Analysis of PALB2 Gene in BRCA1/BRCA2 Negative Spanish Hereditary Breast/Ovarian Cancer Families with Pancreatic Cancer Cases

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Abstract

Background: The PALB2 gene, also known as FANCN, forms a bond and co-localizes with BRCA2 in DNA repair. Germline mutations in PALB2 have been identified in approximately 1% of familial breast cancer and 3–4% of familial pancreatic cancer. The goal of this study was to determine the prevalence of PALB2 mutations in a population of BRCA1/BRCA2 negative breast cancer patients selected from either a personal or family history of pancreatic cancer.

Methods: 132 non-BRCA1/BRCA2 breast/ovarian cancer families with at least one pancreatic cancer case were included in the study. PALB2 mutational analysis was performed by direct sequencing of all coding exons and intron/exon boundaries, as well as multiplex ligation-dependent probe amplification.

Results: Two PALB2 truncating mutations, the c.1653T>A (p.Tyr551Stop) previously reported, and c.3362del (p.Gly1121ValfsX3) which is a novel frameshift mutation, were identified. Moreover, several PALB2 variants were detected; some of them were predicted as pathological by bioinformatic analysis. Considering truncating mutations, the prevalence rate of our population of BRCA1/2-negative breast cancer patients with pancreatic cancer is 1.5%.

Conclusions: The prevalence rate of PALB2 mutations in non-BRCA1/BRCA2 breast/ovarian cancer families, selected from either a personal or family pancreatic cancer history, is similar to that previously described for unselected breast/ovarian cancer families. Future research directed towards identifying other gene(s) involved in the development of breast/pancreatic cancer families is required.
Introduction

Hereditary breast cancer accounts for approximately 5–10% of all breast cancer cases. Mutations in the two main susceptibility genes, BRCA1 and BRCA2, together with mutations in a number of other high-penetrance genes such as TP53 and PTEN, account for 20% of familial breast cancer cases [1–3]. For the remaining 80%, the genetic factors are largely unknown and they are likely to involve mutations in moderate and low penetrance susceptibility genes, plausibly acting together with some environmental or other hereditary factors. Apart from breast and ovarian cancer, BRCA1 and BRCA2 carriers might be at higher risk for additional malignancies such as prostate, colorectal, familial melanoma and pancreatic cancers.

Pancreatic cancers are the fourth most common cause of cancer-related deaths in the Western world. Approximately 5% to 10% of individuals with pancreatic cancer report a history of pancreatic cancer in a close family member. In addition to this, several known genetic syndromes have been shown to be associated with an increased risk of pancreatic cancer. Thus, germline mutations in the BRCA2, p16/CDKN2A, STK11, and PRSS1, that are responsible for familial breast cancer, Familial atypical multiple melanoma, Peutz-Jeghers and Familial pancreatic, respectively, have been clearly associated with an increase risk of pancreatic cancer [4–7]. Additionally, some studies have described pancreatic cancer developing among individuals with HNPCC [8,9].

The PALB2 (partner and localizer of BRCA2) gene was identified by searching for novel components of endogenous BRCA2-containing complexes [10]. PALB2 supports BRCA2 stability and determines its localization in the nucleus after DNA damage [10]. Relocation of PALB2 and BRCA2 to damaged chromatin is regulated by BRCA1. These three proteins form a complex in which PALB2 acts as a bridge between BRCA1 and BRCA2 [11]. This complex is critical for the initiation of homologous recombination in the DNA-damage response [11,12]. In cells depleted of PALB2 the DNA repair pathway dependent on the BRCA1/2 is disrupted [10,11]. Immediately after PALB2 was discovered, evidence showed that it was also a Fanconi anemia gene, known as FANCN [13,14]. Biallelic inactivation mutations in PALB2/FANCN cause Fanconi anemia subtype N, characterized by a severe predisposition to pediatric malignancies such as Wilms tumor, medulloblastoma, AML and neuroblastoma [14]. Interestingly, the gene underlying the D1 subtype of Fanconi anemia, FANCD1 [15], was found to be BRCA2 and biallelic mutations in BRCA2/FANCD1 originate a phenotype with high risk of childhood malignancies, very similar to that produced by PALB2/FANCN biallelic mutations. This supports the proposal that PALB2 is important for BRCA2 tumor suppression activity [16].

