Association between rs61764370, rs9266, and rs140080026 polymorphisms of the *KRAS* gene and breast cancer risk in a Mexican population

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Abstract. – OBJECTIVE: Polymorphisms of the *KRAS* gene have been shown to be associated with cancer. However, their association with breast cancer (BC) has been inconsistent. The purpose of this study was to determine the frequency with which the rs61764370, rs9266, and rs140080026 polymorphisms of the *KRAS* gene are associated with BC in patients of the Mexican population.

PATIENTS AND METHODS: The rs61764370 *A>C or T>G* and rs140080026 *A>G* polymorphisms were determined by Polymerase Chain Reaction (PCR), and the rs9266 *A>G* polymorphism was determined by DNA sequencing of healthy Mexican subjects and BC patients.

RESULTS: We observed that 78% of BC patients are overweight and/or obese, 57% have metastatic lymph nodes, 64% have luminal A/B cancer subtypes, and 61% have stage III-IV cancer. The rs61764370 polymorphism was associated with BC susceptibility when the BC patients and the control group were compared for the *AC* genotype ($p = 0.020$), *AC vs. AA* genotypes (heterozygous model: $p = 0.016$), *AC/CC* genotype (dominant model: $p = 0.002$), and the *C* allele ($p = 0.007$). The *AC/CC* genotype ($p = 0.018$; rs61764370) and *AG/GG* genotype ($p = 0.005$; rs9266) were associated with age in BC patients ≥50 years old. The *AC/CC* (rs61764370) and *AG/GG* (rs9266) genotypes were classified by molecular subtype, TNM stage, miscarriage, lymph node metastasis, ductal type, and Ki-67. These classifications were also associated with BC patients, indicating that these factors may significantly contribute to BC risk. The *AAA* (OR 0.65, 95% CI 0.43-0.98, $p = 0.039$) and *CAA* (OR 3.25, 95% CI 1.13-9.36, $p = 0.021$) haplotypes were also associated with BC susceptibility. In addition, 94 polymorphisms were identified on the 3'UTR of the *KRAS* gene GRCh 38/hg3 (25,209,490-25,209,122) in BC (n = 112) and control (n = 113) samples. However, 92 of these polymorphisms have only expressed the major allele (wild-type allele).

CONCLUSIONS: The rs61764370 polymorphism in the *KRAS* gene was associated with BC susceptibility in the Mexican population. The dominant model of the rs61764370 and rs9266 polymorphisms (classified by molecular subtype, miscarriage, TNM stage, lymph node metastasis, and Ki-67) could significantly contribute to BC risk in patients ≥50 years. The *CAA* haplotype could significantly contribute to BC risk in the Mexican population analyzed.

Key Words:
Breast cancer, KRAS, Rs61764370, Rs9266, Rs140080026, Polymorphism, Mexican.

Introduction

Breast cancer (BC) is the most frequent type of gynecological cancer in the world[1]. BC is one of the principal causes of mortality in adult women, par-

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ticularly in Mexico[1,2]. Epigenetic events are gradual changes that occur in the ducts and lobules of breast tissue and are important to the development of BC[1,3]. Several studies[4-10] have associated the *KRAS* gene with BC. *KRAS* (Kristen-RAS) is part of the *RAS* gene family (*RAS*, *RHO*, *RAB*, *ARF*, *RAC*, and *RAN*). GTP activates the KRAS protein in the plasma membrane which participates in the RAS/RAF-MAPK and PI 3'kinase intracellular signaling pathways. These signals have important functions in cell growth, proliferation, and differentiation[10-13]. The KRAS protein has two active isoforms (KRAS4A and KRAS4B) that are products of alternative splicing in exon 4 of the gene[14]. The *KRAS* gene in humans has two copies: *KRAS2* (locus 12p11.1-12) and *KRAS1* (locus 6p11-12), a pseudogene product resulting from alternative mRNA splicing of *KRAS2*. The *KRAS2* gene contains six exons. Exons 2, 3, and 4 are coding regions[14], and their promotor is regulated by the interaction of proteins and microRNA molecules. More than 2000 microRNAs have been identified in the 3'UTR of the *KRAS* gene, and some functions include cell regulation, proliferation, and differentiation, as well as mRNA destabilization and protein synthesis repression. They have also been seen altered in cancer. These microRNAs are divided into oncomirs (oncogenes) and anti-oncomirs (tumor suppressors)[4].

Polymorphisms in the 3'UTR of the *KRAS* gene are considered by different studies as important genetic biomarkers[15] because these regions are mediated by negative post-transcriptional regulation and bind to complementary sites of target messenger RNAs[15].

The rs61764370 *T>G* (c.*2505, g. 25207290) polymorphism is located in the let-7 complementary site 6, and its function has been associated with the disruption of let-7 binding affinity for KRAS, which results in KRAS inhibition and enhanced tumor growth caused by the *G* allele[4]. Many studies[16-19] have determined that there is an association between the *KRAS* rs61764370 polymorphism and the risk of various cancers, such as lung cancer[16], BC in premenopausal women[17], chronic myeloid leukemia[18], and osteosarcoma[19]. However, these associations have been too inconsistent[20].

