Original Article

Whole-Exome Sequencing and Targeted Gene Sequencing Provide Insights Into the Role of *PALB2* as a Male Breast Cancer Susceptibility Gene

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BACKGROUND: Male breast cancer (MBC) is a rare disease whose etiology appears to be largely associated with genetic factors. *BRCA1* and *BRCA2* mutations account for about 10% of all MBC cases. Thus, a fraction of MBC cases are expected to be due to genetic factors not yet identified. To further explain the genetic susceptibility for MBC, whole-exome sequencing (WES) and targeted gene sequencing were applied to high-risk, *BRCA1/2* mutation–negative MBC cases. **METHODS:** Germ-line DNA of 1 male and 2 female *BRCA1/2* mutation–negative breast cancer (BC) cases from a pedigree showing a first-degree family history of MBC was analyzed with WES. Targeted gene sequencing for the validation of WES results was performed for 48 high-risk, *BRCA1/2* mutation–negative MBC cases from an Italian multicenter study of MBC. A case-control series of 433 *BRCA1/2* mutation–negative MBC and female breast cancer (FBC) cases and 849 male and female controls was included in the study. **RESULTS:** WES in the family identified the partner and localizer of BRCA2 (*PALB2*) c.419delA truncating mutation carried by the proband, her father, and her paternal uncle (all affected with BC) and the *N*-acetyltransferase 1 (*NAT1*) c.97C>T nonsense mutation carried by the proband's maternal aunt. Targeted *PALB2* sequencing detected the c.1984A>T nonsense mutation in 1 of the 48 *BRCA1/2* mutation–negative MBC cases. *NAT1* c.97C>T was not found in the case-control series. **CONCLUSIONS:** These results add strength to the evidence showing that *PALB2* is involved in BC risk for both sexes and indicate that consideration should be given to clinical testing of *PALB2* for *BRCA1/2* mutation–negative families with multiple MBC and FBC cases. ***Cancer*** **2016;000:000–000.** © *2016 American Cancer Society.*

KEYWORDS: genetic susceptibility, male breast cancer, *N*-acetyltransferase 1 (*NAT1*), partner and localizer of BRCA2 (*PALB2*), whole-exome sequencing.

INTRODUCTION

Male breast cancer (MBC) is a rare disease: it accounts for less than 1% of all breast cancers (BCs) and less than 1% of all cancers in men.[1] Approximately 20% of men with BC have a first-degree family history (FH) of BC, and approximately 20% develop a second nonbreast tumor.[2] Overall, these data indicate that genetic factors are associated with the risk of developing MBC.

Inherited mutations in the high-penetrance *BRCA2* gene and, to a lesser extent, in the *BRCA1* gene predispose men to BC and account for approximately 10% of all MBC cases. In high-risk BC families, *BRCA2* mutations are estimated to

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be responsible for 60% to 76% of MBC cases, whereas *BRCA1* mutations account for 10% to 16% of MBC cases.[3,4] Thus, a fraction of high-risk familial MBC cases remain to be assigned to specific genetic factors.

Germ-line mutations in moderate-penetrance BC susceptibility genes such as checkpoint kinase 2 (*CHEK2*) and partner and localizer of BRCA2 (*PALB2*) have also been reported in MBC cases.[4] However, the contribution of these genes to MBC still remains to be precisely assessed. Overall, data on the role of moderate-penetrance genes in MBC susceptibility are sparse and inconclusive, probably because of the low mutation frequency in the general population and the rarity of the disease.[5-8]

Whole-exome sequencing (WES) is a high-throughput technology that allows the sequencing of the 1% to 2% protein-coding subset of the human genome. Recently, it has emerged as a powerful tool for exploring the extent to which rare mutations may explain the heritability of complex diseases, including several types of cancer.[9,10] Rare mutations involved in BC susceptibility have been found in genes newly identified by WES, such as *FANCM* and *RECQL*.[11,12] To our knowledge, no study has performed WES for MBC cases.

To investigate the genetic component of *BRCA1/2* mutation–negative MBC and to identify germ-line mutations that could further explain BC susceptibility, we performed WES in a high-risk, *BRCA1/2* mutation–negative BC pedigree with a first-degree FH of MBC. We complemented this with targeted gene sequencing in a large series of high-risk, *BRCA1/2* mutation–negative MBC cases.

MATERIALS AND METHODS

Patients

Pedigree selected for WES

A high-risk, *BRCA1/2* mutation–negative MBC pedigree was selected for WES analysis from an Italian multicenter study of MBC.[13-15] The selected family had first-degree relatives affected by BC on both the maternal and paternal sides of the proband, a woman with BC. Specifically, 2 first-degree relatives affected with MBC were on the paternal side, whereas 3 female breast cancer (FBC) cases were on the maternal side. In addition, 4 cancer cases other than BC, including 1 with glioma, 1 with stomach cancer, and 2 with lung cancer, were present in the pedigree. Notably, 1 MBC case was also affected by melanoma (Fig. 1A). Three BC cases—the proband, 1 MBC case (the paternal uncle), and 1 FBC case (the maternal aunt)—all tested negative for *BRCA1/2* mutations, were available for the study, and were examined with WES.

