

Original article

A *PALB2* truncating mutation: Implication in cancer prevention and therapy of Hereditary Breast and Ovarian Cancer

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ABSTRACT

Explaining genetic predisposition in Hereditary Breast and Ovarian Cancer (HBOC) families without *BRCA* mutations is crucial. Germline *PALB2* inactivating mutations were associated with an increased risk of HBOC due to its role in DNA repair through cooperation with *BRCA* proteins. The prevalence and penetrance of *PALB2* mutations in Spanish HBOC patients remains unexplained. *PALB2* mutation screening has been conducted in 160 high-risk *BRCA*-negative patients and 320 controls. We evaluated four predicted splicing disruption variants and large genomic rearrangements by multiplex ligation-dependent probe amplification. We have found a frameshift mutation which segregates in an early onset cancer family; and four rare missense variants. None of the variants tested for a predicted splicing disruption showed an aberrant transcript pattern. No large genomic rearrangements were detected. Although *PALB2* truncating mutations are rarely identified, segregation analysis and early onset cancer suggest a significant contribution to HBOC susceptibility in the Spanish population. *PALB2* screening may improve genetic counselling through prevention measures, pedigree management and PARP inhibitor therapy selection.

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Introduction

Hereditary Breast and Ovarian Cancer Syndrome (HBOC) is a genetic condition which predisposes those who have it to develop breast and ovarian cancer. The discovery of *BRCA* mutation's role in HBOC susceptibility marked a milestone in cancer genetics. Progress in the field was hampered by the significant percentage of cases not explained by *BRCA* mutations. Consequently, the need to discover new implicated genes emerges.

As a strategic search, other genes not only involved in DNA repair, but also interacting with *BRCA*s, are suitable candidates. According to this criterion, *PALB2* engaged scientists' attention.

PALB2 cooperates with *BRCA* genes in DNA damage response and truncating mutations in this gene can lead to a defective DNA repair [1]. Under this assumption, germline mutations in *PALB2* would compromise genome stability, which could predispose to cancer through the accumulation of DNA defects [2].

Despite *PALB2* variants being rarely found (1–4% of *BRCA*-negative-families), it is estimated that there is an increased risk comparable to *BRCA2* mutations [3].

As a proof of concept, several studies have ascertained that *PALB2* is an important cause of hereditary breast cancer, according to both the frequency and associated risk [4,5]. Previous reports in the worldwide population have demonstrated the association of the *PALB2* germline pathogenic variants with several familial cancer types, such as male [6] or pancreatic [5,7]. In addition, *PALB2* mutations have also been associated with susceptibility to ovarian cancer [8]. Consequently, *PALB2* has been proposed as a moderate penetrance breast and ovarian cancer gene.

Since the “synthetic lethality” phenomenon has resulted in a personalized therapeutic option in cancer, such as PARP inhibitors (PARPi), looking for mutations that reduce homologous repair is

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mandatory for a better selection of patients. Here, the correlation between *PALB2* mutations and HBOC predisposition is analysed in order to design molecular diagnostic strategies and therapeutic treatment [9].

Materials and methods

Patient selection

We have selected 160 index cases, negative for BRCA1/2 mutations, recruited from the Regional Hereditary Cancer Prevention Program of Castilla & León (Spain). Participants in this study are ovarian cancer patients or breast cancer patients who have first degree relatives with ovarian cancer in the same family side. Ethical committee approval, informed consent, family history and clinical features were collected.

The control population used was from the National DNA Bank, a collection of 320 representative DNA samples of the Spanish population with no familial cancer history. DNA samples were obtained from peripheral blood, using the MagnaPure extraction kit (Roche). Patients and controls were also negative for mutations in other related moderate penetrance genes (*BRIP1*, *RAD51D*, *RAD51C*, *ATM*, and *BARD1*).

Point mutation analysis

The entire coding sequence and splicing sites of *PALB2* was screened using heteroduplex analysis by capillary array electrophoresis (HA-CAE). This method was developed in our laboratory [10].

Genomic fragments covering *PALB2* exons and intron-exon junctions were amplified by multiplex PCR. Primers were designed using Primer3tool software (<http://bioinfo.ut.ee/primer3-0.4.0/>) (accessed November 5, 2017). In total, 17 PCR fragments were required to cover *PALB2* (Supplementary table S1). To optimise the analysis of *PALB2*, amplified fragments with sufficiently different sizes, similar annealing temperature and different fluorophore marker (FAM™ or HEX™) were grouped together in a multiplex PCR. After method optimization, the *PALB2* gene was screened in a total of six multiplex PCR (Supplementary table S2).

