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Variants rs2758346, rs5746094, and rs2758331 of *SOD2* gene: association study with breast cancer in a Mexican population and their analysis *in silico*

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Abstract. – OBJECTIVE: The aim of the study was to analyze the association between the superoxide dismutase 2 (*SOD2*) gene variants rs2758346, rs5746094, and rs2758331 and breast cancer (BC) in the Mexican population as well as to perform *in silico* assessments of the variants' potential impact.

PATIENTS AND METHODS: We performed *in silico* analysis and analyzed 489 healthy women and 467 BC patients using TaqMan assays and Real-Time PCR.

RESULTS: The TT genotype, the T allele of the rs2758346 variant, and the CC genotype of both rs5746094 and rs2758331 were identified as BC risk factors ($p < 0.05$). The TT and CTTT genotype of the rs2758346 variant stratified by the presence of ki-67 (> 20%), TCCC, and estrogen receptor (ER)-positive of the rs5746094 variant, and the CC and CT genotypes of rs2758331 stratified by menopause status and non-chemotherapy response were risk factors. The TTC and TTA haplotypes are risk factors for BC. *In silico* analysis revealed that the rs2758346, rs5746094, and rs2758331 variants could influence *SOD2* gene regulation by transcription factors and circulating RNAs (circRNAs).

CONCLUSIONS: The rs2758346, rs5746094, and rs2758331 variants of the *SOD2* gene were associated with BC risk and could influence *SOD2* regulation by transcription factors and circRNAs.

Key Words:
Breast neoplasms, *SOD2* gene, Variants, Polymorphism, Single nucleotide, *In silico* analysis, Genetic variation, Genetic predisposition to disease, circRNAs.

Introduction

Breast cancer (BC) represents a worldwide health problem and is one of the most common gynecological cancers in women due to its high incidence and mortality rates[1,2]. Although the diagnosis and prognosis of BC have improved in recent years in Mexico, the incidence of BC in women under 40 years of age has been increasing[3-5]. However, studies on genes that participate in some way in different pathways of BC development have led to the recognition of BC as a heterogeneous

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entity in which different molecular subtypes and genetic profiles can be distinguished, allowing for the sectioning of subtype-specific treatments and predicting the response, prolonging life and improving the quality of life of patients[5]. BC is, therefore, a multifactorial disease that is due to an accumulation of genetic and epigenetic changes in the breast tissue that can influence its development and progression[2].

The relationship between cancer development and reactive oxygen species (ROS) has been well documented[6,7]. ROS causes damage to genetic material, which can give rise to chromosomal instability and genetic aberrations, promoting cancer development by increasing oxidative DNA damage that can alter oncogenes and increase cell proliferation[5]. Multiple cellular processes generate the production of ROS, such as estrogens and their metabolites, as well as xenobiotics, which can damage DNA and initiate cancer promotion[2,5].

One of the main lines of defense against ROS is superoxide dismutase (SOD)[2,5-7]. In mammals, three different isoforms of SOD (SOD1, SOD2 and SOD3) have been identified. SOD2 (Mn-SOD; EC 1.15.1.1) uses manganese as a cofactor and participates in the neutralization of ROS generated by the respiratory chain of mitochondria of aerobic cells[2,8]. The *SOD2* gene is in the chromosomal region 6q25, presents GC-rich regions, is a promoter lacking the TATA or CAAT box, and has five exons and four introns. The 3′ region has a binding site for the transcriptional regulatory factor, whose expression is induced by multiplex processes, such as lipopolysaccharides, cytokines, growth factors, cis-elements that recognize intronic regions, and ROS[6]. It has been observed that in malignant cells, the expression of *SOD2* decreases, which leads to an increase in the cell cycle due to an increase in the oxygen levels generated by the ROS, potential cause of DNA damage, cell transformation, and tumorigenesis[6].

Some of the variants identified in *SOD2* are associated with multiple pathologies, including cancer[9-18]. The rs2758346 C>T variant, located in the 5' untranslated region (UTR) (-1221G>A or C>T; c.-115-1526 G>A; c.93-1526; chromosomal location, Chr.6:159694389), has been associated with the development of Alzheimer's disease[9], pancreatic cancer[10], prostatic cancer[11], BC[12], and aerodigestive tract cancer[13]. The reported global frequency of the minor allele T is 50%; in Europeans, it is 47%, in Africans 43%, in Asians 12%-50%, in Americans 58%, and in Japanese 12%[19].

Concerning the rs5746094 T>C intronic variant (intron 1, c.23+93 T>C, c.-115-273, c.93-273, chromosome localization 6:159693136), there are two studies on this variant: one carried out in children with myelomeningocele[14] and another in prostate cancer[11]. The reported global frequency of the C minor allele is 23%; in Africans, it is 15-19%, in Asians 28-38%, in Europeans 25%, in Americans 23-28%, in Japanese 36%, and in Latin Americans 21% (c.406+816 C>A, c.425+816, c.227+816)[20].

The rs2758331 C>A (c.406+816, C>A or G>T, c.425+816, c.227+816, chromosome localization 6:159684038) intron 4 variant has been associated with the development of cardiovascular diseases[15,16], pulmonary function[17], and prostate cancer[18]. Differences in frequency have been reported depending on the study population. In Europeans, the reported frequency of the minor A allele is 46%, in Africans 17%-22%, in Asians 11-14%, in Latin Americans 37-54%, and in Americans 55%[21].

The *SOD2* gene variant may determine BC susceptibility, but the association studies that examined the rs2758346, rs5746094, and rs2758331 variants and BC risk in the Mexican population remain unknown. For this reason, the objective of the present study is to determine the frequency and association of the rs2758346, rs5746094, and rs2758331 variants of the *SOD2* gene in Mexican women with BC. It is also important to use *in silico* studies to understand the possible impact of the aforementioned variants of the *SOD2* gene on potential gene regulation sites and their participation in other cell signaling pathways.

Patients and Methods

Study Groups

The Ethics Committee of the Centro de Investigación Biomédica de Occidente, Instituto Mexicano del Seguro Social approved the study (CLIES #1305; protocol No. R-2021-1305-006). The participants provided written consent and their personal information, and all the procedures performed in the study were in accordance with the Declaration of Helsinki of 1964 and its latest amendments. DNA samples from 467 BC patients (clinically and histologically confirmed) and 489 controls were included in the study.

