Mitochondrial DNA variants and risk of familial breast cancer: An exploratory study

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Abstract. To assess if mitochondrial DNA (mtDNA) variants are associated with mutations in BRCA susceptibility genes and to investigate the possible role of mitochondrial alterations as susceptibility markers in familial breast cancer (BC), 22 patients with or without *BRCA1*/*BRCA2* mutations, 14 sporadic BC patients and 20 healthy subjects were analyzed. In the D-loop and in the *MTND4* region, variants significantly associated with *BRCA1* carriers were identified. Moreover, examination of mitochondrial haplogroups revealed X as the most significantly frequent haplogroup in *BRCA1* carriers (P=0.005), and H as significantly linked to *BRCA2* carriers (P=0.05). Our data suggest the involvement of the mitochondrial genome in the pathogenetic and molecular mechanism of familial BC disease.

Introduction

BRCA1 and *BRCA2* are the most frequently altered susceptibility genes (~20%) in familial breast cancer (BC) (1,2). Women with a deleterious mutation in *BRCA1* or *BRCA2* have approximately a 70% probability of developing BC by the age of 70 (3). The difference in incidence of particular mutations in different ethnic groups is due to a 'founder' effect. Recent evidence has indicated that disease risk in carriers of high-risk mutations (e.g. *BRCA1* and *BRCA2*) could vary also due to other genetic and environmental factors. Genome-wide association studies (GWAS) have identified several common alleles able to modify breast or ovarian cancer risk for *BRCA1* and *BRCA2* mutation carriers, even if more modifiers still remain to be identified (4). Furthermore, other genes or chromosomal regions with moderate and low penetrance have been shown to confer risk of BC (i.e. *CHECK2*, *p53*, *PALB2*, 2q35, 11q13.3) (5,6). However, currently our knowledge of genetic patterns is not sufficient to allow us to identify all hereditary BC.

Due to their role in cell metabolism, mitochondria have long been suspected to be involved in metabolic alterations of tumors (7). In particular, mitochondrial DNA (mtDNA) is highly susceptible to molecular alterations by environmental carcinogens because of a lack of introns and histone-like proteins. Moreover, mtDNA has a high mutation rate due to the close proximity to sources of reactive oxygen species (ROS), complex I (NADH-ubiquinone oxidoreductase, EC 1.6.5.3) and III (ubiquinol: cytochrome c oxidoreductase; EC 1.10.2.2), and also due to the low efficiency of repair systems. These nucleotide modifications have been hypothesized to promote a cascade of events that increases the ROS production thus encouraging cancer development (8,9). Recent studies suggested that several somatic mtDNA mutations, especially in the mitochondrial respiratory complex I genes and in the D-Loop region, may predispose individuals to cancer (10-12) and, in particular, to BC (8,13). The D-Loop is the main regulatory region of mitochondrial transcription and replication that encompasses essential and strongly conserved consensus sequence elements (14) as well as *loci* that rapidly accumulate mutations (i.e., the two hypervariable regions, *HV1* and *HV2*) (15). On the other hand, the high frequency of mutation rate as well as the lack of recombination and the mtDNA polyploid *status* makes it possible to classify individuals through specific mtDNA variants that make up 'haplogroups' (16). Haplogroups, groups of mtDNA haplotypes sharing the same mutational motifs by descent from a common female ancestor, have been shown to possess both geographical- and ethnic-specific differences in prevalence (17). They are extremely common in a continent or even in a single geographic area/population group or completely absent in others (18). Some studies have tried to focus on the hypothetical association between a haplogroup and an increasing BC risk, but without definitive results (19,20). So far, studies have not been carried out to investigate the role of mtDNA alterations in BC heredity. Thus, the role of mtDNA variation needs to be further investigated.

Starting from all these observations, we aimed to verify whether mtDNA alterations and/or a specific mtDNA

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haplogroup could be used as a susceptibility marker for familial BC or as a *BRCA1*/*BRCA2* gene modifier.