As in other Fanconi anemia genes, monoallelic mutations in PALB2 have been associated with increased breast cancer risk [3]. Thus, PALB2 monoallelic mutations have been identified in approximately 1% of hereditary breast cancer families globally, as summarized by Tischkowitz and Xia [16]. It has recently become clear that the PALB2 gene should not only be considered as a susceptibility gene for breast cancer but also for pancreatic cancer. This pancreatic association was based on the identification of a PALB2 mutation by exomic sequencing and the subsequent PALB2 analysis in additional familial pancreatic cancer patients that revealed a prevalence of 3.1% [17]. A similar prevalence (3.7%) was found by Slater et al [18] in European patients with familial pancreatic cancer, whereby PALB2 carriers also had a history of breast cancer.

Given these findings, we aimed to determine the prevalence of PALB2 mutations in a Spanish population of BRCA1/BRCA2-negative breast/ovarian cancer families with either a personal or family history of pancreatic carcinoma.

Materials and Methods

Patients

Index cases from 132 BRCA1/BRCA2 mutation-negative unrelated Spanish breast/ovarian cancer families with a personal history of both breast and pancreatic cancer, or a family history with pancreatic cancer cases, were screened for mutations within the entire coding sequence and splicing sites as well as large genomic rearrangements of PALB2 gene. Patient and family characteristics are summarised in Table 1. Families were enrolled from 11 different Spanish centres (Table S1). Ethical committee approval and informed consent for all participants in the study were obtained.

All index cases had been previously screened for point mutations and large rearrangements in BRCA1 and BRCA2 genes. All were found to be negative.

Mutation analysis of the PALB2 gene

Mutational analysis of PALB2 gene included the complete coding sequencing and flanking intron-exon boundaries along with the analysis of genomic rearrangements, as previously described by Blanco et al [19].

Nomenclature and databases

Sequences used for PALB2 nomenclature were obtained from the NCB1 RefSeq database (NG_007406.1 for genomic, NM_024675.3 for mRNA and NP_078951.2 for protein) (http://www.ncbi.nlm.nih.gov). Standardized nomenclature was reported considering the A of the ATG initiation codon of the coding DNA Reference Sequence as nucleotide position +1.

Potential consequences of missense substitutions were obtained using the prediction software PolyPhen-2 (Polymorphism Phenotyping-2, see http://genetics.bwh.harvard.edu/pph2/), SIFT (Sorting Intolerant From Tolerant, see http://sift.jcvi.org/) and Align-GVGD (Granham score difference, see http://aggd.iarc.fr/) tools. Native alignments of each algorithm were used (see Text S1 for a brief description of the tools).

CEU-population data from 1000 Genomes Project database (http://www.1000genomes.org/) was used to obtain allelic frequency of the identified variants in our samples.

Results

We sequenced all exons and splicing boundaries of PALB2 gene. We also carried out MLPA analysis in 132 index cases from BRCA1/BRCA2-negative families with breast and/or ovarian cancer with either a personal or familial history of pancreatic cancer. Two mutations were identified by sequencing analysis, the
A (p.Tyr551Stop) located at exon 4 with the result of a premature stop codon, and the frameshift c.3362del (p.Gly1121ValfsX3) in exon 13, which is predicted to generate a translation-stop three codons downstream from the first affected amino acid. The PALB2 truncating mutation c.1653T>A (p.Tyr551Stop) was identified in a woman diagnosed with an infiltrating ductal carcinoma (IDC), negative for estrogen receptor (ER), progesterone receptor (PR) and HER2 at the age of 36. Her mother had been diagnosed with breast cancer at 48 and with pancreatic cancer at 72 years of age. Her maternal uncle had been diagnosed with pancreatic cancer at 50 years of age (Figure 1A). Unfortunately, it was not possible to obtain samples from family members to confirm the mutation in the paternal branch of the proband. The frameshift mutation in exon 13, c.3362del produces a stop codon in position 1123 (p.Gly1121ValfsX3) that would cause the loss of the 63 amino acids from the N-terminal PALB2 region. Other truncated mutations in the last codons of the gene have already been described in breast/pancreatic cancer families [3], [20]. It has been shown that residues 836 to 1186 of the PALB2 protein are part of the WD40 repeats C-terminal domain which associates with the N-terminus of BRCA2 [12], [13].

