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1236 C/T and 3435 C/T polymorphisms of the *ABCB1* gene in Mexican breast cancer patients

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ABSTRACT. MDR1, which is encoded by the *ABCB1* gene, is involved in multidrug resistance (hydrophobic), as well as the elimination of xenotoxic agents. The association between *ABCB1* gene polymorphisms

and breast cancer risk in different populations has been described previously; however, the results have been inconclusive. In this study, we examined the association between polymorphisms 3435 C/T and 1236 C/T in the *ABCB1* gene and breast cancer development in Mexican women according to their menopausal status and molecular classification. Molecular subtypes as well as allele and genotype frequencies were analyzed. A total of 248 women with initial breast cancer diagnosis and 180 ethnically matched, healthy, unrelated individuals were enrolled. Polymerase chain reaction-restriction fragment length polymorphism was performed to detect polymorphisms 3435 C/T and 1236 C/T in the *ABCB1* gene. Premenopausal T allele carriers of the 3435 C/T polymorphism showed a 2-fold increased risk of breast cancer with respect to the reference and postmenopausal groups, as well as triple-negative expression regarding the luminal A/B molecular subrogated subtypes. In contrast, the CT genotype of the 1236 polymorphism was a protective factor against breast cancer. We conclude that the T allele carrier of the 3435 C/T polymorphism in the *ABCB1* gene in combination with an estrogen receptor-negative status may be an important risk factor for breast cancer development in premenopausal women.

Key words: Breast cancer; MDR1; Pre- and postmenopausal women; *ABCB1*; Polymorphisms

INTRODUCTION

Breast cancer is the most frequent malignancy in women worldwide. The incidence of this cancer continually increases in low- and middle-income countries such as Mexico (Knaul et al., 2009; Ferlay et al., 2010; Forouzanfar et al., 2011). Breast cancer patients have heterogeneous genetic, biological, and pathological features as well as variable clinical outcomes. Breast cancer oncogenesis implies complex interactions between genes and epigenetic alterations, including susceptibility genes such as *BRCA1*, *BRCA2*, *PTEN*, and *p53*, and somatic genetic alterations such as DNA methylation, chromatin remodeling, and regulation by noncoding RNA (Bièche and Lidereau, 2011). Genetic polymorphism studies are useful for identifying susceptibility genes as well as predictive biomarkers (Peng et al., 2011). The *ABCB1* gene located at 7q21.1 (OMIM: 171050, Gene ID: 5243) codes for the MDR1 protein, which is a member of the superfamily of ATP-binding cassette (ABC) transporters (Couture et al., 2006). These proteins transport molecules across intra/extracellular membranes and are involved in multidrug resistance (hydrophobic) and elimination of xenotoxic agents (Mizutani et al., 2008). Previous studies showed variable results regarding the relationship between *ABCB1* gene polymorphisms and breast cancer risk in different populations, and the clinical relevance of these polymorphisms remains unknown (Lu et al., 2011). The aim of this study was to determine the association between polymorphisms 3435 C/T (rs1045642) and 1236 C/T (rs1128503) in the *ABCB1* gene and breast cancer in Mexican women based on their menopause status and molecular classification. We hypothesized that *ABCB1* gene polymorphisms play a role in breast cancer in Mexican patients and that their polymorphic distributions differ according to their menopausal status and molecular surrogated subtypes.

