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CONTRIBUTION TO FAMILIAL BREAST CANCER OF INHERITED MUTATIONS IN THE BRCA2-INTERACTING PROTEIN PALB2

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Abstract

Inherited mutations in the BRCA2-interacting protein PALB2 are known to be associated with increased risks of breast cancer. In order to evaluate the contribution of *PALB2* to familial breast cancer in the United States, we sequenced the coding sequences and flanking regulatory regions of the gene from constitutional genomic DNA of 1144 familial breast cancer patients with wildtype sequences at *BRCA1* and *BRCA2*. Overall, 3.4% (33/972) of patients not selected by ancestry and 0% (0/172) of patients specifically of Ashkenazi Jewish ancestry were heterozygous for a nonsense, frameshift, or frameshift-associated splice mutation in *PALB2*. Mutations were detected in both male and female breast cancer patients. All mutations were individually rare: the 33 heterozygotes harbored 13 different mutations, 5 previously reported and 8 novel. *PALB2* heterozygotes were 4-fold more likely to have a male relative with breast cancer ($P=0.0003$) and 6-fold more likely to have a relative with pancreatic cancer ($P=0.002$), and 1.3-fold more likely to have a relative with ovarian cancer ($P=0.18$). Compared to their female relatives without mutations, increased risk of breast cancer for female *PALB2* heterozygotes was 2.3-fold (95% CI [1.5–4.2]) by age 55 and 3.4-fold (95% CI [2.4–5.9]) by age 85. Loss of the wildtype *PALB2* allele was observed in laser dissected tumor specimens from heterozygous patients. Given this mutation prevalence and risk, consideration might be given to clinical testing of *PALB2* by complete genomic sequencing for familial breast cancer patients with wildtype sequences at *BRCA1* and *BRCA2*.

INTRODUCTION

Discovery and characterization of the breast cancer susceptibility genes *BRCA1* and *BRCA2* have led to major changes in both prevention and treatment. Genetic testing for inherited mutations offers the opportunity for risk reducing intervention (1). Therapeutic approaches that exploit the biological function of *BRCA1* and *BRCA2* have been proposed (2) and are now showing promise in the clinic (3,4). Given this experience, there has been a great deal of interest in the identification and characterization of other genes responsible for inherited breast cancer (5). Among these genes is PALB2, partner and localizer of BRCA2 (6), first characterized clinically in patients with Fanconi anemia, complementation group N (7,8). It

was soon discovered that heterozygosity for loss of function mutations at *PALB2* increases risk of breast cancer 2- to 6-fold (5,9). Inherited *PALB2* mutations associated with increased risks of breast cancer have been identified in families from many parts of the world (10–18), but thus far, a heterogeneous American population has not been screened.

The purpose of the present project was to estimate the contribution of inherited mutations in *PALB2* to familial breast cancer in a large series of patients from the United States and to characterize the spectrum of inherited breast-cancer-associated mutations in *PALB2* in this heterogeneous population. For this purpose, we sequenced the complete coding and flanking regulatory regions of *PALB2* from constitutional DNA of 1144 familial breast cancer patients, all previously determined to have wildtype sequences at *BRCA1* and *BRCA2*.

Materials and Methods

Subjects

Participants were patients with a primary diagnosis of invasive breast cancer at any age and of any histologic subtype and with at least two first or second degree relatives with invasive breast cancer (19). Two series of participants were identified: one series identifying their ancestry as Ashkenazi Jewish (AJ) and the other series not selected for any specific ancestry. The series of Ashkenazi Jewish patients was enrolled in order to evaluate the possibility of founder mutations in this population in either known or novel breast cancer genes. Enrollment of probands, ascertainment of families, and genomic analysis proceeded identically for the two series. Potential participants were referred by physicians, genetic counselors, or themselves. All potential participants were interviewed prior to enrollment by a certified genetic counselor (J.B.M.) and informed consent was obtained for those meeting the criteria above and interested in enrolling. The consenting participant became the proband for her or his family. For each proband, all genetically informative adult relatives, whether or not affected with breast cancer, were also requested to enroll. Relatives were contacted first by the proband, then interviewed by the genetic counselor and informed consent obtained for those interested. Enrollment began in 1988 and is ongoing. All families in this study have been followed from their time of enrollment to the present by the genetic counselor. For all probands, *BRCA1* and *BRCA2* had been determined to be wildtype, in almost all cases based on commercial sequencing and BART analysis by Myriad Genetics (20). The two series combined included 1144 familial breast cancer patients without mutations in *BRCA1* or *BRCA2*, of whom 172 participants (all female) were of AJ ancestry and 972 participants (959 females and 13 males) were of other ancestries. The study was approved by the University of Washington Human Subjects Division (IRB protocol 34173).