Materials and methods

Subject collection and DNA extraction. After signing informed consent, 56 Caucasian subjects from the Region Puglia were enrolled at the National Cancer Research Centre 'Istituto Tumori Giovanni Paolo II' of Bari, Italy; 32 familial BC patients, 14 sporadic and 10 healthy subjects unrelated to familial BC. Among the 32 familial cases, 22 were affected by BC and 10 were healthy relatives from high risk families. Patients were classified as 'familial' when they satisfied clinical characteristics as previously reported (21). Among the familial cases, 10 subjects were *BRCA1* mutation carriers, 10 *BRCA2* mutation carriers and 12 wild-type for both genes *(BRCAX)*. The *BRCA* molecular screening of patients was performed as reported in our previous study (21). Patients were characterized according to pathological features: TNM classification, nuclear grading, estrogen (ER) and progesterone receptor (PgR) tumor content (Table I).

Members of four different Caucasian families affected by familial breast/ovarian cancer transmitted along the maternal lineage were also analyzed to study the transmission of mitochondrial alterations into each family and their association with the presence of BRCA mutations.

BRCA1 and *BRCA2* mutations were identified in DNA extracted from stored blood by using the DNAeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol.

Amplification and sequencing. Total genomic DNA (1 μg) was extracted from the blood of 36 patients and 20 healthy subjects enrolled at the National Cancer Research Centre of Bari (Italy) and used for amplification with the MitoALL Resequencing kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The *MTND4* (1 of the 7 mitochondrial complex I genes) and the D-Loop mitochondrial region (spanning nucleotides 10760-12137 and 16024-576, respectively) were selectively amplified by PCR. D-loop and *MTND4* were selected because they present the highest mutational rate among the mtDNA genes (22). PCR products were purified with ExoSAP-IT™ (Affymetrix, OH, USA) and then both strands directly sequenced using the BigDye Deoxy Terminator Cycle Sequencing kit (v.3.1, Applied Biosystems Inc.) in an automated sequencer (3130xl Genetic Analyzer; Applied Biosystems Inc.). To evaluate if mtDNA presented different polymorphisms in BC-affected individuals, the sequences were compared for similarities against the revised Cambridge Reference Sequence (NC_012920). The quality of sequences and the coherence of the variants were compared to the international HmtDB database (http://www.hmtdb.uniba.it:8080/hmdb). Mitochondrial haplogroups were identified using several markers as reported at http://www.phylotree.org.

To confirm the data on haplogroup association with the presence of *BRCA1* or *BRCA2* mutations, a second group including 25 subjects, 8 carrying *BRCA1*, 10 carrying *BRCA2* alterations and 7 *BRCAX*, were investigated at 13 *loci* to define to which haplogroup they belonged.

In silico analysis. All mtDNA sequencing results were analyzed *in silico* to verify the possible pathogenetic role of each variant. Mutational analysis was carried out in HmtDB: if the variability index (v.i.) was close to '0' the substitution was considered as a polymorphism; if v.i. was near to '1', the substitution was considered frequent in the population. Polyphen software (http://genetics.bwh.harvard.edu/pph/) was used to predict a possible impact of the evidenced amino acid substitutions on the structure and function of the encoded proteins.

Statistical analysis. Mutations in subgroups were summarized using cross-tabulations and analyzed by the Chi-square test. Associations between mitochondrial alterations or haplogroups and clinicopathological characteristics were assessed using Chi-square tests for categorical variables. All analyses were conducted using SPSS version 17.0 and were considered statistically significant when $p \leq 0.05$.

Results

Identification of mtDNA variants in BC patients compared to healthy subjects. To evaluate if mtDNA presented different polymorphisms in BC affected individuals, mtDNA from the blood of 36 patients and 20 healthy subjects were aligned to the revised Cambridge Reference Sequence (rCRS) and nucleotide variants recorded. DNA sequencing of the D-Loop and *MTND4* regions identified a total of 136 nucleotide variants in all the subjects: 107 in the D-Loop region and 29 in *MTND4*. The over-represented as well as the novel variants are listed in Table II. To assess a potential role in increasing the risk of onset of BC, we searched the variability of the identified SNPs in HmtDB. We found that 17 SNPs presented a variability index (v.i.) equal to '0'; 54 SNPs showed a v.i. ranging from 0.001 to 0.010 when compared to the genome of healthy individuals whereas a v.i. ranging from 0.002-0.026 was revealed when comparing them to subjects affected by any pathology; 58 SNPs presented a v.i. above the highest value of the range. The variants were retrieved also in Mitomap (http://www.mitomap.org/MITOMAP). All resulted as haplogroup markers and no significant difference was shown between BC and healthy subjects. However, 7 variants (149insC, 452delT and m.16026T>C in the D-Loop and m.10799C>T, m.11279C>A, m.11524A>C and m.11925C>A in *MTND4*) were not reported either in HmtDB or Mitomap but were all found at very low frequency (Table III). Among the novel variants retrieved in *MTND4*, the two non-synonymous, m.11279C>A (p.L174M) and m.11524A>C (p.K255N), were analysed by Polyphen (http://genetics.bwh.harvard.edu/pph2/) and were predicted as 'benign', not determining structural variations at protein level.