MATERIAL AND METHODS

Study population

A total of 275 women were recruited as part of the Ella Binational Breast Cancer Study (Ella Study) from 3 public hospitals in Guadalajara, Jalisco, Mexico (Hospital de Especialidades - CMNO-IMSS, Hospital de Gineco-Obstetricia - IMSS, and Instituto Jalisciense de Cancerologia). The Ella Study was a case-control study including women of Mexican descent in the United States and Mexico and has been described previously (Martínez et al., 2010). In this study, we only included patients from the State of Jalisco, Mexico. The Institutional Review Board from each institution approved the study and all study participants provided written informed consent. Eligible patients were Mexican women aged 18 years and older with breast cancer. Patients were classified according to their menopausal status as premenopausal, perimenopausal, and postmenopausal. To characterize luminal A, luminal B, HER2-like, and triple-negative subtypes, ER, PR, HER2, and Ki67 were evaluated by immunohistochemistry (IHC) as previously described (Sotiriou and Pusztai, 2009; Romero et al., 2011). Patients were treated according to the Standard Clinical Practice Guidelines in Oncology according to the clinical stage, histology, hormone receptor, and HER2 status. All breast cancer patients filled out a structured Ella questionnaire. The reference group for this study included a sample of 180 individuals randomly selected from the general population, including men and women at HE-CMNO-IMSS Blood Service, Guadalajara, Mexico.

ABCB1 genotyping

DNA samples were obtained using the Miller isolation method (Miller et al., 1988) and stored at -20°C until use. DNA in the samples was quantified using the Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and adjusted to 100 ng/μL. Previously described polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) protocols were performed to detect 3435 C/T and 1236 C/T polymorphisms (Cascorbi et al., 2001) in the *ABCB1* gene. We used PCR primers, *Taq* polymerase, and dNTPs from Invitrogen (Life Technologies Corporation, Carlsbad, CA, USA). The thermal cycler used for amplification was the PTC-200 (MJ Research, Waltham, MA, USA). For the 3435 C/T polymorphism, the following primers were used: forward 5'-TgTTTTCAgCTgCTTgATgg-3' and reverse 5'-AAggCATgTAT gTTggCCTC-3' at an annealing temperature of 60°C. An amplified fragment of 197 bp was obtained. Genotypes were identified after a 16-h digestion at 37°C with the restriction endonuclease *Mbo*I (Invitrogen), producing bands of 158 and 39 bp for the C allele, and a single band of 197 bp for the T allele. The 1236 C/T polymorphism was detected using the primers forward 5'-TATCCTgTgTCTgTgAATTgCC-3' and reverse 5'-CCTgACTCACCACACCAATg-3' at an annealing temperature of 60°C. A 370-bp amplified fragment was obtained. Genotypes were identified after a 16-h digestion with *Hae*III at 37°C (Invitrogen), which generated a constitutive 273-bp band shared by both genotypes. Additional bands of 62 and 35 bp were observed in the C allele, while the T allele showed a single 97-bp fragment. Quality control was ensured using a blank control in PCR and a positive sample for each allele in RFLP. Restriction enzyme activity was tested with lambda phage digestion (Invitrogen). Polyacrylamide (29:1) 6% gel electrophoresis was performed for 40 min at 180 V with silver staining to observe the genotypes. Gel band sizes were determined using 100- and 50-bp ladder markers (Invitrogen).

Data analysis

Age, menopausal status, IHC markers, histology, and molecular subtypes were analyzed for genetic associations. We determined genotype and allele frequencies in the study groups and tested for Hardy-Weinberg equilibrium. The *ABCB1* 3435 C/T and 1236 C/T polymorphisms were estimated using the χ^2 test or the Fisher exact test and Finetti analysis (Strom and Wienker, 2007). Haplotypes were inferred from genotypes at both polymorphic sites using Haplotype Reconstructor v0.6, and linkage disequilibrium was determined. Haplotype differences among groups were estimated by calculating χ^2.

RESULTS

A total of 275 Mexican breast cancer patients from the State of Jalisco were recruited as part of the Ella study. Of these patients, 248 were included in the current study because genotypes were available. Breast cancer patients were classified according to hormonal status as either premenopausal (60 cases) or postmenopausal (169 cases). Forty-six women were excluded from the analysis because of their menopausal status. The mean age of premenopausal patients was 39 years and that of postmenopausal women was 60 years. The clinical and pathological characteristics for menopausal breast cancer patients are shown in Table 1. The most frequent histological finding was invasive/infiltrating ductal carcinoma followed by lobular and medullar carcinomas. A greater proportion of premenopausal patients were diagnosed during advanced clinical stages compared with postmenopausal patients (47 and 33% at stages III-IV, respectively).