Fragments showing an HA-CAE altered pattern were sequenced with the BigDye Terminator Sequencing Kit v3.1 (Applied Biosystems), with unlabelled forward and reverse primers, on an ABI 3100 DNA Sequencer (four capillaries; Applied Biosystems).

Mutation nomenclature was based on the NM_024675.3 GenBank reference sequences.

RNA isolation and RT-PCR analysis

The patient lymphocyte pellet was stored at -80°C until use. The commercial kit GeneMATRIX Human Blood RNA Purification Kit (EURx) was used for RNA extraction, following the manufacturer's protocol. The final concentration was measured in a NanoDrop™.

Variants predicted as disrupters of splicing in the Human Splicing Finder (HSF) software (<http://www.umd.be/HSF3/>) were selected to perform cDNA-based analysis. The total RNA isolated from lymphocytes was reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche), according to the manufacturer's protocol. Subsequently, we performed a PCR to evaluate the transcription (Fragments and primers are shown in Supplementary Table S3). The PCR product containing wild-type and possible aberrant fragments was visualized in 2% agarose gel.

Detection of large genomic rearrangements (LGRs)

Multiplex ligation-dependent probe amplification (MLPA) analysis was performed with the P260-B1 Kit (MRC-Holland), according to the manufacturer's protocol. The amplified products were separated on ABI3130 and analysed by Coffalyser software (MRC Holland).

In silico analysis

The pathogenicity of variants found with a Minor Allele Frequency (MAF) < 1% was assessed using SIFT (<http://sift.jcvi.org/>) and Polyphen (<http://genetics.bwh.harvard.edu/pph2/>) in silico tools. We also used HSF to evaluate the possibility of splicing alterations, either by exonic splice enhancers or silencers (ESE or ESS, respectively). Those variants predicted as disrupters of splicing were selected to perform cDNA-based analysis.

The frequencies of some of the identified variants were ascertained in ExAc Browser and 1000 Genomes databases. Therefore, we determined the frequencies in our population control group.

Statistical analyses

Carrier frequencies in our 160 cases and 320 population controls were compared using either the Pearson Chi-square test or the Fisher Exact test, as appropriate. All P-values were two-sided. Odds ratios (OR) were generated by a two-by-two table (https://www.medcalc.org/calc/odds_ratio.php; accessed October 21, 2017).

Results

We identified 16 *PALB2* variants (Table 1). Six of the mutations were non-coding; none of them near the intron–exon boundaries. Of the ten coding changes, two were synonymous, seven non-synonymous and one frameshift. All had been previously reported. To obtain specific data for the Spanish population, we decided to screen the 16 variants in our 320-control-population set. Frequencies for both case and control groups are shown in Table 1. The low variant frequencies and the presence of zeros in the control group compromise the statistical power.

To deduce the possible role of missense variants in HBOC pathogenicity, PolyPhen and SIFT predictions were gathered in Table 1. According to the protein position, some of the coding variants may affect certain structural or functional domains of the *PALB2* protein (Fig. 1). Polyphen predicted p.Glu272Lys, p.Pro864Ser, p.Val932Met and p.Leu939Trp changes could significantly alter the protein's properties, endangering the role of *PALB2* in the DNA repair network.

Interestingly, the HSF algorithm predicted three changes as possible splicing disrupters (c.1010T > C, c.1572A > G and c.1676A > G, via creating or breaking Exonic Splicing Enhancers (ESE) and/or Exonic Splicing Silencers (ESS) sites. To verify this prediction, RT-PCR analysis was performed (Supplementary Table 3). In addition, the intronic deletion c.1684 + 39_1684 + 41del was tested to discard a possible splicing alteration. The corresponding cDNA fragments were amplified and loaded in 2% agarose gel. No aberrant transcripts were observed; consequently we rejected any splicing alteration.

The frameshift mutation found in one index case, c.1857delT (p.Phe619LeufsX9), was predicted to create a translation-stop nine codons downstream from the first affected amino acid. We further investigated this mutation in other relatives (Fig. 2). The index case developed ovarian adenocarcinoma at 33 years of age, whereas her sister had breast cancer at age 36. Both affected women carried the mutation while the healthy relatives did not, evidencing that c.1857delT mutation segregates with cancer.