Variant Analysis

Using TaqMan probes (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA)

and Real-Time PCR (qPCR), the rs2758346 variant was analyzed with the probe sequences CGTTTCCGTTGCTTCTTGCAACCCC**[C/T]**GTACAGCCCTCCGAAGGATGACTAG (C___1362042). The rs5746094 variant was analyzed with the sequence CTTGGGGCCGTGACCGGGTCCCCTT**[C/T]**CTTCTCACCCGCACACTGCCCGGCT (C__25474804), and the rs2758331 variant with probe sequences [VIC/FAM]5'-ACATTACTACACAATTATCAAGAAA**[T/C]**CATTACTTTTTGACAAATGGAAATC-3' (C__16288770). The samples were read on a C1000 touch Thermal Cycler, CFX96 Real-Time PCR System (BIO-RAD, Berkeley, CA). As an internal control, 10% of the reactions were analyzed twice to ensure concordance in all analyzed samples.

Analysis In Silico

Prediction of the impact of variants on SOD2 regulation

To predict the impact of different mechanisms of action of the rs2758346, rs5746094, and rs2758331 variants of the *SOD2* gene on different gene regulation sites, the following tools were utilized: HaploReg v4.2[22], RegulomeDB, V2.2[23], and rSNPBASE V3.1[24]. These tools explore databases of the DNA features and regulatory elements in non-coding regions of the human genome using information from the 1000 Genomes Project, linked SNPs, small insertion and deletion, ENSEMBL, Roadmap Epigenomics, and ENCODE projects, as well as sequence conservation across mammals, the effect of SNPs on regulatory motifs, and the effect of SNPs on expression from eQTL studies.

SOD2 Expression Gene Analysis

The *in silico* expression of the *SOD2* gene was analyzed using the online tool Gene Expression Profiling Interactive Analysis[25]. The average values of the expression levels of the *SOD2* gene were evaluated in tumor samples from individuals affected by BC compared to tumors from healthy controls. It is important to highlight that the GEPIA platform uses data from public repositories provided by The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) project for its analyses. During this process, *SOD2* expression levels, previously normalized to the log scale $\log_2$ (TPM + 1), were compared between a total of 1,085 tumor tissue samples from BC and 291 tissue samples considered normal. A significance level was established with a *p*-value threshold of less than 0.01.

SOD2 expression levels were also evaluated by stage using the GEPIA tool. Additionally, overall survival and disease-free survival were analyzed in BC. The expression quantitative trait loci (eQTL) tool, analyzed in the GTEx Portal[26], was used to observe how each variant is associated with the expression of their respective genes. Healthy tissue and breast mammary tissue were used.

Statistical Analysis

The data frequencies were expressed using percentages; the observed and expected genotypes from the control group were compared using the chi-square test for calculating the Hardy-Weinberg equilibrium (HWE). The odds ratios and binary logistic regression in SPSS Statistic Base 24 software (IBM Corp., Armonk, NY, USA) were used to analyze the genotype association. The SHEsis online version of the program was used to analyze pairwise linkage disequilibrium (D') and haplotype frequency[27]. Kaplan-Meier analysis was utilized for survival analysis *in silico*, and a significance threshold of $p \leq 0.01$ was set.

Results

Demographic and Clinical Characteristics of the Study Groups

Table I describes the demographic and clinical variables of the study groups. The average age in the group of patients with BC was 51.92 ± 12.30 years; in the control group, the average age was 52.12 ± 12.28. There were no significant differences in the variables of tobacco and alcohol consumption ($p > 0.05$). The frequency clinical variable most relevant in the BC group was the presence of menopause status (60%), unilateral localization (93%), ductal adenocarcinoma histology (82%), stage II tumor (46%), and luminal A molecular classification (38%).

Analysis of the rs2758346, rs5746094, and rs2758331 Variants of the SOD2 Gene in BC Patients and Control Groups

The TT genotype and recessive model of the rs2758346 variant (OR 2.51, 95% CI 1.72-3.67, $p = 0.0000$), as well as the T allele [odds ratio (OR) 1.39, 95% confidence interval (CI) 1.14-1.70, $p = 0.0009$], were BC risk factors. The CC genotype and recessive model (OR 2.91, 95% CI 1.89-4.48,

Table I. Demographic and clinical data for the study groups.

		BC patients (n=467)		Controls (n=489)		*p*-value
Age (years, average ± SD)		51.92 ±12.30		52.12 ±12.28		0.800*
		n	%	n	%	
≤ 49 years		(196)	42.0	(201)	41.0	1.0**
≥ 50 years		(271)	58.0	(288)	59.0	
Consumption	Tobacco***	(114)	24.0	(123)	25.0	0.80
	Alcohol***	(88)	19.0	(83)	17.0	0.99
Hormonal status	Menopause	(280)	60.0			
	Premenopause	(174)	45.0			
Tumor						
Localization	Unilateral	(447)	96.0			
	Bilateral	(20)	4.0			
Histology (adenocarcinoma)	Ductal	(433)	93.0			
	Lobular	(28)	6.0			
	Mixed	(6)	1.0			
Stage	*In situ*	(3)	1.0			
	I	(31)	6.0			
	II	(214)	46.0			
	III	(126)	27.0			
	IV	(93)	20.0			
Molecular Type	Luminal A	(176)	38.0			
	Luminal B	(124)	27.0			
	Her-2	(58)	12.0			
	Triple-negative	(109)	23.0			
Ki-67	≥ 20%	(302)	64.0			
	< 20%	(165)	36.0			
Chemotherapy status	Response	(252)	54.0			
	No response	(215)	46.0			
Toxicity	Gastric	(246)	53.0			
	Hematologic	(160)	34.0			
	No	(61)	13.0			
	Both	(107)	23.0***			

SD (standard deviation); * Student's *t*-test; **Chi-square test. ***on base to 467.

$p = 0.000$) of the rs5746094 variant were BC risk factors. The CC genotype (OR 1.82, 95% CI 1.31-2.53, $p = 0.0003$) of the rs2758331 was a BC risk factor (Table II).

