Published OnlineFirst February 1, 2011; DOI: 10.1158/0008-5472.CAN-10-3958

Prevention and Epidemiology

Cancer Research

Contribution of Inherited Mutations in the BRCA2-Interacting Protein PALB2 to Familial Breast Cancer

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Abstract

Inherited mutations in the BRCA2-interacting protein PALB2 are known to be associated with increased risks of developing breast cancer. 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 1,144 familial breast cancer patients with wild-type 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 mutations. *PALB2* heterozygotes were 4-fold more likely to have a male relative with breast cancer ($P = 0.0003$), 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 with their female relatives without mutations, increased risk of developing 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 wild-type *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 wild-type sequences at *BRCA1* and *BRCA2*. *Cancer Res; 71(6); 2222–9. ©2011 AACR.*

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 developing breast cancer 2- to 6-fold (5, 9). Inherited *PALB2* mutations associated with increased risks of developing 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 1,144 familial breast cancer patients, all previously determined to have wild-type 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 2 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

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-10-3958

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Published OnlineFirst February 1, 2011; DOI: 10.1158/0008-5472.CAN-10-3958

for any specific ancestry. The series of AJ patients was enrolled 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 2 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 these criteria 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 and 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 wild type, in almost all cases, on the basis of commercial sequencing and BART analysis by Myriad Genetics (20). The 2 series combined included 1,144 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′-untranslated regions (UTR) were evaluated by Sanger sequencing of constitutional genomic DNA from all subjects. Genomic DNA isolated from peripheral blood lymphocytes was amplified by PCR by using flanking intronic primers (Supplementary Table S1). Nested PCR and 4 overlapping amplicons were developed to fully cover the 1,473 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 nonspecific products due to Alu sequences upstream of the splice site, the exon was amplified using nested primers from an outer product and then cycle sequenced from the reverse direction. Similarly, exon 13 was sequenced only in the reverse direction. PCR products were purified and cycle sequenced using the BigDye Terminator Cycle Sequencing chemistry (Applied Biosystems by Life Technologies) and analyzed on an ABI PRISM 3700 Genetic Analyzer (Applied Biosystems by Life Technologies). All sequence variants were confirmed by replicate Sanger sequence and then evaluated for cosegregation with breast cancer in the family of the proband.

Analysis of transcripts

RNA and cDNA were isolated and transcript lengths were evaluated as previously described (19). The possibility of nonsense-mediated decay was evaluated by comparing electropherogram peak heights of mutant and wild-type 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), then by transforming competent *Escherichia coli* strains, and finally by sequencing individual clones. Cloning experiments were carried out in triplicate.

Genotyping family members and controls

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, and c.3022delC and restriction digests were designed for mutations c.196C>T (*Hin*P1I), c.1653T>A (*Hpy*CH4III), c.2718G>A (*Bsm*AI), and c.3113G>A (*Bst*EII). In addition to family members, 960 unrelated anonymous controls were evaluated for each mutation. For TaqMan assays, DNA was diluted to 20 ng, with all wells in the same assay containing the same amount of sample. Two controls without template and positive controls with the proband *PALB2* mutation were run with every plate. After each real-time PCR amplification, an allelic discrimination plate was analyzed using Sequence Detection System software (Applied Biosystems), 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 on the basis of 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.

To determine whether the mutation *PALB2* c.3113 shared a common origin in the 5 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 S2.

Laser-capture microdissection and genotyping breast tumor specimens

Tumor sections of 5 to 8 μm from paraffin-embedded blocks were microdissected with the automated LCM Veritas System (Arcturus Molecular Devices, Life Technologies Corp) as previously described (21). Briefly, for each initial cap placement, laser was focused with the 7.5-μm spot size setting and the 10× objective. The pulse of the capture laser was adjusted to a 1,000- to 1,500-μs duration and 70 mW power. Microdissection was done with a medium laser spot

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size of 12.43 μm and a 10× objective. LOH 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 250 bp (Supplementary Table S3). Sequencing was carried out as described earlier. Basecalls with PHRED scores of more than 30 were included.

Statistical analysis

Frequencies of mutations in subgroups were compared by χ^2 tests or Fisher's exact tests, as appropriate. Risk ratios (RR) and 95% CIs were calculated. Cumulative risks were estimated by Kaplan–Meier methods.

Results

Spectrum of *PALB2* mutations

From genomic DNA of all 1,144 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 probands (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 probands (3.2%) carried a truncating mutation. Of the 172 breast cancer probands of AJ ancestry, none 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 to 11 (Fig. 1).