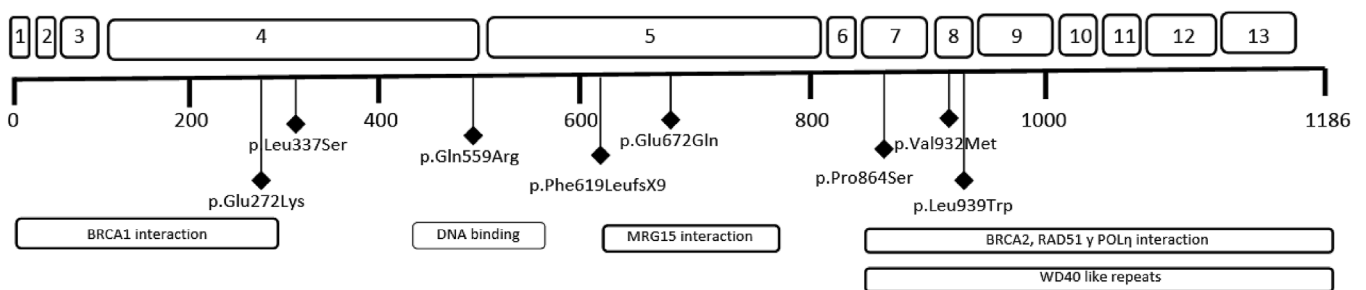
Table 1
PALB2 sequence variants identified in 160 Spanish Breast and Ovarian cancer families.

Nucleotide change ^a	Protein change	dbSNP ^b	Carrier frequency ^c		Odds ratio	95% CI	P-value	Global-MAF	Clinical significance		
			Cases	Controls					ClinVar	SIFT	POLYPHEN
c.-47G > A		rs8053188	0.019 (3/160)	0.025 (8/320)	0.74	0.19–2.84	0.667	0.066 (A)	Benign		
c.49–54C > T		rs515726121	0.013 (2/160)	0/320	10.11	0.48–211.86	0.136	0.001 (T)	Likely Benign		
c.212–183T > G		rs60253267	0.063 (10/160)	0/320	44.72	2.60–768.26	0.009	0.025 (G)	N.A. ^d		
c.212–58A > C		rs80291632	0.063 (10/160)	0/320	44.72	2.60–768.26	0.009	0.014 (G)	Benign		
c.814G > A	p.Glu272Lys	rs515726127	0.006 (1/160)	0/320	6.02	0.24–148.82	0.272	–	Uncertain significance	Tolerated	Possibly damaging
c.1010T > C	p.Leu337Ser	rs45494092	0.025 (4/160)	0.025 (8/320)	1.00	0.29–3.37	1.000	0.007 (C)	Benign (10)/Likely benign (2)/UV (3)	Tolerated	Benign
c.1572A > G	p.Ser524Ser	rs45472400	0.006 (1/160)	0/320	6.02	0.24–148.82	0.272	0.002 (G)	Benign/Likely benign		
c.1676A > G	p.Gln559Arg	rs152451	0.013 (2/160)	0/320	10.11	0.48–211.86	0.136	0.151 (G)	Benign/Likely benign	Tolerated	Benign
c.1684 + 39_1684 + 41del		rs368593832	0.006 (1/160)	0/320	6.02	0.24–148.82	0.272	–	Likely Benign		
c.1857delT	p.Phe619LeufsX9	–	0.006 (1/160)	0/320	6.02	0.24–148.82	0.272	–	N.A.		
c.2014G > C	p.Glu672Gln	rs45532440	0.063 (10/160)	0.056 (18/320)	1.11	0.50–2.48	0.783	0.014 (C)	Benign/Likely benign	Tolerated	Benign
c.2590C > T	p.Pro864Ser	rs45568339	0.006 (1/160)	0/320	6.02	0.24–148.82	0.272	0.001 (T)	Benign/Likely benign	Tolerated	Possibly damaging
c.2794G > A	p.Val932Met	rs45624036	0.006 (1/160)	0.009 (3/320)	0.66	0.06–6.44	0.724	0.001 (A)	Benign/Likely benign	Tolerated	Possibly damaging
c.2816T > G	p.Leu939Trp	rs45478192	0.013 (2/160)	0.003 (1/320)	4.03	0.36–44.87	0.256	0.001 (C)	Benign (1)/Likely benign (5)/UV (5)	Damaging	Possibly damaging
c.3114–51T > A		rs249936	0.100 (16/160)	0/320	73.19	4.36–1228.43	0.003	0.003 (T)	Likely benign		
c.3300T > G	p.Thr1100Thr	rs45516100	0.031 (5/160)	0.047 (15/320)	0.65	0.23–1.83	0.423	0.018 (G)	Benign/Likely benign		

^a GenBank Reference *PALB2* sequence used was NM_024675.3. Nucleotide position +1 corresponds to the A of the ATG start codon. NCBI Single Nucleotide Polymorphism database (dbSNP).