Reported genotype frequency variability of the rs61764370 polymorphism depends on the ethnicity group. The *G* allele showed a frequency of 0.6-15% in control groups among European, African, American, and Asian populations. In populations with Mexican ancestry, a frequency of 3.1% has been reported (dbSNP; http://www.ncbi.nlm.nih.gov/ SNP/rs61764370).

The rs9266 *A>G* (c.*20 A>G, g25209283) polymorphism is located in the 3'UTR of the *KRAS* gene and has been associated with the overexpression of the gene caused by the *G* allele. The complementary sites, including miR-181abcd, miR-4262, and miR-132, have been described[15]. The reported frequency of the *G* allele is 20-88% among European, Asiatic, African, and American populations. In one population with Mexican ancestry, a frequency of 55.5% has been reported (dbSNP; http://www.ncbi.nlm.nih.gov/SNP/rs9266).

There are few cancer association studies of the rs9266 polymorphism; in the Chinese population, the correlation between the rs9266 polymorphism and survival of colorectal cancer was investigated[21]. Its frequency in lung and ovarian cancer has also been described; however, no association has been found[15], and there is no evidence of association in BC studies.

The rs140080026 polymorphism *A>G* (c.*128 A>G; g.25209391) is located in the 3'UTR. However, no functional evidence of the frequency of this variation has been reported in the ClinVar archive. The reported frequency of the *G* allele is 0-5.9% among European, Asiatic, African, American, and Latin American populations. In population with Mexican ancestry, a frequency of 9.4% has been reported (dbSNP; http://www.ncbi.nlm.nih.gov/SNP/rs140080026). There is no evidence of association in cancer studies.

This research aims to determine the frequency with which three *KRAS* gene polymorphisms (rs61764370, rs9266, and rs140080026) are associated with BC in Mexican women, which remains unknown.

Patients and Methods

Study Group Analysis

We analyzed blood samples from 584 patients with clinically and histologically confirmed cases of BC along with 361 healthy women from the general population. Genomic DNA was extracted from blood samples using the Miller method[22]. No familial samples and no age-matched individuals were included in the studied groups. All the procedures performed in the study were in accordance with the 1964 Declaration of Helsinki and the participants provided written informed consent, as approved by Local Ethics Committee CIBO, IMSS (1305). Clinical data (cancer type, molecular type, histological data, TNM stage, and chemotherapeutic pharmacological data) and demographic data (age, weight, personal pathological antecedents, menarche age, hormonal, tobacco and alcohol consumption) were obtained from written questionnaires.

Polymorphism Analysis

The rs61764370, rs9266, and rs140080026 polymorphisms of the 3'UTR of the *KRAS* gene were selected based on data from the SNP database (http://www.ncbi.nlm.nih.gov/SNP/).

Amplification of the rs61764370 polymorphism was performed *via* PCR using the following primers: 5'-CCTGAGTAGCTGGGATTACA-3' and 5'-GGATACCATATACCCAGTGCCTT-3', as previously described[17]. The PCR amplifications were performed for 547 BC samples and 361 control samples. The reaction volume total of 15 µl contained 5 pmol of primers, 0.2 mM dNTPs, 0.75 µl DMSO, 2.1 mM $MgCl_2$, 1.5 U of *Taq* polymerase (Invitrogen, Carlsbad, CA, USA), and 50 ng of genomic DNA. The PCR program used an annealing temperature of 57°C. The PCR product was digested with the Hinf I restriction enzyme and separated using gel electrophoresis with 8% polyacrylamide gels (19:1), followed by silver staining[23]. The 117 and 115 bp fragments were identified as the *AA* genotype, the 117, 115 and 232 bp fragments were identified as the *AC* genotype, and the 232 bp fragments were identified as the *CC* genotype.

The rs9266 and rs140080026 polymorphisms were identified by Sanger sequencing in 112 BC samples and 113 control samples using the following primers: 5'-CCAATTGTGAATGTTG-GTG-3' and 5'-AATGTGAAAAGGAAATGG-3' (selected from dbSNP; http://www.ncbi.nlm.nih.gov/ SNP). The PCR reaction volume total of 15 µl contained 0.25 mM dNTPs (Invitrogen, Carlsbad, CA, USA), 5 pmol of primers, 2.5 mM $MgCl_2$, 0.75 µl DMSO, 2.5 U of *Taq* polymerase (Invitrogen, Carlsbad, CA, USA), and 50 ng of genomic DNA. The annealing temperatures were 55°C, 53°C by 5 sec, and 51°C by 30 sec. The 371 bp fragments were sequenced by capillary sequencing with an Abi Prism 310 Genomic Sequencer, using the BigDyer[R] Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific Inc., Waltham, MA, USA).

When analyzing the sequence of 371 bp, we observed that the rs140080026 polymorphism was recognized by the Pvu II restriction enzyme. We then analyzed the 472/584 BC samples and 207/230 control samples using the RFLP method to complete the total samples reported for this polymorphism. The 371 bp fragments (sequenced using the same primers as mentioned above) were digested by the Pvu II restriction enzyme, and in a previous electrophoretic procedure, the amplified products were separated on 6% polyacrylamide gels (29:1), followed by silver staining[23]. The 103 and 268 bp fragments were identified as the *GG* genotype, the 371, 103 and 268 bp fragments as the *AG* genotype, and 371 bp fragment as the *AA* genotype.

