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**Mutation analysis of *BRIP1* in male breast cancer cases:
a population-based study in Central Italy.**

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ABSTRACT

Breast cancer (BC) in men is rare compared with BC in women, but its incidence is increasing along with attention towards this uncommon disease. Although with some differences, male and female BC share similar genetic predisposition factors, including *BRCA1/2*, *CHEK2* and *PALB2* mutations.

As other *BRCA1/BRCA2* functionally related DNA repair genes, such as *CHEK2* and *PALB2*, *BRIP1* is considered a moderate-penetrance BC susceptibility gene. At present, the role of *BRIP1* on BC susceptibility in men is unknown.

In this study, we aimed to assess whether *BRIP1* variants may contribute to male BC (MBC) risk, by screening 97 MBC cases, all negative for *BRCA1/2*, *CHEK2* and *PALB2* mutations, selected from a population-based series of 126 MBCs from Central Italy. A total of five *BRIP1* germ-line sequence alterations, three coding and two non-coding variants, were detected in our series. The two non-coding variants *IVS4-28G>A* and *3'UTR 4049C>T* were classified as neutral by *in silico* analysis. Of the three coding variants one was a silent variant (*E879E*) and two resulted in amino acid substitution (*R264W* and *P919S*) showing a putative pathogenic role by *in silico* analysis. However, further analysis of tumor-associated loss of heterozygosity and the frequency of variant alleles, tested in 203 male population controls, suggested a neutral effect for both of these variants.

Overall, our results indicate that *BRIP1* variants may not play a relevant role in MBC predisposition.

Keywords: *BRCA1*, *BRCA2*, *BRIP1*, *CHEK2*, germ-line mutations, Male Breast Cancer, *PALB2*.

INTRODUCTION

BRIP1 (BRCA1-interacting protein 1, also known as BACH1 and FANCI), located at 17q22, encodes a DEAH helicase that interacts with the BRCT domain of BRCA1 [1]. *BRIP1* plays a role in DNA damage repair and in BRCA1-dependent checkpoint functions [1]. *BRIP1* is also functionally associated with *BRCA2* in the Fanconi Anemia (FA) pathway [2,3]. Omozygous mutations in *BRIP1* result in FA phenotype while heterozygous truncating mutations confer a 2.0-fold relative risk of developing breast cancer (BC) [2,4,5]. Furthermore, there is evidence that *BRIP1* missense variants may increase BC risk at different extents [6,7].

As other *BRCA1/BRCA2* functionally related DNA repair genes, such as *CHEK2* and *PALB2*, *BRIP1* is considered a moderate-penetrance BC susceptibility gene [4]. First evidence of a role of *BRIP1* as BC susceptibility gene showed that deleterious mutations in *BRIP1* may account for about 1% of *BRCA1/2* negative familial/early-onset BCs [5], however further studies suggested that *BRIP1* contribution to BC susceptibility might be more limited than initially reported [8,9,10].

In recent years, attention is rising towards male BC (MBC) [11]. Carcinoma of the male breast accounts for about 1% of all BC, but its incidence is increasing [12]. As for BC in women, MBC is likely to be caused by hormonal, environmental and genetic risk factors [13]. Although with some differences, MBC and female BC (FBC) share some common genetic predisposition factors. In particular, as in FBC, mutations in the high-penetrance BC genes *BRCA1* and *BRCA2* play a major role in MBC predisposition, with *BRCA2* playing a more significant role in MBC [14].

In addition to mutations in high-penetrance BC genes, variants in moderate-penetrance BC genes, such as *CHEK2* and *PALB2*, are also involved in MBC predisposition [15,16]. However, the contribution of these genes in MBC susceptibility varies depending on ethnic group or cohort analyzed [17,18]. At present the contribution of *BRIP1* variants in MBC predisposition is unknown.