Genomic DNA sequencing

PALB2 exons, flanking intronic regions (50–100 bp in length), and 5'- and 3'-UTRs were evaluated by Sanger sequencing of constitutional genomic DNA from all subjects. Genomic DNA isolated from peripheral blood lymphocytes was amplified by PCR using flanking intronic primers (Supplementary Table 1). Nested PCR and four overlapping amplicons were developed to fully cover the 1473 bp of exon 4. Multiple internal primers were used to sequence exon 5 in both directions. Amplicons were sequenced in both forward and reverse directions except as follows. For exon 9, to overcome the chances of non-specific products due to Alu sequences upstream of the splice site, the exon was amplified using nested primers from an outer product, then cycle-sequenced from the reverse direction. Similarly, exon 13 was only sequenced in the reverse direction. PCR products were purified and cycle-sequenced using the BigDye Terminator Cycle Sequencing chemistry (Applied Biosystems, Life Technologies Corp, Carlsbad, CA) and analyzed on a ABI PRISM® 3700 Genetic Analyzer (Applied Biosystems, Life Technologies Corp, Carlsbad, CA). All sequence

variants were confirmed by replicate Sanger sequence, then evaluated for co-segregation with breast cancer in the family of the proband.

Analysis of transcripts

RNA and cDNA were isolated and transcript lengths evaluated as previously described (19). The possibility of nonsense mediated decay was evaluated by comparing electropherogram peak heights of mutant and wildtype alleles from transcript sequences. Multiple splice variants resulting from single genomic mutations were quantified by cloning PCR-amplified cDNA products into pCR2.1-TOPO cloning vectors (Invitrogen, Carlsbad, CA), then by transforming competent *E. coli* strains, then by sequencing individual clones. Cloning experiments were carried out in triplicate.

Genotyping family members and controls

In order to genotype all informative relatives of each proband with a mutation, TaqMan assays were designed for *PALB2* mutations c.168delTTGT, c.509delGA, c.757delCT, c.1240C>T, c.2386G>T, c.2559C>T, c.2686insT, c.2919delAA, c.3022delC and restriction digests were designed for mutations c.196C>T (*HinPII*), c.1653T>A (*HpyCH4III*), c.2718G>A (*BsmAI*), c.3113G>A (*BstEII*). In addition to family members, 960 unrelated anonymous controls were evaluated for each mutation. For TaqMan assays, DNA was diluted to 20ng, with all wells in the same assay containing the same amount of sample. Two controls without template and positive controls with the proband's *PALB2* mutation were run with every plate. After each real-time PCR amplification, an allelic discrimination plate was analyzed using Sequence Detection System (SDS) software (Applied Biosystems, CA), with the autocaller function enabled and 95% quality interval for allele calling. Fluorescence signals were plotted for test samples, for controls without template, and for positive controls, with X and Y axes representing each allele. Genotypes were called based on location on the scatterplot.

Genotypes of deceased family members and others not available for genotyping were called only if they could be reconstructed with certainty from surviving children, spouses, and siblings. No genotypes were imputed probabilistically.

In order to determine whether the mutation *PALB2* c.3113 shared a common origin in the five patients in which it appears, all probands and their relatives carrying *PALB2* c.3113 were genotyped with microsatellite markers on chromosome 16 developed for this purpose. The markers were selected to flank *PALB2* at chr16:23,614,483–23,652,678 (hg19). The microsatellites were AC23 at chr16:21,732,554–21,732,600 (8 alleles in our series), 23GT at chr16:23,037,671–23,037,716 (9 alleles in our series), and TATG14 at chr16:23,749,512–23,749,567 (4 alleles in our series). Microsatellite sequences, PCR primers, and genotyping conditions are reported in Supplementary Table 2.

Laser capture microdissection and genotyping breast tumor specimens

Tumor sections of 5–8 um from paraffin-embedded blocks were microdissected with the automated LCM Veritas System (Arcturus Molecular Devices, Life Technologies Corp, Carlsbad, CA) as previously described (21). Briefly, for each initial cap placement, laser was focused with the 7.5um spot size setting and the 10× objective. The pulse of the capture laser was adjusted to a 1000–1500 usec duration and 70mW power. Microdissection was performed with a medium laser spot size 12.43um and a 10x objective. Loss of heterozygosity was analyzed by direct sequencing of reconstituted tumor DNA at the site of the mutation. For all the *PALB2* mutations for which tumor specimens were available, sequencing primers were designed to span maximum amplicon sizes of 250bp

(Supplementary Table 3). Sequencing was performed as described above. Basecalls with PHRED scores >30 were included.