Table 1. **BRCA1/BRCA2** mutation-negative Spanish high risk breast/ovarian cancer families with pancreatic cancer cases.

| Type of case | N' of cases (n = 132) | Mean age at cancer diagnosis | Additional family history |
|--------------|-----------------------|----------------------------|--------------------------|
| Personal history of BC and PC | 3 | BC:43.6 | PC in FDR0 |
| Personal history of PC and familiar history of BC and PC | 4 | PC:65 | PC in FDR1 |
| Personal history of OC and familiar history of PC | 9 (1BiOC) | OC:43.6 | PC in FDR3 |
| Personal history of PrC and familiar history of BC and PC | 2 | PrC:54.5 | PC in FDR1 |
| Personal history of BC and familiar history of PC | BC diagnosed <50 | BC diagnosed >50 | |
| | | 92 (13BiBC (1+leukemia, 1+melanoma; 1+OC), 1 MBC, 1+CCR) | |
| | | BC:39.2 | PC in FDR25 (1+BC) |
| | | PC in FDR58.8 | PC in SDR50 |
| | | PC in SDR: 63.8 | BC in FDR: 31 (2 BiBC) |
| | | BC in SDR: 26 (1 BiBC, 1 MBC) | OC in FDR: 1 |
| | | BC in SDR: 77 | BC in FDR: 2 |
| | | BC in SDR: 2 (1 BiBC) | OC in SDR: 1 |
| | | BC diagnosed >50 | |
| | | 22 (4 BiBC, 1MBC, 1+OC, 1+endometrium)BC:58.6 | |
| | | BC:58.6 | PC in FDR: 9 (1+BC) |
| | | PC in FDR: 64.1 | BC in FDR: 12 (1+BC) |
| | | PC in SDR: 65.7 | BC in FDR: 15 (1 BiBC, 1 MBC, 1+PC) |
| | | BC in SDR: 15 | OC in FDR: 1 |
| | | OC in SDR: 1 | OC in SDR: 1 |

**BC**: Breast cancer; **PC**: Pancreatic cancer; **OC**: Ovarian cancer; **BiOC**: Bilateral Ovarian cancer; **PrC**: Prostate cancer; **MBC**: Male Breast cancer; **BiBC**: Bilateral Breast Cancer; **CCR**: Colorectal cancer; **FDR**: First degree relative; **SDR**: Second degree relative.

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cancer present in the family were skin, stomach and CNS in paternal aunts (Figure 1B).

In addition to these two mutations, sequence analysis revealed another 21 different \textit{PALB2} variants and polymorphisms (Table 2), one in the 5'UTR region, 4 in introns and 16 in exons (12 missense and 4 silent coding variants). From the total number of variants identified, seven were novel (c.110G>A (p.Arg37His), c.212-180T>G, c.232G>A (p.Val78Ile), c.262C>T (p.Leu88Phe), c.1431T>C (p.Glu478Lys), c.2587-59T>C, c.2837C>G (p.Ala946Gly)) and observed only once in our samples, whereas the other variants had been previously reported \cite{3}, \cite{19}, \cite{21}, \cite{22}.