Table 1. Clinical and pathological characteristics of breast cancer patients according to menopausal status.

Variables		Premenopausal [N (%)]	Postmenopausal [N (%)]
Age	Mean	39.5	60.2
	Range	27-49	34-95
	SD	5.0	10.9
Histology	Invasive/infiltrating ductal carcinoma	41 (79)	108 (76)
	Invasive/infiltrating lobular carcinoma	5 (9)	24 (17)
	Medullar carcinoma	2 (4)	4 (3)
	Other (mucinuos, mixed, etc.)	4 (8)	6 (4)
Clinical stage	I	4 (8)	20 (14)
	IIa	9 (16)	41 (28)
	IIb	11 (21)	26 (18)
	IIIa	6 (12)	14 (9)
	IIIb	9 (16)	18 (12)
	IIIc	3 (7)	7 (5)
	IV	6 (12)	8 (6)
	Unknown	4 (8)	11 (8)
Molecular subtype*	Luminal A	12 (23)	44 (30)
	Luminal B	12 (23)	17 (12)
	HER2-like	6 (12)	23 (16)
	HER2/luminal B	2 (4)	9 (6)
	Triple negative	12 (23)	14 (9)
	Unclassified	8 (15)	35 (27)

SD = standard deviation. *Surrogate immunohistochemical markers.

Molecular subtyping using surrogate IHC markers showed that triple-negative cancers were 2.5-fold higher in premenopausal patients (23%) than in postmenopausal women (9%). We found a few hybrid cases that represented HER2-like/luminal B expression in both

premenopausal (4%) and postmenopausal patients (6%). In contrast, the luminal A (30%) subtype predominated in postmenopausal patients, followed by HER2-like (16%), luminal B (12%), and triple-negative subtypes (9%).

The allelic and genotypic distributions of polymorphisms 1236 C/T and 3435 C/T of the *ABCB1* gene were estimated in breast cancer cases and compared with the reference group as shown in Table 2. Our results showed that genotypes of *ABCB1* polymorphisms in the reference group were in Hardy-Weinberg equilibrium. The C allele and CT genotype of both 1236 C/T and 3435 C/T polymorphisms were the most frequent in all individuals studied.

Table 2. Genotypic and allelic distributions of *ABCB1* 3435 C/T and 1236 C/T in breast cancer cases *vs* reference group.

Polymorphism group		1236 C/T		3435 C/T	
		BC (N = 248)	RG (N = 137)	BC (N = 248)	RG (N = 152)
Genotypes	CC (%)	76 (0.31)	49 (0.36)	82 (0.33)	56 (0.37)
	CT (%)	111 (0.45)	54 (0.39)	133 (0.54)	72 (0.47)
	TT (%)	61 (0.24)	34 (0.25)	33 (0.13)	24 (0.16)
χ^2		46.32		1.52	
P		<0.0001		0.2176	
Allele	C (%)	263 (0.53)	152 (0.55)	297 (0.60)	184 (0.61)
	T (%)	233 (0.47)	122 (0.45)	199 (0.40)	120 (0.39)
χ^2		0.01		0.03	
P		0.9222		0.8559	
OR (CI)		1.017 (0.731-1.415)		1.027 (0.727-1.303)	

Breast cancer cases (BC), reference group (RG).

A comparison of genotypes among breast cancer patients and the reference group revealed significant differences with respect to the 1236 C/T polymorphism given by the TT genotype (Table 2). Genotype comparisons for the 1236 C/T polymorphism according to menopausal status revealed no significant differences between pre- and postmenopausal women or between these groups with respect to the reference group (Tables 2 and 3). Although we did not find differences in the presence of the 3435 C/T polymorphism between total breast cancer cases and the reference group, significant differences were observed between the reference group and premenopausal breast cancer cases as well as between premenopausal and postmenopausal breast cancer patients (Table 3). Differences were also found when CC was compared with the CT genotype of premenopausal women *vs* the reference group [odds ratio (OR) = 2.274] and premenopausal *vs* postmenopausal women (OR = 2.212) with respect to CT heterozygotes (Table 4).