Statistical analysis

Frequencies of mutations in subgroups were compared by chi-square tests or Fisher's exact tests, as appropriate. Odds ratios (OR), risk ratios (RR) and 95% confidence limits (CI) were calculated. Cumulative risks were estimated by Kaplan-Meier methods.

Results

Spectrum of PALB2 mutations

From genomic DNA of all 1144 participants, complete *PALB2* coding sequences and flanking regulatory regions were evaluated for nonsense mutations, frameshift mutations, and splice mutations leading to out-of-frame message deletions. Of the 972 breast cancer probands of unselected ancestries, 33 (3.4%) carried a truncating mutation in *PALB2* (Table 1). Of the 13 male probands, 2 (16%) carried a truncating mutation; of the 959 female probands, 31 (3.2%) carried a truncating mutation. Of the 172 breast cancer probands of AJ ancestry, zero carried such a mutation. Given the mutation frequency among patients of unselected ancestries, 5.5 carriers would be expected among the AJ patients ($\chi^2=5.9$, $P=0.015$). Thirteen different truncating mutations in *PALB2* were observed, leading to stops in exons 3–11 (Figure 1).

Analysis of alternate transcripts

Identification of aberrant splice mutations was based on analysis of transcripts. For each sample with a single basepair genomic variant in coding sequence or in flanking intronic regions, cDNA was generated from lymphoblast RNA by RT-PCR of *PALB2* message. Of the 19 such variants observed in the cohort, two substitutions, c.2559C>T and c.3113G>A, led to altered splicing, out-of-frame deletions in the *PALB2* message, and hence premature stops (Figure 2). At *PALB2* c.3113G>A in exon 10, three different messages were produced. Cloning and sequencing multiple messages indicated that 56% of mutant messages included an in-frame 117 bp deletion, 40% of messages included an out-of-frame 31 bp deletion, and 4% of messages were an immediate stop at residue 1038 due to the creation of a nonsense codon at this site. Other rare *PALB2* variants in the 1144 probands included silent and missense substitutions in coding sequence, variants in the 5' UTR and flanking intronic sequences, and variants at splice sites leading to in-frame deletions in transcripts (Supplementary Table 4, Supplementary Figure 1).

Breast cancer risks to mutation carriers

As each *PALB2* mutation was identified in a breast cancer proband, all available affected and unaffected relatives were genotyped. For deceased relatives, genotypes were reconstructed as possible from surviving adult children, spouses, and siblings. Genotypes were assigned only if they could be reconstructed with certainty. For 83 female relatives, affected and unaffected, with unambiguous *PALB2* genotypes, cumulative risks of breast cancer were compared for those carrying a *PALB2* mutation versus those with wildtype *PALB2* genotypes. Ratios of these risks provided estimates of relative risks, cumulative by age, associated with *PALB2* truncating mutations. Relative risks were 2.3-fold (95% CI [1.5–4.2]) by age 55 and 3.4-fold (95% CI [2.4–5.9]) at age 85, consistent with those previously reported (4).

Population origins of *PALB2* mutations

Of the 13 *PALB2* mutations identified in this series, five have been previously reported and eight were novel (Table 1). Ancestries of patients carrying *PALB2* mutations, as proportions of all patients of each ancestry in our series, are indicated in Table 2. In some of the patients of German, French, and English origin, we detected mutations originally discovered in patients of the same ancestries (7–9). A mutation previously discovered in a French-Canadian family (12) was not detected in patients of that ancestry in our series. Three mutations offered particularly interesting histories. *PALB2* c.3113G>A appeared in three patients of English ancestry and two patients of African-American ancestry. These five patients shared a haplotype of length >2MB defined by microsatellite markers on chromosome 16 flanking *PALB2*. Among the patients' relatives who were wildtype at c.3113G>A, none shared this complete haplotype. The allele-specific haplotype suggests a single origin for this mutation, which has previously been reported in British families (9). A second historically interesting mutation was c.509-510delGA, carried by seven patients, all of whom reported German ancestry on their familial lineages with histories of breast cancer. Further exploration of these patients' pedigrees revealed a common ancestor who immigrated to the U.S. from Germany in the 19th century. *PALB2* c.509-510delGA has also been reported in families from Poland (17), suggesting this mutation may have a central European origin. Finally, the two patients carrying *PALB2* c.196C>T shared a distant ancestor who immigrated from Scotland.