When we evaluated a possible association between specific SNPs and the BC status, we found that the only variant exclusively present in BC and not in healthy subjects was the m.150C>T (16.7% vs. 0%, P=0.06), whereas four variants resulted more frequently present in healthy than in BC subjects: m.195T>C (55% vs. 13.9%, P=0.002), m.225G>A (20 vs. 2.8%, P=0.05), m.16519T>C (75% vs. 41.7%, P=0.016), m.16189T>C (40% vs. 13.9%, P=0.03).

Microsatellity instability in the D-Loop region, including the two common poly-C stretch variations at nucleotide positions (np) 303-315 (D310) and 568-573, in addition to the

Table I. Clinicopathological characteristics of patients with respect to the different subgroups.

Variables	Familial breast cancer			Sporadic BC n=14
	BRCA1 mutated n=10	*BRCA2* mutated n=10	*BRCAX* n=12	
Gender				
F	7	8	12	14
M	3	2	0	0
Age				
≤45 yrs.	7	6	2	2
>45 yrs.	3	4	10	12
IDC	90% (n=9)	100% (n=10)	75% (n=9)	78% (n=11)
T				
1-2	4	7	8	11
3-4	0	3	4	3
N^+	30% (n=3)	40% (n=4)	90% (n=10)	64% (n=9)
G3	60% (n=6)	70% (n=7)	55% (n=5)	64% (n=9)
ER^+	30% (n=3)	100% (n=10)	67% (n=8)	28% (n=4)
PgR^+	30% (n=3)	80% (n=8)	67% (n=8)	36% (n=5)
Mib^+	80% (n=8)	80% (n=8)	58% (n=7)	71% (n=10)
$ErbB2^+$	50% (n=5)	50% (n=5)	17% (n=2)	28% (n=4)

IDC, infiltrating ductal carcinoma.

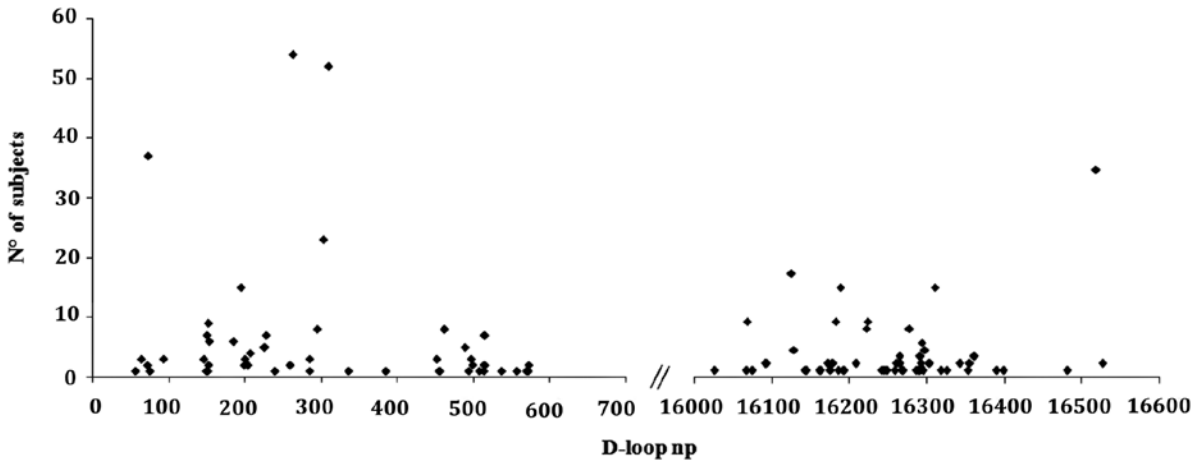

Figure 1. D-Loop variant distribution in the overall BC series. Nucleotide variants identified in the D-loop in *BRCA1, BRCA2, BRCAX* and sporadic subjects clustered in the hypervariable regions *HV1* (16024-16383), *HV2* (57-333) and *HV3* (438-574). np, nucleotide position.