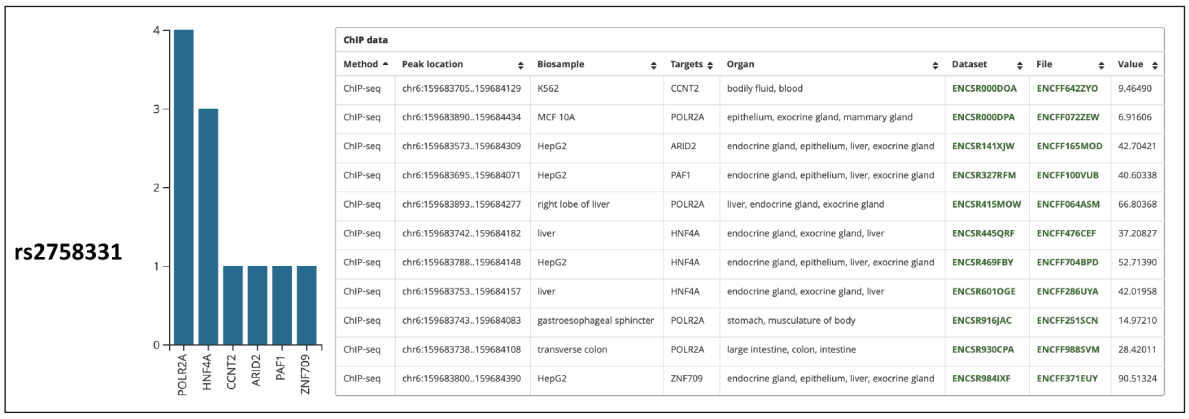
Comparative Analysis of the rs2758346, rs5746094, and rs2758331 Variants of the SOD2 Gene with Clinical Characteristics of BC Patients

In the BC group, the genotype TT (OR 2.5, 95% CI 1.35-4.66, $p = 0.003$) and dominant model CTTT (OR 2.6, 95% CI 1.18-5.71, $p = 0.006$) of the rs2758346 variant with ki-67 <20 were BC risk factors. The dominant model TCCC of the rs5746094 variant was associated with being a risk factor for ER-positive BC (OR 1.91, 95% CI 1.02-3.8, $p = 0.042$). The CC and CA genotypes of the rs2758331 variant were associated with being risk factors in patients of menopause status (OR 1.68, 95% CI 1.04-2.71, $p = 0.034$) those showing a non-chemotherapy response (OR 1.95, 95% CI 1.02-3.71, $p = 0.041$) (Table III).

Haplotypes analysis of rs2758346, rs5746094, and rs2758331variants of SOD2 gene in the study groups

The comparisons between the studied groups showed statistically significant differences in terms of haplotype frequency: CTC (OR 0.60, 95% CI 0.48–0.75, $p = 0.00001$) as a protective factor and TTC (OR 1.40, 95% CI 1.07-1.83, $p = 0.0139$) and TTA (OR 3.45, 95% CI 2.22-5.37, $p = 0.0001$) as BC risk factors (Table IV). The linkage disequilibrium of the rs2758346 and rs5746094 variants showed D' 0.21 and r' = 0.02; for rs2758331 and rs5746094, D' 0.27 and r' = 0.02; and for rs2758346 and rs2758331, D' 0.27 and r' = 0.03 in the control group.

1166



ChIP data

Method	Peak location	Biosample	Targets	Organ	Dataset	File	Value
ChIP-seq	chr6:159683705..159684129	K562	CCNT2	bodily fluid, blood	ENCSR000DOA	ENCFF642ZYO	9.46490
ChIP-seq	chr6:159683890..159684434	MCF 10A	POLR2A	epithelium, exocrine gland, mammary gland	ENCSR000DPA	ENCFF072ZEW	6.91606
ChIP-seq	chr6:159683573..159684309	HepG2	ARID2	endocrine gland, epithelium, liver, exocrine gland	ENCSR141XJW	ENCFF165MOD	42.70421
ChIP-seq	chr6:159683695..159684071	HepG2	PAF1	endocrine gland, epithelium, liver, exocrine gland	ENCSR327RFM	ENCFF100VUB	40.60338
ChIP-seq	chr6:159683893..159684277	right lobe of liver	POLR2A	liver, endocrine gland, exocrine gland	ENCSR415MOW	ENCFF064ASM	66.80368
ChIP-seq	chr6:159683742..159684182	liver	HNF4A	endocrine gland, exocrine gland, liver	ENCSR445QRF	ENCFF476CEF	37.20827
ChIP-seq	chr6:159683788..159684148	HepG2	HNF4A	endocrine gland, epithelium, liver, exocrine gland	ENCSR469FBY	ENCFF704BPD	52.71390
ChIP-seq	chr6:159683753..159684157	liver	HNF4A	endocrine gland, exocrine gland, liver	ENCSR601OGE	ENCFF286UYA	42.01958
ChIP-seq	chr6:159683743..159684083	gastroesophageal sphincter	POLR2A	stomach, musculature of body	ENCSR916JAC	ENCFF251SCN	14.97210
ChIP-seq	chr6:159683738..159684108	transverse colon	POLR2A	large intestine, colon, intestine	ENCSR930CPA	ENCFF988SVM	28.42011
ChIP-seq	chr6:159683800..159684390	HepG2	ZNF709	endocrine gland, epithelium, liver, exocrine gland	ENCSR984IXF	ENCFF371EUY	90.51324

Figure 1. Analysis of the transcription factor of the rs2758331 variant by ChIP-Seq program.

Prediction of the Impact of SNVs

HaploReg provided information about the rs2758346, rs5746094, and rs2758331 variants, revealing their impact on specific regulatory elements. In detail, the tool showed no results for the rs2758346 variant. The rs5746094 variant has an impact on the binding sites of GATA_disc3, SP1_disc3, and p300_disc10 proteins. The tool returned no results for the rs2758346 variant and the rs2758331 variant caused alterations in the anchor points of the Barhl1, Isx, Lhx4, and Prrx2_1 proteins.

In addition, ChIP-Seq analysis of transcription factor (TF) binding sites revealed the following: the rs2758346 variant is located at the binding sites of more than 100 TFs, with some of them located in up to 40 analyzed samples[28]. The rs5746094 variant localizes to the binding sites of more than 200 TFs, some of them verified in more than 100 samples[28]. The rs2758331 variant localizes to the junction of TFs CCNT2 (with a value of 9.46490), POLR2A (present in four samples with values of 6.91606, 66.80368, 14.97210, and 28.42011), ARID2 (with a value of 42.70421), PAF1 (with a value of 40.60338), HNF4A (present in three samples with values of 37.20827, 52.71390, and 42.01958), and ZNF709 (with a value of 90.51324) (Figure 1).