Statistical Analysis

To compare the studied groups, the age variable was expressed as mean ± standard deviation (SD) using an independent *t*-test. Allele and genotype frequencies were obtained by direct counting expressed as a percentage (%), and the BC and control groups were compared by a Chi-squared test. The Hardy-Weinberg equilibrium (HWE) was calculated to compare the observed genotype frequencies with the expected frequencies among the control group. The genetic model (additive, dominant, and recessive) was determined using the Cochran-Armitage test. Odds ratios (OR) and 95% confidence intervals (CI) were calculated using the SPSS v24 software package (Chicago, IL, USA) and considered statistically significant with a $p<0.05$ two-tailed *p*-value. Haplotype analysis was performed by the SHEsis software platform[24].

Results

Demographic and Clinical Characteristics of Studied Groups

Table I shows the demographic and clinical characteristics of the BC patients and the control group. The mean age was statistically different in the BC patients compared to the control group (52.4 *vs.* 49.1; $p<0.05$). The main characteristics of the BC patients include hormonal (oral/injection: 47%) and participation in hormonal replacement therapy (HRT: 11%), body mass index (BMI; normal: 22%, overweight: 34%, obesity I: 28%, obesity II: 12%, obesity III: 4%), presence of DM2 (14%) and systemic arterial hypertension (SAH; 18%), lymph node metastasis (57%), ductal type (92%), luminal A subtype (38%), and stage IV tumor presence (25%).

Frequency of Polymorphisms in the Studied Groups

The genotype distribution of the rs61764370 polymorphism in the *KRAS* gene was significantly

Table I. Demographic characteristics and Clinical data of participants the study groups.

		BC patients$^{(n = 584)}$	Controls$^{(n = 361)}$	*p*-value[1]		
Age$_{\text{(years, mean ± Standard Deviation)}}$		52.44 +/- 11.32	49.15 +/- 12.67	**0.040**[a]		
		(n)	**%**	**(n)**	%	
Tobacco consumption*	Yes	(170)	29	(91)	25	
	No	(414)	71	(270)	75	0.219[b]
Alcohol consumption**						
	Yes	(136)	23	(78)	22	
	No	(448)	77	(283)	78	0.603[b]
Hormonal consumption	Oral/injection[2]	(277)	47			
	Hormonal replacement therapy	(63)	11			
BMI (kg/m^2)***	Normal (18.5-24.9 kg/m^2)[2]	(128)	22			
	Overweight (25-29.9 kg/m^2)[2]	(200)	34			
	Obesity I (30-34.9 kg/m^2)[2]	(163)	28			
	Obesity II (35-39.9 kg/m^2)[2]	(70)	12			
	Obesity III (40-45.9 kg/m^2)[2]	(23)	4			
PPA	Mastitis chronic[2]	(5)	1			
	Breast fibrosis[2]	(71)	12			
	Uterine myomas[2]	(79)	14			
	Type 2 diabetes mellitus (DM2)[2]	(81)	14			
	Systemic Arterial Hypertension (SAH)[2]	(104)	18			
	DM2/SAH[2]	(36)	6			
Metastatic lymph Nodes	Positive[2]	(331)	57			
Type	Ductal	(537)	92			
	Lobular	(45)	7			
	Mixed	(2)	1			
Histological type[3]	Luminal A[2]	(224)	38			
	Luminal B[2]	(149)	26			
	HER2/neu[2]	(105)	18			
	Triple negative[2]	(106)	18			
Stage	I[2]	(46)	8			
	II[2]	(183)	31			
	III[2]	(211)	36			
	IV[2]	(144)	25			

Pathology personal antecedent (PPA), *5 cigarretes-2 pack per day, **3-8 drinks per week, ***Body mass index (BMI), according to OMS classification, [b]Mann-Whitney U test, [a]t student test, [1] ≤ 0.05, [2]positive on base n = 584.

different between the study groups. The *AC* genotype (heterozygous model, OR 1.48, 95% CI 1.07–2.05, $p = 0.020$), *AC vs. AA* genotypes (heterozygous model, OR 1.50, 95% CI 1.08-2.08, $p = 0.016$), *AC/CC* genotype (dominant model, OR 1.64, 95% CI 1.20-2.25, $p = 0.002$), and *C* allele (OR 1.49, 95% CI 1.12-1.99, $p = 0.007$) were observed as risk factors for developing BC (Table II).

The rs9266 and rs140080026 polymorphisms did not show significant differences between the BC and control groups (Table II). Additional data from the 371 bp sequenced segment (part of the 3'UTR of the *KRAS* gene) showed more than 90 polymorphisms (112 BC patients and 113 controls); however, these polymorphisms have only expressed the major allele (wild-type allele) (**Supplementary Table I**). The rs61764370, rs9266, and rs140080026 polymorphisms in the *KRAS* gene were in Hardy-Weinberg equilibrium in the studied groups (Table II, and Figures 1A and 1B).

