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ATM allelic variants associated to hereditary breast cancer in 94 Chilean women: susceptibility or ethnic influences?

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Abstract Besides BRCA1 and BRCA2, two genes accounting for a small proportion of breast cancer cases, ATM has been widely proposed as a low-penetrance susceptibility gene. Several nucleotide changes have been proposed to be associated with breast cancer, still remaining a high controversy in this sense. We screened the *ATM* gene in 94 breast cancer patients selected from 78 high-risk families, not presenting a mutation in BRCA1 or BRCA2. We found three novel allelic variants: IVS64 + 51delT and p.L752L, not showing association with hereditary breast cancer, and p.L694L found in one family in two breast cancer patients. Two amino acid substitutions p.S707P and p.F858L, previously reported to be associated with breast cancer, were present in our study in cases and controls, lacking of association with breast cancer. A positive association of c.5557G>A (p.D1853N) was found (OR 2.52, $P = 0.008$), when analyzed alone and in combination with an intronic variant IVS24-9delT (OR 3.97; $P = 0.0003$). We postulate that our discrepancies with other reports related to the associated ATM alleles to hereditary breast cancer, as well as discrepancies in the literature between other groups, could be explained by the diversity in the ethnic origins of families gathered in a sole study, and the selection of the control group. In relation to this issue, and based on genetic markers, we found that the Chilean group of breast cancer families in this study has a stronger European genetic component than our control sample selected randomly from the Chilean population.

Keywords *ATM* gene · Association analysis · Allelic variants · Genetics of breast cancer · Ethnic influences

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Introduction

The screening of BRCA1 and BRCA2 as the two main susceptibility genes for hereditary breast cancer has been pursued for several groups during the last decade. These studies revealed that germline mutations in these genes account for a small proportion of the affected families, strongly suggesting that other genes should be involved. We have previously screened for germline mutations in BRCA1 and BRCA2, 66 Chilean families that presented several cases of breast cancer [1]. Only 20% of these families presented a mutation in either one of these two genes. The ATM gene has been frequently involved in hereditary breast cancer as a low-penetrance susceptibility gene [2], and several reports describe that heterozygous women for mutations in this gene have an increased susceptibility to breast cancer [3, 4]. Mutations in the *ATM* gene caused ataxia–telangiectasia (A–T), an autosomal recessive disorder characterized by a progressive cerebellar ataxia, oculocutaneous telangiectasia, immunodeficiency, mental retardation, chromosomal instability, an increased susceptibility to ionizing radiation [5], and a 100-fold higher risk for cancer for A–T relatives [6, 7]. The most common cancers in A–T patients are lymphomas and

leukemia, although breast cancer is found in higher rates among heterozygous relatives [3].

The *ATM* gene has been mapped to chromosome 11q22-23 [8]; it spans 150 kb and comprises 66 exons [9]. The gene encodes a 350-kDa serine–threonine kinase that plays a central role in DNA repair caused by ionizing radiation [9, 10]. ATM is highly related to BRCA1, since both are localized mainly in the nucleus [11, 12] and respond to the DNA damage salvage pathway [13, 14]. Also, BRCA1 is phosphorylated by ATM in response to DNA double-strand breaks [15]. Other tumor suppressors phosphorylated by ATM are P53 and CHEK2 [16–18] which have also been associated with breast cancer [19–22]. The frequency of ATM heterozygotes in the general population has been estimated to be around 1–3% [23]. It has been reported that women carrying one mutated allele have a fivefold increased risk of developing breast cancer, compared to noncarriers [3]. Several studies based on population case–control series of women with family history of breast cancer have shown an association of missense mutations in the *ATM* gene with an increased risk to develop breast cancer [2].

This study describes the screening of the entire *ATM* gene in 42 index patients with breast cancer, and without mutations in BRCA1 or BRCA2. In addition, we analyzed the presence of seven ATM-specific allelic variants in a total of 108 patients including relatives and BRCA1 or BRCA2 carriers.