The rSNPBase tool revealed how each variant is linked to different regulatory elements of the gene. This tool showed that the rs2758346 variant is found at the site of the topologically associated domain TADs in the regions chr6:159600001-159640000, chr6:160080001-160120000, chr6:160080001-160120000, chr6:16104 0001-161080000 and chr6:160080001-160120000, chr6:161120001 -161160000, with the data extracted from the following cell lines: SK-N-MC, SK-N-MC, and NCI-H460 respectively. Furthermore, this variants was identified in different cell cultures in interacting chromatin regions between the blocks chr3:160283657..160284290-chr6:160114899..160115726,2 (Hela-S3), chr11:17229113..17229713-chr6:160114465..160115464,2 (Hela-S3), chr22:36424592..36425093-chr6:160114609..160115499,2 (HCT-116).

These findings were obtained using the Pol2 antibody experiments in Hela cell lines -S3, Hela-S3, and HCT-116, respectively. In addition, the rs2758346 variant localizes to the long noncoding RNA or circulating RNA (lncRNA, circRNA) lncRNA hsa-circ-WTAP-antisense junction.2.

According to rSNPBase, the rs5746094 variant is located in the same three TADs as the previous variant in addition to four interactive chromatin regions in the genomic blocks chr14:64108084_64108793, chr6:160113968_160114719, chr10:120863166_120863863, chr6:160114037_160114961, chr15:91445291_91445962, chr6:160114063_160114670, and chr1:149859086_149859973, chr6:160113946_16011 4930, where Pol2 antibodies were used in Hela-S3 cell lines Hela-S3, HCT-116, and Hela-S3, respectively. Likewise, this variant is located in the regions of two different IncRNAs: hsa-circ-SOD2-overlap.2 and hsa-circ-WTAP-antisense.2.

The rs2758331 variant is located in the same TADs mentioned for the anterior variants as well as in the regions of seven different IncRNAs: hsa-circ-SOD2-antisense.4, hsa-circ-SOD2-overlap.42, has-circ-SOD2.22, has-circ-SOD2.3, has-

Table II. Genotype and allelic distribution of the rs2758346, rs5746094, and rs2758331 variants of the *SOD2* gene in BC patients and a control group.

Variants			BC		Controls*			
rs2758346	**Model**	**Genotypes**	**(n=424)**	**%**	**(n=457)**	**%**	**OR 95%(CI)**	***p*-value**
		CC	(190)	45	(219)	48	1.0	
		CT	(139)	33	(191)	29	0.67 (0.51-0.89)	0.007
		TT	(95)	22	(47)	23	2.51 (1.72-3.67)	0.0000
	Dominant	CC	(190)	45	(219)	48		
		CT+TT	(234)	55	(238)	52	1.13 (0.87-1.47)	0.355
	Recessive	TT	(95)	22	(47)	23	2.51 (1.73-3.67)	0.005
		CC+TC	(329)	78	(410)	77		
		Alleles	**(2n=848)**		**(2n=914)**			
		C	(519)	0.6120	(629)	0.6881	0.71 (0.58-0.87)	0.0009
		T	(329)	0.3880	(285)	0.3119	1.39 (1.14-1.70)	0.0009
rs5746094	**Model**	**Genotypes**	**(n=466)**	**%**	**(n=475)**	**%**		
		TT	(309)	66	(259)	54	1.0	
		TC	(76)	16	(184)	39	0.30 (0.22-0.41)	0.0000
		CC	(81)	18	(32)	7	2.91 (1.89-4.48)	0.0000
	Dominant	TT	(309)	66	(259)	54		
		TC+CC	(157)	34	(216)	46	0.60 (0.47-0.79)	0.0012
	Recessive	CC	(81)	18	(32)	7	2.91 (1.89-4.48)	0.0000
		TT+TC	(385)	82	(443)	93		
		Alleles	**(2n=932)**		**(2n=950)**			
		T	(694)	0.7446	(702)	0.7389	1.03 (0.83-1.26)	0.818
		C	(238)	0.2554	(248)	0.2611	0.97 (0.78-1.19)	0.818
rs2758331	**Model**	**Genotypes**	**(n=427)**	**%**	**(n=404)**	**%**		
		CC	(124)	29	(74)	18	1.82 (1.31-2.53)	0.0003
		CA	(151)	35	(199)	49	0.56 (0.42-0.74)	0.00006
		AA	(152)	36	(131)	33	1.15 (0.86-1.53)	0.372
	Dominant	CC	(124)	29	(74)	18		
		CA+AA	(303)	71	(330)	82	0.54 (0.39-0.76)	0.0003
	Recessive	AA	(152)	36	(131)	33	1.15 (0.86-1.53)	0.334
		CC+CA	(275)	64	(273)	67		
		Alleles	**(2n=854)**		**(2n=808)**			
		C	(399)	0.4672	(347)	0.4294	1.16 (0.96-1.41)	0.134
		A	(455)	0.5328	(461)	0.5706	0.85 (0.70-1.04)	0.134

OR (odds ratio), CI (confidence intervals, *p*-value adjusted (significant < 0.05). *Hardy-Weinberg equilibrium (HWE) for the control group: variants rs2758346 (Chi-square test = 0.312, $p = 0.575$), rs5746094 (Chi-square test = 0.007, $p = 0.929$), and rs2758331 (Chi-square test = 0.0107; $p = 0.917$).

Table III. *SOD2* gene variants (rs2758331, rs5746094, and rs2758346) and their association with the clinical-pathologic variant of the BC group.

Variant	Genotype	Variable	OR	95% (CI)	*p*-value
rs2758346	TT	ki-67<20%	2.5	(1.35-4.66)	0.003
	CTTT	ki-67<20%	2.6	(1.18-5.7)	0.006
rs5746094	TCCC	ER-positive	1.91	(1.02-3.8)	0.042
rs2758331	CC	Menopause status	1.68	(1.04-2.71)	0.034
	CA	Non-chemotherapy response	1.95	(1.02-3.7)	0.041

Bivariate analysis, OR (odds ratio), CI (confidence intervals), *p*-value adjusted (significant < 0.05).

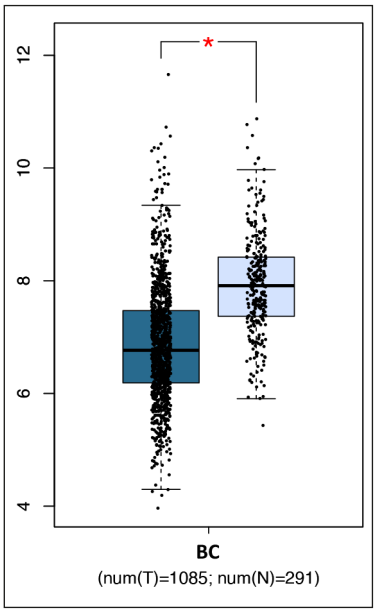


Figure 2. Analysis of expression in BC and control group tissues.

circ-SOD2.4, has-circ-SOD2.7, and has-circ-intronic.33538.