Cases selected for validation

A subset of 48 high-risk, *BRCA1/2* mutation–negative MBC cases from the Italian multicenter study of MBC was selected as a validation series[13-15] on the basis of the following criteria: first-degree breast/ovarian cancer (BC/OC) FH, MBC onset at $\leq$60 years, and bilateral MBC. Notably, 44 cases (91.7%), including 3 patients with a first-degree FH of MBC, had a first-degree FH of BC/OC, and 28 (58.3%) had a first-degree FH of cancer or cancers at sites other than the breasts and ovaries (mostly prostate and colon cancer). Overall, 24 cases (50%) presented with a first-degree FH of both BC/OC and cancer at other sites. One case presented with contralateral BC, and 7 (14.6%) had a personal history of cancer or cancers at sites other than the breasts. The age at first BC diagnosis ranged from 38 to 83 years; the mean age was 60.4 years.

A case-control series of 331 *BRCA1/2* mutation–negative MBC cases unselected for FH, including the 48 high-risk MBC cases,[13-15] and 631 healthy male controls was used for validation of the results. An additional series of 102 *BRCA1/2* mutation–negative FBC patients[16] and 218 healthy female controls was also included as a validation series. Male and female control samples were obtained from individuals enrolled under research or clinical protocols and from blood donors. For each study participant, blood or DNA samples, together with clinicopathologic features for cases, were collected. DNA from blood samples was extracted and quantified as previously described.[13] For some cases, formalin-fixed, paraffin-embedded (FFPE) tumor sections were available. Tumor DNA was extracted from microdissected FFPE sections to ensure at least 60% to 70% of tumor cells with the AllPrep DNA/RNA FFPE kit (Qiagen, Hilden, Del) according to the manufacturer's instructions.

The study was approved by the local ethics committee (Sapienza University of Rome, Protocol 264/12).

WES

Genomic DNA was extracted from whole blood samples with the QIAamp DNA Mini Kit according to the manufacturer's instructions (Qiagen). DNA quantity and quality were measured with both fluorometric quantitation by Qubit (Invitrogen, Carlsbad, Calif) and spectrophotometric quantitation by NanoDrop (Thermo Scientific, Waltham, Mass). A total of 10 μg of DNA for each sample was used to prepare in vitro DNA libraries with the TruSeq DNA sample preparation kit and the TruSeq exome enrichment kit (Illumina, San Diego, Calif). Libraries were sequenced on the Illumina HiSeq instrument with a 100-bp paired-end protocol (2×100 bp).