Association of clinical and demographic variables with polymorphisms in BC patients

Significant differences were observed between BC patients and controls ($p < 0.05$) when comparing the rs61764370 and rs9266 polymorphisms of the *KRAS* gene when stratified by age (≥50 years old) (Table III).

Significant differences were observed when comparing the *AC/CC* genotype of the rs61764370 polymorphism in BC patients with clinical stage III-IV cancer and chemotherapy partial response (OR 4.2, 95% CI 1.33-13.75, $p = 0.014$), human epidermal growth factor receptor 2 (*HER2*) molecular subtype and lymph node positive (OR 3.4, 95% CI 1.24-9.84, $p = 0.018$), *HER2* molecular subtype and miscarriage (OR 4.5, 95% CI 1.15-17.5, $p = 0.006$), presence of luminal A subtype and miscarriage (OR 2.3, 95% CI 1.01-5.31, $p = 0.047$), and presence of luminal A subtype and Ki-67 expression ≥ 20% (OR 4.5, 95% CI 2.22-9.13, $p = 0.001$) (Table IV).

The *AG/GG* genotype of the rs9266 polymorphism showed significant statistical differences in BC patients with triple negative breast cancer (TNBC) and miscarriage (OR 4.3, 95% CI 1.07-17.47, $p = 0.039$), and clinical stage III-IV cancer and ductal type (OR 2.9, 95% CI 1.27-6.57, $p = 0.017$) (Table IV).

Association of Haplogenotypes of KRAS Polymorphisms in the Study Groups

The haplotype frequency of *KRAS* polymorphisms and their association with the study groups are presented in Table V. The most frequent haplotype was *AGA* (45% of BC patients and 41% of the control group). However, no statistical differences were observed between the study groups. Nonetheless, evident differences were observed between the *AAA* (OR 0.65, 95% CI 0.43-0.98, $p = 0.039$) and *CAA* (OR 3.25, 95% CI 1.13-9.36, $p = 0.021$) haplotypes.

We observed that the D' and r2 values of rs61764370 vs. rs9266 were 0.206 and 0.007, respectively; rs61764370 *vs.* rs140080026 were 0.023 and 0.001, respectively; and rs9266 vs. rs140080026 were 0.863 and 0.056, respectively (Figure 2).

Haplogenotype association between BC patients and controls has shown that the *AAAAAA* haplogenotype was associated with protective susceptibility (OR 0.38, 95% CI 0.16-0.87, $p = 0.032$) (Table VI). The association between clinical variables and haplogenotypes in the BC patients did not show statistically significant differences (data not shown).

Discussion

Demographic and Clinical Characteristics of BC

In Mexico and around the world, BC is one of the main causes of death and gynecological disease in adult women[1,2]. BC was observed to occur at an average age of 50 years[2,3,10,25], which is consistent with data from this study, since the mean age was 52.44 years. In this study, prevalent patrons were observed as predominant clinical characteristics in BC patients, including the presence

Table II. Genotype and allelic distributions of rs61764370, rs9266 and rs140080026 polymorphisms of the *KRAS* gene in BC patients and controls.

Polymorphism		BC*		Controls*		OR	95%(CI)	*p*-value
Rs61764370	***Genotype***	(n=547)	%	(n=361)	%			
	AA	(393)	72	(288)	80	1.0		
	AC	(142)	26	(69)	19	1.48	(1.07-2.05)	**0.020**
	CC	(12)	2	(4)	4	1.20	(0.79-1.84)	0.920
Heterozygous	*AC vs. AA*	(142)	26	(69)	19	1.50	(1.08-2.08)	**0.016**
Dominant	AC/CC	(164)	28	(73)	23	1.64	(1.20-2.25)	**0.002**
	Allele (2n1094)			(2n=722)				
	A	(928)	0.8482	(645)	0.8933	0.66	(0.50-0.89)	**0.007**
	C	(166)	0.1518	(77)	0.1067	1.49	(1.12-1.99)	**0.007**
Rs9266	**Genotype**	(n=112)	%	(n=113)	%			
	AA	(23)	21	(30)	27	1.0		
	AG	(56)	59	(54)	48	1.09	(0.64-1.84)	0.842
	GG	(33)	29	(29)	25	1.21	(0.67-2.17)	0.625
	Allele(2n=224)			(2n=226)				
	A	(102)	0.4553	(114)	0.5044	0.82	(0.56-1.18)	0.343
	G	(122)	0.5447	(112)	0.4956	1.21	(0.84-1.76)	0.343
Rs140080026	***Genotype***	(n=584**)	%	(n=320**)	%			
	AA	(536)	91.7	(300)	94	1.0		
	AG	(47)	8	(20)	6	1.31	(0.76-2.26)	0.393
	GG	(1)	0.3	(0)	0			
	Allele(2n=514)			(2n640)				
	A	(1119)	0.9580	(620)	0.9687	0.73	(0.43-1.25)	0.313
	G	(49)	0.0420	(20)	0.0313	1.58	(1.09-2.29)	0.313

Odds ratio (OR), confidence intervals (CI), significant *p*-value < 0.05. *Hardy-Weinberg equilibrium in BC patients of rs61764370 (chi-square test=0.038, p=0.843) and controls (chi-square test=0.003 p=0.953), BC patients of rs9266 (chi-square test=0.007, p=0.932 and controls (chi-square test=0.224, p=0.638), and BC patients of the rs140080026 (chi-square test = 0.0008, p=0.977) and controls (chi-square test=0.332, p=0.5639) of *KRAS* gene polymorphisms.
**(n=584 BC patients 112 were sequencing and 472 by RFLPs analyzed; n=320 controls 113 were sequencing and 207 by RFLPs analyzed).