The results of bioinformatic predictions for intronic and missense variants are represented in Table 3. Four of the missense variants, c.1010T>C (p.Leu337Ser), c.2816T>G (p.Leu939Trp), c.2837C>G (p.Ala946Gly) and c.2993G>A (p.Gly998Gln), were predicted to likely affect \textit{PALB2} protein function by all the tested algorithms. Variants c.2816T>G (p.Leu939Trp) and c.2837C>G (p.Ala946Gly) are not present in CEU 1000 Genome Project samples, whereas 1010T>C (p.Leu337Ser) and 2993G>A (p.Gly998Gln), have a 2% frequency in CEU population (Table 2). For variant c.2816T>G (p.Leu939Trp), a similar frequency in controls and cases was observed \cite{22}–\cite{24}. For the missense variant c.110 G>A (p.Arg37His) two of the three prediction programs considered the variant as deleterious, whereas only one prediction tool considered variants c.2014 G>C (p.Glu672Gln), c.2590 C>T (p.Pro864Ser) and 2794 G>A (p.Val932Met) as deleterious (see Table 3). The bioinformatic splicing analyses showed consensus site score variations for variants c.110 G>A (p.Arg37His) (three programs), c.2837C>G (p.Ala946Gly) (two programs) and c.2993G>A (p.Gly998Gln) (one program). A destruction of a cryptic splice site was predicted for variants c.47G>A and c.2794G>A (p.Val932Met) by three and two programs, respectively, as well as an increase in the score of a cryptic sites by all programs for the variant c.2794G>A (p.Val932Met) and by two programs for the variant c.2590C>T (p.Pro864Ser).

\section*{Discussion}

Mutations in \textit{PALB2} gene were originally associated with an increased risk for breast cancer and later, with pancreatic cancer. We analyzed a large series of hereditary breast/pancreatic cancer families analysed for \textit{PALB2} mutations. We identified two germline truncating mutations, the nonsense c.1653T>A (p.Tyr551Stop) located at exon 4 and the novel frameshift mutation c.3362del (p.Glu1121ValfsX3) in exon 13. These mutations were considered to be pathogenic, since they all create a stop codon that is predicted to cause a truncation of the \textit{PALB2} protein. The nonsense mutation c.1653T>A (p.Tyr551Stop) had been previously reported in a Fanconi anemia patient as well as in a familial breast cancer case \cite{13}, \cite{25}. Truncating mutations in \textit{PALB2} are rare in individuals without cancer. In fact, they had not been identified in 1084 healthy individuals analysed \cite{3}. In our series, c.1653T>A (p.Tyr551Stop) and c.3362del (p.Glu1121ValfsX3) were identified in index cases diagnosed with breast cancer under 50 years of age and at least one first degree relative diagnosed with pancreatic cancer. No ovarian cancer was present in these families. Considering these two \textit{PALB2} variants as causal mutations, the prevalence of \textit{PALB2} mutation in our BRCA1/BRCA2 breast and pancreatic cancer series is 1.5% (2/132). Previous studies of
breast/pancreatic cancer families have described prevalences from 0% (77 families analysed in Studler et al [26], 45 in Adank et al [27], 29 in Guirroz et al [28] and 28 in Harinck et al [29]), 2.1% (Hofstatter et al [24], 2 mutations in 94 families), to 4.8% (Peterlongo et al [20], 3 mutations in 62 families) reviewed in Table 4. Considering these studies with our data, the global prevalence of PALB2 mutation in breast/pancreatic cancer families is 1.5% (7 mutations in 467 families).

We recently, estimated a PALB2 mutation prevalence of 0.75% for Spanish breast/ovarian cancer families with at least one male breast cancer case [19]. Although we identified twice as many carriers in families with pancreatic cancer cases than in families with male breast cancer cases, both prevalences are similar to the 1% reported for families with breast cancer unselected for other cancers [3], [30], [31]. Importantly, for most of the breast/pancreatic cancer series analysed, index cases were breast cancers. Similarly, in our study only 7 from the 132 index cases were pancreatic cancer. The selection of index cases could therefore be introducing a bias in the estimate of the prevalence of PALB2 mutations in these families.

