SPANDIDOS PUBLICATIONS

Gene polymorphisms in TYMS, MTHFR, p53 and MDR1 as risk factors for breast cancer: A case-control study

L.A. HENRÍQUEZ-HERNÁNDEZ[1,2], A. MURIAS ROSALES[2,3], A. HERNÁNDEZ GONZÁLEZ[2,4], A. CABRERA DE LEÓN[2,4,5], B.N. DÍAZ-CHICO[2,6], M. MORI DE SANTIAGO[3] and L. FERNÁNDEZ PÉREZ[1,2]

[1]Clinical Science Department, Universidad de Las Palmas de Gran Canaria, C/Dr. Pasteur s/n, CP 35016, Las Palmas de Gran Canaria; [2]Canary Institute for Cancer Research (ICIC); [3]Medical Oncology Service, Hospital Universitario Insular de Gran Canaria, Avda. Marítima del Sur s/n, 2nd floor, CP 35016, Las Palmas de Gran Canaria; [4]Research Unit, Hospital Universitario de La Candelaria, Carretera de El Rosario 145, Hospital de La Candelaria, 38010 Santa Cruz de Tenerife, Tenerife; [5]Preventive Medicine and Public Health Unit, Universidad de La Laguna; [6]Physiology, Biochemistry and Molecular Biology Department, Universidad de Las Palmas de Gran Canaria, Spain

Received May 21, 2009; Accepted August 20, 2009

DOI: 10.3892/or_00000584

Abstract. Breast cancer (BC) is a complex disease influenced by environmental and genetic factors. The disease has important genetic and environmental components, most of them are still unknown. An important role of gene polymorphisms related to the risk of developing BC has been reported. However, the results have been controversial. We investigated the association of TSER, MTHFR C677T, p53 codon 72 and MDR1 C3435T gene polymorphisms with breast carcinoma in women from Canary Islands (Spain). Blood samples collected from 135 patients with BC and 304 healthy controls all of them Caucasian, were analyzed through polymerase chain reaction-restriction fragment length polymorphism. Subsequently, a structured questionnaire including patient history and risk factors in relation to BC development was filled out. Allelic frequencies of these genetic variations were: TSER, (2) 0.55 and (3) 0.45 in cases, 0.49 and 0.51 respectively in controls (P=0.240); MTHFR C677T, (C) 0.63 and (T) 0.37 in cases, 0.60 and 0.40 respectively in controls (P=0.568); p53 Arg72Pro, (Arg) 0.74 and (Pro) 0.26 in cases and controls (P=0.910); MDR1 C3435T, (C) 0.52 and (T) 0.48 in cases, 0.55 and 0.45 respectively in controls (P=0.523). We did not observe any gene polymorphism as a risk factor to develop BC. A statistical association was observed between p53 codon 72 polymorphism and family history of breast cancer in both groups, as well as between MDR1 C3435T and smoking habits in cases (P<0.05). Gene polymorphisms vary by regions. The present study contributes to the characterization of the genetic pattern of the Canary population.

Correspondence to: Dr L.A. Henríquez-Hernández, Universidad de Las Palmas de Gran Canaria, C/Dr. Pasteur s/n, CP 35016, Las Palmas de Gran Canaria, Spain
E-mail: lhenriquez@dcc.ulpgc.es

Key words: breast cancer, polymorphism, PCR-RFLP, risk factors, TSER, MTHFR, p53, MDR1

Introduction

Breast cancer (BC) is the most common cancer in women. Incidence rates of the disease vary considerably, with the highest rates seen in North America (99.4 per 100,000 women) and Europe (62.3 per 100,000 women) (1). Canary Islands have one of the highest rates of the disease compared with the rest of the Spanish territory (http://www.gobiernodecanarias.org/sanidad/scs/3/3_6/cancer/ppal.jsp). In several ways, Canary population seems to have special characteristics: it is a closed population with less exchange of genetic information (2), dietary habits are enriched by fat and carbohydrates (Nutrition Survey of Canary, ENCA. http://www.gobiernodecanarias.org/sanidad/scs/1/plansalud/enca/ppal_enca.htm) and there is an inadvertent exposure to persistent pesticides (3). These factors must be related with others, such as genetic factors, to explain the rates of BC in our population. In the last ten years many risk factors for the development of BC (4) have been identified, although the aetiology of the disease is poorly understood. Risk factors that may modulate the development of BC include age, ethnicity, reproductive events, exogenous hormones, lifestyle, bone density and genetic factors (5). Some molecular markers related to BC have been elucidated as risk factors (6). Thymidylate synthase (TYMS) is a key enzyme that participates in folate metabolism and catalyzes the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) in the process of DNA synthesis (7). This conversion is essential for the provision of thymidine, a nucleotide needed for DNA synthesis and DNA repair (8). A tandem repeat polymorphism has been identified in the 5'-UTR enhancer region of the TYMS promoter (TSER), the immediate upstream of the ATG codon initiation start site, which contains triple (TSER 3R) or double (TSER 2R) repeats of a 28-bp sequence as well as

several rare alleles containing 4, 5 or 9 repeats (9). *In vitro* and *in vivo* studies showed that TYMS expression was TSER genotype-dependent and that the 3R allele was associated with a higher TYMS expression level (9). TYMS variants are thought to be functionally relevant and are hypothesized to be associated with risk of breast cancer (10) and other cancers (11).

Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in the folate metabolic pathway. It catalyses the conversion of 5,10-methylenetetrahydrofolate (5,10-methylene-THF) to 5-methylenetetrahydrofolate, which is the predominant form of folate in plasma and provides the methyl group for *de novo* methionine synthesis (12). C677T is a common single nucleotide polymorphism (SNP) in exon 4 at the folate binding site of the MTHFR gene which results in alanine to valine substitution at codon 222 (13). Individuals with the MTHFR 677TT genotype have been shown to have only 30% of *in vitro* MTHFR enzyme activity compared with the wild-type (14). Reduced activity of the MTHFR, due to C677T polymorphism, influences the general balance between DNA synthesis, repair and methylation processes (15). MTHFR 677TT has been associated with risk for many different types of cancer, including endometrial, gastric, leukemia, lung, head and neck and colorectal cancers (16-20). Nevertheless, several case-control studies investigated the association of MTHFR C677T and BC risk with controversial findings (15).

