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PRECLINICAL STUDY

Mutation analysis of *PALB2* gene in French breast cancer families

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Abstract Several population-based and family-based studies have demonstrated that germline mutations of the *PALB2* gene (Partner and Localizer of BRCA2) are associated with an increased risk of breast cancer. Distinct mutation frequencies and spectrums have been described depending on the population studied. Here we describe the first complete *PALB2* coding sequence screening in the French population. We screened the complete coding sequence and intron–exon boundaries of *PALB2,* using the EMMA technique, to assess the contribution of pathogenic mutations in a set of 835 familial breast cancer cases and 662 unrelated controls from the French national study GENESIS and the Paul Strauss Cancer Centre, all previously tested negative for *BRCA1* and *BRCA2* pathogenic mutations. Our analysis revealed the presence of four novel deleterious mutations: c.1186insT, c.1857delT and c.2850delC in three cases, c.3418dupT in one control. In addition, we identified two in-frame insertion/deletion, 19 missense substitutions (two of them predicted as pathogenic), 9 synonymous variants, 28 variants located in introns and 2 in UTRs, as well as frequent variants. Truncating *PALB2* mutations were found in 0.36 % of

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familial breast cancer cases, a frequency lower than the one detected in comparable studies in other populations (0.73–3.40 %). This suggests a small but significant contribution of *PALB2* mutations to the breast cancer susceptibility in the French population.

Keywords PALB2 · Familial breast cancer · Germline mutations · Genetic testing

Introduction

Breast cancer molecular diagnostic tests include in a growing number of countries the mutational analysis of *PALB2* (Partner and Localizer of BRCA2), in addition to *BRCA1* and *BRCA2*. Nevertheless, the debate about the use of *PALB2* mutation status as a pre-symptomatic biomarker in genetic counselling is still open in several countries [1]. Interestingly, the frequency of *PALB2* deleterious mutations appears to be variable among different populations. Thus, the precise knowledge of mutations spectrum and frequencies in each specific population is important in order to improve the molecular analysis strategy and genetic counselling.

PALB2 is one of the additional cancer predisposition genes for which germline loss-of-function mutations have been the most frequently identified, although some studies failed to identify *PALB2* mutations in breast cancer series from specific populations [2–4]. The first *PALB2* family-based association study, conducted in the UK, estimated the relative risk conferred by *PALB2* mutations at 2.3 [CI 95 %: 1.4–3.9] [5]. Nevertheless, subsequent population-based studies estimated the risk associated with at least some *PALB2* mutations as higher [6, 7]. Two recent analyses conducted on large series of *PALB2* mutations carriers showed that *PALB2* mutations confer to their carriers a risk overlapping with that estimated for *BRCA2* mutation carriers, supporting the classification of *PALB2* as a high-risk breast cancer gene [8]. Knowing the contribution of *PALB2* mutations to familial breast cancer in specific populations is warranted to be able to implement *PALB2* analysis in genetic testing, especially as the frequency of *PALB2* mutations vary depending on the population [5, 9–15]. Several rare recurrent mutations have been described in Australia [7], the North of Italy [16], China [17], Poland [18], Quebec [19] and Finland [6]. Nevertheless, no data are available from French population.

In the present study, we screened for germline *PALB2* mutations 835 breast cancer patients from breast/ovarian cancer families and 662 unrelated controls recruited in the French national study GENESIS and in the oncogenetic clinic of the Paul Strauss Cancer Centre of Strasbourg (CPS series).

Methods

Study subjects

The study was conducted on a subgroup of subjects from the GENESIS (GEne SISters) French national study (Sinilnikova et al. submitted) and a series of patients selected at the oncogenetic clinics of the Paul Strauss Cancer Centre in Strasbourg (CPS). GENESIS index cases are women diagnosed with infiltrating mammary adenocarcinoma with a family history of breast cancer, having at least one breast cancer-affected sister. The recruitment was done from April 2007 to December 2013 through the French national network of cancer genetics clinics (Groupe "Génétique et Cancer" (GGC)) covering the entire national territory. The controls are unaffected friends or colleagues of index cases matched by age (±3 years). Other family members were included in the study if they consented to participate. Information about ethnic origin is self-reported by study subjects. In the present study we analysed the first 641 index cases and 592 controls included for which blood samples were available.

