amplifiedBCL2 gene. Bands were fractionated by electrophoresis on a 1.5 % agarose gel (2 h., 5V/cm², 0.5X TBE buffer) and visualized under U.V. light after staining with ethidium bromide staining. Lane: M:100bp ladder; Lane: 1-11 product forBCL2gene (252bp).

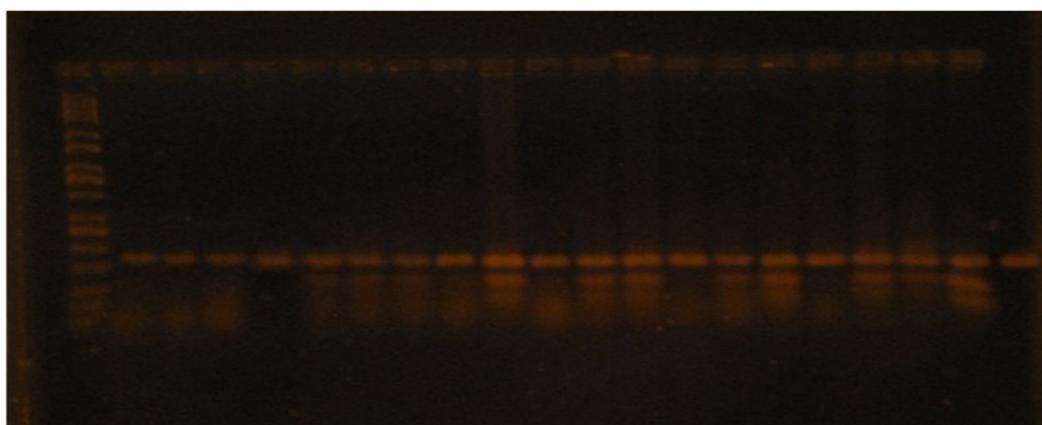


Figure 2: Bcl-2 (-938C > A) genotyping results. BCL-2 (-938C > A) polymorphism genotyping with PCR-RFLP, Undigested 252 bp products represent GG genotype sample number 1,2,3,4,13,16,20 , and TG heterozygous genotype showed both the undigested 252 bp band and digested 154 and 98 bp bands sample number 5,6,7,8,9,10,11,12,14,15,17,18,19.

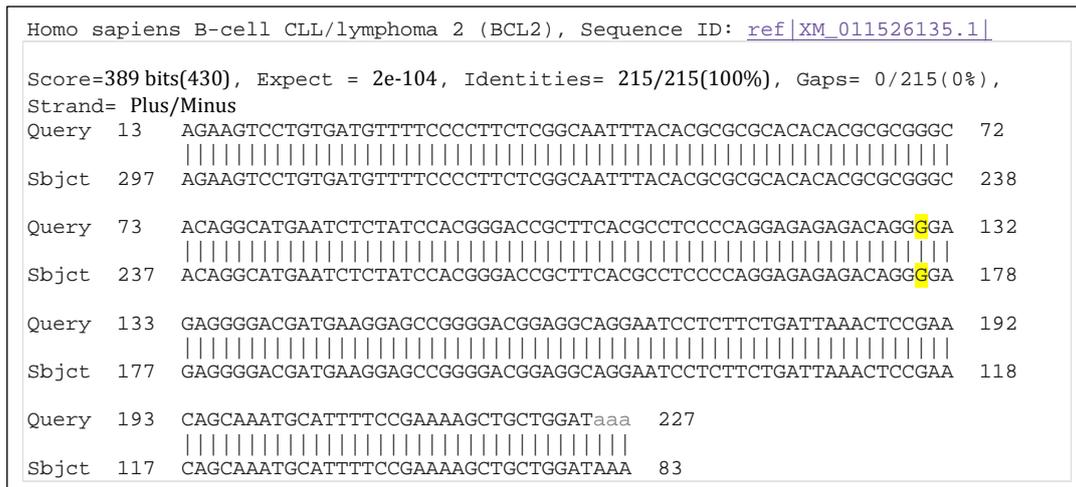


Figure 3 : Sequencing of sense (BCL2)gene for controls with homozygous (G/G) as compared with wild type (BCL2) obtained from Gene Bank.