We found a significant difference in the 3435 C/T polymorphism between the luminal A surrogated phenotype and the reference group [TT *vs* CT, OR = 2.417, 95% confidence interval (CI) = 1.011-5.778, P = 0.0430], genotype comparison (χ^2 = 5.21, P = 0.0225), as well as compared to luminal B + HER2-like + triple-negative patients (TT *vs* CT, OR = 0.383, 95%CI = 0.154-0.956, P = 0.0357) by the C allele contribution. We also observed differences when we tested triple-negative patients with respect to all ER+ cases that cover luminal A and B surrogated subtype patients by the T allele (CC *vs* CT, OR = 2.378, 95%CI = 1.018-5.553, P = 0.0477).

The haplotypes of the studied groups were inferred. We observed the 4 expected haplotypes and found that H1 was the most frequent (Table 5). Linkage disequilibrium (D') values

were calculated based on the estimated haplotypes of *ABCB1* polymorphisms. The reference group had high linkage disequilibrium compared to breast cancer patients, including menopausal classification. Haplotype distributions from the reference group were significantly different regarding breast cancer, premenopausal, and postmenopausal breast cancer patients. Nevertheless, premenopausal and postmenopausal comparisons showed no differences (χ^2 = 0.96, P = 0.3272). In addition, the H4 haplotype showed a significant difference in postmenopausal women compared to the reference group (χ^2 = 4.07, P = 0.043, OR = 2.21, 95%CI = 1.008-4.877), but no differences were observed in pre- or postmenopausal women.

Table 3. Genotypic distribution and frequencies in 3435 C/T and 1236 C/T polymorphisms of *ABCB1* in pre- and postmenopausal women.

Polymorphism		1236 C/T		3435 C/T	
Group		PreM (N = 56)	PostM (N = 134)	PreM (N = 56)	PostM (N = 125)
Genotypes	CC (%)	15 (0.27)	39 (0.29)	13 (0.23)	45 (0.36)
	CT (%)	27 (0.48)	57 (0.42)	38 (0.68)	60 (0.48)
	TT (%)	14 (0.25)	38 (0.28)	5 (0.09)	20 (0.16)
vs RG	χ^2	1.7	0.39	6.92	0.11
	P	0.1923	0.5323	0.0085	0.7401
PreM *vs* PostM	χ^2	0.74		6.05	
	P	0.3897		0.0139	

Premenopausal (PreM), postmenopausal (PostM). RG = reference group. Fisher exact test.

Table 4. Analysis of *ABCB1* 3435 C/T and 1236 C/T polymorphisms in pre- and postmenopausal women.

Polymorphism	Variant	χ^2	P	OR (95%CI)
	PreM *vs* RG			
1236 C/T	CC↔CT	0.67	0.4123	1.633 (0.779-3.424)
	CC↔CT/TT	1.45	0.2290	1.522 (0.766-3.025)
3435 C/T	CC↔CT	5.13	0.0234	2.274 (1.107-4.671)
	CC↔CT/TT	3.43	0.0640	1.929 (0.956-3.895)
	PreM *vs* PostM			
1236 C/T	CC↔CT	0.74	0.390	0.726 (0.349-1.510)
	CC↔CT/TT	0.61	0.4334	0.762 (0.385-1.507)
3435 C/T	CC↔CT	4.80	0.0284	2.212 (1.078-4.540)
	CC↔CT/TT	3.43	0.0641	1.128 (0.956-3.895)

PreM = premenopausal cases; PostM = postmenopausal cases; RG = reference group; OR = odds ratio; CI = confidence interval.

Table 5. Haplotype frequencies of *ABCB1* 3435 C/T and 1236 C/T polymorphisms in the groups studied*.