poly-CA stretch at 514-523, was also detected (Table II). We found that the 36 BC subjects showed length variations at 514-523 and 568-573 with a higher frequency than in healthy subjects: 16.7 and 5% vs. 1 and 0%, respectively; on the other hand, D310 resulted the most frequently polymorphic site in the D-Loop both in BC and in healthy subjects. When considering the variability of the D-loop region, as expected, most of the variants were found in the D-Loop hypervariable regions; 48/107 variants (45%) in *HV1*; 31/107 (29%) in *HV2* and 20/107 (18.7%) in *HV3* (Fig. 1). No differences between BC and healthy subjects were found.

Haplogroup analysis of the overall 36 BC patients showed that H and J/T were the most represented haplogroups (33 and 25%, respectively) whereas haplogroup X was absent in our series of healthy subjects.

D-Loop and MTND4 variants with respect to BC features. In order to evaluate a possible association between mtDNA

Table II. mtDNA variants and relative frequencies in the D-loop and *MTND4* in BC subjects.

D-loop	%
D310	**97.2**
m.263A>G	94.4
m.73A>G	61.0
m.16519T>C	41.7
m.16126T>C	27.8
m.462C>T	17.0
m.150C>T; m.295C>T; m.16069C>T; m.16311T>C	16.7
m.195T>C; m.228G>A; m.515delAC; m.16189T>C; m.16224C>T	13.9
m.152T>C; m.185G>A	11.0
m.93A>G; m.T146C; m.153A>G; m.G207G>A; m.C285C>T; m.T489T>C; m.A16183A>C; m.16278C>T, m.16292C>T; m.16294C>T; m.16298T>C; m.16362T>C	8.3
m.494delC	6.3
m.200A>G; m.452delT; m.499G>A; m.557insC; m.568-573; m.16017T>C; m.16093T>C; m.16129G>A; m.16209T>C, m.16223C>T; m.16304T>C; m.16343A>G; m.16527T>C	5.6
m.456C>T	2.9
m.56insT; m.64C>T; m.72T>C; m.149insC; m.151C>T; m.199T>C; m.203G>A; m.204T>C; m.225G>A; m.226T>C; m.239T>C; m.250T>C; m.285insA; m.336T>C; m.385A>G; m.497C>T; m.514C>T; m.514insC; m.515insA; m.16026T>C; m.16067C>T; m.16092T>C; m.16114C>T; m.16162A>G; m.16172T>C; m.16179C>T; m.16192C>T; m.16249T>C; m.16261C>T; m.16265A>T; m.16270C>T; m.16287C>T; m.16293A>G; m.16326T>C; m.16354C>T; m.16356T>C; m.16399A>G; m.16482A>G	2.8
MTND4	**%**
m.11719G>A	47.2
m.11251A>G	25.0
m.11467A>G	22.2
m.10822C>T; m.11299T>C; m.11812A>G	8.3
m.10799C>T; m.10915T>C; m.11176G>A; m.10927T>C; m.10907T>C; m.11009T>C; m.11017T>C; m.11167A>G; m.11253T>C; m.11279C>A; m.11332C>T; m.11353T>C; m.11362A>G; m.11377G>A; m.11476C>T; m.11485T>C; m.11524A>C; m.11840C>T; m.11869C>A; m.G11887G>A; m.G11914A; m.11925C>A; m.12127G>A	2.8

Nucleotide position of microsatellite instability (MSI) and the relative frequency are highlighted in bold.

alterations and some BC characteristics, we correlated mtDNA variants with tumor grade differentiation (G1, G2 and G3). Several D-Loop and *MTND4* alterations indeed resulted to be linked to specific cancer pathological features. Particularly, m.11719G>A and m.16519T>C appeared to be linked to scarcely differentiated tumors: 57% in G3 vs. 12.5% in G2 (P=0.006) and 57% in G3 vs. 12.5% in G2 (P=0.05), respectively. m.16126T>C frequently occurred in hormone receptor-negative subjects: 47% in ER^- and PgR^- vs. 12% in ER^+ and PgR^+ (P=0.035). Regarding haplogroup analysis, haplogroup H was more represented in G2 than in G1 patients (75 vs. 24%, P=0.02), thus suggesting a possible correlation between the mtDNA background and tumor differentiation grade.