Mutations in the p53 gene are the most common genetic alterations in human cancer and they can be found in 20-30% of the sporadic BC (21). In addition to gene mutations, some polymorphisms in p53 gene have been suggested to play a role in BC (22). Studies have been focused on a common single-base-repair polymorphism at codon 72, resulting in either a proline (Pro) residue (CCC) or an arginine (Arg) residue (CGC) at this position (23). The two polymorphic variants have been shown to have structural differences and different biological properties (24,25). The Pro-rich domain of p53 has been shown to be an important component in the apoptotic function of p53. Arg72 form of wild-type p53 harbours a greater apoptosis-inducing potential than the Pro72 variant (24), and may alter the tumour response to systemic chemotherapy by influencing the apoptotic capacity (26). The association between codon 72 gene polymorphism and cancer risk has been reported in different populations, although results with regards to most cancers remain controversial (23,27-29).

The multidrug resistance gene 1 (MDR1) product P-glycoprotein (Pgp) represents the most widely studied membrane protein of the large mammalian ABC transporter family (30). Although the physiological role of Pgp is not fully understood, it is conceivable that Pgp may prevent intracellular accumulation of potentially toxic substances and metabolites (31). Its wide tissue distribution (adrenal gland, breast, placenta, brain, etc.) suggests that Pgp has a fundamental role in normal cellular metabolism (32). The MDR1 gene is polymorphic and more than 40 SNPs have been identified so far. The C3435T mutation in exon 26 of the MDR1 gene is a silent mutation encoding the amino acid isoleucine. This polymorphism affects the expression and function of the Pgp in many ways (33,34). C3435T polymorphism in the MDR1 gene may limit the local detoxification activity in breast tissue and be a risk factor for cancer development and behaviour (35). Different studies have been associated this polymorphism in MDR1 gene with smoking, high body mass index and risk of BC (35,36).

Considering the above background and observations, we studied the local distribution of TSER, MTHFR C677T, p53 codon 72 and MDR1 C3435T gene polymorphisms in BC patients from Gran Canaria (Canary Islands, Spain) looking for genetic risk factors that could be related with other risk factors (e.g., environmental factors), to help to understand the rate of the disease in our population.

Materials and methods

Patients. Between March 2005 and March 2008, a total of 135 patients diagnosed with primary breast cancer at our Service (Medical Oncology Service, Hospital Insular de Gran Canaria, Gran Canaria, Spain) were enrolled in this study. Patients in clinical stages I-IV were included in the study. The diagnosis of the disease was confirmed by histological examinations. All participants were Caucasians of Canary origin. A second group of 304 healthy age-matched women from the same geographical area enrolled in the 'CDC de Canarias' cohort study, was formed as a control group (37). The Ethics Committee of our Hospital approved the present study and written informed consent was obtained from each participant before biological samples were obtained. All women in this trial filled out a structured questionnaire including patient history and risk factors in relation to BC development. Age, anthropometrical variables expressed by body mass index (BMI kg/m^2), smoking habits, reproductive and menstrual history, exposure to estrogens and family history data were collected in cases and controls. For final analysis of disease risk, only women with >1 year of oral contraceptive consumption were used for cancer risk expression. Demographic characteristics of cases and controls are shown in Table IA and B.

Genotyping. Polymorphic sites in TYMS (TSER), MTHFR (C677T), p53 codon 72 (Arg/Pro) and MDR1 (C3435T) were examined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. Genomic DNA was extracted from whole blood (5 ml) using a DNA Isolation Kit for mammalian blood (Roche). DNA was then quantified and stored at -20˚C. PCR was performed on each DNA sample using the primer sequences detailed in Table II. The PCR reactions contained 0.2 mM dNTPs (Amersham Pharmacia), 1X PCR buffer (Applied Biosystems), 1.5 mM $MgCl_2$ (Applied Biosystems), 1 μM forward and reverse primers (EcoGene), 1 U ampliTaq Gold DNA polymerase (Applied Biosystems) and 200 ng DNA in a 20-μl reaction volume. PCR amplification protocol for TSER included 10% of DMSO. For the amplification of the DNA regions containing the polymorphic sites in TSER, the following program was used (38,39): an initial denaturation step at 95˚C for 10 min, 40 cycles of 30 sec at 95˚C, 30 sec at 63˚C, 30 sec at 72˚C, and a final extension step of 7 min at 72˚C. The PCR amplification conditions for the polymorphism MTHFR C677T consisted of (39) an initial denaturation step at 95˚C for 10 min, followed by 40 cycles

Table I-A. Demographic characteristics of cancer patients and controls (continuation).

Characteristics	Cases (%)	Controls (%)	P-value
Total	135 (100)	304 (100)	
Age (years)			
≤49	59 (43.7)	179 (58.9)	<0.001
50-59	35 (25.9)	88 (28.9)	
≥60	36 (26.7)	36 (11.8)	
ND	5 (3.7)	1 (0.3)	
BMI (kg/m^2)			
≤25	53 (39.3)	99 (32.6)	0.190
25.1-30	45 (33.3)	113 (37.2)	
≥30	30 (22.2)	90 (29.6)	
ND	7 (5.2)	2 (0.7)	
Smokers			
No	249 (81.9)	110 (81.5)	0.537
Yes	54 (17.8)	20 (14.8)	
ND	1 (0.3)	5 (3.7)	
Menopause			
No	49 (36.3)	188 (61.8)	<0.001
Yes	81 (60.0)	115 (37.8)	
ND	5 (3.7)	1 (0.3)	
Early menarche (<11 years old)			
No	124 (91.9)	227 (91.1)	0.148
Yes	6 (4.4)	26 (8.6)	
ND	5 (3.7)	1 (0.3)	
Oral contraceptives exposure (>1 year of exposure)			
No	58 (43.0)	121 (39.8)	0.332
Yes	71 (52.6)	182 (59.9)	
ND	6 (4.4)	1 (0.3)	
Estrogens exposure after menopause			
No	66 (81.5)	83 (72.8)	0.080
Yes	13 (16.0)	31 (27.2)	
ND	2 (2.5)	1 (0.3)	
Descendants			
No	22 (16.3)	34 (11.2)	0.105
Yes	108 (80.0)	269 (88.5)	
ND	5 (3.7)	1 (0.3)	
Lactation			
No	28 (29.7)	95 (31.3)	0.079
Yes	80 (59.3)	174 (57.2)	
Family history[a]			
No	116 (85.9)	253 (83.2)	0.123
Yes	14 (10.4)	50 (16.4)	
ND	5 (3.7)	1 (0.3)	

[a]Family history of breast cancer in first degree relatives. ND, non-determined.