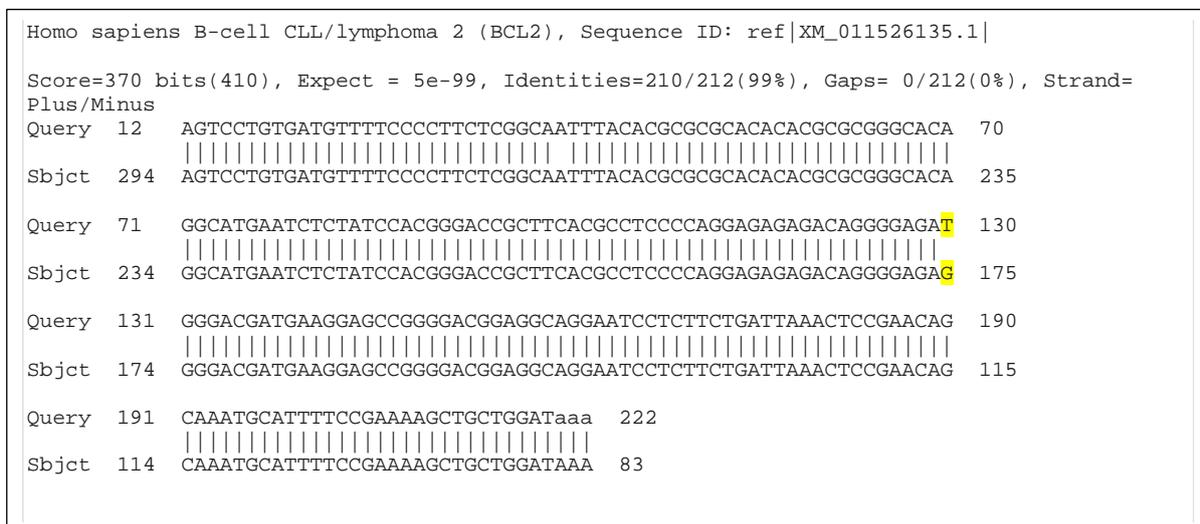


Figure 4 : Sequencing of sense (BCL2)gene for patients with heterozygous (G/T) as compared with wild type (BCL2) obtained from Gene Bank.

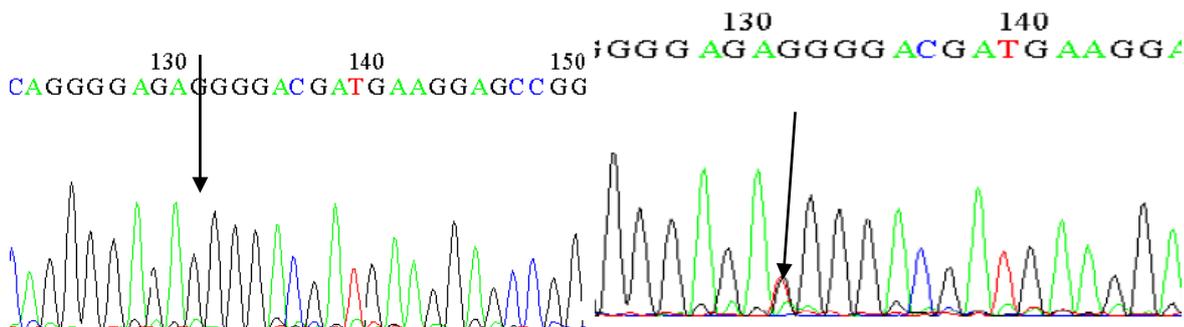


Figure 5: Nucleotide sequence chromatogram representing two does genotype of BCL-2 (-938C>A) gene. The left figure is a GG homozygote, and the right figure is a G/T heterozygote

Table 1: Distribution of alleles A and C among different population.