The CPS series consist of a total of 194 patients selected from the routine clinical testing, on basis of familial breast and/or ovarian cancers. For four of them, the index case was a breast cancer-affected male. 70 unrelated anonymous controls were evaluated.

All index cases analysed were tested negative for *BRCA1/2* point mutations and large rearrangements.

The characteristics of study subjects are presented in Table 1.

Ethics statement

All participants gave written informed consent. The GENESIS study protocol was approved by the appropriate ethics committee (CCP Ile-de-France III) and by the French data protection authority (CNIL).

DNA extraction

For GENESIS, genomic DNA was extracted from blood samples using the DNA extractor Autopure-LS (Qiagen), and DNA handling (normalization and aliquoting) was done using a TECAN EVO instrument.

For CPS, genomic DNA was isolated from 3 ml of blood using the manual extraction method Flexigene (Qiagen).

PALB2 mutation screening

The 13 coding exons of *PALB2* (NCBI reference sequence NM_024675.3) were screened using the EMMA technique

Table 1 Characteristics and distribution of study participants

(a) GENESIS study

	Index cases	Controls
Age at diagnosis/ascertainment		
≤40	106 (16.5 %)	35 (5.9 %)
41–50	262 (40.9 %)	148 (25 %)
51–60	185 (28.9 %)	223 (37.7 %)
61–70	79 (12.3 %)	152 (25.7 %)
≥71	9 (1.4 %)	34 (5.7 %)
Total	641 (100.0 %)	592 (100.0 %)
Ethnicity		
Caucasians	619 (97 %)	580 (98 %)
Non-caucasians	16 (2 %)	7 (1 %)
Non reported	6 (1 %)	5 (1 %)
Total	641 (100 %)	592 (100 %)

(b) CPS series

Age at diagnosis/ascertainment	Index cases
≤40	56 (28.9 %)
41–50	59 (30.4 %)
51–60	44 (22.7 %)
61–70	19 (9.8 %)
≥71	16 (8.2 %)
Total	194 (100 %)

Type of family	Total	Mean age at diagnosis
Female breast cancer only	117	47 (32–73)
Breast and ovarian cancers	55	51 (28–74)
Male and female breast cancers	22	58 (33–83)
Total	194	

(c) Breast and ovarian cancers families

Cancer/family	Number of families (%)	
	GENESIS	CPS
Breast		
1	–	8 (4.12)
2	165 (25.74)	36 (18.56)
3	276 (43.06)	52 (26.80)
4	131 (20.44)	56 (28.87)
5	51 (7.96)	28 (14.43)
6	14 (2.18)	11 (5.67)
7	2 (0.31)	2 (1.03)
8	1 (0.16)	1 (0.52)
9	1 (0.16)	–
Total number of BC	2052	688
Mean number of BC/family	3.20	3.54
Ovary		
0	609 (95.01)	136 (70.01)
1	28 (4.37)	46 (24.23)
2	4 (0.62)	9 (4.64)
Total number of OC	36	64
Mean number of OC/family	0.06	0.33

(Enhanced Mismatch Mutation Analysis, Fluigent), based on heteroduplex analysis by capillary electrophoresis in a specific high-resolution polymer [20, 21] (see Supplementary methods for details).

In silico analyses

The in silico analyses of *PALB2* missense variants were performed using the freely available web-based programs Sorting Intolerant From Tolerant (SIFT) [22, 23], Align Grantham Variation Grantham Deviation (Align-GVGD) [24, 25] and Polymorphism Phenotyping version 2 (PolyPhen2), HumDiv-trained model [26, 27]. The protein multiple sequence alignment (PMSA) that we used for SIFT and Align-GVGD is a manually curated alignment using 10 species in which the most divergent sequence is that of the fish *Danio rerio*. This alignment is available at the Align-GVGD website (http://agvgd.iarc.fr/alignments.php).

All variants with a Minor Allele Frequency (MAF) <1 % have been tested for their potential effect on splicing using five different splicing variant predictors included in the AlaMut program (Alamut, Interactive Biosoftware, Rouen, France): SSF [28], Max-EntScan (MES) [29], Splice site predictor by neural network (NNSPLICE) [30], GeneSplicer [31] and Human Splicing Finder (HSF) [32]. A prediction was considered positive when the score of the new consensus site was at least 15 % lower than the wild-type score using MES or 5 % lower using SSF and when at least one of the other tools showed a reduction in the consensus score [33]. We considered the prediction to be indicative of the creation of a new splice site if its score is at least equal to 50 % of the wild-type score.