Methods

Families and patients

We selected 78 families with breast, or breast and ovarian, cancers which were screened for mutations in *BRCA1* and *BRCA2* genes. Families were chosen by any of the following criteria: three cases of breast cancer in first-degree relatives; or two cases of breast cancer in first-degree relatives one diagnosed before age 40; or one breast cancer and one ovarian cancer in first-degree relatives. The index patients were recruited from three cancer centers in Santiago, Chile. After signing an informed consent, blood samples were obtained from affected individuals. DNA was isolated from peripheral blood lymphocytes, as previously described [24]. Among these families, we choose 42 index patients without mutations in BRCA1 or BRCA2, and we screened the *ATM* gene for mutations. Blood from 200 healthy control individuals was obtained from a blood bank, Santiago, Chile.

Mutational screening

Polymerase chain reaction (PCR) amplification covering coding sequences and intron–exon boundaries of the *ATM* gene was performed following standard methods, using primers described by Castellví-Bel et al. [25] and Sandoval et al. [26]. The localization of intronic primers allowed the amplification of more than 50 bp of intronic regions for each fragment, allowing the analysis of almost 10,265 bp of intronic regions in the *ATM* gene. Amplified products larger than 350 bp were digested with an appropriate restriction enzyme before being analyzed. All fragments were analyzed by single-strand conformational polymorphism (SSCP). Briefly, PCR products were denatured at 95°C for 5 min, cooled on ice for 5 min, and submitted to electrophoresis on MDE 0.5X (FMC Bioproducts) at 18°C, 3 W for 14 h. For some cases, glycerol was used to enhance the resolution of the technique at 18°C, 6 W for 16 h. The electrophoresis time was dependent on the size of the PCR fragments. The gels were silver stained after electrophoresis. PCR products showing an abnormal migration by SSCP were subjected to direct sequencing using the fmol DNA cycle sequencing system (Promega) and γ^{32}P-ATP. Electrophoresis was carried out onto 6% acrylamide/bisacrylamide (19:1) gels in the presence of 8 M urea. Gels were dried and subjected to autoradiography for 24–48 h. The following variants were screened directly by restriction digestion: IVS10-6T>G and IVS38-8T>C with *Rsa*I; c.2082T>C with *Alw*26I; and c.2119T>C with *Eco*81I.

Statistical analyses

Analyses of genotype and allelic frequencies for each polymorphism were based on all cases ($n = 94$) and controls ($n = 200$). With the purpose to evaluate the deviations from the genetic frequencies predicted by Hardy-Weinberg, the χ^2 test was used. Odds ratios (ORs) and 95% confidence limits (CIs) were calculated by standard methods. The Fisher's exact test was used to compare allelic frequencies distribution in cases and controls. The SAS software (SAS Institute, Inc., Cary, NC, USA) was used for statistical analysis. The IVS24-9delT/c.5557A haplotype phase was determined by segregation analysis in the parents. We performed contingency table analyses of heterogeneity among populations with the algorithms contained in the BIOSYS software [27]. The genetic affinities among populations were evaluated from the allele frequencies obtained, using the genetic distances of Nei [28, 29] and modified [30]. Although all three distances gave essentially the same results, we report here the modified Rogers distance, which allows the analysis of populations with different sizes and is more sensitive in the study of recent demographic events [31].

Results

The complete screening of the *ATM* gene in the 42 index patients revealed the presence of several polymorphisms

and allelic variants, as shown in Table 1. Among these, one intronic polymorphism IVS64 + 51delT and two silent mutations c.2082T>C (p.L694L) and c.2256A>G (p.L752L) have not been previously described. Some of the variants found were analyzed in all accessible relatives with breast cancer and in 14 patients with mutations in BRCA1 or BRCA2; these are seven exonic nucleotide changes, and two intronic variants: IVS24-9delT and IVS38-8T>C. We analyzed a total of 108 patients, 94 of these not mutated in BRCA1 or BRCA2 (Table 2).

Nine of the intronic polymorphisms detected in introns (Table 1A) have been already described in different populations [25, 26, 32, 33]. A novel polymorphism in intron 64 IVS64 + 51delT was found with frequencies 0.01 in 42 cases and 0.0025 in 200 controls, showing no association ($P = 0.684$).