Table I-B. Demographic characteristics of cancer patients and controls (continuation).

Characteristics	Cases	Controls	P-value
Total	135 (100)	304 (100)	
Age (years)			
Mean/Range	51.91/32-76	47.32/34-65	<0.001
BMI (kg/m^2)			
Mean/Range	27.24/18-41	27.78/16-59	0.340
Smoke (years)			
Mean/Range	19.66/2-45	21.21/2-45	0.378
Age of menarche			
Mean/Range	12.68/9-16	12.65/8-18	0.887
Contraceptives exposure (years)			
Mean/Range	7.21/1-32	6.85/1-28	0.674
No. of children			
Mean/Range	2.22/0-10	2.45/0-13	0.238
Age at first labour			
Mean/Range	25.88/16-42	23.59/14-35	<0.001
Lactation (months)			
Mean/Range	8.99/0-63	6.52/0-72	0.042

of 1 min at 95˚C, 1 min at 63˚C, 1 min at 72˚C and a final extension step at 72˚C for 7 min. The PCR amplification conditions for the polymorphism at codon 72 in p53 consisted of (26) an initial denaturation step at 94˚C for 2 min, followed by 35 cycles of 30 sec at 94˚C, 45 sec at 60˚C, 30 sec at 72˚C and a final extension step at 72˚C for 10 min. The PCR amplification conditions for the polymorphism in MDR1 C3435T consisted of (36) an initial denaturation step at 94˚C for 2 min, followed by 35 cycles of 30 sec at 94˚C, 30 sec at 60˚C, 30 sec at 72˚C and a final extension step at 72˚C for 4 min. Except for TSER, the amplified fragments were digested with the appropriate restriction endonucleases (New England BioLabs) listed in Table II. After incubation at the optimal temperature, 20 μl of digested product was analyzed by gel electrophoresis on a 2-3% low melting agarose gel and visualized under ultraviolet light in a ChemiDoc System (Bio-Rad) after staining with ethidium bromide (Pierce). Electrophoresis identification of the alleles by their PCR product length is shown in Fig. 1.

Statistical analysis. The difference in allele or genotype frequencies between controls and BC patients was determined using the χ^2 test, as well for determination of the deviation from the Hardy-Weinberg equilibrium. A binary logistic regression model was used to calculate the odds ratio (OR) and the corresponding 95% confidence interval (CI) to set the association between the polymorphisms and cancer risk. T-test was used to explore the association between continuous variables. χ^2 test was used in the case of categorical

Table II. Technical details of PCR-RFLP analysis.

Gene ref. seq.	Polymorphism	PCR primers	PCR product size (bp)	Restriction enzyme (T[a])	Fragment identifying genotypes (bp)
TYMS	TSER	F[a]: GTGGCTCCTGCGTTTCCCCC			2R/2R=215; 3R/3R=243
NT_010859		R[b]: GCTCCGAGCCGGCCACAGGCATGGCGCGG			2R/3R=243, 215
MTHFR	C677T	F[a]: TGAAGGAGAAGGTGTCTGCGGGA	198	HinfI, 37˚C	C/C=198; T/T=176
NT_021937		R[b]: AGGACGGTGCGGTGAGAGTG			C/T=198, 176
p53	Arg72Pro	F[a]: TCCCCCTTGCCGTCCCAA	279	BstUI, 60˚C	Arg/Arg=160, 119; Pro/Pro=279
NT_010718		R[b]: CGTGCAAGTCACAGACTT			Arg/Pro=279, 160, 119
MDR1	C3435T	F[a]: TGCTGGTCCTGAAGTTGATCTGTGAAC	248	MboI, 37˚C	C/C=170, 60; T/T=238
NT_007933		R[b]: ACATTAGGCAGTGACTCGATGAAGGCA			C/T=238, 170, 60

[a]Forward primer. [b]Reverse primer.