Results

Subjects included in the analysis

Of the 1275 GENESIS samples screened, 42 (23 cases and 19 controls) were excluded from the analysis because their amplicon failure rate was greater than 20 %. The distribution of the remaining 641 cases and 592 controls by age and self-reported ethnic origin is detailed in Table 1a. All

the CPS samples (194 index cases and 70 controls) were successfully screened. The characteristics of GENESIS and CPS families are detailed in Table 1a and b, respectively.

The cases studied belonged to breast cancer families, of which 75 % (74 % in GENESIS, 77 % in CPS) had at least 3 breast cancer cases (Table 1c). Families including women affected with ovarian cancer were more represented in CPS (55/194 = 28 %) versus GENESIS (32/641 = 5 %).

Mutations identified

Mutation screening of the *PALB2* coding region and flanking intronic boundaries performed in 1497 subjects (835 cases, 662 controls) identified four new deleterious germline truncating mutations: c.1186insT, c.1857delT and c.2850delC in three index cases and c.3418dupT in a control (Table 2; Figs. 1 and 2).

We also identified 32 different exonic rare variants, 12 of which were novel: two in-frame (one insertion and one deletion), 19 missense, 9 synonymous and the remaining two were single-base-pair substitutions in the 5′-UTR sequence (Table 3; Supplementary Table S1). All the 19 exonic missense variants with a reported MAF < 1 % were analysed for their effect on protein function using SIFT [23], Align-GVGD [25] and Polyphen-2 [27] programs (Table 3). Two missense variants (Fig. 1) were classified as potentially deleterious by the three bioinformatics tools: c.2816T > G and c.3128G > C. The pedigrees of the carriers are shown in Supplementary Fig. S1 and S2A, respectively. c.2816T > G, p.Leu939Trp, was detected in 5 index cases (MAF = 0.30 %) and 2 unaffected controls (MAF = 0.15 %). The c.3128G > C, p.Gly1043Ala variant, localized at a position well conserved in the WD repeat (see Discussion), was found in a 51-year-old healthy control with no history of breast cancer in the family. These two missense mutations have already been reported in cases and/or controls with a relatively similar frequency [2, 5, 10, 14, 16, 34–37]. One missense substitution, c.3410T > C, classified as potentially deleterious by two

Table 2 *PALB2* deleterious mutations identified

Type	Location	DNA change	Protein change	1000G/EVS European Americans MAF	Number of heterozygous carriers in index cases (MAF %)	Number of heterozygous carriers in controls (MAF %)
Frameshift	Exon 4	c.1186insT	p.Cys396Leufs*5	–	1 (0.06)	0 (0)
Frameshift	Exon 5	c.1857delT	p.Phe619Leufs*9	–	1 (0.06)	0 (0)
Frameshift	Exon 9	c.2850delC	p.Ser951Leufs*2	–	1 (0.06)	0 (0)
Frameshift	Exon 13	c.3418dupT	p.Trp1140Leufs*17	–	0 (0)	1 (0.08)