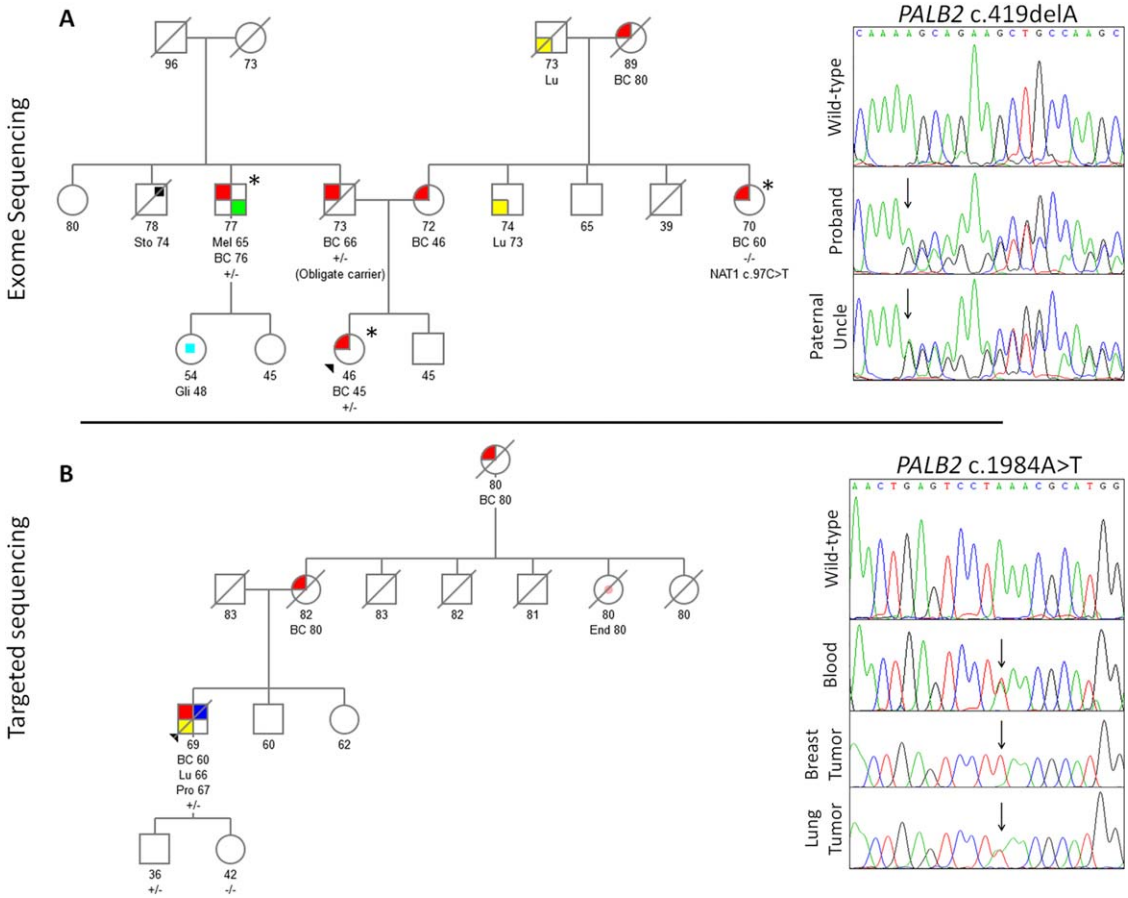

Figure 1. Pedigrees of *PALB2* mutation carriers. Probands are indicated by an arrow head. Individuals analyzed by whole-exome sequencing are indicated by an asterisk. For each individual in the pedigrees, the age at diagnosis, the age at the current time or death, or both are reported. Tested family members are marked with +/– for heterozygous mutation carriers and with –/– for the wild type. (A) Pedigree of the *PALB2* c.419delA carrier and partial electropherogram of *PALB2* exon 4 confirming the presence of *PALB2* c.419delA in the germ-line DNA of the proband and her paternal uncle. (B) Pedigree of the *PALB2* c.1984A>T carrier and partial electropherogram of *PALB2* exon 5 showing the presence of *PALB2* c.1984A>T in the germ-line DNA of the proband and the loss of the wild-type allele in his breast tumor sample but not in his lung tumor sample. BC indicates breast cancer (red); End, endometrial cancer (pink); Gli, glioma (light blue); Lu, lung cancer (yellow); Mel, melanoma (green); *NAT1, N*-acetyltransferase 1; *PALB2*, partner and localizer of BRCA2; Pro, prostate cancer (blue); Sto, stomach cancer (black).

Data Analysis

The adopted next-generation sequencing data analysis was close to the one described by D'Antonio et al.[17] In particular, the quality check of the sequenced reads was performed with the NGS QC toolkit (version 2.3.3).[18] This tool discarded low-quality reads after a quality check and removed primer/adaptor sequences. After a quality-control step, the filtered short reads were mapped to the reference genome (hg19) with BWA (version 0.7.10).[19] This is a fast and memory-efficient read aligner based on a data-compression algorithm (the Burrows-Wheeler transform). SAMtools was used in the variant calling preprocessing phase to order the reads by chromosomal coordinates and merge the paired and unpaired BAM files of the same sample.[20] The realigner tool of the Genome Analysis Tool Kit (GATK; version 2.8-1) was used to perform a local realignment around insertion/deletions (indels) to correct misalignments (due to indels); consensus indels were obtained, and false-positives were reduced.[21,22] The GATK recalibration tool was adopted to recalibrate the quality score after indel realignment.[23] The detection of single-nucleotide and indel polymorphisms was performed with the main algorithm of GATK on the basis of a Bayesian statistical model for the calculation of the probability of the genotype.[23] As confirmation of the accuracy of each call, the Phred-like quality score was associated with the variant results. Furthermore, each detected variant was annotated with information such as the genome position and functional effect with the ANNOVAR tool (version 2014, November 12).[24]

TABLE 1. Exome Variant Filtering Steps

	Proband	Paternal Uncle	Maternal Aunt
Variants called in coding regions	22,855	22,998	22,876
Heterozygous variants	14,238	14,188	14,407
Variants after removal of synonymous variants	7183	7148	7281
Truncating variants (frameshift or stop-gain mutations)	149	143	132
Novel or MAF $<1\%$	44	48	39
Filtering for gene function (previous association with breast cancer)	1	1	1
Shared with proband	—	1	0

Abbreviation: MAF, minor allele frequency.

The filtering of variants after annotation was performed with an in-house–developed script in R software (https://www.r-project.org/). Because a dominant inheritance pattern was expected, homozygous variants were filtered out. Moreover, only variants predicted to cause a truncated protein product (frameshift or stop-gain mutations) were chosen. We also removed variants with an allelic frequency $\geq 1\%$ in the 1000 Genomes Project (http://www.1000genomes.org/), the Exome Variant Server (http://evs.gs.washington.edu/), and dbSNP (http://www.ncbi.nlm.nih.gov/SNP/). Variants were further considered on the basis of a gene's previously known biological function in BC.

Sanger Sequencing

Sanger sequencing of polymerase chain reaction products was performed on both strands with the BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Foster City, Calif) in the ABI-Prism 3130XL genetic analyzer (Applied Biosystems). Polymerase chain reaction fragments and sequencing reactions were purified with the MultiScreen HTS vacuum manifold (Millipore, Billerica, Mass). The primer pairs used are available upon request.

Variants were named according to Human Genome Variation Society nomenclature (http://www.hgvs.org/) and were identified as new by reference to the Leiden Open Variation Database (version 3.0; http://databases.lovd.nl/), the Exome Aggregation Consortium browser (http://exac.broadinstitute.org/), and the Exome Variant Server.

Genotyping

Genotyping was conducted with a high-resolution melting analysis performed with the 7500 Fast real-time polymerase chain reaction system (Applied Biosystems). Primers were designed with the Primer3Plus platform and are available upon request. Samples from individuals carrying and not carrying the selected variant were used as positive and negative controls in each high-resolution melting experiment. Melting curves were analyzed with High Resolution Melting software (Applied Biosystems). Fragments showing aberrant melting curves were analyzed with Sanger sequencing to determine the presence of genetic variations.