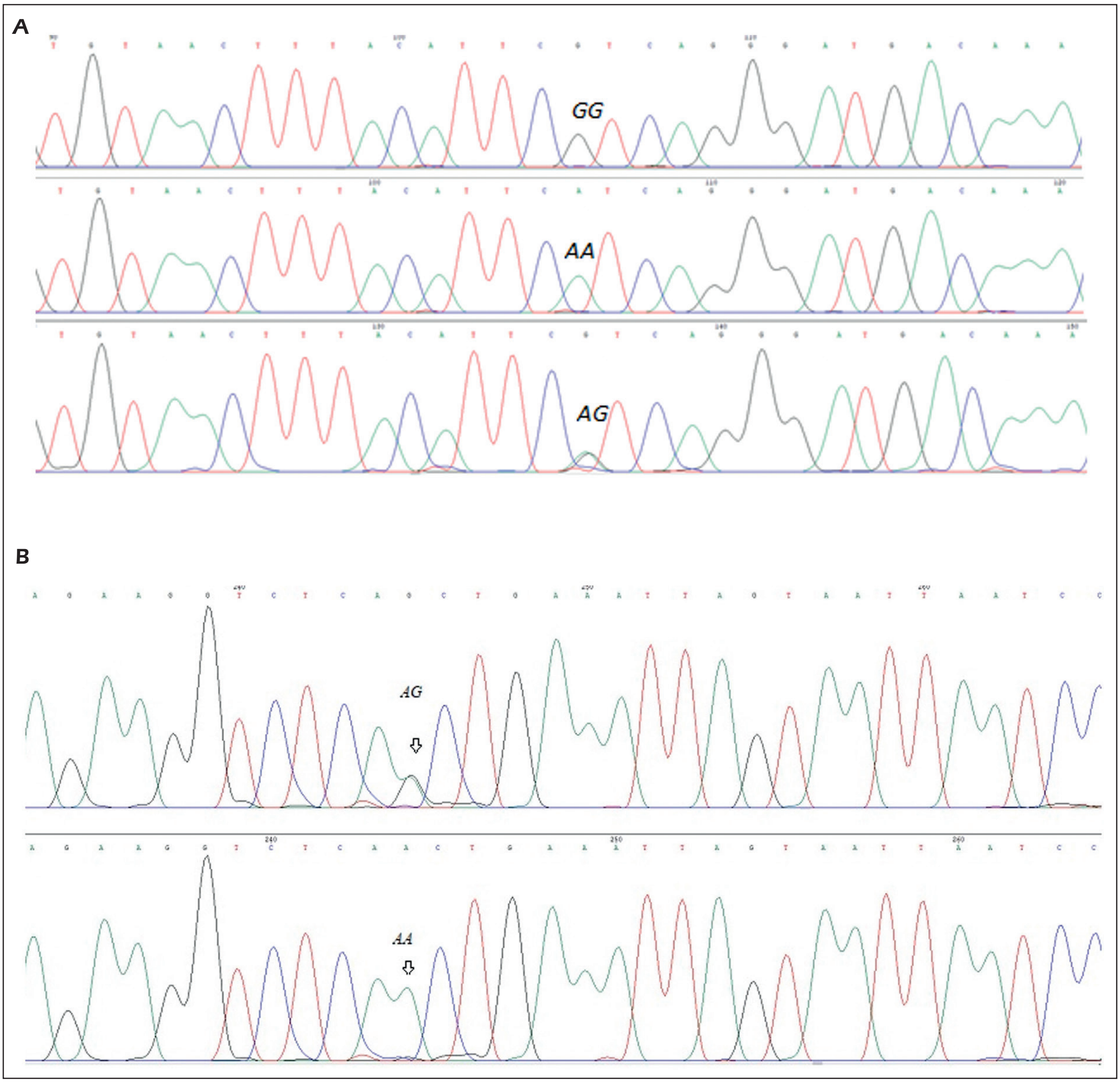

Figure 1. *A,* Identification of rs9266 polymorphism on complementary sequence. GG (polymorphic homozygous), GA (heterozygous), AA (wild homozygous). **B,** Identification of rs140080026 polymorphism on complementary sequence. GA (heterozygous) and AA (wild homozygous).

of obesity, lymph node metastasis, presence of luminal A/B subtype, and advanced cancer stages (III-IV). Reynoso et al[25] reported similar clinical dates in a study that included 4,300 Mexican BC patients from different regions of Mexico. In the BC groups, some risk factors were observed, such as obesity, low rate of breastfeeding and hormonal exposure, and being diagnosed in advanced cancer stages. In this context, better medical care strategies are required for BC patients in the Mexican population to reduce the rate of disease progression and improve the quality of life of patients.