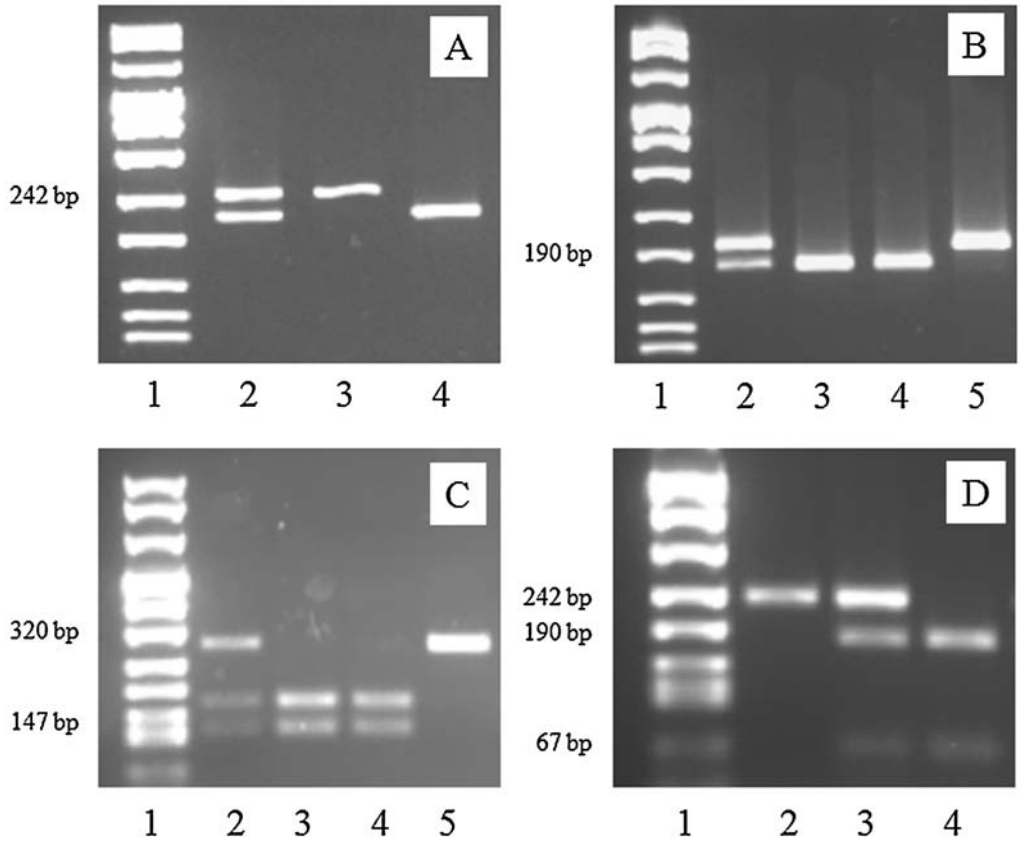

Figure 1. Agarose gel electrophoresis of TSER (A) PCR product and MTHFR^{C677T} (B), p53^{Arp72Pro} (C) and MDR1^{C3435T} (D) PCR products digested with the appropriate restriction enzyme. Lane 1 in all images corresponds to DNA Molecular Weigh Marker VIII (Roche). (A) Lane 2, heterozygote 2R/3R (215 and 243 bp); lane 3, homozygote 3R/3R (243 bp); lane 4, homozygote 2R/2R (215 bp). (B) Lane 2, heterozygote C/T (198 and 176 bp); lanes 3 and 4, homozygote TT (176 bp); lane 5, homozygote CC (198 bp). (C) Lane 2, heterozygote Arg/Pro (279, 160 and 119 bp); lanes 3 and 4, homozygote Arg/Arg (160 and 119 bp); lane 5, homozygote Pro/Pro (279 bp). (D) Lane 2, homozygote TT (238 bp); lane 3, heterozygote CT (238, 170 and 60 bp); lane 4, homozygote CC (170 and 60 bp).

variables. All tests were two-sided and differences were considered significant at $p<0.05$. All analysis were performed with SPSS for Windows (version 15.0; SPSS, Chicago, IL), in April 2009.

Results

The study population consisted of 135 women with histologically confirmed breast carcinoma with an age range from

Genotype and allelic frequencies of gene polymorphisms in this study.

	n	Genotypes (n/%)				Alleles (n/%)		
TS 28 bp STR		**2/2**	**2/3**	**3/3**	**P-value**	**2**	**3**	**P-value**
Cases	133	44 (0.33)	59 (0.44)	30 (0.23)	0.109	73.5 (0.55)	59.5 (0.45)	0.240
Controls	283	66 (0.24)	145 (0.51)	72 (0.25)		138.5 (0.49)	144.5 (0.51)	
MTHFR C677T		**C/C**	**C/T**	**T/T**		**C**	**T**	
Cases	135	52 (0.39)	65 (0.48)	18 (0.13)	0.754	84.5 (0.63)	50.5 (0.37)	0.568
Controls	292	107 (0.36)	138 (0.48)	47 (0.16)		176 (0.60)	116 (0.40)	
p53 codon72		**Arg/Arg**	**Arg/Pro**	**Pro/Pro**		**Arg**	**Pro**	
Cases	135	73 (0.54)	54 (0.40)	8 (0.06)	0.290	100 (0.74)	35 (0.26)	0.910
Controls	295	167 (0.56)	100 (0.34)	28 (0.10)		217 (0.735)	78 (0.264)	
MDR1 C3435T		**C/C**	**C/T**	**T/T**		**C**	**T**	
Cases	135	35 (0.26)	70 (0.52)	30 (0.22)	0.567	70 (0.52)	65 (0.48)	0.523
Controls	301	85 (0.28)	162 (0.54)	54 (0.18)		166 (0.55)	135 (0.45)	

Table IV. Association between p53 genotype and alleles with family history and MDR-1 with smoking.

	Group	Genotype/ factor	Family history (n/%)	Non-family history (n/%)	P-value	Allele/ factor	Family history (n/%)	Non-family history (n/%)	P-value
p53 Arg72Pro	Control	Arg/Arg	21 (43.7)	145 (58.9)	0.024	Arg	30.5 (63.5)	185.5 (75.4)	0.096
		Arg/Pro	19 (39.6)	81 (32.9)		Pro	17.5 (36.5)	60.5 (24.5)	
		Pro/Pro	8 (16.7)	20 (8.1)					
	Case	Arg/Arg	4 (28.6)	66 (56.9)	0.031	Arg	8 (57.1)	88 (75.8)	0.132
		Arg/Pro	8 (57.1)	44 (37.9)		Pro	6 (42.9)	28 (24.1)	
		Pro/Pro	2 (14.3)	6 (5.2)					
			Smokers	Non-smokers			Smokers	Non-smokers	
MDR1 C3435T	Case	C/C	2 (10.0)	31 (28.2)	0.038	C	10 (50.0)	58 (52.7)	0.822
		C/T	16 (80.0)	54 (49.1)		T	10 (50.0)	52 (47.3)	
		T/T	2 (10.0)	25 (22.7)					

32 to 76 years (mean 51.9 years) at the time of diagnosis. The majority of women were post-menopausal (60%). Control group consisted in 304 women with a mean age of 47.3 years and 37.8% of menopausal women. Demographic characteristics of cases and controls are detailed in Table IA and B. There was a significant difference between menopausal frequencies in cases compared with controls (χ^2 test, P<0.001). Mean age at first labor was 25.88 and 23.59 years in cases and controls, respectively, with a significant difference between means (t-test, P<0.001). In our study population, patients with BC gave birth >2 years later than control women. There was no significant difference in relation to lactation, although the mean of months of lactation was higher in cases (8.99) compared with controls (6.52) (t-test, P=0.042). TSER, MTHFR C677T, p53 Arg72Pro and MDR1 C3435T alleles and genotype frequencies were estimated for control group and patients with BC in the current study. More than 95% of the polymorphism determinations were successful. Genotype and allelic frequencies are shown in Table III. No significant differences were observed in any case, and there were no statistical changes of genotype distribution of polymorphisms in cases and controls when stratified by age (<50 vs. 50-59 vs. >60; <50 vs. ≥50 years) or by BMI (≤25 vs. 25.1-30 vs. >30) (data not shown). The genotype distributions among cases and controls were in Hardy-Weinberg equilibrium (P=NS). We did not observe incremented risk of BC in relation with any genotype of the genes under study (P=NS, binary logistic regression model used). We observed a significant association between p53 Arg72Pro genotype and family history of BC in control group (χ^2 test, P=0.024). Allele Pro was present in 36.5% of healthy people with family history of the illness compared with the 24.5% of allelic frequency detected in people with non-familial history (χ^2 test, P=0.096). The same pattern was observed in the group of patients with BC (P=0.031). Allele Pro was present in 42.9% of patients with family history compared with the 24.1% of patients with non-family history of BC (χ^2 test, P=0.132). These data are shown in Table IV.