Analysis of alternative transcripts

Identification of aberrant splice mutations was done on the basis of analysis of transcripts. For each sample with a single bp genomic variant in coding sequence or in flanking intronic regions, cDNA was generated from lymphoblast RNA by reverse transcriptase-PCR of *PALB2* message. Of the 19 such variants observed in the cohort, 2 substitutions, c.2559C>T and c.3113G>A, led to altered splicing, out-of-frame deletions in the *PALB2* message, and hence premature stops (Fig. 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 1,038 due to the creation of a nonsense codon at this site. Other rare *PALB2* variants in the 1,144 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 S4 and Supplementary Fig. S1).

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 developing breast cancer were compared for those carrying a *PALB2* mutation versus those with wild-type *PALB2* genotypes. Ratios of these risks provided estimates of

Table 1. Inherited truncating mutations in *PALB2*

Genomic locale (hg19)	Exon	DNA mutation	Type	RNA mutation
chrl6:23,649,207-210	3	c.172-175 del TTGT	Frameshift	–
chrl6:23,649,186	3	c.196 C>T	Nonsense	–
chrl6:23,647,357-358	4	c.509-510 del GA	Frameshift	–
chrl6:23,647,109-110	4	c.757–758 del CT	Frameshift	–
chrl6:23,646,627	4	c.1240 C>T	Nonsense	–
chrl6:23,646,214	4	c.1653 T>A	Nonsense	–
chrl6:23,641,089	5	c.2386 G>T	Nonsense	–
chrl6:23,640,552	6	c.2559 C>T	Splice	r.2558–2586 del 29
chrl6:23,637,618	7	c.2686 ins T	Frameshift	–
chrl6:23,637,587	7	c.2718 G>A	Nonsense	–
chrl6:23,634,364-365	9	c.2920-2921 del AA	Frameshift	–
chrl6:23,632,770	10	c.3026 del C	Frameshift	–
chrl6:23,632,683	10	c.3113 G>A	Splice	(a) r.2997-3113 del 117 (b) r.3083-3113 del 31 (c) –

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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, 5 have been previously reported and 8 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 3 patients of English ancestry and 2 patients of African American ancestry. These 5 patients shared a haplotype of length more than 2 Mb defined by microsatellite markers on chromosome 16 flanking *PALB2*. Among the patients' relatives who were wild type 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 7 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 United States from Germany in the 19th century. *PALB2* c.509-510delGA has also been reported in families from Poland (17), suggesting that this mutation may have a central European origin. Finally, the 2 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 $\pm$ 11.9 years among *PALB2* heterozygotes vs. 50.2 $\pm$ 6.8 years among patients not carrying *PALB2* mutations). Patients with *PALB2* mutations were more likely to be probands of families with at least 6 cases of female breast cancer, 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 (17/33) were not probands of these extremely high-incidence families.

LOH in tumors from patients with inherited *PALB2* mutations

For 7 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 LOH at *PALB2*. In each of these specimens, loss of the wild-type *PALB2* allele was observed (Fig. 3). At least for these patients, LOH of the wild-type *PALB2* allele suggests that *PALB2* acts as a conventional inherited tumor suppressor gene.

Table 1. Inherited truncating mutations in *PALB2 (Cont'd)*

Protein mutation	Stop codon	Frequency of cases (n = 972)[a]	Frequency of controls (n = 960)	Ancestry
S58fsX8[b]	66	1	0	Irish
Q66X[b]	66	2	0	Scots
R170fsX13	183	7	0	German
L253fsX2	255	4	0	German
R414X[b]	414	3	0	German
Y551X	551	2	0	French
G796X	796	1	0	English
G853fsX20[b]	873	1	0	German
S896fsX31[b]	927	3	0	German
W906X[b]	906	1	0	English
K974fsX4[b]	978	1	0	English
P1009fsX5[b]	1,014	2	0	German
p.999-1038del (56%)	–	5	0	African
G1028fsX2 (40%)	1,030			American (2)
W1038X (4%)	1,038			English (3)

[a]Series 2.
[b]Mutation not previously reported.