Some mtDNA variants enhanced the risk of carrying BRCA1 mutations. Familial and sporadic BC subjects did not show differences in number or type of D-loop and *MTND4* alterations (Fig. 2). The sporadic patients presented 14 substitutions in the *MTND4* gene, and 60 substitutions in the D-Loop region: 100% showed a variation at D310, 62% at 11719, 50% at 16519, and 44% at 303 np. Among the familial subjects, the *BRCA1* mutated group presented 9 substitutions in the *MTND4* gene, and 38 substitutions in the D-Loop region: 100% of patients showed D310 variation, 90% at 16519, 70% at 11719, 60% at 195, 50% at 303 and 40% at 153, 225, 226, 16183, 16189 and 16278 np. *BRCA2* mutated subjects showed 9 substitutions in the *MTND4* gene, and 35 substitutions in the D-Loop region: 80% presented variation at D310, 40% at 11467 and 150,

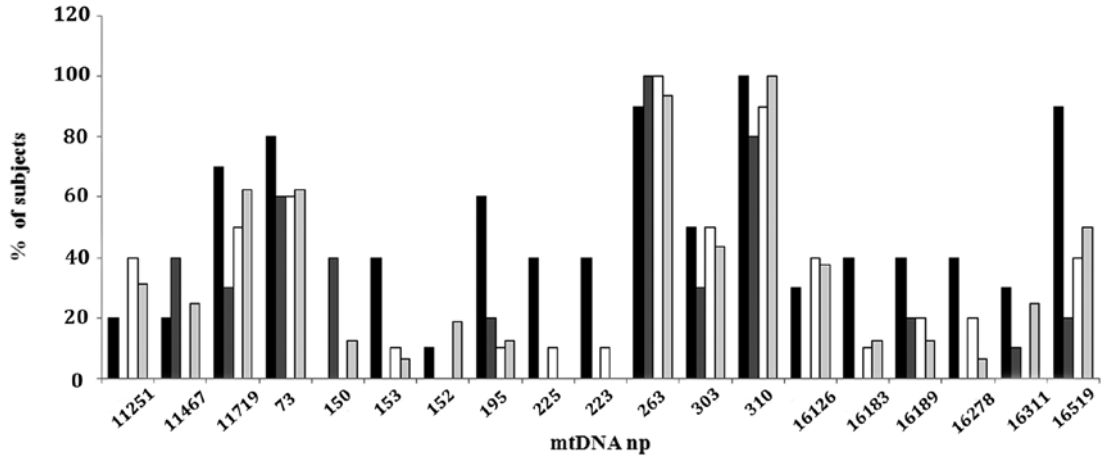

Figure 2. Frequency of mtDNA mutations in the overall series of subjects. The most represented SNPs in the D-Loop and *MTND4* in the patients carrying *BRCA1* mutations (black), *BRCA2* mutations (grey), with familial BC but without *BRCA* mutations [*BRCAX* (white)] and with sporadic BC (light grey) are shown.

Table III. D-Loop and *MTND4* SNPs in *BRCA1* mutation carrier and non-carrier patients.

mtDNA SNPs	*BRCA1* carrier vs. *BRCA1* non-carrier	P-value	OR (95% IC)
m.153A>G	50% vs. 5%	0.009	19 (1.8-201.9)
m.195T>C	60% vs. 40%	0.04	6 (1.12-31.99)
m.225G>A	40% vs. 5%	0.03	12.7 (1.18-136.28)
m.226T>C	40% vs. 5%	0.03	12.7 (1.18-136.28)
m.16183A>C	40% vs. 5%	0.03	12.7 (1.18-136.28)
m.16278C>T	40% vs. 8%	0.03	7.3 (1.3-41.4)
m.16519T>C	90% vs. 30%	0.003	21 (2.15-204.6)
m.11719G>A	70% vs. 15%	0.005	13.2 (2.13-82.13)

The reported alterations confer a higher risk to be significantly associated with *BRCA1* mutations.

and 30% at 303 and 11719 np. The *BRCAX* group revealed 4 substitutions in the *MTND4* gene, and 37 substitutions in the D-Loop region: 90% presented variation at D310, 50% at 303 and 11719, and 40% at 11251, 16126 and 16519 np (Fig. 2).