Table V. p53 codon 72 gene polymorphism in healthy women from different populations compared to Canary individuals (χ^2 test).

Population	n	Arg/Arg (%)	Arg/Pro (%)	Pro/Pro (%)	P-value	Author
Canary	295	167 (0.56)	100 (0.34)	28 (0.10)		Present study
China	160	56 (0.35)	72 (0.45)	32 (0.20)	<0.001	Siddique *et al* (54)
Greece	51	10 (0.20)	32 (0.63)	9 (0.17)	<0.001	Kalemi *et al* (55)
Nigeria	122	15 (0.12)	60 (0.49)	47 (0.39)	<0.001	Beckman *et al* (51)
Peru	127	65 (0.51)	46 (0.37)	16 (0.12)	0.497	Klug *et al* (56)
Portugal	145	92 (0.63)	40 (0.28)	13 (0.09)	0.342	Santos *et al* (57)
Swedish	689	375 (0.54)	253 (0.37)	61 (0.089)	0.733	Sjalander *et al* (58)
USA	164	90 (0.55)	62 (0.38)	12 (0.07)	0.605	Madeleine *et al* (59)
Germany	151	84 (0.56)	57 (0.38)	10 (0.066)	0.514	Klaes *et al* (60)
Italy	172	86 (0.50)	71 (0.41)	15 (0.087)	0.229	Rezza *et al* (61)
Russia	249	130 (0.52)	105 (0.42)	14 (0.056)	0.069	Suspitsin *et al* (28)
Tunisia	34	13 (0.39)	19 (0.56)	2 (0.059)	0.044	Mabrouk *et al* (62)
Israel	162	24 (0.15)	134 (0.83)	4 (0.025)	<0.001	Arbel-Alon *et al* (63)

Related to this gene, we made a comparison of codon 72 polymorphism in healthy women from different populations of the world (Table V) (23). We found significant differences between genotype distribution in our population compared with people from China, Greece, Nigeria, Tunisia and Israel. On the other hand, our population seems to have a similar genotype distribution of p53 Arg72Pro to healthy women from Peru, Portugal, Sweden, USA, Germany, Italy and Russia. Regarding MDR1 C3435T, we observed a significant association between this gene polymorphism and smoking, but only in patients with BC (P=0.038). In patients, a slight reduction of allele T was detected in non-smokers compared with smokers (47.3 and 50%, respectively). In any case, this change in allelic distribution was statistically significant (Table IV). No significant association was observed between MDR1 and smoking in the control group (data not shown).

Discussion

In the present study we explored the role of TSER, MTHFR C677T, p53 Arg72Pro and MDR1 C3435T gene polymorphisms in Canary population, looking for genetic findings that could help to understand the specific characteristics related to breast cancer observed in our population. All the gene polymorphisms were selected in order to investigate the relation with breast cancer risk.

TSER is a single tandem repeat polymorphism in the 5'-UTR enhancer region of the thymidylate synthase promoter (TYSM). The role of TSER in the response to fluoropyrimidine-based chemotherapy has been reported and accepted (40). However, its implication in cancer as a risk factor is actually not clear. Ko *et al* observed an incremented risk of cholangiocarcinoma in 47 carriers of TSER 2R (+) in combination with MTHFR 677CC compared with 204 healthy controls in a Korean population (11). Related to breast cancer, a case-control study was made in 432 patients and 473 free of cancer controls from China (10). Different gene polymorphisms in TYMS were determined. Authors suggested that the *TS3'-UTR del6* polymorphism may play a role in the etiology of breast cancer. No association was observed in relation with TSER. Genetic polymorphisms vary between ethnic groups. In the study of Zhai *et al* (10), the frequencies of TSER 3R/3R, 2R/3R and 2R/2R genotypes were 64.5, 30.2 and 5.3%, respectively, among 473 control subjects, which were significantly different from those among the 283 controls presented in the present study (P<0.0001). The genotype distribution is quite different between Chinese and Canary populations. Despite this, our findings do not contradict the results of this large study, because we did not observe any relation between breast cancer risk and TSER. In the other hand, genotype frequencies of TSER in our study was similar to other Caucasian populations (χ^2 test, P=NS) (41,42).

The role of MTHFR C677T gene polymorphism in breast cancer risk is very controversial. Allele T seems to be involved in cancer risk. There are some studies in favor of this argument (43), most of them in people from Turkey and oriental countries (44,45). This hypothesis is refuted by different authors in different populations (46,47). Moreover, there are studies that support a possible benefit of this polymorphism in the prevention of breast cancer (48). Several meta-analysis indicate that this gene polymorphism is a possible modifier of breast cancer risk, specially in association with other risk factors such as estrogen exposure in premenopausal women. Furthermore, there is some evidence that high folate intake may counterbalance the effect of this SNP in increasing breast cancer risk (15,49). We did not observe any association between MTHFR C677T gene polymorphism and breast cancer risk when cases and control subjects were stratified by menopausal status, even by oral contraceptive exposure. Notwithstanding, a borderline significance was observed in allele distribution in premenopausal women (χ^2 test, P=0.074). Allele T was less frequent in patients with breast cancer compared with controls (29 and 42%, respectively). The same pattern in T allele frequency was observed in other publications (48,50). No significant increment in cancer risk

cted related to this finding. Genotype and allele ons change depending on the population studied. This is why we compared genotype and allele frequencies from our study with others (15). We did not observe differences comparing with people from UK, USA, Austria, Germany or Italy (χ^2 test, P=NS). Nevertheless, a significant difference was observed between our population compared with Turkish and white non-Hispanic people from USA (χ^2 test, P<0.05). Sample size, experimental design and patient selection, end-points considered (folate intake) and intrinsic characteristics of each population could help to explain these findings.