In Silico Analysis of SOD2 Gene Expression

When it was demonstrated that the analyzed variants were related to regulatory elements of the *SOD2* gene, an analysis was carried out with the GEPIA tool and an eQTL analysis with the GTEX tool. The GEPIA tool showed that the mean levels of *SOD2* expression in samples from BC patients have statistically significant differences ($p < 0.01$) when compared to samples from healthy controls, with a mean expression of 6.77 in samples from patients with BC patients compared to the mean of 7.91 in samples from healthy controls (Figure 2).

When the comparative analysis of the mean expression of *SOD2* by stage was performed in the samples from patients with BC, it was shown that there were no statistically significant differences in the mean expression by tumor stage ($F = 4.01$, $Pr(>F) = 0.0031$) (Figure 3). Similarly, in the survival analysis performed using GEPIA, no statistically significant differences were observed in terms of overall survival ($p = 0.91$) or disease-free survival ($p = 0.85$) (Figure 4).

The eQTL analysis obtained by the GTEx tool showed that the genotypes of the rs2758346,

Table IV. Haplotype frequency of the rs2758346, rs5746094, and rs2758331 variants of the *SOD2* gene.

Genotype			BC (2n=794)		Controls (2n=774)			
rs2758346	rs5746094	rs2758331	n	%	n	%	OR 95% (CI)	*p*-value
C	C	C	(67)	8	(68)	9	0.95 (0.67-1.36)	0.8767
C	C	A	(57)	7	(54)	7	1.03 (0.70-1.51)	0.9541
C	T	C	(188)	24	(262)	34	0.60 (0.48-0.75)	0.00001
C	T	A	(188)	24	(171)	22	1.09 (0.86-1.38)	0.4923
T	T	C	(154)	20	(113)	15	1.40 (1.07-1.83)	0.0139
T	T	A	(83)	10	(28)	3	3.45 (2.22-5.37)	0.00001
T	C	A	(57)	7	(78)	10	0.69 (0.48-0.98)	0.05049

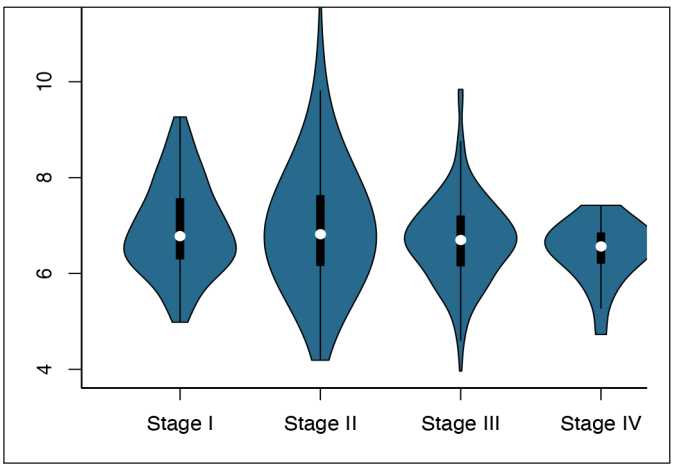


Figure 3. Expression analysis of *SOD2* in BC tissue stratified by stage.

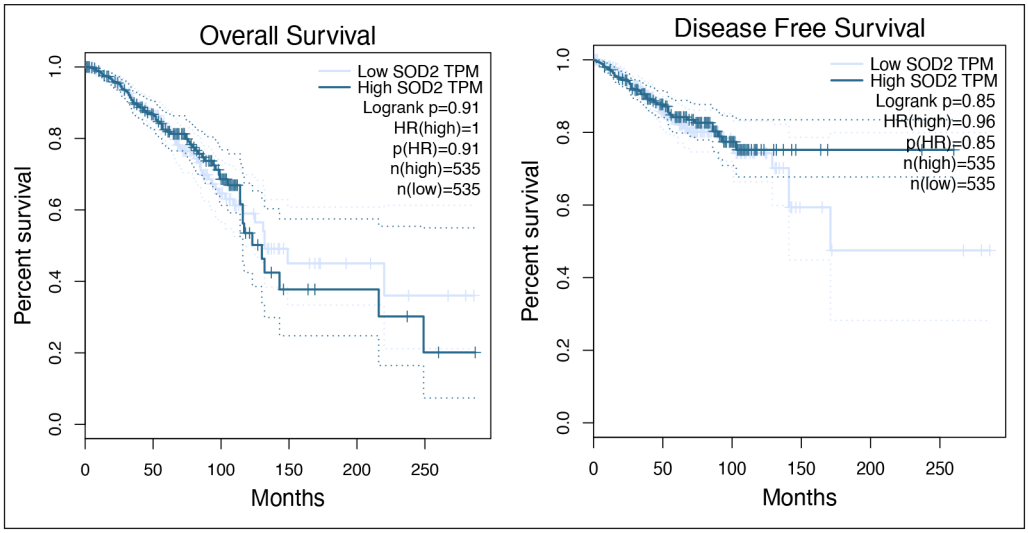


Figure 4. Survival analysis of *SOD2* expression in BC.

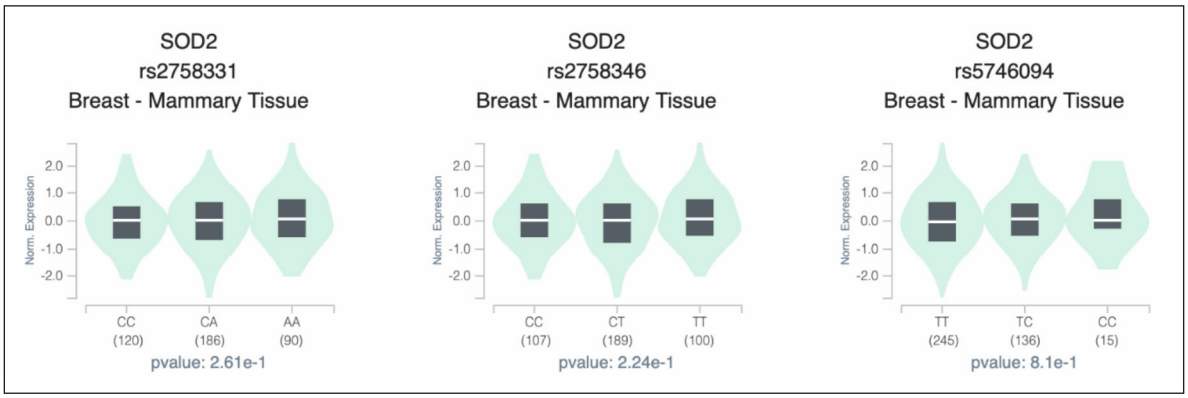


Figure 5. Expression analysis of *SOD2* gene in healthy tissue of rs2758346, rs5746094, and rs2758331 variants by genotype.

rs5746094, and rs2758331 variants are not associated with changes in the expression of the *SOD2* gene in healthy mammary tissue ($p > 0.05$: rs2758331; $p = 0.26$, rs2758346; $p = 0.22$ and rs5746094; $p = 0.81$) (Figure 5).