^b Absolute frequencies for cases and controls are presented. OR, odds ratio; CI, confidence interval.

^c N.A. Non available.



The non-synonymous variants found in this study are overlaid on the exonic structure of the gene, with the possible affected functional domains and structural motifs according to the position at amino acid level.

Fig. 1. Schematic representation of *PALB2* gene. The non-synonymous variants found in this study are overlaid on the exonic structure of the gene, with the possible affected functional domains and structural motifs according to the position at amino acid level.

At a practical level, the clinical and pedigree management followed the Genetic Counselling guidelines. Several family members were recruited to ascertain the mutation status in order to implement prevention measures; among them, only the two affected women presented the mutation. At clinical level, both women, underwent surgery and chemotherapy treatment.

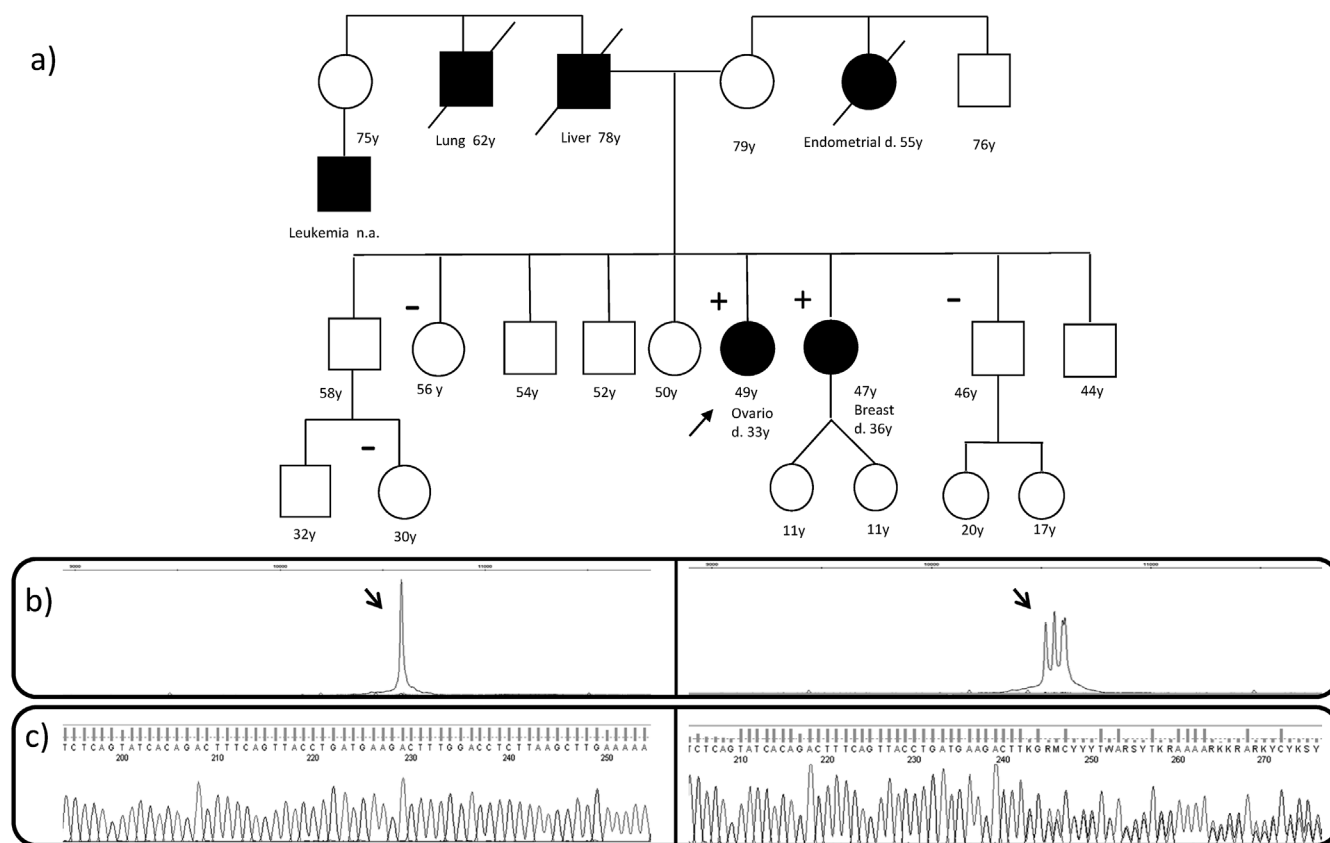
In order to carry out a comprehensive *PALB2* mutation screening, we performed MLPA to identify germline exon deletions or duplications undetectable by HA-CAE. No large genomic rearrangements were found.