Group	Haplotype**				D'	χ^2	P
	CC (H1)	TT (H2)	TC (H3)	CT (H4)			
BC	0.3874	0.2672	0.2053	0.1399	0.57	4.14	0.0419
PreM	0.3748	0.3021	0.1979	0.1251	0.64	3.94	0.0472
PostM	0.3862	0.2414	0.2206	0.1516	0.47	5.83	0.0158
RG	0.45533	0.30669	0.16291	0.07507	0.84		

*Linkage disequilibrium value is described in right column as D'. **Position 1: mdr1236 C/T; position 2: mdr3435C/T, BC = all breast cancer cases. PreM = premenopausal cases; PostM = postmenopausal cases; RG = reference group.

DISCUSSION

Breast cancer is a complex multifactorial disease caused by genetic and environmental factors. Recently, Zhang et al. (2011) analyzed genes associated with breast cancer risk, including germline mutations in *BRCA1* and 19 common loci as breast-cancer susceptibility variants. However, these genes explain only 28% of the disease heritability. In this study, we analyzed the role of the 1236 C/T and 3435 C/T polymorphic regions in the *ABCB1* gene as risk factors for breast cancer. We hypothesized that polymorphism distributions in the *ABCB1* gene may differ according to menopausal status and molecular subtypes in Mexican breast cancer patients, contributing to cancer susceptibility. In agreement with previous reports, we observed significant differences in molecular subtypes between premenopausal and postmenopausal breast cancer patients (Perou et al., 2000; Cleator et al., 2007; Sotiriou and Pusztai, 2009). Our results showed that the distributions of molecular subtypes differed between premenopausal and postmenopausal breast cancer patients. For example, triple-negative cases were more frequent in premenopausal patients, suggesting that breast cancer is a heterogeneous spectrum of different diseases. This is the first study to simultaneously explore the association between the *ABCB1* 1236 C/T and 3435 C/T polymorphisms and breast cancer risk according to menopausal status and molecular subtypes. Previous reports focused on breast cancer risk, recurrence probability, and clinicopathological characteristics with different results depending on ethnic background and the study design (Vaclavikova et al., 2008; Henríquez-Hernández et al., 2009; Lu et al., 2011; Teh et al., 2011). In this study, the 3435 C/T polymorphism was associated with a 2-fold cancer increase in risk in heterozygotes for the T allele (CT) and premenopausal breast cancer cases compared to the reference group and postmenopausal breast cancer women that were T allele carriers. In addition, luminal A patients that were C allele carriers showed a 2-fold increased risk for breast cancer compared to the reference group; however, luminal-A patients regarding any other molecular subrogated subtype showed a 0.3-fold increased risk for breast malignancy development. This may explain the protective contribution of the C allele based on characteristics in luminal-A patients with respect to other molecular subtypes. Triple-negative (ER-) patients showed a 2-fold increased risk compared to luminal A/B (ER+) patients, suggesting that estrogen receptor-negative status and T allele carrier is a risk factor for breast cancer.

The polymorphisms 1236 C/T and 3435 C/T of the *ABCB1* gene did not result in a change in amino acid sequence, but generated a conformational change in the mRNA (Wang and Sadée, 2006), leading to instability that may change the half-life of the protein. This alteration may influence the metabolism and elimination of some toxic or carcinogenic substances, allowing intracellular accumulation of metabolites causing cellular damage, apoptosis alteration, immune defects, or cancer development (Johnstone et al., 2000a,b). Our results suggest that the T allele of the 3435 C/T polymorphism in the *ABCB1* gene is an important risk factor for developing breast cancer in premenopausal women and triple-negative subrogated subtypes. It has been reported that premenopausal breast cancer differs from postmenopausal breast cancer. For example, unfavorable tumor features such as a higher frequency of being multifocal, high grade, and lymphatic involvement characterizes breast cancer in young women (Fredholm et al., 2009). Moreover, it is well-known that steroids such as estrogen and progesterone can increase the protein levels of the *ABCB1* gene and function as substrates for the MDR1 protein (Kim and Benet, 2004; Coles et al., 2009). This partially explains the differential association between 3435 C/T polymorphisms and breast cancer in our study ac-