The regulatory factor and circRNA binding sites for the rs2758346, rs5746094, and rs2758331 variants of the *SOD2* gene are shown in Figure 6.

Discussion

In Mexico, as in the rest of world, BC is a public health problem and represents the leading cause of death from tumors in women[1-3]. Its highest frequency has been observed in women from approximately 50 years of age[1,2]. This is consistent with the average age data in this study. However, different risk factors related to the presence of BC in this respect have been noted: menopause status, unilateral localization, ductal type histology, stage advancer III-IV, luminal A, and ki-67 > 20%. In Mexico, in recent years, important campaigns for the early detection and management of BC have been carried out; however, BC-related medical care continues to be a health problem in the country, and there has been a recent increase in BC in women under 40 years of age[1-4]. Therefore, more studies are needed to better understand the Mexican population's genomics and the biological mechanisms of BC2.

In the regulation process of oxidative stress in the tumoral cell, different cellular mechanisms of the participation of *SOD2* have been proposed[2,5-8]. One of these mechanisms is that of circulating oxidant species in the cell damaging signaling, transcription,

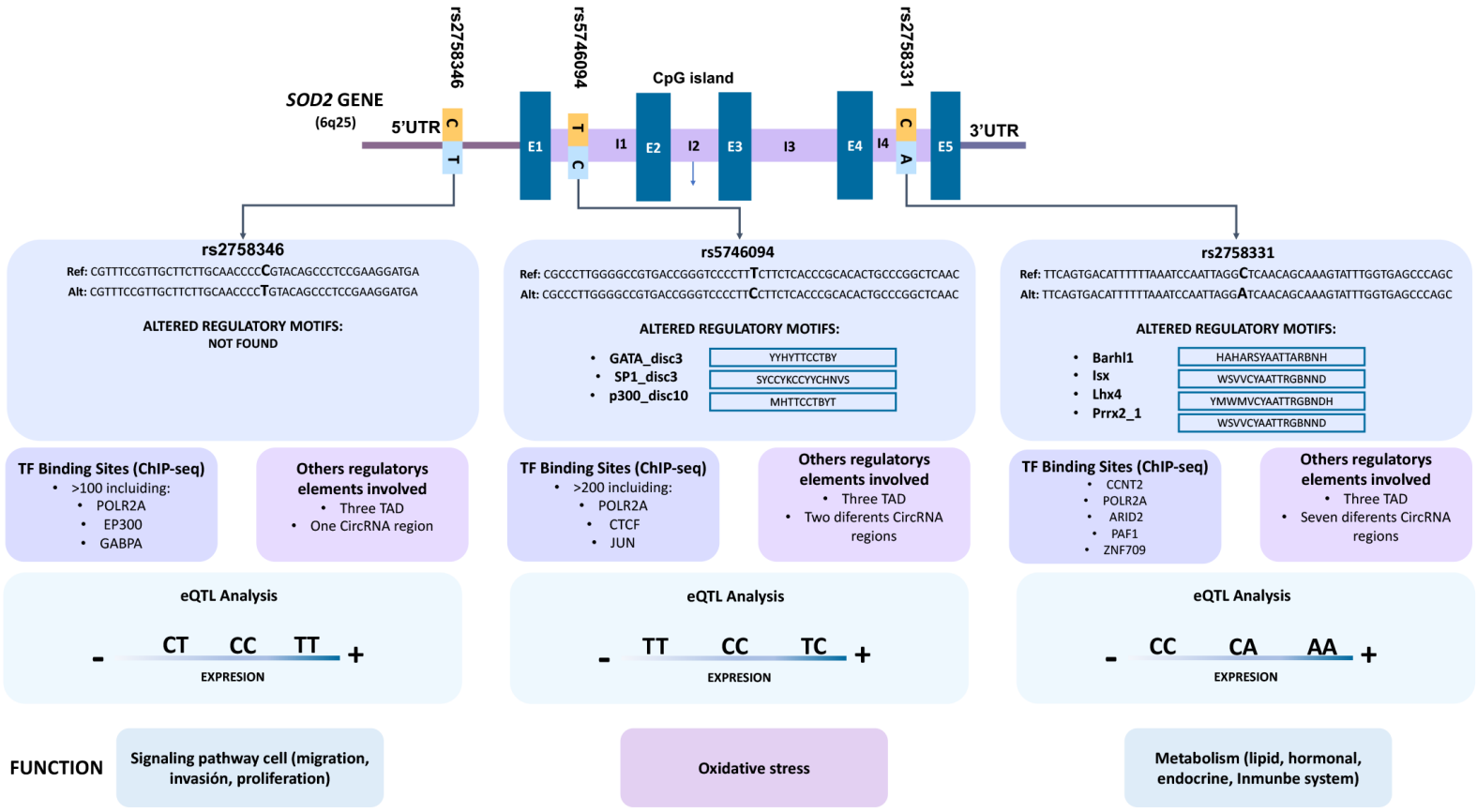


Figure 6. Union sites of regulation factor and circRNA at rs2758346, rs5746094, and rs2758331 variants of the *SOD2* gene.

and protein factors with deleterious consequences, such as mutations or cell death[2,5]. It is thought that the generation of ROS accumulates oxygen[2] and other oxidants, which consequently generates an imbalance of *SOD2* expression and increases DNA damage, initiating a malignant cellular transformation and giving rise to tumorigenesis[5].