Discussion

In the present study, 160 index cases from Spanish BRCA1/BRCA2 negative families and 320 non-disease controls were screened for *PALB2* mutations.

One truncating mutation in *PALB2* was identified in our familial set, revealing prevalence similar to those previously obtained [11,12]. In contrast with other studies [13,14], we have not found founder mutations, probably explained by the limited number of analysed high-risk HBOC individuals.

The *PALB2* germline deletion c.1857delT, located in exon 5, leads



a) Family Pedigree. Probands screened for *PALB2* mutations are indicated by a black figure. The arrow indicates the index case analysed. Segregation analysis was performed in two affected probands and other healthy family members. Confirmed mutation carriers are indicated by a "+" sign, non carriers with a "-" sign. Age at diagnosis for cancer patients and type of cancer is indicated when known. A slashed circle/square indicates a deceased individual. b) HA-CAE pattern of exon 5 *PALB2* fragment. On the left, a negative proband. On the right, the index case with the mutation. c) Analysis of *PALB2* c.1857delT variant in germline DNA: on the left, a negative proband. On the right, the index case with the mutation.

Fig. 2. Characterization of a breast cancer family carrying the *PALB2* c.1857delT (p.Phe619LeufsX9) variant. a) Family Pedigree. Relatives screened for *PALB2* mutations are indicated by a black figure. The arrow indicates the index case analysed. Segregation analysis was performed in two affected probands and other healthy family members. Confirmed mutation carriers are indicated by a "+" sign, non carriers with a "-" sign. Age at diagnosis for cancer patients and type of cancer is indicated when known. A slashed circle/square indicates a deceased individual. b) HA-CAE pattern of exon 5 *PALB2* fragment. On the left, a negative proband. On the right, the index case with the mutation. c) Analysis of *PALB2* c.1857delT variant in germline DNA: on the left, a negative proband. On the right, the index case with the mutation.

to the formation of a stop codon nine residue downstream of the mutation (p.Phe619LeufsX9). The premature stop codon might result in nonsense-mediated mRNA decay, verifying the protein truncation. The mutation was identified in a woman diagnosed at 33 years of age with an ovarian adenocarcinoma. The mutation also segregated in her affected sister, who developed breast cancer at the age of 36 years, but it is not present in the non-affected family members. No pathogenic mutations were found in both sisters any other ovarian cancer penetrance genes (*BRIP1*, *RAD51D*, *RAD51C*, *ATM*, and *BARD1*). The early onset cancer in the carriers supports the idea that *PALB2* confers a substantial risk of HBOC in this family. It is important to note that the heterozygous status of the *PALB2* germline mutation would be sufficient to cause DNA replication and damage response defects [7]. This assumption would be in accordance with Antoniou et al., who concluded that cancer risk for *PALB2* mutation carriers may overlap with that for *BRCA2* mutation carriers [15]. Indeed, the breast carcinoma expressed oestrogen and progesterone receptors, concurring with the *BRCA2* tumour phenotypic [13–15].

One strength of our study is that we selected a significant number of ovarian cancer cases, focusing on the connection between *PALB2* and ovarian cancer. In fact, most of the studies have focused on breast cancer predisposition while we have detected *PALB2* mutations in breast and ovarian cancer families, emphasising

the role of this gene in ovarian cancer predisposition. Interestingly, the frequency in our population- 0.63%- is slightly higher than those reported by others (0.2–0.6%) [2].