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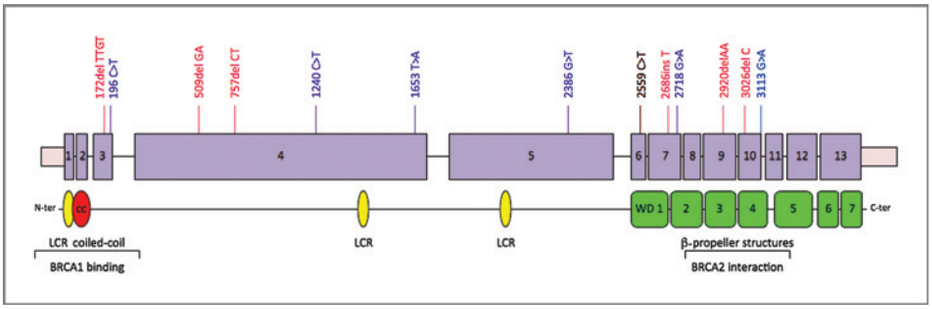

Figure 1. Truncating mutations in *PALB2*. Thirteen different truncating mutations in *PALB2* were detected in familial breast cancer patients. On the *PALB2* gene sequence, frameshift mutations are indicated in red, nonsense mutations in purple, and splice mutations in blue. On the PALB2 protein, yellow symbols indicate low-complexity regions (LCR), the red symbol the coiled coil domain, and green symbols the WD40-like repeats that comprise β-propeller structures.

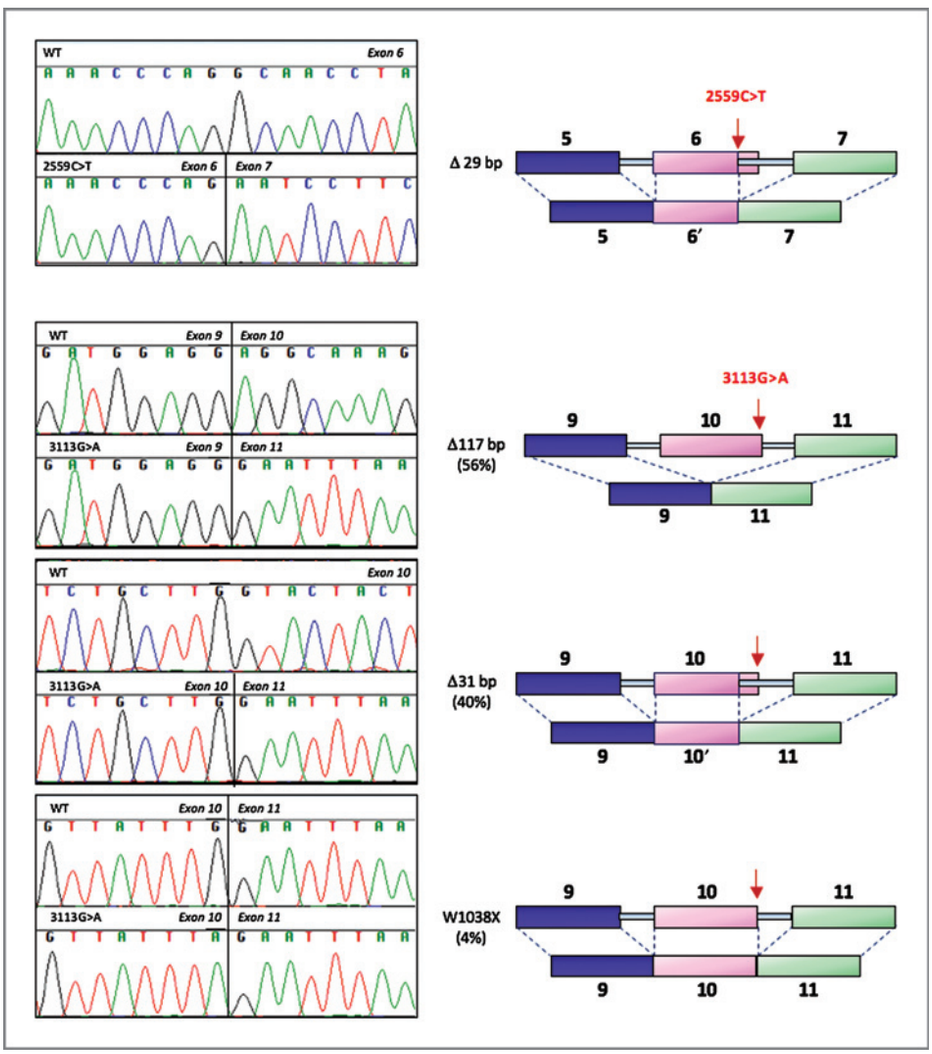

Figure 2. Splice mutations in *PALB2*. Effects on splicing of single bp genomic substitutions in *PALB2* were evaluated by sequencing transcripts. *PALB2* c.2559C>T leads to altered splicing and deletion of 29 bp in the *PALB2* message. *PALB2* c.3113G>A produces 3 different *PALB2* messages: complete deletion of exon 10 (117 bp), use of an alternative splice site within exon 10, and deletion of 31 bp, and an immediate stop at codon 1,038.