cording to menopausal status and molecular subtype. A previous study in a Turkish population showed that T carriers of the 3435 C/T polymorphism have a higher risk (OR = 1.5 in T allele) of developing breast cancer, but there were no associations between clinical and pathological parameters (Turgut et al., 2007). In another study of Czech breast cancer patients, the prognostic values of the *ABCB1* genotype and phenotype were assessed, including the 1236 C/T and 3435 C/T polymorphisms. The results revealed no significant correlation between *ABCB1* mRNA expression levels and clinical or pathological characteristics, but the T allele homozygous as well as T allele carriers showed an increased ER-negative status compared with wild-type carriers (Vaclavikova et al., 2008). No association was found in the genotype of the 3435 C/T polymorphism ($P = 0.744$) or allele frequencies ($P = 0.590$) between breast cancer patients and controls in an Iranian population (Tatari et al., 2009). A study of the Chinese population for 1236 C/T, 3435 C/T, and 2677 T/A polymorphisms in the *ABCB1* gene showed that the TT 3435 genotype was associated with susceptibility to breast cancer (OR = 1.386), but no association was found for the 1236 polymorphism (Wu et al., 2012). We compared the frequencies of these polymorphisms with our results and found differences in the 3435 C/T polymorphism genotypes with respect to patients from Turkey, the Czech Republic, Brazil, and Asian countries ($P < 0.05$). Our observed allelic frequencies were different from those for patients studied in Turkey, Brazil, and the Czech Republic ($P < 0.05$), but not significantly different from Asiatic populations. The 3435 C/T polymorphism in the *ABCB1* gene has been widely studied in many diseases and in different populations, and has mainly been linked to treatment response (Eichelbaum et al., 2004; Lu et al., 2011; Wang et al., 2011). Regarding the 1236 polymorphisms, we observed differences only in Asiatic populations ($P = 0.018$). To establish specific allele risks, it is necessary to perform genotyping in each population.

Haplotypes were inferred using a mathematical algorithm and D' was estimated. Premenopausal women showed higher linkage disequilibrium (D' = 0.64) compared to postmenopausal women (D' = 0.53). However, the reference group showed the highest linkage disequilibrium (D' = 0.84), likely because of the increased H1 (C allele in both loci) and decreased H4 (1236 C allele and 3436 T allele). This suggests that although 1236 C/T showed no differences alone, but when its haplotype was combined with the 3435 C/T polymorphism, there was an additive effect for breast cancer susceptibility, particularly in premenopausal women. The haplotype H4 contains the T 3435 allele, which may be a risk allele, and the 1236 C allele. This agrees with our findings because in breast cancer cases, H4 is 2 times as frequent in breast cancer patients compared to the reference group. Postmenopausal H4 haplotype carriers showed a 2-fold higher risk of developing breast cancer. Previously, Wu et al. (2012) studied the polymorphisms 1236 C/T, 3435 C/T, and 2677 T/A from the *ABCB1* gene in breast cancer and found that 3435T-1236T-2677T haplotype carriers were associated with an increased breast cancer risk (OR = 1.7). This finding is different from ours pertaining to the 1236 polymorphism, and we did not analyze the 2677 polymorphism. However, the 3435T allele appears to be a strong factor alone or in haplotype in breast cancer susceptibility.

CONCLUSIONS

Our results suggest that the T 3435 *ABCB1* polymorphism in Mexican premenopausal and triple-negative women is a risk factor for breast cancer development, while the CT genotype of the 1236 polymorphism is a protective factor for the same tumor. In addition, the H4

(1236 C-3436 T) haplotype in postmenopausal woman carriers increases the risk of breast cancer development by 2-fold.

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