The association between p53 codon 72 gene polymorphism and cancer risk has been reported in different populations, although results with regard to most cancer remain incomplete (27-29). In our population, we did not observed any differences in genotype and allele distributions between cases and controls, and no increment in breast cancer risk was detected. Studies that support the hypothesis related to this polymorphism as a cancer risk observed a relative frequency of the Arg allele higher in breast cancer patients (23). This finding was not reproduced in the present study. These contradictory results may be attributed to the ethnic differences, chance deviations, or publication bias toward 'positive' findings. There are considerable variations in the distribution of the codon 72 genotypes in several populations. This SNP seems to be maintained by natural selection influenced by environmental factors, such as the degree of exposure to sunlight UV-B component (51). Population-based studies indicate that the Arg allele is most prevalent in individuals with light complexion, with a clear decline in the prevalence of Pro allele with increasing north latitude (51). We compared the genotype and allele distribution of our population with other ones, concluding significant differences with people from China, Greece, Nigeria, Tunisia and Israel. Unexpected, because the Canary population has a light complex, the genotype distribution of this gene polymorphism was similar to other populations from Europe, USA and Peru, indicating that genotype distribution in our region has it own characteristics. We observed a significant association between p53 Arg72Pro genotype and family history of BC in cases and controls. This SNP seems to be relevant in the modulation of apoptotic capacity of cells, with Pro allele reducing apoptosis potential (24). Because this association was observed in cases and controls in the same pattern (Table IV), this finding may be explained, at least in part, by the natural selection and genetic inheritance of p53 Arg72Pro gene polymorphism.

MDR1 C3435T has been involved in susceptibility and prognosis of some cancers with more or less positive association. The results varied in level of statistical significance and role of genotype consequence. Whereas the T/T form was in majority associated with higher risk of disease origin, the carriers of C/C genotype were supposed to have a worse prognosis (35). We did not observe significant differences in this gene polymorphism between patients with breast cancer compared with controls. These findings are in contrast with other studies, although the literature on the 'functional' effects of SNPs in the MDR1 gene is replete with contradictory findings (52). Turgut *et al* demonstrated a 1.5-fold increased risk for development of breast cancer in T allele carriers, in a study with 57 patients and 50 healthy subjects in a Turkish population (36). In the present study, T allele in cases was slightly higher than in controls, but this finding was not significant. Sample size and intrinsic characteristics of each population could explain this result. The human multidrug resistance gene 1 encodes a plasma membrane P-glycoprotein (Pgp) that functions as the transmembrane efflux pump for various structurally unrelated anticancer agents and toxins. Functional polymorphism C3435T in the MDR1 gene alters the detoxification protective function of Pgp in breast tissue cells exposed to environmental carcinogens in tobacco smoke. This SNP occurs in high incidence among women with breast carcinoma where C allele carriers have increased risk of developing cancer when exposed to toxic substances (35). No significant differences were observed in genotype and allelic frequencies between this publication and the present study. A significant association was detected between MDR1 C3435T genotype frequencies and smoking in cases. T allele was present in high frequency in smoker patients than in non-smokers, but this slight increase in T allele distribution was not significant. No statistical increase in breast cancer risk was detected. It was reported that C/T and T/T carriers have low levels of Pgp expression, provoking an abnormal accumulation of harmful substances into the cells with subsequent cell damage (53). The meaning of this association in our population should be explored in detail.

In conclusion, TSER, MTHFR C677T, p53 Arg72Pro and MDR1 C3435T gene polymorphisms were determined in 135 patients with primary breast cancer and in 304 healthy controls from the Canary Islands. No significant differences were observed in genotype and allele frequencies between case and control groups, and no breast cancer risk factor was observed related to these polymorphisms. A statistical association was seen between p53 codon 72 gene polymorphism and family history of breast cancer in both groups, as well as between MDR1 C3435T gene polymorphism and smoking habits in cases (35). We introduced new data that could help to characterize the Canary population. Further experiments are needed to understand the incidence of breast cancer in our region.

Acknowledgements

We thank Fátima Gillén and Elia García for their crucial role in sample and questionnaire collection. Dr Jesús García Foncillas and Dr Ruth Zárate for technical support and protocol assistance. Dr Laura López Ríos for her inestimable help in the statistical analysis. This study was supported by a grant from the Foundation of the Canary Institute for Cancer Research (FICIC). Henríquez-Hernández LA was supported by a grant of the Consejería de Industria from the Canary Government and the Canary Foundation for Investigation and Health (FUNCIS).

References

1. Kamangar F, Dores GM and Anderson WF: Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. J Clin Oncol 24: 2137-2150, 2006.