Polymorphism Associations Between Study Groups

We analyzed the importance of the rs61764370, rs9266, and rs140080026 polymorphisms of the *KRAS* gene and their association with BC risk in a sample Mexican population.

It has been observed that the associated differences in polymorphisms on the *KRAS* gene, especially within the 3'UTR, where multiple complementary sites for this miRNA have been determined and have been related to the risk of cancer. The rs61764370 polymorphism (classified as a germline and functional polymorphism in the *KRAS* 3'UTR) had diana sites in the let-7 complementary site 6. It has been also observed that this site disrupts the let-7 binding affinity for *KRAS* and stimulates the growth and progression of the tumor[4].

There is contradictory data on the association between the rs61764370 polymorphism and the risk of cancer development. Some studies have not reported an association[20], while others have documented an association with non-small cell lung cancer, colorectal cancer, prostate cancer, oral cancer, gastric cancer, ovarian cancer, and BC[6-11,14-19]. Specifically in BC, Mohthash et al[26] reported statistically significant differences in the distribution frequency of the rs61764370 polymorphism between BC cases and controls from the South Indian population, indicating that the *KRAS* gene could be an important risk factor in the development of BC. Sanaei et al[4] observed that the heterozygous genotype and variant allele increased the risk of BC in a southeast Iranian population.

Similar results were observed in the present study; the rs61764370 polymorphism also showed an association with BC susceptibility. The *AC* genotype (OR 1.48, 95% CI 1.07-2.05, $p = 0.020$), *AC vs. AA* genotypes (heterozygous model; OR 1.50, 95% CI 1.08-2.08, $p = 0.016$), *AC/CC* genotype (dominant model; OR 1.64, 95% CI 1.20-2.25, $p = 0.002$), and *C* allele (OR 1.49, 95% CI 1.12–1.99, $p = 0.007$) of the rs61764370 polymorphism have shown statistically significant differences between BC patients and control groups and were associated with a risk of developing BC.

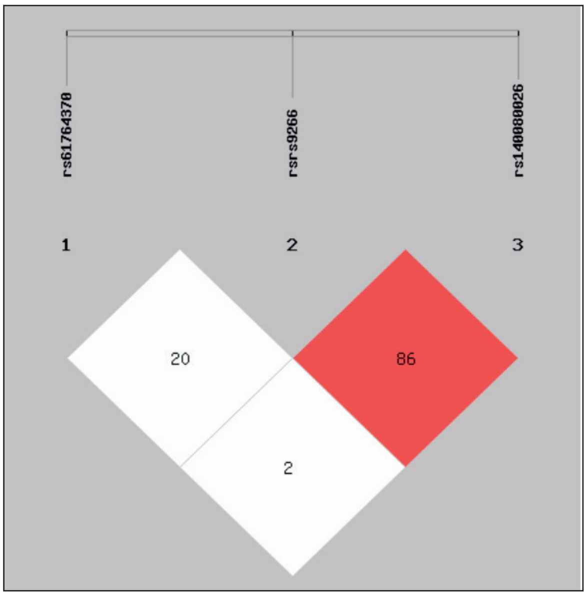

Figure 2. Linkage disequilibrium plot of polymorphism the rs61764370, rs9266 and rs140080026 of KRAS gene.

Table III. Association of the rs61764370 and rs9266 polymorphisms of the *KRAS* gene with age in BC patients and controls.

Polymorphism		Genotype*	Variables	OR	95%(CI)	*p*-value
rs61764370	3'UTR 25,207290	*AC/CC*	≥50 years old	1.4	(1.09-1.98)	0.018
rs9266	3'UTR 25,209280	AG/GG	≥50 years old	3.2	(1.46-7.31)	0.005

*Dominant model

Table IV. Association of the rs61764370 and rs9266 polymorphisms of the *KRAS* gene with clinical characteristics of BC patients, classified by stage.

Polymorphism		Genotype*	Stage	Variables	OR	95%(CI)	*p*-value
rs61764370	3'UTR 25,207290	*AC/CC*	III-IV	Partial response**	4.2	(1.33-13.75)	0.014
			HER2	Metastasis node lymph	3.4	(1.24-9.84)	0.018
				miscarriage	4.5	(1.15- 17.5)	0.006
			Lum A	miscarriage	2.31	(1.01- 5.31)	0.047
				Ki-67 ≥20%***	4.5	(2.22- 9.13)	0.001
rs9266	3'UTR	*AG/GG*	Triple negative	miscarriage	4.3	(1.07-17.47)	0.039
	25,209280	*AG/GG*	III-IV	ductal	2.9	(1.27-6.57)	0.017

*Dominant model. **According to the pathological Ryan's classification (non-chemotherapy response, non-chemotherapy response by recurrence). Non-response to chemotherapy treatment with anthracyclines, taxanes, and trastuzumab was evaluated. ***The cut-off point can be discriminated in these tumors with a low Ki-67 expression[35].