Five amino acid changes were detected in exons 13, 15, 19, and 39 (Table 1B). In exon 19, c.2572T>C leading to p.F858L was found in 1 of 94 cases and 4 of 200 controls, giving frequencies of 0.005 and 0.01, respectively (OR 0.53; 95% CI 0.07–3.55; $P = 1.000$) (Table 3B). This variant has been previously described as a polymorphism in different cases/controls studies, from Sweden, UK, Germany, and Finland [34–37], although Stredrick et al. [38] found association in a US Study with a significant increased risk of 2.03. Exon 13 presented a nucleotide substitution c.1744T>C (p.F582L), previously described in two individuals (Africa and Middle East) from diverse human populations [39]. In this study, p.F582L was found in four cases and two controls, giving frequencies of 0.02 and 0.005, respectively (OR 4.32; 95% CI 0.92–20.3; $P = 0.086$) showing no association (Table 3B). A nucleotide change in exon 15 c.2119T>C (p.S707P) was found in one case and eight controls with frequencies of 0.005 and 0.02, respectively (OR 0.26; 95% CI 0.04–1.63; $P = 0.284$). Atencio et al. [32] described this variant as a mutation found in 1 of 52 women affected by breast cancer, being not screened in controls. However, two different

Table 1 Nucleotide changes found in the *ATM* gene

Intron/exon	Nucleotide change	Effect	rs number	Described as
A. Intronic changes				
4	IVS 4 + 36 insAA	Unknown	rs2066734	Polymorphism[a]
17	IVS 17-56 G>A	Unknown	rs672655	Polymorphism[a,b]
22	IVS 22-77 T>C	Unknown	rs664677	Polymorphism[a,b]
24	IVS 24-9 del T	Unknown	rs3218698	Polymorphism[a,b]
25	IVS 25-13 insA	Unknown	rs3218681	Polymorphism[b]
25	IVS 25-35 T>A	Unknown	–	Polymorphism[b]
38	IVS 38-8 T>C	Unknown	rs3092829	Polymorphism[a]
48	IVS 48-69 insATT	Unknown	rs3212322	Polymorphism[b]
62	IVS 62 + 8 A>C	Unknown	rs4986839	Polymorphism[b]
64	IVS 64 + 51 del T	Unknown	–	Not described[c]
B. Missense mutations				
13	c.1744 T>C	p.F582L	rs2235006	Allelic variant[d]
15	c.2119 T>C	p.S707P	rs4986761	Allelic variant[e,f]
19	c.2572 T>C	p.F858L	rs1800056	Polymorphism[g]
39	c.5557 G>A	p.D1853N	rs1801516	Polymorphism[a,b]
39	c.5558 A>T	p.D1853V	rs1801673	Allelic variant[f]
C. Silent mutations				
15	c.2082 T>C	p.L694L	–	Not described[c]
17	c.2256 A>G	p.L752L	–	Not described[c]

The rs numbers are from the Human SNP Ensembl database. GenBank Accession No. U82828

[a] Sandoval et al. [26]

[b] Castellví-Bel et al. [25]

[c] This study

[d] Thorstenson et al. [39]

[e] Izatt et al. [35]

[f] Dork et al. [36]

[g] Vorechovsky et al. [34]

Table 2 Breast cancer families in this study

	BRCAX	BRCA1/2
No. of patients analyzed	94	14
No. of families	67	11
Patients with breast cancer	84	10
Patients with bilateral breast cancer	8	4
Patients with ovarian cancer	2	0
Age at diagnosis (%)		
<30	7 (7.45)	1 (7.14)
30–49	50 (53.2)	8 (57.14)
50–69	33 (35.1)	5 (35.71)
>70	4 (4.25)	0 (0)
Ethnicity		
Chilean	45	6
Spanish	7	3
Arabian	1	0
Other Europeans	14	2