RESULTS

WES in a High-Risk, BRCA1/2 *Mutation–Negative MBC Family*

WES was performed in a high-risk, *BRCA1/2* mutation–negative MBC family selected from the ongoing Italian multicenter study of MBC. Three family members, including the proband, her paternal uncle, and her maternal aunt (Fig. 1A), were analyzed. The mean coverage for all cases was $>70\times$. Results from the WES bioinformatics analysis are reported in Table 1.

As a result of the variant filtering, 1 heterozygous truncating mutation, *PALB2* c.419delA (p.Lys140SerfsTer37), which was shared by the proband and her paternal uncle (both affected with BC), was considered as a causal pathogenic mutation. The mutation, located in exon 4 of *PALB2*, was successfully validated with Sanger sequencing (Table 2 and Fig. 1A).

The proband was diagnosed with an estrogen receptor–positive, progesterone receptor–positive ductal carcinoma in situ at the age of 45 years; her father, a mutation obligate carrier, was diagnosed with a ductal carcinoma at the age of 66 years; and her paternal uncle was diagnosed with melanoma at the age of 65 years and with an estrogen receptor–positive, progesterone receptor–positive ductal carcinoma at the age of 76 years.

A nonsense, potentially deleterious variant in *N*-acetyltransferase 1 (*NAT1*; c.97C>T; p.Arg33Ter; rs56318881), a BC-related gene, was identified by WES in the proband's maternal aunt, who was diagnosed with BC at the age of 60 years. The mutation was confirmed by Sanger sequencing and was not detected in the proband.

Overall, *NAT1* and *PALB2* variants were observed on the maternal and paternal sides of the family, respectively.

TABLE 2. *PALB2* Genetic Variants Found in High-Risk MBC Cases

	Location	Nucleotide Change[a]	Protein Change[a]	rs Number	dbSNP MAF	ExAC Browser MAF (European Population)	Heterozygous MBC Cases, n/N (%)	In Silico Analysis			Studies or Projects Reporting Variant
								SIFT	PolyPhen-2	AGVGD	
Exome sequencing	**Exon 4**	**c.419delA**	**p.Lys140SerfsTer37**	–	–	–	**2/2**	–	–	–	–
Targeted sequencing	Exon 4	c.1676A>G	p.Gln559Arg	rs152451	0.1508	0.0955	6/48 (12.5)	Tolerated	Benign	Neutral	6, 25-29
	Exon 5	c.1960 A>G	p. Ile654Val	rs749842477	–	0	1/48 (2.1)	Tolerated	Benign	Neutral	ExAC browser
	Exon 5	c.1984 A>T	p.Lys662Ter	–	–	–	1/48 (2.1)	–	–	–	30
	Exon 5	c.2014 G>C	p.Glu672Gln	rs45532440	0.0144	0.0283	2/48 (4.2)	Tolerated	Benign	Neutral	25-29
	Exon 9	c.2993G>A	p.Gly998Glu	rs45551636	0.0086	0.0214	2/48 (4.2)	Damaging	Probably damaging	Likely deleterious	25-29
	Exon 12	c.3300 T>G	p.Thr1100=	rs45516100	0.0176	0.0283	2/48 (4.2)	–	–	–	25-29
	5′-UTR	g.5154G>A	–	rs8053188	0.0663	0.0236	1/48 (2.1)	–	–	–	6, 27, 28, 31
	Intron 3	g.9966A>C	–	rs80291632	0.0142	–	1/48 (2.1)	–	–	–	6, 27, 28, 31
	3′-UTR	**g.43066G>A**	–	–	–	–	**1/48 (2.1)**	–	–	–	–

Abbreviations: AGVGD, Align Grantham Variation-Grantham Deviation; ExAC, Exome Aggregation Consortium; MAF, minor allele frequency; MBC, male breast cancer; *PALB2*, partner and localizer of BRCA2; SIFT, Sorting Intolerant From Tolerant; PolyPhen-2, Polymorphism Phenotyping version 2; UTR, untranslated region.

[a] The positions of variants are reported with reference to RefSeq NG_007406.1, NM_024675.3, and NP_078951.2 (National Center for Biotechnology Information). Novel mutations are indicated in bold.

PALB2 *Mutations in High-Risk MBC Cases*

On the basis of the WES findings, we further performed a targeted sequencing of the complete coding region and intron/exon boundaries of *PALB2* for 48 high-risk MBC patients negative for *BRCA1/2* pathogenic mutations. As shown in Table 2, a total of 9 *PALB2* variants were identified by targeted sequencing.

The *PALB2* c.1984A>T (p.Lys662Ter) nonsense mutation was found in a man diagnosed with breast, lung, and prostate cancers at 60, 66, and 67 years of age, respectively (Fig. 1B). This case had also an FH of BC on the maternal side of the family. Specifically, his mother and grandmother were both diagnosed with BC at 80 years of age. He also had a maternal aunt diagnosed with endometrial cancer at 80 years of age.