Male breast cancer, pancreatic cancer, and ovarian cancer in *PALB2* families

The cancer profiles of *PALB2* families (Table 3) were similar to cancer profiles of *BRCA2* families. Male or female patients with *PALB2* mutations were significantly more likely than patients without *PALB2* mutations to have a relative with male breast cancer or with pancreatic cancer. In contrast, *PALB2* genotype was not significantly associated with the likelihood of having a relative with ovarian cancer or with age at onset of female breast cancer (50.0±11.9 years among *PALB2* heterozygotes vs 50.2±6.8 years among patients not carrying *PALB2* mutations). Patients with *PALB2* mutations were more likely to be probands of families with at least six cases of female breast cancer, perhaps reflecting the possibility that some families in our series with relatively few cases of breast cancer do not carry mutations in any susceptibility genes. It should be noted that most of the patients with *PALB2* mutations (17 of 33) were not probands of these extremely high-incidence families.

Loss of heterozygosity in tumors from patients with inherited *PALB2* mutations

For seven female breast cancer patients with *PALB2* mutations, paraffin embedded breast tumor specimens were available, enabling dissection of tumor cells by laser capture microscopy and evaluation of loss of heterozygosity at *PALB2*. In each of these specimens, loss of the wildtype *PALB2* allele was observed (Figure 3). At least for these patients, loss of heterozygosity of the wildtype *PALB2* allele suggests that *PALB2* acts as a conventional inherited tumor suppressor gene.

DISCUSSION

In a series of familial breast cancer patients of unselected ancestry, with wildtype sequences of *BRCA1* and *BRCA2*, the prevalence of inherited *PALB2* mutations was 3.4%. Thirteen different protein truncating mutations were identified, all of which were individually rare. Five mutations had been previously reported; eight were encountered for the first time in this series. Both the occurrence of *PALB2* mutations among male and female breast cancer patients and the 4-fold higher frequency of male breast cancer among relatives of *PALB2* heterozygotes suggest that *PALB2* predisposes to both male and female breast cancer. Cancer profiles of these families are also consistent with the suggestion that *PALB2* is a

susceptibility gene for pancreatic cancer (22). Ovarian cancer was more common among relatives of *PALB2* heterozygotes (55% of *PALB2* families included a relative with ovarian cancer) than among relatives of other cases (41% of non-*PALB2* families included a relative with ovarian cancer), but this difference was not significant given this sample size. Direct evaluation of large numbers of ovarian cancer patients will be useful in determining the role of inherited mutations in *PALB2* in this malignancy. The distributions of ages at diagnosis of breast cancer patients with *PALB2* mutations were very similar to those of the other familial breast cancer patients in the series, suggesting that in the context of familial breast cancer, *PALB2* does not lead to significantly younger diagnosis. It is very unlikely that there is a founder allele of *PALB2* among patients of Ashkenazi Jewish ancestry, but any family, of any ancestry, could harbor a private cancer-predisposing allele of *PALB2*.

The prevalence of inherited mutations in *PALB2* in non-*BRCA1*, non-*BRCA2* familial breast cancer patients is approximately the same as the prevalence of inherited mutations in *CHEK2* in a similar cohort (19). It has been suggested that genetic testing for *CHEK2* c.1100delC in the clinical setting is now timely, with targeted surveillance and medical follow up for mutation carriers (23). We agree and join others (24) in suggesting that similar consideration be extended to *PALB2*. The breast cancer risk associated with protein truncating mutations in *PALB2* appears greater than the risk associated with *CHEK2* c.1100delC. The challenge is that in the U.S., no one mutation of *PALB2* is sufficiently frequent to represent a substantial fraction of *PALB2* mutations, so that the entire gene must be sequenced to capture the responsible alleles. Simultaneous detection of all mutations in all known breast cancer genes will make such expanded screening more feasible (25).

Testing for *PALB2* and other breast cancer susceptibility genes will add complexity to the clinical interpretation of results. In particular, variants of uncertain significance will be identified. This study focused on *PALB2* mutations leading to protein truncation. Individual missense mutations in *PALB2* will ultimately need to be evaluated as they have been for *BRCA1* and *BRCA2* (26,27).

The possibility of genetic testing for *PALB2* provides another example of the complexity of the transition from research laboratory to clinic. The involvement of medical geneticists and genetic counselors in assessing risk is particularly critical when, as here, consequences of genetic testing can entail substantial medical and surgical decisions (28–30).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Truncating mutations in *PALB2*. Thirteen different truncating mutations in *PALB2* were detected in familial breast cancer patients. On the *PALB2* gene sequence, frameshift mutations are indicated in red, nonsense mutations in purple, and splice mutations in blue. On the PALB2 protein, yellow symbols indicate low complexity regions (LCR), the red symbol the coiled coil domain, and green symbols the WD40-like repeats that comprise beta propeller structures.