BRCAX: non-BRCA1/2 mutation carriers

cases/controls studies, one of them with 1,331 breast cancer women and 649 controls, report no association of this missense mutation to breast cancer [35, 40]. It is interesting to note that only one study made in a homogeneous group of 1,000 breast cancer cases and 500 controls from a region of North of Germany [36] reported an association of S707P with breast cancer (OR 2.4; $P = 0.05$) and a frequency five times higher in women with bilateral breast cancer, compared to random individuals. Two amino acid changes in the same codon, p.D1853N and p.D1853V, were found in exon 39 (Table 1B). The variant p.D1853N has been described in different studies and populations as a common polymorphism [35–37, 41], however in this study we found frequencies of 0.111 and 0.054 in cases and controls, respectively (OR 2.52; 95% CI 1.33–4.77; $P = 0.008$), evidencing an association with hereditary breast cancer in Chilean patients. The next variant p.D1853V was found in one case and is not present in 200 controls. This amino acid substitution, found in a family with Italian ancestors from our study, has been previously described in two reports from Germany and Finland [36, 37]. In both studies, the frequencies of p.D1853V in cases and controls are too low to be classified as polymorphisms (<0.01), however, the allelic frequencies in cases are 1.5 and 2.7 times higher than controls, respectively.

Two novel exonic nucleotide substitutions were found in exons 15 and 17; p.L694L and p.L752L, respectively (Table 1C). The variant p.L752L was found at frequencies of 0.012 in cases and 0.02 in controls, not being associated with breast cancer. The variant p.L694L was found in two cases and was absent in 200 controls.

Discussion

The participation of *ATM* as a susceptibility and low-penetrance gene in breast cancer has been widely discussed in the last years. The model described by Gatti et al. [42] proposes that the ATM mutations causing susceptibility to breast cancer are missense substitutions, rather than truncating mutations occurring in A–T patients. The diverse studies performed in breast cancer cases, with a variety of selection criteria, have been inconclusive in defining specific variants associated with breast cancer at the moment.

In this study, we screened, through SSCP and direct sequencing, all exons and intron–exon boundaries including at least 50 bp of the intronic sequence, of the *ATM* gene, in 42 cases and 200 controls. We found 10 intronic sequence changes and seven exonic nucleotide substitutions (Table 2). Nine of the intronic variants have been previously reported in the literature as polymorphisms, however, we decided to test two of these variants IVS38-8T>C and IVS24-9delT in 94 non-BRCA1/BRCA2 cases and 14 BRCA1/2 mutation carriers, for the following reasons: the intronic variant IVS38-8T>C in combination with c.5557G>A (p.D1853N) was associated to bilateral breast cancer, in a study of 185 women with breast or ovarian cancer belonging to 121 families from Finland [37]. In our screening, these two variants were found separately in a number of patients as shown in Table 3, and combined in the same individual in five cases (Table 3C). The haplotype estimation could not be done due to the absence of available relatives. Nevertheless, we estimated the frequencies of the two variants combined in cases and controls and did not find association with breast cancer cases or any specific feature as bilateral breast cancer, ovarian cancer, or age at onset. As it has been stated by Langholz et al. [43], the most probable explanation is that the haplotype constitutes a genetic marker in linkage disequilibrium with a different risk locus.

The other interesting intronic variant IVS24-9delT was not associated with breast cancer in the group of 94 cases, showing an OR of 1.74 with no significance ($P = 0.067$) (Table 3A). However, we did find association for the combination of IVS24-9delT and c.5557G>A (p.D1853N) which presented haplotype frequencies of 0.1 in cases and 0.02 in controls, with an OR of 3.97 (95% CI 1.87–8.40; $P = 0.0003$) (Table 3C). This haplotype was only present in 1 of 14 BRCA1/BRCA2 carriers. The amino acid change p.D1853N has been reported to be lack of association with breast cancer in several studies [36, 37], however, in the Chilean high-risk breast cancer families study we found an increased risk of 2.52 (Table 3B). Our group of patients selected by hereditary breast cancer is the only one showing association with this variant by itself, and in combination with the intronic IVS24-9delT, increasing the