When we considered only *BRCA1* mutation carriers compared to non-carriers we found a significant association with 7 D-Loop SNPs (m.153A>G, m-195T>C, m.225G>A, m.226T>C, m.16183A>C, m.16519T>C, m.16278C>T) and one SNP in the *MTND4* gene (m.11719G>A), as reported in Table III. The mutations conferring *BRCA2* inactivation were not associated with any of the mtDNA variants detected, with the exception of the variation at positions 514-523 that was found at higher frequency (60%) in the *BRCA2* carriers. Furthermore, when considering the contemporary alterations of the two mtDNA regions in each patient, all subjects showed mutations in the D-Loop with at least 3 changes up to 16, while 72.5% of subjects had alterations in the *MTND4* gene up to 6 alterations at the same time. As expected, the D-Loop was much more variable than the *MTND4* gene; however, while mutation distribution in the D-Loop was independent of the presence of *BRCA* mutations, non-carrier familial BCs presented fewer changes in the *MTND4* gene. This result was not, however, statistically significant (Fig. 3).

Haplogroup analysis in patients carrying and not carrying *BRCA* mutations showed that the X and H haplogroups are able to discriminate between *BRCA1* and *BRCA2* carriers. Haplogroup X was significantly more frequent in *BRCA1* carriers and completely absent in *BRCA2* carriers (P=0.005), while haplogroup H was significantly less represented in *BRCA1* carriers (P=0.04) and mostly present in *BRCA2* carriers (P=0.05) (Fig. 4). To confirm the association between a specific haplogroup and *BRCA1* or *BRCA2* mutations, a further subset of 25 subjects were analyzed. The results confirmed the association demonstrating that seven *BRCAX* subjects belonged to haplogroup X (n=2) and H (n=5). Haplogroup X resulted in 100% of eight *BRCA1* carriers and haplogroup H in 100% of ten *BRCA2* carriers.

Mitochondrial DNA variants in the BRCA families. Members of four different families affected by familial breast/ovarian cancer transmitted along the maternal lineage were also analyzed (Table IV). In each family, all the analyzed members presented, as expected, specific variants in both the D-Loop and *MTND4* regions independently of the presence of a tumor. However, some alterations were associated to *BRCA* mutation type being present in members of different *BRCA1*