ss#	Sample Ascertainment		Genotype Detail			Alleles				
	Population	Individual Group	Chrom. Sample Cnt.	Source	A/A	A/C	C/C	HWP	A	C
ss1361473805	EAS		1008	AF					0.41769999	0.58229995
	EUR		1006	AF					0.56659997	0.43340001
	AFR		1322	AF					0.17019999	0.82980001
	AMR		694	AF					0.58930004	0.41069999
	SAS		978	AF					0.31190002	0.68809998
ss137429571	ENSEMBL_Watson		2	IG	1				1	
ss168385600	CEU	European	2	IG		1			0.5	0.5
ss203388312	BUSHMAN_POP2		2	IG		1			0.5	0.5
ss227920630	pilot_1_YRI_low_coverage_panel		118	AF					0.11016949	0.88983053
ss237511604	pilot_1_CEU_low_coverage_panel		120	AF					0.46666666	0.53333334
ss243751036	pilot_1_CHB+JPT_low_coverage_panel		120	AF					0.41666666	0.58333331
ss3216615	JBIC-allele		1490	AF					0.36250001	0.63749999
ss6904557	PDR90	Global	150	IG	0.25333	0.4133	0.333333	0.150222	0.46000001	0.54000002
ss76894403	HapMap-HCB	Asian	90	IG	0.22222	0.4222	0.355556	0.371093	0.43333334	0.56666666
	HapMap-JPT	Asian	88	IG	0.13636	0.5909	0.272727	0.200325	0.43181819	0.56818181
	HapMap-YRI	Sub-Saharan African	120	IG	0.01667	0.1667	0.816667	0.583882	0.1	0.89999998

Table (2): The genotype and allele frequency in studied groups

Polymorphisms	Groups								X ²	P-value	OR	CI 95%	EF	PF
	Control N=30				Patients N=60									
	H-W observed frequency	%	H-W expected frequency	%	H-W observed frequency	%	H-W expected frequency	%						
AA	0	0	0.53	13.4	0	0	0.02	0.03	0	0	0	0	-	-
CC	22	73.3	22.54	46.5	2	3.33	1.97	3.28	50.1	2.4x10 ⁻¹²	79.8	15.97-398.32	0.72	
AC	8	26.7	6.93	40.1	58	96.67	58.01	96.69	50.1	2.4x10 ⁻¹²	0.01	0.0-0.06	-	0.96
Total	30	100	30	100	60	100	60	100						
X ²	0.710				52.51									
P-value	0.3994				0.0000									
Allele frequency														
A	0.13				0.48									
C	0.87				0.52									

The main function of BCL2 protein as anti-apoptotic regulatory component and aberrant expression of BCL2 gene activate tumorigenesis by preventing death of malignant cells. The response to chemotherapy and radiotherapy was also determining by expression of BCL2, making it a candidate gene in prediction of cancer prognosis. The central role of SNPs in genes regulating essential pathways such as apoptosis, and angiogenesis in modifying the expression pattern and consulting the risk for cancer development has been reported [8, 16, 17]. It was observed that AA genotype was also associated with high Ki67 proliferation index, advanced stage breast

tumors, and lymph node positive status which indicates that altered expression of BCL2 gene in the presence of A allele might be associated with decreased apoptosis and enhanced proliferation rate. Flowcytometry analysis also revealed reduced apoptosis level among patients with AA genotype compared to those with CC genotype. Hence, it can be concluded the failure of apoptosis due to enhanced expression of antiapoptotic protein BCL2 might promote malignant growth. (3) Zhang et al., Concluded that AA genotype of BCL-2 (-938C > A) is associated with susceptibility of breast cancer, and this genotype is only associated with the nodal

status and pathological diagnosis of breast cancer. The polymorphism has an effect on Bcl-2 expression(2)

The - 938C allele in comparison with the A allele displayed significantly increased inhibition of BCL-2 promoter activity and binding of nuclear proteins (Nuckel et al. 2007). Thus, the BCL-2-938 AA genotype is associated with an increase in Bcl-2 expression This SNP (rs2279115) has been associated with an improved survival rate in some type of tumours such as breast or renal cancer (25)

However Meta-analysis was performed regarding the relationship between Bcl-2 promoter single nucleotide polymorphisms (SNPs) and cancer susceptibility and prognosis. Up to August 2016, 32 original publications were identified covering several SNPs including Bcl-2 promoter SNPs (rs2279115). these results showed statistically significant association between rs2279115 and cancer susceptibility and prognosis in all four genetic models. Subgroups analysis indicated that rs2279115 was associated with a significantly higher risk of cancer susceptibility in Asia but not in Caucasian. Furthermore, rs2279115 was associated with a significantly higher risk in digestive system cancer and endocrine system cancer but not in breast cancer, respiratory cancer and hematopoietic cancer. Simultaneously, rs2279115 was correlated with a significantly higher risk of cancer prognosis in Asia but not in Caucasian. Considering these promising results, rs2279115 may be a tumor marker for cancertherapy in Asia.(4)In conclusion, our results suggest that, patients AA genotype of BCL2-938C>A polymorphism might increased risk of breast cancer development and progression.

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