Further studies[10-18] on the rs2758346, rs5746094, and rs2758331 variants of *SOD2* and cancer have shown different various types of susceptibility. However, little is known about their association with Mexican BC patients. In this study, the authors observed that the frequency of the TT genotype and the T allele of rs2758346 and the CC genotype of both the rs5746094 and rs2758331 variants of *SOD2* showed statistically significant differences between the BC patients and the controls ($p < 0.05$) and were associated with the risk of developing BC.

A few studies in the literature[10-18] have shown an association of the *SOD2* variants with cancer and other types of diseases. One of these studies showed that the AA genotype of the rs2758346 variant was associated with an increased risk for pancreatic cancer among those with low dietary vitamin E[10]. The findings observed in this study agree with previous data[10-18]; however, our study was focused on BC. Another study[18] was carried out on cancers of the upper aerodigestive tract across 10 European countries, although no risk association with the rs2758331 variant of *SOD2* was found[29]. Another study[9] carried out in patients with Alzheimer's disease showed that the alleles transmitted more frequently to cases with AD were T, C, G, and G, studied as a haplotype (rs2758346, rs4880, rs2855116, and rs5746136 in the 3'UTR). On the other hand, regarding the rs5746094 variant, there are only two studies: one carried out in patients with meningocele where no association was observed[14] and another in osteoporosis patients where they observed a risk association with the G allele of the rs5746094 variant[30]. Regarding the rs2758331 variant, a study[31] analyzed the association between treatment-related ototoxicity in cisplatin-treated pediatric medulloblastoma with different variants of the *SOD2* gene, including the rs2758331 variant, and found no association. The relationship between variants of the *SOD2* gene, including the rs2758331 variant, and platinum-based chemotherapy in patients with advanced non-small cell lung cancer, was also investigated, with no risk association observed between them[32]. One study[33] also found no association of this variant with osteoarthritis. However, an association study was carried out with different variants of the *SOD2* gene, including the rs2758331 variant, with survival outcomes after radiation therapy for prostate cancer and observed an association between rs2758331 ($p = 0.04$) and lethal prostate cancer[34]. It should be noted that this is the first study carried out in the Mexican population where variants of the *SOD2* gene with BC are analyzed. There are no studies of other populations where these variants are analyzed with BC, so, unfortunately, we cannot match the findings observed in the present study.

The association analysis of the clinical variables of the BC patients showed that the TT genotype and the dominant model (CTTT) of the rs2758331 variant was associated with ki-67(≥ 20%). The TCCC genotype of the rs5746094 variant was associated with ER-positive, and the genotypes CC and CA of the rs2758346 variant were associated with menopause and non-chemotherapy response, respectively, and were seen as a risk factor. In this sense, different mechanisms have been proposed for the development of carcinogenesis[2,5-8]. However, regarding the association of Ki-67, considered a marker of the cellular proliferation of BC and other types of cancer, one study[34] revealed that low levels of expression of antioxidant enzymes, such as SOD, are associated with the proliferation of tumors. Therefore, many intrinsic factors in the tumor microenvironment can affect SOD expression levels, such as inflammation, oxidative stress, and altered cellular metabolism[2,35,36].

Neoplastic cells are characterized by elevated levels of ROS (produced mainly by mitochondria)[2,5-8,36]. The important antioxidant *SOD2* is regulated by SIRT3 (deacetylase), and recent research has indicated that SIRT3 is decreased in 87% of BC[36]. In addition, it has been shown that ROS participate in cellular metabolic reprogramming towards glycolysis and are also the main promoters of oncogene activation. This includes Ras, which regulates the release of *SOD2* and, in turn, *SOD2* is regulated by *SOD1* in BC cells. *SOD2* to *SOD1* switch in BC[36].

We also observed that the patients with ER-positive BC were associated with the rs5746094 variant. In this regard, it has been shown that the differentiation of the breast epithelium is the product of the oxidative stress generated by the estrogens that are part of the adipose tissue of the breast[2]. Consequently, a decreased antioxidant enzyme activity of the SOD family can occur and produce DNA damage, which could cause cancer[2,5-8,36]. Regarding the rs2758331 variant, we observed an association between women with menopausal BC

and those who did not respond to chemotherapy. In this regard, it has been described that one of the main factors that contribute to the symptoms of menopause is oxidative stress in the form of free radicals and antioxidant deficiencies, which is directly related to the decrease in estrogen during reproductive aging. Low concentrations of estrogen have been shown to have pro-oxidant effects (high concentrations of proinflammatory cytokines, base oxidation, DNA adduct formation, and breaks in genetic material) that can trigger carcinogenesis[37].

On the other hand, it has been described that circulating levels of *SOD2* increase when patients with BC respond to treatment according to the reduction of the tumor during neoadjuvant chemotherapy. Therefore, it is inferred that, in patients who do not respond to chemotherapy, the expression levels of SOD in the microenvironment of the tumor are low and, consequently, the growth of the tumor is accelerated[38].

It is suggested that a comprehensive understanding of molecular interactions in pathways is important for the integration of multi-omics data (genome, transcriptome, epigenome, metabolome, and immunome) and can help in understanding and proposing the development of combination therapies with potential therapeutic use suitable for BC[2,5,36,38].

The *SOD2* variants analyzed in this study were not shown to be in linkage disequilibrium. In the study groups analyzed, the frequent haplotypes of rs2758346, rs5746094, and rs2758331 observed as a protective factor against susceptibility to the development of BC were CTC (present in 34% of the controls and 24% of the patients with BC), TTC (present in 20% of BC and 15% of the controls), and TTA (present in 10% of BC and 3% of the controls) as a susceptibility risk factor. Unfortunately, no study on BC has analyzed this association. Thus, combining the two *SOD2* variants is important information that identifies the haplotypes that confer risk against development susceptibility to BC. It should be noted that more population studies are necessary to demonstrate this association.

In Silico Analysis

Using open-access databases, we identified seven regulatory motifs in transcription factors that could be modulated by the studied variants. Specifically, the C alleles of the rs5746094 variant could affect the binding with the motifs GATA_disc3, SP1_disc3, and p300_disc10, while the A allele of the rs2758331 variant could affect the binding of the Barhl1, Isx, Lhx4, and Prrx2_1 proteins. In this regard, these factors are known to participate in many cellular processes, such as cell growth immune responses, cell differentiation, apoptosis, chromatin remodeling, and response to DNA damage, in addition to post-translational modifications, such as glycosylation, acetylation, and phosphorylation[5-8,36]. These have been proposed as biomarkers of different pathologies, such as Alzheimer's disease, high-grade glioma, and triple-receptor negative BC as well as hypothyroidism due to deficient transcription factors involved in pituitary development or function[5-8,9,12,36].