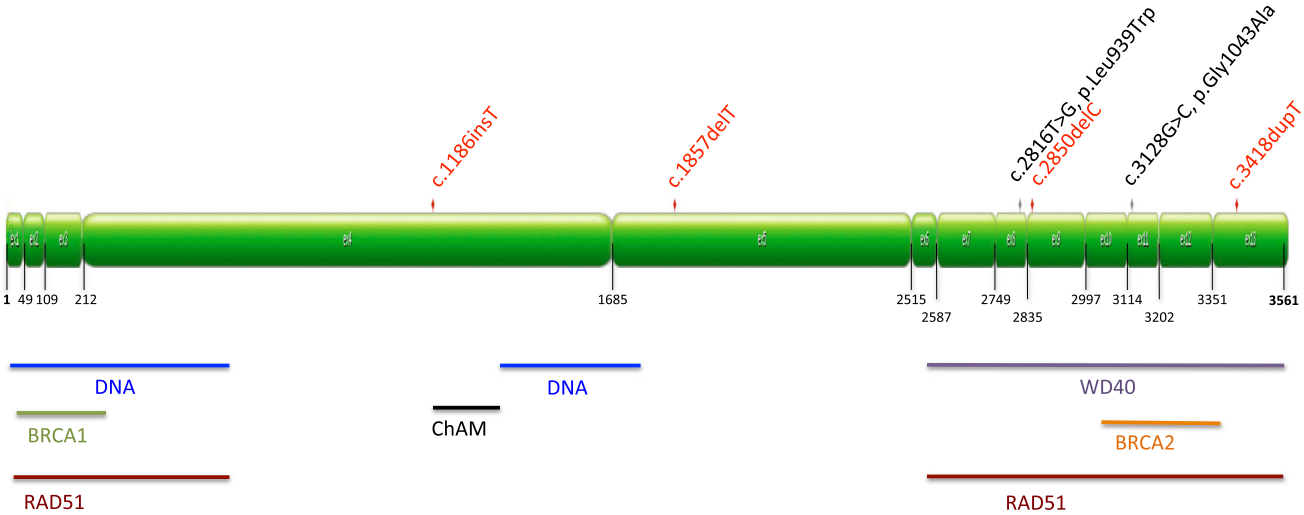

Fig. 1 *PALB2* truncating mutations and potentially deleterious missense variants. *Upper part* Schematic diagram of the *PALB2* coding sequence with the 4 truncating mutations (in *red*) and the 2 missense variants classified as deleterious (in *black*) with the in silico tools. Numbers correspond to nucleotides of the coding sequence. *Lower part* depiction of sequences coding for functional domains (ChAM (chromatin-association motif), WD40 domains) as well as for regions of interaction with DNA and the principal PALB2 protein partners as reported in Uniprot website (http://www.uniprot.org/uniprot/Q86YC2)