In our previous studies, performed on a population-based series of 108 MBC cases from Central Italy, we reported that high-penetrance *BRCA1/2* mutations account for about 9% of MBC cases [19] and that germ-line mutations in the moderate-penetrance BC genes *CHEK2* and *PALB2* are unlikely to account for a significant proportion of MBCs [20,21]. The aim of the present study is to assess whether and to what extent *BRIP1* variants may contribute to MBC risk, by screening a population-based series of 97 Italian MBC cases all found negative for *BRCA1/2*, *CHEK2* and *PALB2* mutations.

MATERIAL AND METHODS

A total of 97 MBC patients selected from a well-characterized population-based series of 126 MBCs from Central Italy were tested for *BRIP1* mutations. All 97 MBCs have been previously screened for *BRCA1*, *BRCA2*, *CHEK2* and *PALB2* mutations and found negative [19,20,21]. Age at first BC diagnosis ranged between 35 and 90 years (mean 63.9 years). A positive first-degree family history (FH) of breast/ovarian cancer was reported in 25.8% of the cases and 14.4% of the patients had a personal history of cancers other than BC.

A series of 203 healthy adult males from the same area of Central Italy was also included in this study as population controls. For each study participant a signed informed consent form and a blood sample were obtained.

DNA was extracted from peripheral blood lymphocytes by using QIAamp DNA Blood mini kit (Qiagen, Venlo, The Netherlands) and following manufacturer instructions.

The entire coding region and intron-exon boundaries of *BRIP1* were screened for germ-line mutations by using Single Strand Conformation Polymorphism (SSCP) analysis. Primer pairs were as previously described [5]. Cases displaying abnormal SSCP patterns were evaluated by direct sequencing with the ABI Prism-3100 Genetic Analyzer (Applied Biosystems, Warrington, UK). Briefly, PCR fragments and sequencing reactions were purified with the MultiScreen_{HTS} Vacuum Manifold (Millipore, Billerica, MA) and sequencing primers were the same as for SSCP analysis.

Loss of heterozygosity (LOH) analysis was performed by direct sequencing analysis of DNA extracted from micro-dissected formalin-fixed paraffin-embedded breast tumor samples in order to assess the likely pathogenicity of the variants identified.

In silico analysis was also performed to assess the likely pathogenicity of variants by using PolyPhen (<http://genetics.bwh.harvard.edu/pph>), SIFT (<http://blocks.fhcrc.org/sift/SIFT.html>), Align-GVGD (<http://agvgd.iarc.fr>) and Pmut (<http://mmb2.pcb.ub.es:8080/PMut>) and Splice site predictions (http://www.fruitfly.org/seq_tools/splice.html).

Population controls were screened for specific variants detected through mutational screening by using High Resolution Melting (HRM) analysis performed with the 7500 Fast Real-Time PCR (Applied Biosystems, Warrington, UK). Primer sets used for HRM were the same as described above for SSCP analysis.

Comparisons of the frequencies of variant alleles and genotypes between cases and controls were assessed using Pearson's Chi Square test and Fisher's exact test.

RESULTS

Mutational analysis of the complete coding region and intron/exon boundaries of *BRIP1* was performed on 97 MBC patients selected from a population-based series of 126 Italian MBCs. All 97 patients were previously found to be negative for *BRCA1*, *BRCA2*, *CHEK2* and *PALB2* mutations [19,20,21]. Results are summarized in Table 1. Overall, no deleterious *BRIP1* mutations were found among the 97 MBC patients analyzed. A total of five germ-line sequence alterations, three coding and two non-coding variants, all previously described in FBC cases [5,8], were detected in our MBC series. Of the three coding variants two resulted in amino acid substitution (R264W and P919S) and one was a silent variant (E879E).

The missense variant R264W was identified in one patient (1/97, 1%) diagnosed with BC at 76 years of age and with negative FH. R264W occurs in a well-conserved helicase domain and was predicted to be likely pathogenic by *in silico* analysis performed with PolyPhen, SIFT, AGVGD and Pmut. The likely pathogenicity of R264W was further examined by tumor-associated LOH analysis. Sequencing of DNA extracted from the micro-dissected breast tumor sample of the R264W carrier showed that no loss of the wild-type allele had occurred in tumor cells (Figure 1). The presence of R264W was also assessed in 203 (406 chromosomes) healthy male controls by HRM analysis and one subject (1/203, 0.5%) was found to carry the variant. No statistically significant difference in frequency of the variant 264W allele between cases (1/194, 0.5%) and controls (1/406, 0.2%) emerged ($p=0.54$).