2. Maca-Meyer N, Villar J, Perez-Mendez L, Cabrera de Leon A and Flores C: A tale of aborigines, conquerors and slaves: Alu insertion polymorphisms and the peopling of Canary Islands. Ann Hum Genet 68: 600-605, 2004.
3. Zumbado M, Goethals M, Alvarez-Leon EE, *et al*: Inadvertent exposure to organochlorine pesticides DDT and derivatives in people from the Canary Islands (Spain). Sci Total Environ 339: 49-62, 2005.
4. Veronesi U, Boyle P, Goldhirsch A, Orecchia R and Viale G: Breast cancer. Lancet 365: 1727-1741, 2005.
5. Dumitrescu RG and Cotarla I: Understanding breast cancer risk - where do we stand in 2005? J Cell Mol Med 9: 208-221, 2005.
6. Freisinger F and Domchek SM: Clinical implications of low-penetrance breast cancer susceptibility alleles. Curr Oncol Rep 11: 8-14, 2009.
7. Sharp L and Little J: Polymorphisms in genes involved in folate metabolism and colorectal neoplasia: a HuGE review. Am J Epidemiol 159: 423-443, 2004.
8. Rustum YM, Harstrick A, Cao S, *et al*: Thymidylate synthase inhibitors in cancer therapy: direct and indirect inhibitors. J Clin Oncol 15: 389-400, 1997.
9. Kawakami K, Omura K, Kanehira E and Watanabe Y: Polymorphic tandem repeats in the thymidylate synthase gene is associated with its protein expression in human gastrointestinal cancers. Anticancer Res 19: 3249-3252, 1999.
10. Zhai X, Gao J, Hu Z, *et al*: Polymorphisms in thymidylate synthase gene and susceptibility to breast cancer in a Chinese population: a case-control analysis. BMC Cancer 6: 138, 2006.
11. Ko KH, Kim NK, Yim DJ, *et al*: Polymorphisms of 5,10-methylenetetrahydrofolate reductase (MTHFR C677T) and thymidylate synthase enhancer region (TSER) as a risk factor of cholangiocarcinoma in a Korean population. Anticancer Res 26: 4229-4233, 2006.
12. Kim YI: Folate and carcinogenesis: evidence, mechanisms, and implications. J Nutr Biochem 10: 66-88, 1999.
13. Goyette P, Sumner JS, Milos R, *et al*: Human methylenetetrahydrofolate reductase: isolation of cDNA mapping and mutation identification. Nat Genet 7: 551, 1994.
14. Frosst P, Blom HJ, Milos R, *et al*: A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 10: 111-113, 1995.
15. Macis D, Maisonneuve P, Johansson H, *et al*: Methylenetetrahydrofolate reductase (MTHFR) and breast cancer risk: a nested-case-control study and a pooled meta-analysis. Breast Cancer Res Treat 106: 263-271, 2007.
16. Boccia S, Boffetta P, Brennan P, *et al*: Meta-analyses of the methylenetetrahydrofolate reductase C677T and A1298C polymorphisms and risk of head and neck and lung cancer. Cancer Lett 273: 55-61, 2009.
17. Esteller M, Garcia A, Martinez-Palones JM, Xercavins J and Reventos J: Germ line polymorphisms in cytochrome-P450 1A1 (C4887 CYP1A1) and methylenetetrahydrofolate reductase (MTHFR) genes and endometrial cancer susceptibility. Carcinogenesis 18: 2307-2311, 1997.
18. Graziano F, Kawakami K, Ruzzo A, *et al*: Methylenetetrahydrofolate reductase 677C/T gene polymorphism, gastric cancer susceptibility and genomic DNA hypomethylation in an at-risk Italian population. Int J Cancer 118: 628-632, 2006.
19. Iacopetta B, Heyworth J, Girschik J, Grieu F, Clayforth C and Fritschi L: The MTHFR C677T and DeltaDNMT3B C-149T polymorphisms confer different risks for right- and left-sided colorectal cancer. Int J Cancer 125: 84-90, 2009.
20. Pereira TV, Rudnicki M, Pereira AC, Pombo-de-Oliveira MS and Franco RF: 5,10-Methylenetetrahydrofolate reductase polymorphisms and acute lymphoblastic leukemia risk: a meta-analysis. Cancer Epidemiol Biomarkers Prev 15: 1956-1963, 2006.
21. Borresen-Dale AL: TP53 and breast cancer. Hum Mutat 21: 292-300, 2003.
22. Papadakis EN, Dokianakis DN and Spandidos DA: p53 codon 72 polymorphism as a risk factor in the development of breast cancer. Mol Cell Biol Res Commun 3: 389-392, 2000.
23. Damin AP, Frazzon AP, Damin DC, *et al*: Evidence for an association of TP53 codon 72 polymorphism with breast cancer risk. Cancer Detect Prev 30: 523-529, 2006.
24. Dumont P, Leu JI, Della Pietra AC III, George DL and Murphy M: The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. Nat Genet 33: 357-365, 2003.
25. Harris N, Brill E, Shohat O, *et al*: Molecular basis for heterogeneity of the human p53 protein. Mol Cell Biol 6: 4650-4656, 1986.
26. Xu Y, Yao L, Ouyang T, *et al*: p53 codon 72 polymorphism predicts the pathologic response to neoadjuvant chemotherapy in patients with breast cancer. Clin Cancer Res 11: 7328-7333, 2005.
27. Koushik A, Platt RW and Franco EL: p53 codon 72 polymorphism and cervical neoplasia: a meta-analysis review. Cancer Epidemiol Biomarkers Prev 13: 11-22, 2004.
28. Suspitsin EN, Buslov KG, Grigoriev MY, *et al*: Evidence against involvement of p53 polymorphism in breast cancer predisposition. Int J Cancer 103: 431-433, 2003.
29. Zhang ZW, Laurence NJ, Hollowood A, *et al*: Prognostic value of TP53 codon 72 polymorphism in advanced gastric adenocarcinoma. Clin Cancer Res 10: 131-135, 2004.
30. Borst P and Elferink RO: Mammalian ABC transporters in health and disease. Annu Rev Biochem 71: 537-592, 2002.
31. Schinkel AH: The physiological function of drug-transporting P-glycoproteins. Semin Cancer Biol 8: 161-170, 1997.
32. Hitchins RN, Harman DH, Davey RA and Bell DR: Identification of a multidrug resistance associated antigen (P-glycoprotein) in normal human tissues. Eur J Cancer Clin Oncol 24: 449-454, 1988.
33. Hoffmeyer S, Burk O, von Richter O, *et al*: Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo. Proc Natl Acad Sci USA 97: 3473-3478, 2000.
34. Sauna ZE, Kimchi-Sarfaty C, Ambudkar SV and Gottesman MM: Silent polymorphisms speak: how they affect pharmacogenomics and the treatment of cancer. Cancer Res 67: 9609-9612, 2007.
35. Zubor P, Lasabova Z, Hatok J, Stanclova A and Danko J: A polymorphism C3435T of the MDR-1 gene associated with smoking or high body mass index increases the risk of sporadic breast cancer in women. Oncol Rep 18: 211-217, 2007.
36. Turgut S, Yaren A, Kursunluoglu R and Turgut G: MDR1 C3435T polymorphism in patients with breast cancer. Arch Med Res 38: 539-544, 2007.
37. Cabrera de Leon A, Rodriguez Perez Mdel C, Almeida Gonzalez D, *et al*: Presentation of the 'CDC de Canarias' cohort: objectives, design and preliminary results. Rev Esp Salud Publica 82: 519-534, 2008.
38. Jakobsen A, Nielsen JN, Gyldenkerne N and Lindeberg J: Thymidylate synthase and methylenetetrahydrofolate reductase gene polymorphism in normal tissue as predictors of fluorouracil sensitivity. J Clin Oncol 23: 1365-1369, 2005.
39. Salgado J, Zabalegui N, Gil C, Monreal I, Rodriguez J and Garcia-Foncillas J: Polymorphisms in the thymidylate synthase and dihydropyrimidine dehydrogenase genes predict response and toxicity to capecitabine-raltitrexed in colorectal cancer. Oncol Rep 17: 325-328, 2007.
40. Russo A, Corsale S, Cammareri P, *et al*: Pharmacogenomics in colorectal carcinomas: future perspectives in personalized therapy. J Cell Physiol 204: 742-749, 2005.
41. Iacopetta B, Grieu F, Joseph D and Elsaleh H: A polymorphism in the enhancer region of the thymidylate synthase promoter influences the survival of colorectal cancer patients treated with 5-fluorouracil. Br J Cancer 85: 827-830, 2001.
42. Marsh S, Collie-Duguid ES, Li T, Liu X and McLeod HL: Ethnic variation in the thymidylate synthase enhancer region polymorphism among Caucasian and Asian populations. Genomics 58: 310-312, 1999.
43. Stevens VL, McCullough ML, Pavluck AL, *et al*: Association of polymorphisms in one-carbon metabolism genes and postmenopausal breast cancer incidence. Cancer Epidemiol Biomarkers Prev 16: 1140-1147, 2007.
44. Deligezer U, Akisik EE and Dalay N: Homozygosity at the C677T of the MTHFR gene is associated with increased breast cancer risk in the Turkish population. In Vivo 19: 889-893, 2005.
45. Lee SA, Kang D, Nishio H, *et al*: Methylenetetrahydrofolate reductase polymorphism, diet, and breast cancer in Korean women. Exp Mol Med 36: 116-121, 2004.
46. Hekim N, Ergen A, Yaylim I, *et al*: No association between methylenetetrahydrofolate reductase C677T polymorphism and breast cancer. Cell Biochem Funct 25: 115-117, 2007.
47. Tao MH, Shields PG, Nie J, *et al*: DNA promoter methylation in breast tumors: no association with genetic polymorphisms in MTHFR and MTR. Cancer Epidemiol Biomarkers Prev 18: 998-1002, 2009.