The selection of a gene to be included in a routine genetic test would be, in part, based on the risk it confers. However, the risk associated with deleterious mutations in genes like PALB2 is not easily determined since deleterious alleles are extremely rare in the population, and the number of mutation carriers in published studies are small [32]. Mutations in PALB2 gene were originally associated with a moderate (2-3 fold) increase risk for breast cancer [3]. As higher risks were increasingly suggested, at least for specific mutations [33], [34], the inclusion of this gene in hereditary breast cancer tests would be justified.

In our study both breast tumors from our PALB2 mutant breast cancer patients displayed a more aggressive tumor phenotype, including triple-negative disease, higher tumor grade (c.3362del, p.Gly1121ValfsX3). It has been shown that some PALB2-associated breast cancers display a more aggressive tumor phenotype, including triple-negative disease, higher tumor grade and higher Ki67 expression [35]. Thus, tumors of the 1592delT PALB2 mutation carriers presented triple negative phenotype more often (54.5%, P<0.0001) than those of other familial (12.2%) or sporadic (9.4%) breast cancer patients [35]. In fact, nearly 40% of the PALB2-associated breast tumors identified to date displayed a triple-negative phenotype, regardless of the specific PALB2 mutation [16]. This representation of triple negative tumors, more akin to BRCA1- than BRCA2-related tumors, could be related to

| NUCLEOTIDE CHANGE* | PROTEIN CHANGE | Name of SNP | AA | AB | BB | A (A%) | B (B%) | A% | B% |
|---------------------|----------------|-------------|----|----|----|--------|--------|----|----|
| S’ upstream sequence | c.-47G->A | - | rs8053188 | 127 | 5 | 0 | 259(98.1) | 5(1.9) | 98 | 2 |
| EXON 3 | c.110G->A | p.Arg37His | rs202194596 | 131 | 1 | 0 | 263(99.6) | 1(0.4) | 100 | 0 |
| INTRON 3 | c.212-58A->C | - | rs80291632 | 123 | 9 | 0 | 255 (96.6) | 9(3.4) | 96 | 4 |
| EXON 4 | c.232G->A | p.Val78Ile | - | 131 | 1 | 0 | 263(99.6) | 1(0.4) | 100 | 0 |
| EXON 5 | c.2587-59T | - | rs4554034 | 131 | 1 | 0 | 263(99.6) | 1(0.4) | 98 | 2 |
| INTRON 6 | c.1194G->A | p.Asp219Gly | - | 131 | 1 | 0 | 263(99.6) | 1(0.4) | 100 | 0 |
| EXON 7 | c.1653T->A | p.Tyr551Stop | rs118203997 | 131 | 1 | 0 | 263(99.6) | 1(0.4) | 100 | 0 |
| EXON 8 | c.2590C->T | p.Leu88Phe | - | 131 | 1 | 0 | 263(99.6) | 1(0.4) | 98 | 2 |
| EXON 9 | c.212-180T->C | - | rs80291632 | 123 | 9 | 0 | 255 (96.6) | 9(3.4) | 96 | 4 |
| EXON 10 | c.2587-59T | - | rs4554034 | 131 | 1 | 0 | 263(99.6) | 1(0.4) | 100 | 0 |
| EXON 11 | c.2587-59T | - | rs4554034 | 131 | 1 | 0 | 263(99.6) | 1(0.4) | 100 | 0 |
| EXON 12 | c.2587-59T | - | rs4554034 | 131 | 1 | 0 | 263(99.6) | 1(0.4) | 100 | 0 |
| EXON 13 | c.2587-59T | - | rs4554034 | 131 | 1 | 0 | 263(99.6) | 1(0.4) | 100 | 0 |

*Allelic frequency is the percentage of n/N, where n is the number of minor alleles and N is the total number of alleles.