The missense variant P919S, occurring in the BRCA1 binding domain, was previously identified as a common polymorphism with a putative association with an increased BC risk [7]. In our series, P919S was detected in 60 (60/97, 61.8%) MBCs. In particular, homozygous P919P genotype was observed in 37 (37/97, 38.3%) cases, heterozygous P919S genotype in 49 (49/97, 51%) cases and rare homozygous S919S genotype in 11 (11/97, 10.7%) cases. We also assessed the presence of P919S in 203 (406 chromosomes) healthy male controls by HRM analysis and 121 (121/203, 59.6%) controls were found to carry the variant. In particular, homozygous P919P genotype was observed in 82 (82/203, 40.4%) controls, heterozygous P919S genotype in 95 (95/203, 46.8%) controls and rare homozygous S919S genotype in 26 (26/203, 12.8%) controls. Overall, no statistically significant difference in the distribution of the frequency of the rare allele ($p=0.9$) and of the three specific *BRIP1* P919S genotypes ($p=0.7$) between cases and controls emerged.

The two non-coding variants IVS4-28G>A and 3'UTR 4049C>T were predicted to not affect splicing by the analysis with Splice site predictions and were classified as neutral.

Overall, our results indicate that *BRIP1* variants identified in this study do not play a significant role in MBC susceptibility.

DISCUSSION

The close functional interaction between *BRIP1* and both *BRCA1* and *BRCA2* in DNA damage repair and FA pathways led us to the hypothesis that, as for FBC, *BRIP1* mutations may explain a percentage of MBC cases that cannot be attributed to *BRCA1/2* alterations. Thus, we evaluated the contribution of *BRIP1* variants in a population-based series of 97 Italian MBCs found to be negative for mutations in the most relevant BC genes, including *BRCA1*, *BRCA2*, *CHEK2* and *PALB2*. To our knowledge, this is the first report about the prevalence of *BRIP1* mutations in a MBC series.

At present, a total of eight *BRIP1* truncating mutations have been identified worldwide. Overall, 11 *BRCA1/2* mutation negative FBC cases, including 9/1212 British [5], 1/75 Australian [22] and 1/49 Italian [23] patients, were found to carry *BRIP1* truncating mutations. We screened 97 *BRCA1/2*, *CHEK2* and *PALB2* mutation negative Italian MBCs and no *BRIP1* truncating mutation was found in our patients, thus suggesting that *BRIP1* may not play a significant role in MBC susceptibility. Our results, based on a relatively large MBC series, are consistent with results from previous studies performed on 96 French Canadian [8], 357 Chinese [9] and 214 Finnish [10] *BRCA1/2* mutation negative FBC cases in which no *BRIP1* truncating mutation were found. Overall, these results suggest that the contribution of *BRIP1* to BC predisposition in both females and males is less consistent compared to other moderate BC susceptibility genes such as *CHEK2* and *PALB2*. Interestingly, no deleterious *BRIP1* mutations were also found in 94 patients from 94 chromosome 17q-linked hereditary prostate cancer families, including 88 men with prostate cancer and 6 women with BC [24]. These findings are particularly relevant since prostate cancer and MBC share common risk factors, including *BRCA1/2* and *PALB2* mutations [25,26].

In this study we performed mutational analysis of the complete coding region and intron/exon boundaries of *BRIP1* by SSCP analysis. SSCP has been also used in a previous study allowing the detection of a novel *BRIP1* truncating mutation consisting in a 4-nucleotide deletion [23]. We failed to detect any *BRIP1* truncating mutation in our series. Since SSCP is a low sensitive technique to detecting base substitutions, it can not be excluded that our results may be underestimated. Nonetheless, we succeeded in the identification of five *BRIP1* gene variants.