PALB2 has a role in the DNA damage response network: interacting with breast and ovarian cancer proteins, such as *BRCA*s, and activating the repair network through its functional domains. With the goal of providing new insights in the interpretation of *PALB2* variants, we integrated information about protein predictions, mapped functional domains or structural motifs of *PALB2* and cases/control frequencies (Table 1). Fig. 1 represents the different mutations over a schematic *PALB2* protein, connecting the amino acid position with the possible affected domains: p.Glu272Lys would affect the physical interaction with *BRCA1* [16], p.Gln559Arg is located in the DNA binding domain [17], p.Glu672Gln is located in the MRG15 interaction domain [18]. MRG15 mediates the DNA-damage-response functions of the *BRCA* complex in chromatin, so the DNA repair efficiency could be questioned [19]. The variants p.Pro864Ser and p.Leu939Trp could be disrupters of the interaction among *BRCA2*, *RAD51* and *POI* η , as well as the *PALB2* WD40 domain [17].

Specifically, Park et al. [20] characterized the effects of the p.Leu939Trp missense mutant of the *PALB2* WD40 domain, concluding that it partially disrupts the *PALB2*-*RAD51C*-*BRCA2* complex in cells. Functionally, this mutant displayed a decreased

capacity for DNA double-strand break-induced HR and an increased cellular sensitivity to ionizing radiation. In contrast, another functional assay [21] concluded that this mutation does not disrupt the HR-mediated DNA repair activity of *PALB2*.

As a consequence of the c.1857delT mutation, a shorter *PALB2* protein is expected, lacking of the MGR15 and C-terminal domain with the WD40 repeat-like segments. The WD40 motifs are necessary for BRCA2/*PALB2* complex formation while MGR15 domain is necessary for the *PALB2* interaction with the histone binding protein MGR15. MGR15 plays a key role in tethering *PALB2* to active genes. Thus, a failure of MGR15/*PALB2* interaction leads to genome instability [22].

All these inquiries could suggest a functional consequence, based on the importance of the *PALB2* interaction with the other proteins of DNA repair machinery. In fact, alterations of the *PALB2* and BRCA2 interaction result in severe homologous recombination repair defects [17].

Large deletions or duplications were not found in *PALB2*, concurring with other similar studies. Hence, it seems that genomic rearrangements in *PALB2* rarely contribute to the HBOC predisposition in our population.

Genetic counselling is greatly desired in unexplained HBOC families; consequently, we attempted to study the four mutations predicted by HSF as splicing disrupters at transcriptional level. This putative effect on the splicing mechanism is tested here for the first time. None of the four variants (c.1010T > C, c.1572A > G, c.1676A > G and c.1684 + 39_1684 + 41del) causes aberrant splicing.

Mutations in *PALB2* cause a defective DNA repair which could be exploited as a treatment opportunity. The alteration of *PALB2* functional domains could impair the coordination with BRCA5 resulting in a hypersensitivity to DNA interstrand crosslinking agents [23]. Defects in DNA repair caused by *PALB2* mutations could determine synthetic lethal interactions with PARP and CHK1 inhibitors. Smith et al. have been described in vivo hypersensitivity to BMN 673, a potent inhibitor of PARP [23]. Likewise, Chen et al., suggested CHK1 inhibition as strategy for targeting Fanconi Anemia deficient tumours [24]. This experimental evidence would support the use of PARP and CHK1 inhibitors as a targeted therapy, alone or in combination with DNA crosslinking agents.

In this study, the two affected women who carry the truncating *PALB2* mutation, showed sensitivity to carboplatin (a DNA cross-linking agent) without any relapse to date. This response would be consistent with the hypothesis that germline mutations in genes involved in homologous recombination, including *PALB2*, could indicate a response to DNA crosslinking agents [2]. Therefore, we are dealing to an actionable mutation, which allows a better clinical management of this family, enabling future preventive measures especially in the underage twin daughters.

In accordance with other authors [8,15,25], our results endorse the clinical testing of *PALB2* in patients with HBOC without BRCA mutations. Larger case-control studies are required to evaluate the implication of *PALB2* mutations in Breast and Ovarian Cancer predisposition as well as to detect recurrent mutations in the Spanish population. Furthermore, the functional characterization of the variant spectrum is needed to unveil a possible defective DNA repair and subsequently the role of *PALB2* in genesis and cancer treatment.

Conclusions

Our study confirms that *PALB2* mutations are present in our population at around 1% of BRCA negative HBOC cases. The cosegregation of c.1857delT supports this mutation as the cause of the cancer cases in this family.

PALB2 screening should be encouraged in women from HBOC families to implement genetic counselling in the case of a strong family cancer history and possible selection to receive PARP inhibitors. In conclusion, identifying carriers allows us to establish a familial management plan and follow-up decision-making.

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Conflicts of interest

The authors declare no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.breast.2018.11.010>.

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