The mutation carrier presented with an estrogen receptor–negative, progesterone receptor–positive breast tumor. A sequencing analysis of tumor DNA obtained from his breast and lung tumors showed a loss of the wild-type allele in the breast tumor sample but not in the lung tumor sample (Fig. 1B). We did not search for a different second-hit *PALB2* mutation in the lung tumor sample.

Testing of *PALB2* c.1984A>T in the mutation carrier's healthy offspring revealed that his son was a mutation carrier, whereas his daughter was wild-type (Fig. 1B).

In addition to the *PALB2* nonsense mutation, 4 *PALB2* missense variants (p.Gln559Arg, p.Ile654Val, p.Glu672Gln, and p.Gly998Glu) and 1 synonymous variant (p.Thr1100=), all previously reported, were identified in the analyzed series (Table 2). The variants p.Gln559Arg, p.Ile654Val, and p.Glu672Gln were predicted to be neutral by in silico analysis, whereas the variant p.Gly998Glu was predicted to have a damaging effect. Furthermore, 3 noncoding variants—2 previously reported (g.5154G>A and g.9966A>C) and 1 novel (g.43066G>A)—were detected (Table 2). An in silico analysis predicted that these variants would not affect splicing.

NAT1 *c.97C>T in MBC and FBC*

High-resolution melting was performed to genotype the *NAT1* c.97C>T nonsense variant in a case-control study including 331 *BRCA1/2*-negative MBC cases, 631 healthy male controls, 102 *BRCA1/2*-negative FBC cases, and 218 healthy female controls (Fig. 2). The *NAT1* c.97C>T variant was not found in any of the cases and controls analyzed.

DISCUSSION

In this study, we investigated the genetic causes of high-risk, *BRCA1/2* mutation–negative MBC cases combining exome sequencing and targeted gene sequencing. We

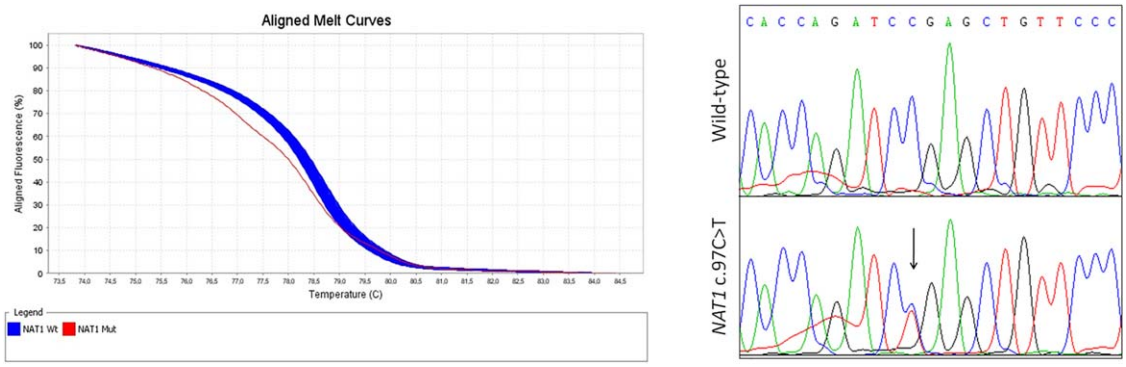

Figure 2. Genotyping of the *NAT1* c.97C>T mutation. (A) High-resolution melting curves for *NAT1* exon 5. The case harboring the c.97C>T genotype (positive control) is in red. (B) Partial electropherogram of *NAT1* exon 5 showing the Wt sequence and the nonsense mutation c.97C>T in the germ-line DNA of the family member indicated in Figure 1. Mut indicates mutant; *NAT1, N*-acetyltransferase 1; Wt, wild type.

selected a high-risk, *BRCA1/2* mutation–negative family with 2 first-degree MBC cases and 4 FBC cases to be analyzed by WES. This peculiar selection allowed the identification of a pathogenic *PALB2* germ-line mutation associated with BC susceptibility in both sexes. We found that the proband, her father, and her paternal uncle, all affected with BC, were carriers of the *PALB2* c.419delA truncating mutation; to our knowledge, this mutation has not been previously reported.

In a previous study, we performed *PALB2* genetic screening in a population-based series of 97 MBC cases unselected for FH, and we did not identify any pathogenic mutation.[6] Here, we performed *PALB2*-targeted sequencing in a selected series of 48 high-risk, *BRCA1/2* mutation–negative MBC patients from the ongoing Italian multicenter study of MBC.[13-15] In this series, we identified a *PALB2* truncating mutation, c.1984A>T (p.Lys662Ter), in 1 MBC case, who presented with an FH of BC and a personal history of lung and prostate cancer. This mutation was previously reported in a French woman diagnosed with bilateral BC who presented with a personal history of colon cancer and an FH of prostate and pancreatic cancer.[30] We could show the loss of the wild-type allele in the breast tumor of our mutation carrier, and this further confirmed the pathogenic effect of the *PALB2* c.1984A>T mutation.