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Table 2. Ancestries of participants

Origin of lineage with breast cancer, as reported by the participant	Cases genotyped	Cases with *PALB2* mutations
Series 1: AJ 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	
Welsh	6	
Series 2 total	972	33

Discussion

In a series of familial breast cancer patients of unselected ancestry, with wild-type 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; 8 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 cancers. 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 AJ 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* seems greater than the risk associated with *CHEK2* c.1100delC. The challenge is that in the United States, 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).

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Table 3. Cancers in relatives of participants

Cancer in relatives	*PALB2* genotype of participant			
	Mutation ($n = 33$)	Wild type ($n = 939$)	RR (95% CI)	*P*
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
≥6 female breast cancers	16	280	1.63 (1.13–2.34)	0.036

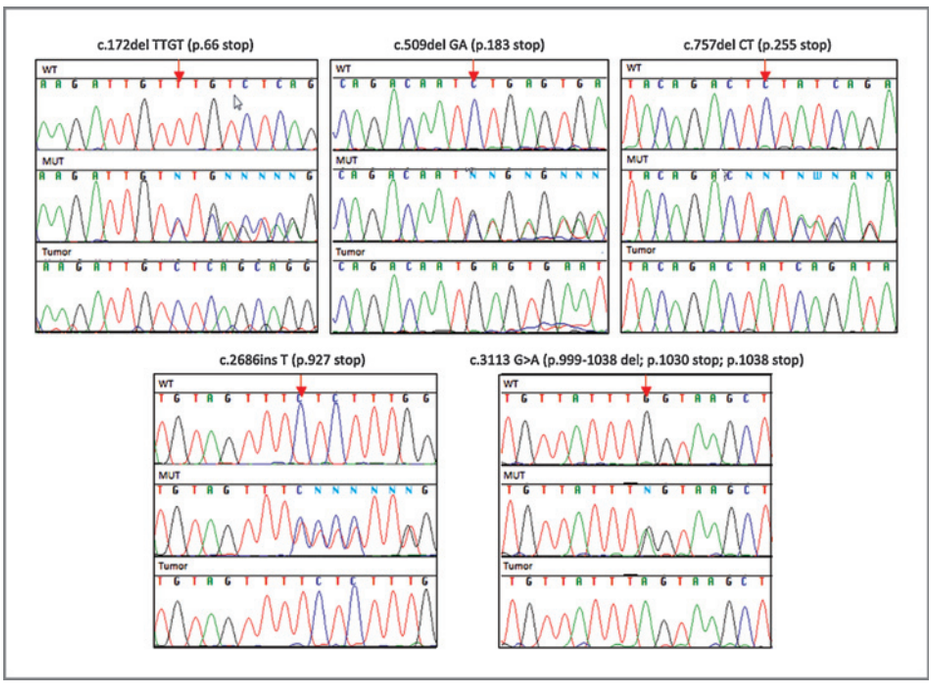

Figure 3. LOH of wild-type *PALB2* alleles in tumor tissue. Tumor tissue was available from 7 patients with 5 different mutations. In all cases, cancer cells isolated by laser dissection carried only the mutant *PALB2* allele. Each panel indicates the sequence at the mutant allele (arrow) for a wild-type control (WT), for constitutional genomic DNA from the carrier (MUT), and for laser-dissected tumor cells (Tumor).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

Supported by the Breast Cancer Research Foundation, Komen Foundation for the Cure, and NIH grants 5R01ES013160 (M-C. King, S.C. Casadei, B.M. Norquist, T. Walsh, S. Stray, J.B. Mandell, and M.K. Lee) and 5U01ES017156 (J.A. Stamatoyannopoulos).

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Received October 30, 2010; revised January 20, 2011; accepted January 25, 2011; published OnlineFirst February 1, 2011.

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Published OnlineFirst February 1, 2011; DOI: 10.1158/0008-5472.CAN-10-3958

Cancer Research
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Cancer Res 2011;71:2222-2229. Published OnlineFirst February 1, 2011.

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