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Table 3. Results of bioinformatic analysis for PALB2 variants.

| Location, SS, Distance | Splice Signal Detection | Proximal Cryptic/De novo (%) Variation |
|------------------------|-------------------------|---------------------------------------|
| Location, SS, Distance | Splice Signal Detection | Proximal Cryptic/De novo (%) Variation |
| Location, SS, Distance | Splice Signal Detection | Proximal Cryptic/De novo (%) Variation |
| Location, SS, Distance | Splice Signal Detection | ProximalCryptic/De novo (%) Variation |
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| Location, SS, Distance | Splice Signal Detection | Proximal Cryptic/De novo (%) Variation |
| Location, SS, Distance | Splice Signal Detection | Proximal Cryptic/De novo (%) Variation |
| Location, SS, Distance | Splice Signal Detection | Proximal Cryptic/De novo (%) Variation |

The table reports score modifications due to the detected variants in PALB2 (for greater clarity, when the variants didn’t change the score, the corresponding tool is not indicated). Proximal cryptic sites are indicated with the corresponding tool, when the variants led to de novo site it is also indicated. N/A = not applicable as the change is synonymous or intronic. Location indicates exon/intron, SS stands for splice site and distance to the nearest splice site is indicated in base pairs.

aCpDNA analysis was performed. No additional products in the carrier sample compared to control samples has been identified (data not shown).

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the nature of the interaction and/or certain functional similarities between PALB2 and BRCA1 [11], [12], or a direct transcriptional activation of the estrogen receptor by PALB2 as has been shown for PALB2 [11], [12], or a direct transcriptional activation of the estrogen receptor by PALB2 as has been shown for BRCA1 [36]. However, larger numbers of PALB2-related tumors will need to be studied before any firm conclusions can be drawn.

As shown in Table 3, different prediction tools gave contradictory results. For instance, missense variant c.656 A>G (p.Asp219Gly) and c.2590 C:T (p.Pro864Ser) were predicted as C65 likely to be pathogenic) by A-GVGD and as Benign and Tolerated by Polyphen and Sift. Prediction of individual variants can be sensitive to the alignment used; both the number and type of orthologues aligned at the mutation site can affect prediction of pathogenicity [37]. Since we used default alignments for each tool, we cannot rule out that the different outcomes we have for these variants is related to this. However, an optimum alignment is difficult to identify and is likely to vary depending on the variant tested [37]. It has been previously shown that the accuracy of the result improves when multiple algorithms give the same prediction [38]. Considering this as well as such consensus predictions for all consensus sites described by Cartegni et al [40], 11 bases for the 5’ site (from the 3 last exonic to the 8 first intronic bases) and 14 bases for the 3’ site (from the 12 last intronic to the first 2 exonic bases), would have reliable predictions with these bioinformatic tools [39]. In our study, this would mean that the unique reliable prediction is for variant c.110 G>A (p.Arg37His), at the second exonic base from the 3’ site. However, the reduction in the score predicted by the algorithms, all lower than 10%, and the absence of variations near cryptic splice sites would suggest that c.110 G>A (p.Arg37His) is not a variant producing a major impact in splicing process. The RNA analysis of the variant confirmed this prediction (Table 3).

### Conclusions

In summary, we found that PALB2 mutations occur with a prevalence of 1.5% in a population of BRCA1/2-negative breast cancer patients specifically selected from a personal and/or familiar history of pancreatic cancer. This is not much different from the prevalence described for families not selected for the presence of pancreatic cancer. However, we cannot rule out a higher prevalence of 1.5% in a population of BRCA1/2-negative breast cancer patients specifically selected from a personal and/or familiar history of pancreatic cancer. However, we cannot rule out a higher prevalence of pancreatic cancer index cases.

PALB2 mutations seem to explain only a small fraction of the clustering of both pancreatic and breast cancer. It is therefore, crucial that future research aims to identify other gene(s) that are involved in the development of familial breast/pancreatic cancer cases.

### Supporting Information

**Table S1** Participating centers and families from Spain. (DOCX)

**Text S1** This document summarizes the meanings of scores of bioinformatic programs used. (DOCX)
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Author Contributions

Conceived and designed the experiments: AB AV. Performed the experiments: AB AV. Analyzed the data: AB AV. Contributed reagents/materials/analysis tools: MH AO OD MDM MI CMB AT AL GL J. Brunet BG MPS MJG SGE AC MIT EAV MTC J. Balka J. Benitez TC. Wrote the paper: AB AV.

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