In this study, we also identified 4 *PALB2* missense variants (p.Gln559Arg, p.Ile654Val, p.Glu672Gln, and p.Gly998Glu) considered not pathogenic on the basis of either the previously reported polymorphic frequency in controls[6] or the neutral effect on protein function predicted by an in silico analysis. Nevertheless, it cannot be excluded that some of these variants may represent low-penetrance susceptibility polymorphisms, as recently suggested for the p.Gln559Arg variant.[29]

If we consider the 2 MBC cases in the family analyzed by WES and the case identified in the series of 48 high-risk MBC cases, 3 cases from 2 high-risk families were carriers of pathogenic *PALB2* mutations. Overall, 2 of 49 unrelated high-risk MBC cases (4%) were *PALB2* mutation carriers.

To date, only a limited number of studies have investigated the presence of *PALB2* mutations in MBC.[26-28,31-34] Previous studies, including a small number of high-risk MBC cases (up to 13 cases), showed a variable mutation frequency ranging from 1% to 16%.[26,28,31,34] Our data, based on a series of high-risk MBC cases larger than those analyzed thus far, provide a more precise assessment of the contribution of *PALB2* mutations in high-risk MBC cases. Notably, the frequency of *PALB2* pathogenic mutations in high-risk MBC cases reported here was higher than that observed in high-risk FBC cases (ie, 4% vs 1%).[35]

Our data show that MBC cases harboring *PALB2* mutations were also affected by other tumors, including melanoma, prostate cancer, and lung cancer, and they had a FH of breast and other cancers, including stomach cancer. *PALB2* mutations were frequently observed in pedigrees with cases of melanoma, pancreatic cancer, prostate cancer, lung cancer, and stomach cancer in addition to BC.[25-31,34,36] Overall, these observations suggest that *PALB2*-related families may resemble *BRCA2*-like families, in which MBC and several other cancers may be found in addition to FBC. However, further studies are needed to evaluate the risk conferred by *PALB2* mutations in cancers other than BC.

Because of the high BC risk recently associated with *PALB2* mutations,[37] the identification of *PALB2* mutation carriers in MBC is likely to be of significant clinical relevance. Recently introduced National Comprehensive Cancer Network practice guidelines suggest that female *PALB2* mutation carriers should undergo enhanced breast screening.[38] Notably, in our study, we identified a young, healthy male *PALB2* mutation carrier in a high-risk MBC family. We suggest that in the presence of *PALB2* pathogenic mutations and a significant cancer FH, it is important to consider BC awareness and surveillance in male *PALB2* mutation carriers, as recently indicated for male *BRCA1/2* mutation carriers.[38] Thus, men with *PALB2* mutations also may be candidates for personalized medical decisions according to the best available information about their cancer risk.

In this study, we also identified the rare nonsense variant *NAT1* c.97C>T (rs56318881), also known as *NAT1**19, in an FBC case. *NAT1* encodes for an *N*-acetyltransferase involved in the metabolism of xenobiotics and carcinogens, and the *NAT1* c.97C>T variant encodes for a truncated protein with impaired functionality.[39,40] It has been shown that *NAT1* variants may increase the risk of lung cancer and BC.[41-43] Notably, we found the *NAT1* c.97C>T variant on the side of the pedigree in which 3 cases of BC and 2 cases of lung cancer were present. Unfortunately, DNA from family members affected with lung cancer was not available for testing. There is also evidence that *NAT1* may have a possible role in MBC because *NAT1* has been suggested to be a prognostic marker in MBC patients.[44] In this study, we investigated the potential role of *NAT1* c.97C>T in genetic susceptibility to BC by genotyping a large case-control series. The variant was not found in any of the cases and controls analyzed, and this suggests that it may represent a rare, family-specific predisposition factor. However, given the low frequency of this variant in the population (minor allele frequency from the Exome Aggregation Consortium browser = 0.00004), we cannot exclude a small effect of this variant on BC risk.

In conclusion, our results add strength to the accumulating evidence showing that *PALB2* is involved in BC risk (particularly MBC) and suggest that MBC cases may be helpful in the identification of *PALB2* families, as is the case for *BRCA2* families.[45] *PALB2* seems now to be ready for clinical translation, and *PALB2* genetic testing should be considered for *BRCA1/2* mutation–negative BC families with MBC and/or other cancers. Overall, results from this study highlight the importance of a good selection of high-risk BC families for genetic testing with the aim of improving the clinical management of MBC and FBC patients and their relatives.

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CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