The potential role of the coding and non-coding variants of *BRIP1* remains largely controversial. Several studies identified specific missense alterations, including P47A, R173C and Q944E that may confer BC susceptibility [1,9,27]. P47A was first identified in an early-onset BC patient with a positive FH and was shown to be associated with loss of BRIP1 helicase activity *in vitro* [1]. However, subsequent studies on large BC series did not

confirm this association [5,22]. R173C and Q944E were identified only in studies on Swedish and Chinese populations and might represent two rare population-specific BC susceptibility alleles [9,27].

Here, we identified one rare missense variant, R264W, previously described in FBC cases [5]. This variant was predicted to be pathogenic by *in silico* analysis performed with PolyPhen, SIFT, AGVGD and Pmut. We detected R264W variant in one MBC case (1/97, 1%) and in one control individual (1/203, 0.5%). These findings are consistent with data reported in FBC cases showing R264W both in affected women (5/1212, 0.41%) and in controls (6/2081, 0.29%) [5]. In this study we further evaluated the likely pathogenicity of the variant by LOH analysis and showed that no loss of the wild-type allele had occurred in the breast tumor of the R264W carrier. Overall, our data indicate that R264W is not likely to be associated with an increased risk of BC.

We also identified a common missense variant, P919S, frequently reported in FBC series. The role of this variant in BC susceptibility has been investigated in several case-control studies, with contrasting results [5,7,28]. In our study, we found a comparable allele frequency and genotypes distribution between cases and controls, suggesting that P919S is not associated with MBC predisposition in our population.

In addition, we detected two already described non-coding variants, IVS4-28G>A and 3'UTR 4049C>T, and a synonymous variant, E879E, that were classified as neutral polymorphisms, according to previous studies [5,8].

In conclusion, although *BRIP1* is considered a moderate-penetrance BC susceptibility gene, our study provides no evidence that germ-line variants in *BRIP1* might contribute to MBC predisposition, in contrast to what is observed for other moderate-penetrance BC genes such as *CHEK2* and *PALB2* [15,16,21]. Larger studies are needed to further confirm these findings.

To our knowledge, at present our series represents the largest population-based MBC series extensively screened for the most important BC susceptibility genes, including *BRCA1*, *BRCA2*, *CHEK2*, *PALB2* and *BRIP1* [19,20,21] and, notably, only about 10% of MBC cases have been assigned to specific genetic alterations suggesting that the presence of additional genetic and, possibly, epigenetic factors deserve to be further investigated.

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Figure Legends

Fig. 1 Sequence analysis of the *BRIP1* R264W variant.

A) *BRIP1* nucleotide sequence at position 790 in the DNA of a control individual. B) Sequence analysis of genomic DNA from the MBC case with an abnormal *BRIP1* exon7 SSCP pattern showing the heterozygous C>T substitution at nucleotide 790 (R264W). C) Sequence analysis of DNA extracted from the breast tumor sample of the R264W carrier showing no loss of the wild-type allele.

Table 1 *BRIP1* genetic variants found in a population-based series of 97 *BRCA1/2*, *CHEK2* and *PALB2* mutation negative MBC cases and 203 healthy adult male population controls from Central Italy.

Location	Nucleotide change ^a	Amino Acid substitution	Allelic frequencies (%)		<i>In silico</i> analysis
			Cases	Controls	
<i>Coding</i>					
Exon 7	c.790C>T	p.R264W	1/194 (0.5)	1/406 (0.2)	Probably damaging
Exon 19	c.2637G>A	p.E879E	69/194 (35.6)	–	Synonymous
Exon 19	c.2755C>T	p.919S	71/194 (36.6)	147/406 (36.2)	Probably neutral
<i>Non-coding</i>					
Intron 4	c.380-28G>A	–	2/194 (1)	–	Neutral
3'UTR	c.4049C>T	–	2/194 (1)	–	Neutral

^a Nucleotide ID: NG_007409.1, NCBI