YC, Wu MH, Yu JC, *et al*: Genetic polymorphisms of the methylenetetrahydrofolate reductase gene, plasma folate levels and breast cancer susceptibility: a case-control study in Taiwan. Carcinogenesis 27: 2295-2300, 2006.
49. Zintzaras E: Methylenetetrahydrofolate reductase gene and susceptibility to breast cancer: a meta-analysis. Clin Genet 69: 327-336, 2006.
50. Justenhoven C, Hamann U, Pierl CB, *et al*: One-carbon metabolism and breast cancer risk: no association of MTHFR, MTR, and TYMS polymorphisms in the GENICA study from Germany. Cancer Epidemiol Biomarkers Prev 14: 3015-3018, 2005.
51. Beckman G, Birgander R, Sjalander A, *et al*: Is p53 polymorphism maintained by natural selection? Hum Hered 44: 266-270, 1994.
52. Ambudkar SV, Kimchi-Sarfaty C, Sauna ZE and Gottesman MM: P-glycoprotein: from genomics to mechanism. Oncogene 22: 7468-7485, 2003.
53. Penson RT, Oliva E, Skates SJ, *et al*: Expression of multidrug resistance-1 protein inversely correlates with paclitaxel response and survival in ovarian cancer patients: a study in serial samples. Gynecol Oncol 93: 98-106, 2004.
54. Siddique MM, Balram C, Fiszer-Maliszewska L, *et al*: Evidence for selective expression of the p53 codon 72 polymorphs: implications in cancer development. Cancer Epidemiol Biomarkers Prev 14: 2245-2252, 2005.
55. Kalemi TG, Lambropoulos AF, Gueorguiev M, Chrisafi S, Papazisis KT and Kotsis A: The association of p53 mutations and p53 codon 72, Her 2 codon 655 and MTHFR C677T polymorphisms with breast cancer in Northern Greece. Cancer Lett 222: 57-65, 2005.
56. Klug SJ, Wilmotte R, Santos C, *et al*: TP53 polymorphism, HPV infection, and risk of cervical cancer. Cancer Epidemiol Biomarkers Prev 10: 1009-1012, 2001.
57. Santos AM, Sousa H, Catarino R, *et al*: TP53 codon 72 polymorphism and risk for cervical cancer in Portugal. Cancer Genet Cytogenet 159: 143-147, 2005.
58. Sjalander A, Birgander R, Hallmans G, *et al*: p53 polymorphisms and haplotypes in breast cancer. Carcinogenesis 17: 1313-1316, 1996.
59. Madeleine MM, Shera K, Schwartz SM, *et al*: The p53 Arg72Pro polymorphism, human papillomavirus, and invasive squamous cell cervical cancer. Cancer Epidemiol Biomarkers Prev 9: 225-227, 2000.
60. Klaes R, Ridder R, Schaefer U, Benner A and von Knebel Doeberitz M: No evidence of p53 allele-specific predisposition in human papillomavirus-associated cervical cancer. J Mol Med 77: 299-302, 1999.
61. Rezza G, Giuliani M, Garbuglia AR, *et al*: Lack of association between p53 codon-72 polymorphism and squamous intraepithelial lesions in women with, or at risk for, human immunodeficiency virus and/or human papillomavirus infections. Cancer Epidemiol Biomarkers Prev 10: 565-566, 2001.
62. Mabrouk I, Baccouche S, El-Abed R, *et al*: No evidence of correlation between p53 codon 72 polymorphism and risk of bladder or breast carcinoma in Tunisian patients. Ann NY Acad Sci 1010: 764-770, 2003.
63. Arbel-Alon S, Menczer J, Feldman N, Glezerman M, Yeremin L and Friedman E: Codon 72 polymorphism of p53 in Israeli Jewish cervical cancer patients and healthy women. Int J Gynecol Cancer 12: 741-744, 2002.