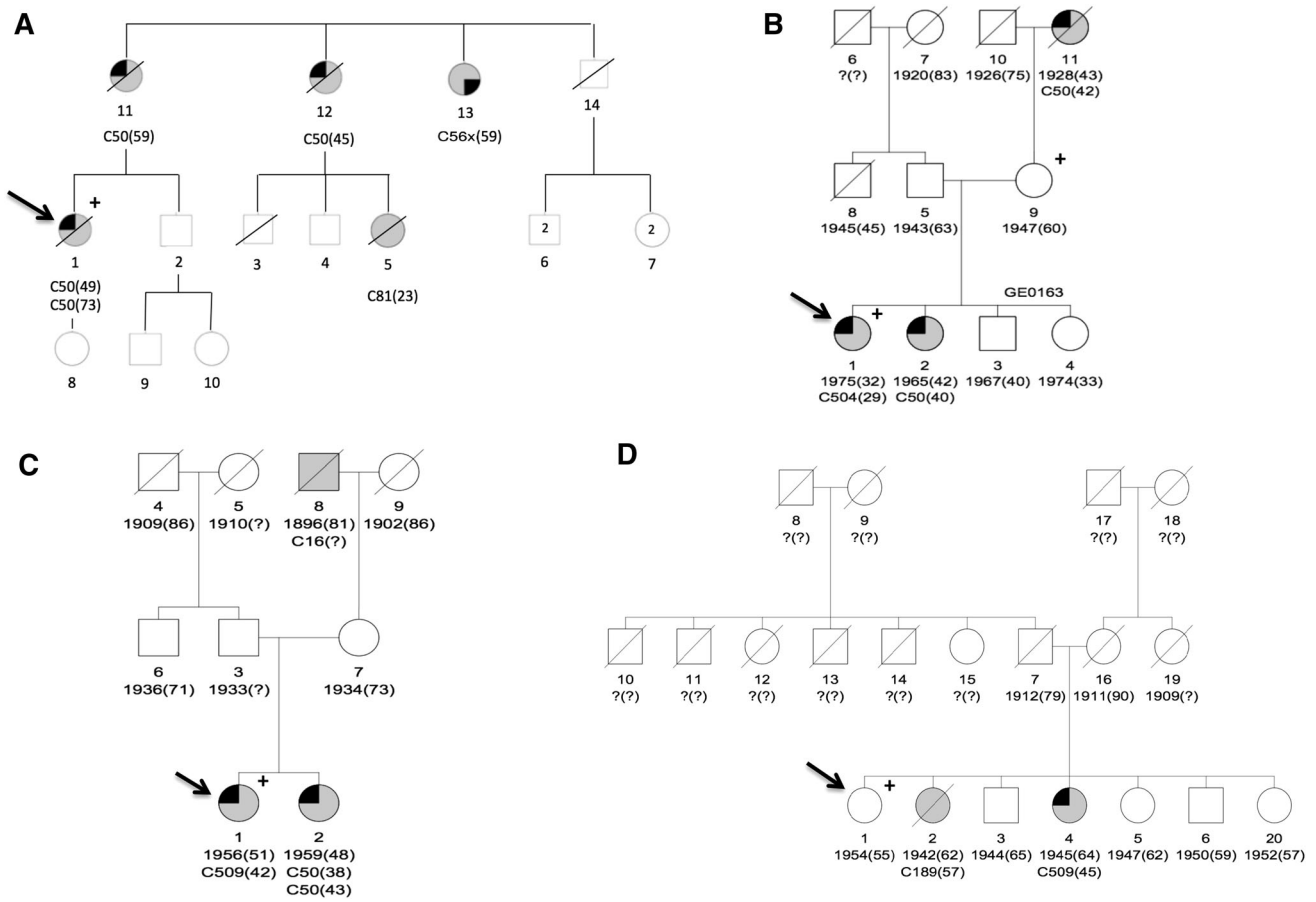

Fig. 2 Pedigrees of truncating mutation carriers. The pedigrees of the 4 truncating mutation carriers are shown. **A** c.1186insT, p.Cys396Leufs*5, **B** c.1857delT, p.Phe619Leufs*9, **C** c.2850delC, p.Ser951Leufs*2, **D** c.3418dupT, p.Trp1140Leufs*17. For each individual, year of birth and age at inclusion (in *brackets*) are indicated, if known. *Grey symbols* with an *upper black* corner on the *left* indicate breast cancer patients; for women *grey circles* with a *lower black* corner on the *right* indicate ovarian cancer patients, full *grey symbols* indicate patients with other cancer types. The plus signs indicate the status of the tested individuals. For each cancer patient, the type of cancer is indicated with the International Statistical Classification of Diseases and Related Health Problems (ICD) codes (*C50** breast cancer, *C16* stomach cancer, *C189* colon cancer, *C56* ovarian cancer, *C81* Hodgkin lymphoma). The age of diagnosis is specified in brackets. The *arrows* indicate the screened subjects (index cases or control)

tools, has been found in an index case and never reported (Supplementary Fig. S2B).

In addition, we detected 28 intronic different rare variants, 16 reported here for the first time (Supplementary Table S1): 6 were small insertions and/or deletions and 22 were single-base changes.

All the variants, except the truncating mutations, have been tested for their potential effect on splicing using five different splicing variant predictors: Splice Site Finder (SSF) [28], MES [29], NNSPLICE [30], GeneSplicer [31] and HSF [32] (Supplementary Table S2). None of them was predicted to be a *bona fide* splicing variant. The exonic variant, c.3350G > A changes the last base of exon 12, potentially weakening the canonical donor splicing site, but only SSF predicts that the substitution could affect splicing (reduction of wild-type score by 13 %).

Eleven well-known SNPs with a reported Minor Allele Frequency (MAF) >=1 % were also found (Supplementary Table S3).

Discussion

Biallelic mutations in *PALB2* (also called *FANCN*) cause Fanconi anaemia [37], a rare chromosome instability syndrome marked by congenital anomalies, bone marrow failure and severe paediatric cancer susceptibility. As it is the case for other Fanconi anaemia genes, monoallelic loss-of-function mutations in *PALB2* have been shown to increase the risk of developing breast cancer [5, 13]. The PALB2 protein interacts with BRCA1 through its coiled-coil N-terminal domain and with BRCA2 through the

Table 3 In silico assessment of the effect of *PALB2* missense variants on protein function