AUTHOR CONTRIBUTIONS

Valentina Silvestri: Bioinformatic analysis and interpretation of data, drafting of the manuscript, data acquisition, review of the manuscript, and agreement to submit the manuscript for publication. **Veronica Zelli**: Drafting of the manuscript, data acquisition, review of the manuscript, and agreement to submit the manuscript for publication. **Virginia Valentini**: Drafting of the manuscript, data acquisition, review of the manuscript, and agreement to submit the manuscript for publication. **Piera Rizzolo**: Data acquisition, review of the manuscript, and agreement to submit the manuscript for publication. **Anna Sara Navazio**: Data acquisition, review of the manuscript, and agreement to submit the manuscript for publication. **Anna Coppa**: Data acquisition, review of the manuscript, and agreement to submit the manuscript for publication. **Simona Agata**: Data acquisition, review of the manuscript, and agreement to submit the manuscript for publication. **Cristina Oliani**: Data acquisition, review of the manuscript, and agreement to submit the manuscript for publication. **Daniela Barana**: Data acquisition, review of the manuscript, and agreement to submit the manuscript for publication. **Tiziana Castrignanò**: Bioinformatic analysis and interpretation of data, data acquisition, review of the manuscript, and agreement to submit the manuscript for publication. **Alessandra Viel**: Data acquisition, review of the manuscript, and agreement to submit the manuscript for publication. **Antonio Russo**: Data acquisition, review of the manuscript, and agreement to submit the manuscript for publication. **Maria Grazia Tibiletti**: Data acquisition, review of the manuscript, and agreement to submit the manuscript for publication. **Ines Zanna**: Data acquisition, review of the manuscript, and agreement to submit the manuscript for publication. **Giovanna Masala**: Data acquisition, review of the manuscript, and agreement to submit the manuscript for publication. **Laura Cortesi**: Data acquisition, review of the manuscript, and agreement to submit the manuscript for publication. **Siranoush Manoukian**: Data acquisition, review of the manuscript, and agreement to submit the manuscript for publication. **Jacopo Azzollini**: Data acquisition, review of the manuscript, and agreement to submit the manuscript for publication. **Bernard Peissel**: Data acquisition, review of the manuscript, and agreement to submit the manuscript for publication. **Bernardo Bonanni**: Data acquisition, review of the manuscript, and agreement to submit the manuscript for publication. **Paolo Peterlongo**: Data acquisition, review of the manuscript, and agreement to submit the manuscript

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REFERENCES

1. Ly D, Forman D, Ferlay J, Brinton LA, Cook MB. An international comparison of male and female breast cancer incidence rates. *Int J Cancer.* 2013;132:1918-1926.
2. Korde LA, Zujewski JA, Kamin L, et al. Multidisciplinary meeting on male breast cancer: summary and research recommendations. *J Clin Oncol.* 2010;28:2114-2122.
3. Frank TS, Deffenbaugh AM, Reid JE, et al. Clinical characteristics of individuals with germline mutations in BRCA1 and BRCA2: analysis of 10,000 individuals. *J Clin Oncol.* 2002;20:1480-1490.
4. Rizzolo P, Silvestri V, Tommasi S, et al. Male breast cancer: genetics, epigenetics, and ethical aspects. *Ann Oncol.* 2013;24(suppl 8): viii75-viii82.
5. Falchetti M, Lupi R, Rizzolo P, et al. BRCA1/BRCA2 rearrangements and CHEK2 common mutations are infrequent in Italian male breast cancer cases. *Breast Cancer Res Treat.* 2008;110:161-167.
6. Silvestri V, Rizzolo P, Zanna I, et al. PALB2 mutations in male breast cancer: a population-based study in central Italy. *Breast Cancer Res Treat.* 2010;122:299-301.
7. Silvestri V, Rizzolo P, Falchetti M, et al. Mutation screening of RAD51C in male breast cancer patients. *Breast Cancer Res.* 2011;13: 404.
8. Silvestri V, Rizzolo P, Falchetti M, et al. Mutation analysis of BRIP1 in male breast cancer cases: a population-based study in central Italy. *Breast Cancer Res Treat.* 2011;126:539-543.
9. Snape K, Ruark E, Tarpey P, et al. Predisposition gene identification in common cancers by exome sequencing: insights from familial breast cancer. *Breast Cancer Res Treat.* 2012;134:429-433.
10. Sokolenko AP, Suspitsin EN, Kuligina ES, et al. Identification of novel hereditary cancer genes by whole exome sequencing. *Cancer Lett.* 2015;369:274-288.
11. Kiiski JI, Pelttari LM, Khan S, et al. Exome sequencing identifies FANCM as a susceptibility gene for triple-negative breast cancer. *Proc Natl Acad Sci U S A.* 2014;111:15172-15177.
12. Cybulski C, Carrot-Zhang J, Kluzniak W, et al. Germline RECQL mutations are associated with breast cancer susceptibility. *Nat Genet.* 2015;47:643-646.
13. Ottini L, Silvestri V, Saieva C, et al. Association of low-penetrance alleles with male breast cancer risk and clinicopathological characteristics: results from a multicenter study in Italy. *Breast Cancer Res Treat.* 2013;138:861-868.
14. Silvestri V, Rizzolo P, Scarno M, et al. Novel and known genetic variants for male breast cancer risk at 8q24.21, 9p21.3, 11q13.3 and 14q24.1: results from a multicenter study in Italy. *Eur J Cancer.* 2015;51:2289-2295.
15. Ottini L, Silvestri V, Rizzolo P, et al. Clinical and pathologic characteristics of BRCA-positive and BRCA-negative male breast cancer patients: results from a collaborative multicenter study in Italy. *Breast Cancer Res Treat.* 2012;134:411-418.
16. Ottini L, D'Amico C, Noviello C, et al. BRCA1 and BRCA2 mutations in central and southern Italian patients. *Breast Cancer Res.* 2000;2:307-310.
17. D'Antonio M, D'Onorio De Meo P, Paoletti D, et al. WEP: a high-performance analysis pipeline for whole-exome data. *BMC Bioinformatics.* 2013;14(suppl 7):S11.
18. Patel RK, Jain M. NGS QC toolkit: a toolkit for quality control of next generation sequencing data. *PLoS One.* 2012;7:e30619.
19. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics.* 2009;25:1754-1760.
20. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics.* 2009;25:2078-2079.
21. McKenna A, Hanna M, Banks E, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res.* 2010;20:1297-1303.
22. DePristo MA, Banks E, Poplin R, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat Genet.* 2011;43:491-498.
23. Li R, Li Y, Fang X, et al. SNP detection for massively parallel whole-genome resequencing. *Genome Res.* 2009;19:1124-1132.
24. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res.* 2010;38:e164.
25. Rahman N, Seal S, Thompson D, et al. PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. *Nat Genet.* 2007;39:165-167.
26. Ding YC, Steele L, Kuan CJ, Greilac S, Neuhausen SL. Mutations in BRCA2 and PALB2 in male breast cancer cases from the United States. *Breast Cancer Res Treat.* 2011;126:771-778.
27. Blanco A, de la Hoya M, Balmana J, et al. Detection of a large rearrangement in PALB2 in Spanish breast cancer families with male breast cancer. *Breast Cancer Res Treat.* 2012;132:307-315.
28. Vietri MT, Caliendo G, Casamassimi A, et al. A novel PALB2 truncating mutation in an Italian family with male breast cancer. *Oncol Rep.* 2015;33:1243-1247.
29. Thompson ER, Gorringe KL, Rowley SM, et al. Prevalence of PALB2 mutations in Australian familial breast cancer cases and controls. *Breast Cancer Res.* 2015;17:111.
30. Nguyen-Dumont T, Hammet F, Mahmoodi M, et al. Mutation screening of PALB2 in clinically ascertained families from the Breast Cancer Family Registry. *Breast Cancer Res Treat.* 2015; 149:547-554.
31. García MJ, Fernandez V, Osorio A, et al. Analysis of FANCB and FANCN/PALB2 Fanconi anemia genes in BRCA1/2-negative Spanish breast cancer families. *Breast Cancer Res Treat.* 2009;113: 545-551.
32. Sauty de Chalon A, Teo Z, Park DJ, et al. Are PALB2 mutations associated with increased risk of male breast cancer? *Breast Cancer Res Treat.* 2010;121:253-255.
33. Casadei S, Norquist BM, Walsh T, et al. Contribution of inherited mutations in the BRCA2-interacting protein PALB2 to familial breast cancer. *Cancer Res.* 2011;71:2222-2229.
34. Adank MA, van Mil SE, Gille JJ, Waisfisz Q, Meijers-Heijboer H. PALB2 analysis in BRCA2-like families. *Breast Cancer Res Treat.* 2011;127:357-362.
35. Fernandes PH, Saam J, Peterson J, et al. Comprehensive sequencing of PALB2 in patients with breast cancer suggests PALB2 mutations explain a subset of hereditary breast cancer. *Cancer.* 2014;120:963-967.
36. Lu C, Xie M, Wendl MC, et al. Patterns and functional implications of rare germline variants across 12 cancer types. *Nat Commun.* 2015;6:10086.
37. Antoniou AC, Casadei S, Heikkinen T, et al. Breast-cancer risk in families with mutations in PALB2. *N Engl J Med.* 2014;371:497-506.
38. National Comprehensive Cancer Network. National Comprehensive Cancer Network guidelines Genetic/Familial High-Risk Assessment: Breast and Ovarian, version 2.2016. https://www.nccn.org/professionals/physician_gls/pdf/genetics_screening.pdf. Accessed June, 2016.
39. Fretland AJ, Doll MA, Zhu Y, Smith L, Leff MA, Hein DW. Effect of nucleotide substitutions in N-acetyltransferase-1 on N-acetylation (deactivation) and O-acetylation (activation) of arylamine