Table V. Polymorphism the rs61764370, rs9266 and rs140080026 haplotype frequencies of the *KRAS* gene in the study groups.

Haplotypes*				Frequency					
	rs61764370	rs9266	rs140080026	BC$^{(n=198)}$ (n)	%	Controls$^{(n=214)}$ (n)	%	OR (95% CI)	*p*-value
1	A	A	A	(66)	33	(92)	43	0.65 (0.43-0.98)	0.039
2	A	A	G	(12)	6	(7)	3	1.91 (0.73-4.99)	0.178
3	A	G	A	(89)	45	(87)	41	1.18 (0.80-1.76)	0.391
4	C	A	A	(14)	7	(5)	2	3.25 (1.13-9.36)	0.021
5	C	A	G	(3)	1.5	(4)	2	0.80 (0.17-3.65)	1.0
6	C	G	A	(14)	7	(18)	8.5	0.77 (0.37-1.59)	0.491
7	C	G	G	(1)	0.5	(1)	0.5	1.08 (0.06-17.4)	1.0

* rs61764370 *vs*. rs9266 D'= 0.206, r^2=0.007; rs61764370 *vs*. rs140080026 D'= 0.023, r^2=0.001, and rs9266 *vs*. rs140080026 D'=0.863, r^2=0.056.

Additional data from the present study included 94 polymorphisms within the 3'UTR region between position 25,209,122-25,209,490 of the *KRAS* gene and were also analyzed. However, 92 of the polymorphisms showed no risk association to BC. With these polymorphisms, we only observed the presence of the major or wild-type allele in both BC patients and control groups. Despite this, it has been suggested that the 3'UTRs of the *KRAS* gene play an important role in tumorigenesis because it has multiplex binding sites for different mRNAs and miRNAs[27]. However, in the 3'UTR of the *KRAS* gene analyzed in this study sample, such variability was not evident except for the rs9266 and rs140080026 polymorphisms. We also determined that the genotypic and allelic distributions of the rs9266 and rs140080026 polymorphisms are located in the 3'UTR of the *KRAS* gene; however, in our analysis, we observed similar frequencies between the BC and control groups, and the statistical analysis showed no risk association with BC. When the genotypic frequency of the rs9266 polymorphism was compared with data reported in https://www.ncbi.nlm.nih.gov/projects/SNP/, similitudes (people of Mexican ancestry from Los Angeles, California, and Utah residents with Northern and Western European ancestry; $p > 0.05$) and differences (Maasai in Kinyawa and Luhya in Webuye from Kenya; and Toscans in Italy; $p < 0.05$) in genotypic frequency distribution were observed between the control group from the data of this study. The rs140080026 polymorphism allelic frequency was also compared with data reported in https://www.ncbi.nlm.nih.gov/projects/SNP/. Similitudes (EAS population; $p > 0.05$) and differences (AMR, AFR, SAS, EUR populations; $p < 0.05$).

The association of the rs9266 and rs140080026 polymorphisms with BC is the first to be analyzed in the Mexican population and in other populations of the world, so there are no reference studies in BC.

Table VI. Haplogenotypes frequencies of rs61764370A>C, rs9266A>G and rs140080026A>G polymorphisms of the *KRAS* gene in the study groups.

Haplotypes*				Frequency					
	rs61764370	rs9266	rs140080026	BC$^{(n=100)}$ (n)	%	Controls$^{(n=107)}$ (n)	%	OR (95% CI)	*p*-value
1	***AA***	***AA***	***AA***	(9)	9	(22)	20	0.38 (0.16-0.87)	**0.032**
2	*AA*	*AA*	*AG*	(6)	6	(3)	3	2.21 (0.53-9.09)	0.431
3	*AA*	*AG*	*AA*	(30)	30	(35)	33	0.96 (0.53-1.73)	1.0
4	*AA*	*AG*	*AG*	(4)	4	(3)	3	1.44 (0.31-6.62)	0.927
5	*AA*	*GG*	*AA*	(20)	20	(18)	17	1.23 (0.61-2.50)	0.681
6	*AC*	*AA*	*AA*	(7)	7	(2)	2	3.95 (0.80-19.4)	0.142
7	*AC*	*AA*	*AG*	(0)	0	(1)	1		
8	*AC*	*AG*	*AA*	(12)	12	(9)	8	1.68 (0.65-4.31)	0.386
9	*AC*	*AG*	*AG*	(5)	5	(3)	3	1.82 (0.42-7.84)	0.646
10	*AC*	*GG*	*AA*	(7)	7	(9)	8	0.81 (0.29-2.29)	0.904
11	*CC*	*AG*	*AA*	(0)	0	(1)	1		
12	*CC*	*AG*	*AG*	(0)	0	(1)	1		

Association of polymorphisms by age between BC patients and control groups

The association between the dominant model of the rs61764370 (*AC/CC*) and rs9266 (*AG/GG*) polymorphisms and BC risk is stratified by age (≥50 years old) and was also demonstrated between BC patients and controls (p <0.05). There are no other studies that demonstrate this association; however, Wu et al[28] analyzed 18 cancer types by pan-cancer transcriptome and observed significant aging-associated molecular patterns in 16 cancer types, which included BC. In addition, they observed that aging was associated with cancer in important cell regulation pathways, such as xenobiotic metabolism, hypoxia, *KRAS* signaling, p53 pathways, and others.