Location	DNA change	Protein change	rs number	1000G overall MAF (%)	EVS European Americans MAF	Number of heterozygous carriers in index cases (MAF %)	Number of heterozygous carriers in controls (MAF %)	Studies or projects reporting the missense variant	SIFT prediction	AGVGD prediction	Polyphen2 prediction
Exon 1	c.11C>T	p.Pro4Leu	rs45619737	–	0.06	1 (0.06)	1 (0.08)	[37]; EVS; 1000G	**Affect protein function (*P* = 0.04)**	Class C0	Benign (0.090)
Exon 2	c.53A>G	p.Lys18Arg	rs138789658	0.5	–	1 (0.06)	0 (0)	[14, 44, 48, 49]; EVS; 1000G	Tolerate ($P = 0.46$)	Class C0	**Probably damaging (1)**
Exon 3	c.194C>T	p.Pro65Leu	rs62625272	–	0.01	1 (0.06)	0 (0)	EVS; 1000G	Tolerated ($P = 0.54$)	Class C0	Benign (0.007)
Exon 4	c.232G>A	p.Val78Ile	rs515726085	–	–	1 (0.06)	1 (0.08)	[34, 36]	Tolerated ($P = 0.61$)	Class C0	Benign (0.009)
Exon 4	c.338C>G	p.Pro113Arg	rs374425261	–	0.01	1 (0.06)	0 (0)	EVS; 1000G	Tolerated ($P = 0.35$)	Class C0	Benign (0.020)
Exon 4	c.344G>T	p.Gly115Val	rs145598272	–	0.02	0 (0)	1 (0.08)	[19, 37]; EVS; 1000G	**Affect protein function (*P* = 0.03)**	Class C0	Benign (0.002)
Exon 4	c.365A>G	p.Asp122Gly	–	–	–	1 (0.06)	0 (0)	–	Tolerated ($P = 0.45$)	Class C0	Benign (0.004)
Exon 4	c.656A>G	p.Asp219Gly	rs45594034	–	0.01	2 (0.12)	0 (0)	[5, 10, 12, 18, 34, 50]; EVS; 1000G	Tolerated ($P = 0.4$)8	Class C0	Benign (0)
Exon 4	c.1377C>G	p.Asp459Glu	–	–	–	1 (0.06)	0 (0)	–	Tolerated ($P = 1.00$)	Class C0	Benign (0.041)
Exon 7	c.2590C>T	p.Pro864Ser	rs45568339	0.1	0.31	21 (1.26)	10 (0.76)	[2, 5, 10, 12, 16, 34–37, 49, 51–54]; EVS; 1000G	Tolerated ($P = 0.92$)	Class C0	**Possibly damaging (0.578)**
Exon 7	c.2596G>A	p.Gly866Ser	–	–	–	1 (0.06)	0 (0)	–	**Affect protein function (*P* = 0.02)**	Class C15	Benign (0.122)
Exon 7	c.2606C>G	p.Ser869Cys	–	–	–	1 (0.06)	0 (0)	–	Tolerated (P = 0.08)	Class C15	**Probably damaging (1)**
Exon 8	c.2794G>A	p.Val932Met	rs45624036	0.1	0.59	13 (0.78)	6 (0.45)	[5, 16, 35, 36, 43, 44, 53–57]; EVS; 1000G	Tolerated (P = 0.24)	Class C0	**Probably damaging (1)**
Exon 8	**c.2816T>G**	**p.Leu939Trp**	**rs45478192**	**0.1**	**0.23**	**5 (0.3)**	**2 (0.15)**	**[2, 5, 10, 14, 16, 34–37, 54]; EVS; 1000G**	**Affect protein function (*P* = 0.00)**	**Class C55**	**Probably damaging (1)**
Exon 11	**c.3128G>C**	**p.Gly1043Ala**	**rs377713277**	–	**0.01**	**0 (0)**	**1 (0.08)**	**[10, 35]; EVS; 1000G**	**Affect protein function (*P* = 0.00)**	**Class C55**	**Probably damaging (1)**
Exon 12	c.3235G>T	p.Ala1079Ser	–	–	–	1 (0.06)	0 (0)	–	Tolerated ($P = 0.56$)	Class C0	**Possibly damaging (0.679)**
Exon 12	c.3350G>A	p.Arg1117Lys	–	–	–	1 (0.06)	0 (0)	–	Tolerated ($P = 0.55$)	Class C0	**Probably damaging (0.996)**
Exon 13	**c.3410T>C**	**p.Ile1137Thr**	–	–	–	**1 (0.06)**	**0 (0)**	–	**Affect protein function (*P* = 0.02)**	**Class C25**	**Probably damaging (0.998)**
Exon 13	c.3428T>A	p.Leu1143His	rs62625284	–	0.01	1 (0.06)	0 (0)	[16, 58]; EVS; 1000G	Tolerated ($P = 0.119$)	Class C0	**Probably damaging (0.995)**

Positive predictions are highlighted in bold, as well as variants classified as potentially deleterious by the three bioinformatics tools

seven-bladed WD40-type C-terminal region (Fig. 1), acting as a bridge between these two proteins [38]; PALB2 mediates BRCA2 recruitment to DNA damage sites and is therefore essential for BRCA2 function in double-strand break repair by homologous recombination [39]. As attested by numerous publications (Supplementary Table S4), *PALB2* is one of the breast cancer susceptibility genes for which germline loss-of-function mutations have been the most frequently identified in breast cancer families of different populations.