Figure 2. Splice mutations in *PALB2*. Effects on splicing of single bp genomic substitutions in *PALB2* were evaluated by sequencing transcripts. *PALB2* c.2559C>T leads to altered splicing and deletion of 29 bp in the *PALB2* message. *PALB2* c.3113G>A produces three different *PALB2* messages: complete deletion of exon 10 (117 bp), use of an alternate splice site within exon 10 and deletion of 31 bp, and an immediate stop at codon 1038.

Figure 3.

Loss of heterozygosity of wildtype *PALB2* alleles in tumor tissue. Tumor tissue was available from seven patients with five different mutations. In all cases, cancer cells isolated by laser dissection carried only the mutant *PALB2* allele. Each panel indicates the sequence at the mutant allele (arrow) for a wildtype control (WT), for constitutional genomic DNA from the carrier (MUT), and for laser-dissected tumor cells (Tumor).

Table 1

Inherited truncating mutations in *PALB2*

Genomic locale (hg19)	Exon	DNA mutation	Type	RNA mutation	Protein mutation	Stop codon	Freq cases (N=972)*	Freq controls (N=960)	Ancestry
chr16:23,649,207-210	3	c.172-175 del TTGT	frameshift	-	S58fsX8**	66	1	0	Irish
chr16:23,649,186	3	c.196 C>T	nonsense	-	Q66X**	66	2	0	Scots
chr16:23,647,357-358	4	c.509-510 del GA	frameshift	-	R170fsX13	183	7	0	German
chr16:23,647,109-110	4	c.757-758 del CT	frameshift	-	L253fsX2	255	4	0	German
chr16:23,646,627	4	c.1240 C>T	nonsense	-	R414X**	414	3	0	German
chr16:23,646,214	4	c.1653 T>A	nonsense	-	Y551X	551	2	0	French
chr16:23,641,089	5	c.2386 G>T	nonsense	-	G796X	796	1	0	English
chr16:23,640,552	6	c.2559 C>T	splice	r.2558-2586 del 29	G853fsX20**	873	1	0	German
chr16:23,637,618	7	c.2686 ins T	frameshift	-	S896fsX31**	927	3	0	German
chr16:23,637,587	7	c.2718 G>A	nonsense	-	W906X**	906	1	0	English
chr16:23,634,364-365	9	c.2920-2921 del AA	frameshift	-	K974fsX4**	978	1	0	English
chr16:23,632,770	10	c.3026 del C	frameshift	-	P1009fsX5**	1014	2	0	German
chr16:23,632,683	10	c.3113 G>A	splice	(a) r.2997-3113 del 117 (b) r.3083-3113 del 31 (c) -	p.999-1038del (56%) G1028fsX2 (40%) W1038X (4%)	- 1030 1038	5	0	African American (2), English (3)

* Series 2

** Mutation not previously reported

Table 2

Ancestries of participants

Origin of lineage with breast cancer, as reported by the participant	Cases genotyped	Cases with PALB2 mutations
Series 1: Ashkenazi Jewish ancestry		
Ashkenazi Jewish	172	
Series 2: Unselected ancestry		
African American	18	2
American Indian or Inuit	33	
Austrian	4	
Basque	3	
Belgian	2	
Chinese	4	
Czech	7	
Danish	13	
Dutch	20	
English	225	6
European not otherwise known	3	
Filipino	1	
French	25	2
French Canadian	21	
German	257	20
Greek	6	
Hungarian	3	
Icelandic	2	
Irish	116	1
Italian	65	
Japanese	1	
Korean	2	
Lebanese	2	
Mexican	13	
Norwegian	12	
Polish	11	
Portuguese	4	
Puerto Rican	2	
Romanian	3	
Russian	3	
Scots	9	2
Scots Irish	31	
Slovenian	4	
Swedish	22	
Swiss	17	
Turkish	2	

Origin of lineage with breast cancer, as reported by the participant	Cases genotyped	Cases with PALB2 mutations
Welsh	6	
Series 2 total	972	33

Table 3

Cancers in relatives of participants

Cancer in relatives	PALB2 genotype of participant			
	Mutation N=33	Wildtype N=939	RR [95% CI]	P value
Male breast cancer	9	62	4.13 [2.25, 7.58]	0.0003
Pancreatic cancer	5	24	5.93 [2.41, 14.56]	0.002
Ovarian cancer	18	388	1.32 [0.96, 1.82]	0.18
≥ female breast cancers	16	280	1.63 [1.13, 2.34]	0.036