It has been observed that the rs61764370 polymorphism is associated with an increase in MAPK signaling in different tumors. It is also associated with ER/PR negative premenopausal BC patients, implying that age and hormonal status are important risk factors in the development of BC[7].

Association of polymorphisms with clinical characteristics in BC

In this study, it was also observed that the *AC/CC* genotype of the rs61764370 polymorphism was a risk factor in BC with TNM stages III-IV and partial chemotherapy response, HER2 with lymph node and miscarriage, presence of the luminal A subtype with miscarriage, and Ki-67 expression (≥20%). Therefore, it has been described that the *KRAS* variant might be an important factor in diagnosing BC with a worse prognosis[7].

Thus, an explanation of the results observed in this study would be that the *C* allele is probably a consequence of multiplex binding sites for different mRNAs and miRNAs. This may lead to recognition that regulates the transcription of the *KRAS* gene and impacts the deregulation of progesterone with implications in the gestational process. Moreover, it has been demonstrated that the activated *KRAS* oncogene epigenetically targets genes involved in the progesterone resistance, which has been related with poor reproductive outcomes[29,30].

In addition, we also observed an association between the *AC/CC* genotype of the rs61764370 polymorphism and luminal A subtype risk along with miscarriage rate and Ki-67 expression (>20%). In this sense, the association of *KRAS* mRNA expression with BC prognosis in the luminal A subtype has been documented[31]. It is also known that the high expression of Ki-67 may contribute to the growth of cancer cells through the S, G2, and M phases[32].

In addition, we observed an association between the *AG/GG* genotype of the rs9266 polymorphism and TNBC risk along with miscarriage rate, TNM stages III–IV, and ductal histological type. Though there are no studies described in the literature that support these findings, it has been hypothesized that the increase in *KRAS* signaling decreased the survival rate in TNBC. It has also been observed that the rs9266 polymorphism participates in the regulation of the *KRAS* gene by disrupting complementary sites, which promotes tumor progression[15,33].

Haplotype and Haplogenotype Distribution in the Study Groups

The haplotype and haplogenotype associations of the rs61764370, rs9266, and rs140080026 polymorphisms of the *KRAS* gene were determined between BC patients and control groups. A linkage disequilibrium plot showed high D’ (0.863) in rs9266 *vs.* rs140080026 and low D’ in rs61764370 *vs.* rs9266 (0.206) and rs140080026 (0.023). We also observed that the *AAA* (OR 0.65; 95% CI 0.43–0.98, p = 0.039) and *CAA* (OR 3.25; 95% CI 1.13–9.36, p = 0.021) haplotypes and the *AAAAAA* haplogenotype (OR 0.38; 95% CI 0.16-0.87, p = 0.032) were associated with protection from BC.

To our knowledge, this is the first study to report the association between the rs61764370, rs9266, and rs140080026 polymorphisms of the *KRAS* gene and BC by subtypes and TNM stages. As has been shown in other studies[34], the progression of cancer is associated with the modify the expression of different pathways of cellular regulation.

Investigations on the expression of microRNAs have shown different molecular subtypes of BC, including luminal A, HER2, and TNBC expression profiles of the different microRNAs. These depend on the time of therapy, time before operation, or time after chemotherapy and radiotherapy, which could be predictive factors for the prognosis of patients with BC. The findings suggested that microRNAs can be a useful tool in the diagnosis of BC[10].

Conclusions

Our results showed an association between risk for BC compared to controls in the *AC* genotype, *C* allele, *AC vs. AA genotypes* (heterozygous model), and *AC/CC* genotype (dominant model) and the rs61764370 polymorphism. It also showed that the *AG/CC* genotype of the rs61764370 polymorphism and *AG/GG* genotype of the rs9266 polymorphism were associated with age in BC patients over 50 years old. However, the differences were also observed in patients with the *AC/CC* genotype of the rs61764370 polymorphism with (1) TNM stages III–IV with partial chemotherapy response, (2) HER2 with lymph node metastasis, (3) HER2 with miscarriage, (4) luminal A subtype with miscarriage, and (5) luminal A subtype with Ki-67 expression (≥20%).

In addition, the *AG/GG* genotype of the rs9266 polymorphism was associated with (1) TNBC and miscarriage and (2) TNM stages III-IV and histological ductal type. The haplotypes *AAA* and *CAA* were observed to be factors for susceptibility to BC. Previous evidence confirms that these findings significantly contribute to BC susceptibility in the analyzed sample from the Mexican population.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Authors' Contributions

GAMP contributed to the design, analysis, experimentation, data collection, and financing; GVPM and MTMT contributed to the analysis experimentation and analysis of the manuscript; and FLE, ZGGM, GMBC, RRMA, and AMPP contributed to the design and analysis of the manuscript. All the authors have read and approved the final manuscript.

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