Table 3 Analysis of ATM nucleotide changes and breast cancer risk

Nucleotide change/effect	Genotype	Cases (%)	Controls (%)	Allelic frequencies of cases/controls	OR	95% CI	*P* value
A. Intronic changes							
IVS24-9delT	T/T	71 (67)	172 (86)	0.127/0.07	1.74	0.99–3.05	0.067
	(-T)/T	22 (31)	25 (12.5)				
	(-T)/(-T)	1 (2.4)	3 (1.5)				
IVS38-8 T>C	TT	84 (89.4)	180 (90)	0.053/0.055	0.96	0.45–2.05	1.000
	TC	10 (10.6)	18 (9)				
	CC	0 (0)	2 (1)				
B. Amino acid substitutions							
c.1744 T>C, p.F582L	TT	90 (95.8)	198 (99)	0.02/0.005	4.32	0.92–20.3	0.086
	TC	4 (4.2)	2 (1)				
	CC	0 (0)	0 (0)				
c. 2119 T>C, p.S707P	TT	93 (99)	192 (96)	0.005/0.02	0.26	0.04–1.63	0.284
	TC	1 (1)	8 (4)				
	CC	0 (0)	0 (0)				
c. 2572 T>C, p.F858L	TT	93 (99)	196 (98)	0.005/0.01	0.53	0.07–3.55	1.000
	TC	1 (1)	4 (2)				
	CC	0 (0)	0 (0)				
c.5557 G>A, p.D1853N	GG	74 (78.7)	183 (91.5)	0.111/0.04	2.52	1.33–4.77	0.008*
	GA	19 (20.3)	15 (7.5)				
	AA	1 (1)	2 (1)				
C. Compound alleles							
IVS24-9delT, c.5557G>A	TG/TG	75 (80)	190 (95)	0.101/0.02	3.97	1.87–8.41	0.0003*
	(-T)A/TG	19 (20)	9 (4.5)				
	(-T)A/(-T)A	0 (0)	1 (0.5)				
IVS38-8T>C, c.5557G>A	TG/TG	89 (94.6)	194 (97)	0.02/0.01	1.53	0.51–4.64	0.535
	CA/TG	5 (5.4)	5 (2.5)				
	CA/CA	0 (0)	1 (0.5)				

(-T): deletion of T

* Significant *P* value

risk to breast cancer to 3.97 ($P = 0.0003$). This discrepancy with other previously reported could be explained by linkage disequilibrium with another locus, as it has been proposed for the IVS38-8T>C in combination with c.5557G>A (p.D1853N) for the Finnish families [43] or by differences in genetic origins between the group of cases and controls, discussed later in this paper. The other amino acid substitution p.D1853V, found in one patient and in none controls in this study, is a rare variant also reported previously [36, 37, 41], in very low frequencies in cases as in controls. The inability to perform association studies with this variant gives very scarce information about its possible role in breast cancer. For this variant, we analyzed the expected effect of the two sequence changes c.5557G>A and c.5558A>T [44] on exonic splicing enhancer (ESE) sequences. Both nucleotide changes affect an ESE sequence, suggesting a possible alteration of normal splicing. This finding has been previously noted [37, 45], but no functional studies have been reported until today.

Among the exonic variants found in our study, p.S707P failed to present association with breast cancer, as reported formerly [37, 40]; however, Dork et al. [36] concluded that this amino acid substitution is associated with a significant high risk to breast cancer. Interestingly, an alignment of the different *ATM* gene sequences from human, pig, *Xenopus laevis*, and mouse, performed at the SIB using the BLAST network service, shows a serine in codon 707 in human, mouse, and Xenopus, but a proline is present in the ATM pig sequence [46]. The pig amino acid sequence shows the highest amino acid identity with the human (86%), followed by mouse (82%), Xenopus (63%) [46], strongly suggesting that the missense substitution p.S707P would not affect the ATM cellular function. The IVS10-6T>G variant has been reported in two studies in Caucasian populations [47–50], as a mutation involved in A–T and

Table 4 Analysis of genetic distances between five populations

Population	EU	AS	BC	CO	AM
EU	********				
AS	0.08	****			
BC	0.029	0.065	****		
CO	0.065	0.032	0.043	***	
AM	0.079	0.008	0.063	0.027	****

Allelic frequencies for European and Asian populations were obtained from the International HapMap Project, http://www.hapmap.org. Allelic frequencies for the Amerindian group were obtained from the genotyping analysis of 100 Chilean aborigines