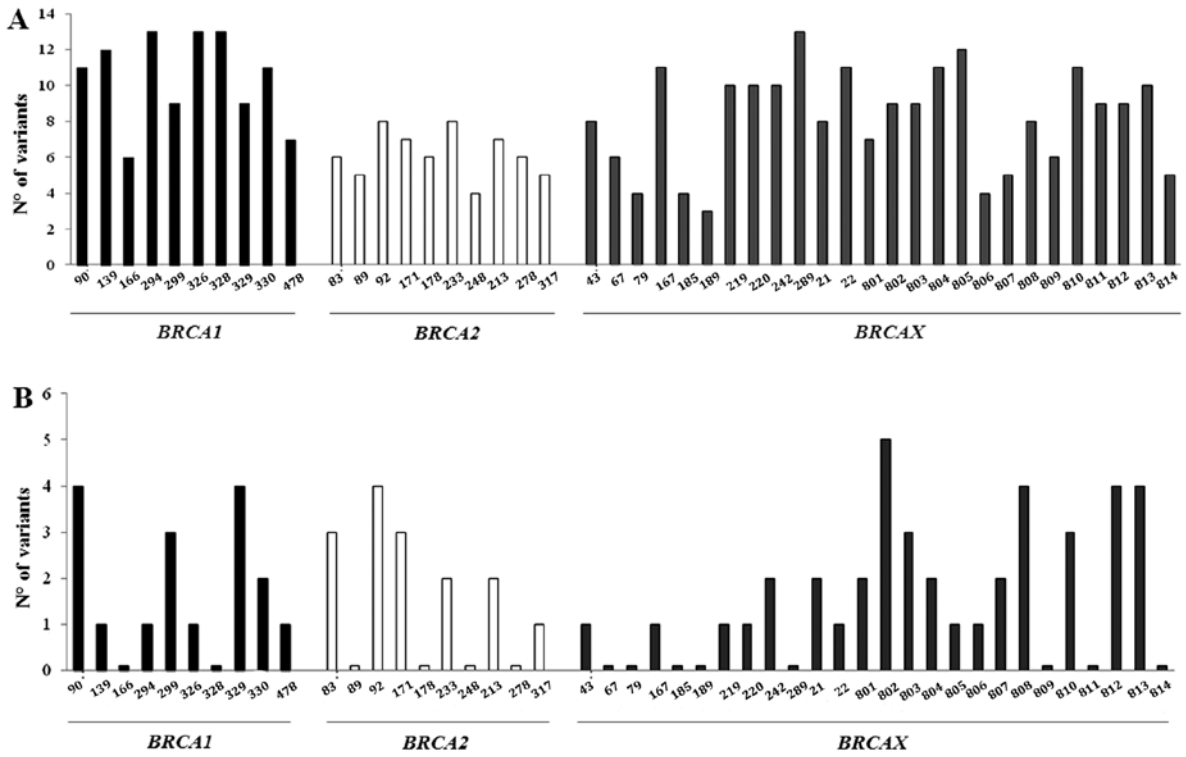

Figure 3. Variants in mtDNA regions with respect to *BRCA* mutational status. Frequency of nucleotide alterations in the D-Loop (A) and *MTND4* (B) with respect to *BRCA* mutations for each patient is shown. X-axis reports the patient ID and Y-axis the number of variants.

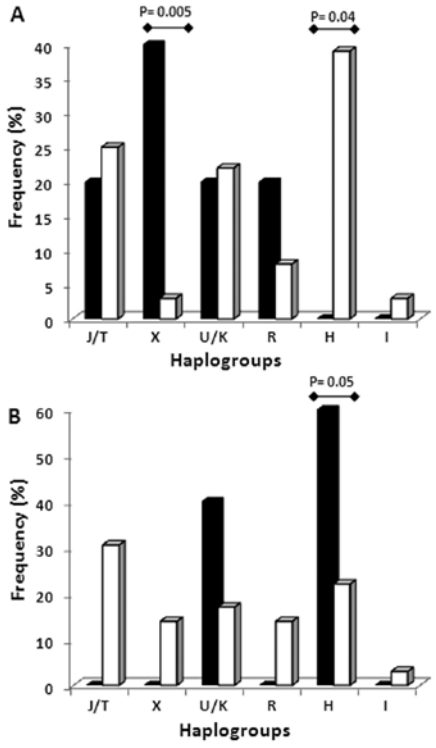

Figure 4. Mitochondrial haplogroup distribution in *BRCA1* and *BRCA2* carriers. Mitochondrial haplogroups were determined in *BRCA1* (A) and in *BRCA2* carriers (B) and compared to subjects not carrying *BRCA* mutations. Black, *BRCA*-mutated cases; white, cases not carrying *BRCA* mutations.

carrier families while others were specific of a familial group (m.64C>T, m.16183A>C, m.16189T>C, m.11812A>G). In addition, these results were consistent with the association between specific haplogroups and *BRCA* mutations as found in the above 25 subjects.

Discussion

In this study, we have shown that the mitochondrial regions D-Loop and *MTND4* display a panel of mtDNA *loci* significantly associated with the presence of mutations in the *BRCA1* gene, and that specific mitochondrial haplogroups are present in patients with mutations in *BRCA1* or *BRCA2*. As expected, the vast majority (72.7%) of the mtDNA mutations identified in our study were in the D-Loop region where microsatellite instability (MSI) was also detected. Conflicting hypotheses have been proposed about the frequency of various MSI in the mitochondrial genome (23), being identified in various types of cancer (24,25). D310, which is located in one of the two highly conserved regions (Conserved Sequence Block I, CSB I) and regulates mtDNA replication (26), has been previously suggested to be involved in carcinogenesis and included in a set of SNPs that could be considered as cancer susceptibility biomarkers (27). Recently, the relatively high frequency of alterations (32.5%) detected in D310 has been associated with clinicopathological parameters of breast cancer (BC). Specifically, 66.7% of the alterations were observed in stage II BC, indicating that D310 instability may be a critical feature for early progression of BC (28). Moreover, other recent results highlighted D310 in some unrelated BC harboring *BRCA1* mutations (29). These data would support an implication of this mitochondrial allelic variant in cell assessment associated with *BRCA1* mutations if we had not found a high percentage of mutations even in healthy controls. Similarly,

Table IV. Mutation clustering in members of hereditary BC families.