The presence of seven different circulating RNAs associated with the variants studied, mainly rs2758331, was also demonstrated. In this sense, it is known that circRNA are non-coding RNAs that can have both sense and antisense orientations, which have been proposed as cancer biomarkers since these circRNAs could probably decrease pre-mRNA splicing and mRNA translation, altering cell migration, invasion, proliferation, colony formation, and tumorigenesis. They have been proposed as biomarkers of therapeutic response, participating in lipid metabolism and oxidative stress[39-41].

The *in silico* study shows differences in the mRNA expression levels between the BC tissue and normal tissue. It is possible that these transcription factors and circRNAs may be involved in the regulation of the gene, since the *in silico* analysis showed that different transcript factors and at least seven circRNAs had an association with the alleles of the rs2758346, rs5746094, and rs2758331 variants analyzed in the present study. New studies are recommended to validate this information.

BC, as a multifactorial disease, is characterized by the accumulation of environmental and epigenetic genetic changes that increase the risk of the disease, making it even more difficult to understand the molecular mechanisms that lead to the growth and progression of tumor metastasis[2,5-8]. Oxidative stress from ROS generation has been proposed as an important factor for the activation of redox-sensitive signaling pathways and transcription factors in tumor cells that regulate angiogenesis, proliferation, and metastasis. In breast tissue, estrogen and SOD play an important role in cell differentiation; however, an imbalance between them can lead to cancer development[2,5,36]. In addition, the low activity of *SOD2,* depending on

genetic variants, may contribute to the process of breast carcinogenesis[5,36,42]. These variants are usually in regulatory regions of the gene and affect gene expression[2,5,36,42]. Various studies[2,5-8,35,36,37-42] have described altered expression levels of *SOD2* in tumor tissues. Both low and high expression levels have been described in comparison to normal tissues depending on the type of cancer, stage, chemotherapy, tumor grade, and ER. It has been described that the decreased expression of the *SOD2* gene in cancer cells may be due, in part, to defects in the transcriptional regulation of the gene due to gene mutations, epigenetic processes, or the high expression of repressive factors, microRNAs, and circRNA. The differential expression of the *SOD2* gene between normal mammary epithelium and early-stage breast tumors is thought to be mediated by ROS.

In addition, *in silico* studies[43,44] have analyzed the benefits of the so-called "genomic era", where the utility of the complete sequencing of the human genome has led to significant studies on cancer and its understanding, highlighting the usefulness of TCGA. Where the amount of data generated has impacted new knowledge about the behavior of cancer, the above has helped to verify theories put forward by different scientists and the generation of new hypotheses in different metabolic pathways, growth, inhibition, apoptosis and their behavior of cancer. Furthermore, the constant improvement of tools, such as TCGA and PCAWG, has been of great benefit and will have a direct impact on the generation of new knowledge about cancer and other pathologies.

On the other hand, there are other studies[45] showing that the presence of mutations in *SOD* genes may potentially influence drug susceptibility, in this regard, a study carried out on BC cell lines observed that quercetin and raloxifene showed synergistic anticancer effects on cell viability, nitric oxide production, cell migration and apoptotic genes such as *Bcl2*, *p53*, *MMP2* and *MMP9*. Another study[46] was conducted on curcumin and its effects on cytotoxicity, mitochondrial membrane potential (MMP), glutathione (GSH), generation of intracellular ROS, apoptosis, and DNA damage in BC cell lines. It was shown that the cytotoxicity, genotoxicity, and apoptosis of curcumin in BC cells induced an increase in intracellular ROS and decreased GSH and MMP[46]. Similarly, a study conducted by our group[47], showed the association of the *SOD1* gene variants rs4817415, rs2070424, and rs1041740 with BC risk.

In addition, in the context of precision oncology, a growing field that tailors cancer treatment to individual genetic profiles, a study[48] analyzed the molecular targets for predicting the prognosis of histological subtypes of BC, and found that each molecular subtype of BC is associated with a specific expression profile that alters the response to treatment. Therefore, additional gene expression profiling of each subtype is essential to provide information on the more precise behavior of different breast tumors, thus offering hope for even more specific precision oncology[49,50]. Furthermore, through an *in silico* study[50] using the Oncomine database, the expressions of the *CDK* gene in BC were examined. It was observed that *CDK1*, *CDK2c*, *CDK4*, *CDK5*, *CDK7*, *CDK8*, and *CDK20* can be used as molecular markers for BC patients or as potential targets for BC therapy by targeting CDKs.

Conclusions

Our results showed that the TT genotype and T allele of the rs2758346 variant and the CC genotype of both rs5746094 and rs2758331 were associated with a risk for BC when compared with the control group. Furthermore, differences were observed in the patients with BC stratified by the TT and CTTT genotypes of the rs2758346 variant and the presence of ki-67 ($\geq$ 20%). The rs5746094 variant was also a risk factor for BC patient-group carriers of the TCCC and ER-positive, and carriers of the CC and CT genotypes of the rs2758331 variant were risk factor-stratified by menopause status and non-chemotherapy response. The presence of the TTC and TTA haplotypes is an associated risk susceptibility factor in BC. The identification of multiple transcription factors and seven circRNAs interacting with the analyzed variants of the *SOD2* gene *in silico,* as well as different signaling pathways, is important. More studies are needed to confirm the observed findings.

Conflict of Interest

The authors declare that they have no conflict of interests.

Ethics Approval

The Ethics Committee of the Centro de Investigación Biomédica de Occidente, Instituto Mexicano del Seguro Social approved the study (CLIES #1305; protocol No. R-2021-1305-006). The integrity of the patients was respected in accordance with good ethical practices and the Declaration of Helsinki and its amendments.

Informed Consent
A written informed consent was obtained from control subjects and patients.

Availability of Data and Materials
Data and materials are available in the article.

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Authors' Contributions
MPGA, AFGR, LEF, BCGM, GMZG, AMPP: study conception and/or design. MPGA, AFGR, LEF, ASA, BCGM, GMZG, JIDS, ARC, IPDR, AVRR, AMPP: analysis and interpretation of results. MPGA, AFGR, LEF, BCGM, GMZG, AMPP: critical revision or editing of the article. MPGA, AFGR, LEF, BCGM, GMZG, AMPP: analysis, experimentation. and data collection. MPGA, AFGR, LEF, BCGM, GMZG, AMPP: supervision. MPGA, LEF, GMZG, BCGM, AMPP: financing support.

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