EU European; *AS* East Asia; *BC* breast cancer cases; *CO* controls; *AM* Amerindian (Chile)

also associated to breast cancer. We did not find this nucleotide substitution in neither 108 cases nor 200 controls from the Chilean population. The IVS10-6T>G nucleotide change has been determined as a very ancient mutation, which probably occurred once during human evolution at least 50,000 years ago [48]. The fact that we did not find this mutation in our control group could be explained by the possibility that this variant was not carried with Amerindians entering the Americas, from East Asia, through the Behring corridor around 35,000 years ago [51]. In this sense, the contribution of Amerindian genes to diseases as breast cancer is a very interesting issue to analyze. In a previous report, we have noted remarkable ethnic differences, determined by mitochondrial DNA markers, between our group of families with high risk to breast cancer and the admixed population from Chile (control group) [1]. We revealed a relevant non-Amerindian genetic component in hereditary breast cancer in relation to *BRCA1* and *BRCA2* genes. This finding is in agreement with a recent report by Joslyn et al. [52] describing a lower frequency of breast cancer in Hispanic versus non-Hispanic women in the US. It is interesting to mention that the Chilean population is considered to be ethnically composed by an admixture of 30% Amerindian origin and 70% European, mostly Spanish, which colonized this part of the Continent among the XVI and XVIII centuries [53, 54]. All the breast cancer families selected in this study lived in Chile for three or more generations, and 61 of 78 had at least two surnames from Spanish origin (Table 2). Other families with different European origins were also included in this study and classified by the ethnic ancestry from the family branch carrying breast or ovarian cancer (Table 2). In this study, we screened five exonic allelic variants of the *ATM* gene shown in Table 1, in a sample of 100 Chilean aborigines (Amerindians) in order to determine the presence of these in an ethnic group strongly contributing to the admixed Chilean population. We found interesting differences in the frequency distribution of alleles from cases, controls, and Amerindians (data not shown). We performed a genetic distance analysis taking the allelic frequencies for these three groups and allelic frequencies described for Europeans and Asian individuals obtained from the Hap Map site [55]. The 5×5 contingency table analyses shows nonsignificant differences between the population samples analyzed for c.2119 (p.S707P) and c.2572 (p.F858L) variants (data not shown). These variants are not associated with breast cancer in our group of study showing higher frequencies in the control group than in cases. The most interesting result is the one obtained for c.5557G>A (p.D1853N), whose differences were statistically significant among the five groups analyzed ($\chi^2 = 64.308$, $P < 10^{-5}$). The distance matrix (Table 4) shows that the group of Chilean breast cancer families is closer to the European than to the control group from a random sample from Chile. The control group shows a genetic distance in between breast cancer group and Amerindians, the latter equivalent to East Asian (Table 4). These results are in agreement with the hypothesis that Chilean breast cancer group presents a stronger European genetic component than the admixed Chilean population, most probably from Spanish origin. In relation to this hypothesis, we need to discuss the association of the variant c.5557G>A (p.D1853N) found in our group of patients respect to our control group, chosen among Chilean inhabitants with no cancer, which we supposed to be an appropriate control group. This variant has the following frequencies: European 0.175; Asian 0.00; Chilean cases 0.134; control sample (Chile) 0.04; control sample (Spain) 0.143; Chilean aborigines 0.00. Thinking on a bias to a stronger Spanish genetic component in the group of patients, compared to the control sample, we analyzed the possible association of the c.5557G>A (p.D1853N) in our cases, and taking as a control the Spanish frequency for this variant, which is 0.143. This analysis was negative for association ($P = 0.269$). These findings lead us to discuss about the best control sample to choose for association studies in countries having diverse genetic and ethnic origins. Even more, could we know

which the best control sample for an association study is? In this regard, the controversy in the literature about the association in some reports, or no association in others, for ATM allelic variants such as p.S707P, F858L, D1853N, IVS10-6T>G, could be explained by the diversity in the ethnic origins of all samples, either cases or controls, in studies gathering research centers from different countries. Association studies are statistically more significant when the number of individuals is higher, but increasing the number of individuals by mixing people from different ethnicities could give stronger association, in some cases, with no physiological sense. Functional testing of all associated or not associated ATM variants has to be done in order to determine a real participation of these variants in breast cancer development.

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