This study is the first report of a complete mutation screening of *PALB2* in French population. Four new germline truncating mutations were found: c.1186insT, c.1857delT, c.2850delC and c.3418dupT. The first three mutations are clearly pathogenic because they create a premature stop codon expected to trigger nonsense-mediated mRNA decay (NMD) [40]. The c.3418dupT mutation is located in the last exon, therefore it is not supposed to trigger NMD. However, it induces the loss of the last 45 amino acids that are part of the WD40 domain in the RAD51-interacting region, shown to be essential for a fully functional PALB2 protein (Fig. 1). At least two confirmed *PALB2* pathogenic truncating mutations associated with breast cancer and/or Fanconi anaemia map to the last 150 nucleotides: c.3459C > G [41] and c.3497delG [42]. Thus, all four new truncating mutations detected in this study can be considered as pathogenic. One of these truncating mutations (c.3418dupT) has been found in a healthy control (55 years) whose sister was diagnosed with breast cancer at 45 (not tested) (Fig. 2d). The presence of truncating mutations at a low frequency (0.08–0.2 %) in healthy controls has already been described in two studies screening for the recurrent mutations c.509-510delGA in Poland [18] and c.1592delT in Finland [43] (Supplementary Table S4). In addition, the reported penetrance of *PALB2* mutations is increasing from 45 to 80 years of age [42]. Thus, the presence of c.3418dupT in a 55-year-old control does not preclude its causality.

In our study, *PALB2* mutations were detected in 2 breast cancer only families and 1 breast/ovarian cancer family but not in the 22 families with cases of male breast cancer. There are few reports of *PALB2* pathogenic mutations in male breast cancer patients and no evidence of association with increased risk [34, 44, 45]. Similarly, *PALB2* mutations in familial pancreatic cancer seem to be very rare [46], and indeed, we did not find any *PALB2* mutation in four pancreatic cancer families that we also screened (data not shown).

To date, at least 37 publications reported *PALB2* screening in breast cancer cases from different populations and cohorts (Supplementary Table S4). Ten studies analysed more than 500 cases in populations without founder mutation effect and revealed a mutation frequency spanning from 0.73 %, [CI95 %: 0.27–1.59], to 3.40 %, [CI95 %: 2.35–4.73]. In our screening, truncating *PALB2* mutations have been found with a frequency of 0.36 %, [CI95 %: 0.07–1.05] (Supplementary Table S4). The lower frequencies we observed could be a consequence of ascertainment bias. On the other hand, the hypothesis that, in the French population, the frequency of *PALB2* mutations in breast cancer families is lower than in other countries cannot be excluded, but further data are necessary to confirm these differences.

Two missense variants were classified as potentially deleterious with all the three in silico predictors used, but as yet, no genetic epidemiologic data support the possible pathogenicity of these variants, even if two of them mutate amino acids of the WD40 domain (c.3128G > C and c.3410T > C). It has to be noticed that to date, no *PALB2* missense variants could be classified as definitely pathogenic. This does not rule out the possibility that some may be deleterious, but suggests that they should be very rare and very large cohorts of cases and controls will be needed to determine their associated risks [47].

Finally, we did not identify any founder mutation, thereby confirming the necessity of *PALB2* full-gene sequencing in the diagnostic screening.

This study shows that *PALB2* mutations make a small contribution to the heritable breast cancer susceptibility in French population. Nevertheless, screening for inherited loss-of-function mutations in *PALB2* is recommended to enter clinical practice in France as in other countries. The generalization of inclusion of *PALB2* in diagnostic gene screening panels will allow obtaining a precise evaluation of mutation frequency and will provide more data for penetrance and risk estimate for breast and ovarian cancer, an essential step to get an exhaustive cancer predisposition counselling and mutation-targeted personalized therapies.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflicts of interest.

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