carcinogens: implications for cancer predisposition. *Cancer Detect Prev.* 2002;26:10-14.
40. Fretland AJ, Doll MA, Leff MA, Hein DW. Functional characterization of nucleotide polymorphisms in the coding region of N-acetyltransferase 1. *Pharmacogenetics.* 2001;11:511-520.
41. Wikman H, Thiel S, Jager B, et al. Relevance of N-acetyltransferase 1 and 2 (NAT1, NAT2) genetic polymorphisms in non–small cell lung cancer susceptibility. *Pharmacogenetics.* 2001;11:157-168.
42. Lee KM, Park SK, Kim SU, et al. N-acetyltransferase (NAT1, NAT2) and glutathione S-transferase (GSTM1, GSTT1) polymorphisms in breast cancer. *Cancer Lett.* 2003;196:179-186.
43. Zheng W, Deitz AC, Campbell DR, et al. N-acetyltransferase 1 genetic polymorphism, cigarette smoking, well-done meat intake, and breast cancer risk. *Cancer Epidemiol Biomarkers Prev.* 1999;8:233-239.
44. Johansson I, Nilsson C, Berglund P, et al. Gene expression profiling of primary male breast cancers reveals two unique subgroups and identifies N-acetyltransferase-1 (NAT1) as a novel prognostic biomarker. *Breast Cancer Res.* 2012;14:R31.
45. Azzollini J, Scuvera G, Bruno E, et al. Mutation detection rates associated with specific selection criteria for BRCA1/2 testing in 1854 high-risk families: a monocentric Italian study. *Eur J Intern Med.* 2016;32:65-71.