Families	Pathological status	*BRCA* mutation	D-Loop mutations	*MTND4* mutation	Haplogroup
DA					
Sister #1	H	B1	**C64T**, A73G, A153G, T195C,	-	X
Sister #2	H	B1	G225A, T226C, D310, A16183C,	-	
Brother	H	B1	C16226T, C16278T, T16519C	-	
PA					
Aunt	A	B1	A73G, A153G, T195C, G225A,	**A11812G**	X
Nephew	H	B1	T226C, D310, A16183C, **T16189C**,		
Niece	H	B1	**C16223T**, C16278T, T16519C		
PR					
Sister #1	A	BX	A73G, G185A, G228A, C295T,	T11253C	J
Sister #2	A	BX	D310, C462T, 514-523, C16069T		
SS					
Sister #1	A	B2	G207A, 514-523, G16129A	-	H
Sister #2	A	B2		-	

Mutations in each *BRCA* family member are reported. Mutations specifically associated to each family are reported in bold. H, healthy subject; A, family members affected by BC; B1, *BRCA1* mutation; B2, *BRCA2* mutation; BX, *BRCAX*.

no significant association between alterations frequently retrieved in the D-loop (m.263A>G) or previously described as a highly polymorphic marker (m.73A>G, m.16519T>C) (19) and risk of developing BC were found. When analyzing *MTND4*, we found that similarly to that detected in BC cell lines (30) m.11251A>G and m.11719G>A were highly frequent in BC subjects but equally represented in healthy controls. However, considering the clinicopathological features of the patients, this is the first report on the association between the presence of specific mitochondrial polymorphisms and tumor differentiation grade (m.11719G>A and m.16519T>C) or hormone receptor status (m.16126T>C).

Hypothesizing that the mitochondrial localization of BRCA1 protein could have a role in regulating both mtDNA damage and apoptotic activity and that its mislocalization could cause cancer, this study focused on a possible relationship between mtDNA variants and germinal *BRCA1* mutations. In literature, a diagnostic algorithm showed that *BRCA* mutations accelerate the onset of mtDNA alterations, leading to phenotypic expression of premature aging and BC (31). We have shown that seven D-Loop and one *MTND4* variations are associated with an increased risk of harboring also *BRCA1* mutations. Of note, among these, m.16183A>C is located at the 3' sequence of the Termination Associated Sequence (TAS) and the DNA 7S binding site which is involved in the regulation of mtDNA synthesis (32). The alteration m.16519T>C, retrieved in 90% of *BRCA1* carriers compared to non-carriers (30%), was previously found in literature to increase familial BC risk (19). Our result confirms that m.16519T>C is a hot spot mutation playing a relevant role in developing BC. All the other alterations associated with *BRCA1* significant mutation risk have also been described in literature in different cancer tissues, such as oral, prostate, thyroid, breast, ovarian cancer and melanoma (29,33). Conversely, variants m.153A>G and m.225G>A, previously associated with breast carcinogenesis (34) have been found as mitochondrial polymorphisms.

In literature, scant data are available on the relationship of specific mitochondrial haplogroups and an increasing BC risk in Europe. Although haplogroup H is common in Europe, representing 40% of European mtDNAs (35,36), our haplogroup analysis revealed that X and H significantly discriminate *BRCA1* or *BRCA2* mutated subjects. These data suggested a possibly higher susceptibility role for these mitochondrial haplogroups to present specific alterations in the genes predicting familial BC risk. When determining the nucleotide sequence in all the available members of two families, the mutations detected in the D-Loop resulted significantly associated with the presence of *BRCA1* mutations since they are transmitted in all family members and identify the X haplogroup. Consequently, they could be considered as dominantly inherited alterations and as representing predicting factors in terms of susceptibility to familial BC risk.

The identification of significant mtDNA variants associated with BC suggests the involvement of mtDNA as a genetic modifier considering both single polymorphisms and specific haplogroups. To better understand the intrinsic clinical implications of the specific mitochondrial biomarkers and haplogroups herein identified, further data involving subjects with and without cancer need to be collected and analyzed. The intriguing results presented in this study can contribute to facilitating the possibility of their application in identifying high risk individuals for developing a specific screening approach for early diagnosis of BC.

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