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► To cite this version:

Heide Hellebrand, Christian Sutter, Ellen Honisch, Eva Gross, Barbara Wappenschmidt, et al.. Germline mutations in the PALB2 gene are population specific and occur with low frequencies in familial breast cancer. Human Mutation, 2011, 32 (6), <10.1002/humu.21478>. <hal-00616697>

HAL Id: hal-00616697

<https://hal.science/hal-00616697v1>

Submitted on 24 Aug 2011

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Journal:	<i>Human Mutation</i>
Manuscript ID:	humu-2010-0309.R2
Wiley - Manuscript type:	Mutation in Brief
Date Submitted by the Author:	19-Jan-2011
Complete List of Authors:	<p>Hellebrand, Heide; Technische Universität München, Obstetrics and Gynaecology Sutter, Christian; University of Heidelberg, Institute of Human Genetics Honisch, Ellen; Heinrich Heine Universität, Obstetrics and Gynaecology Gross, Eva; Technische Universität München, Obstetrics and Gynaecology Wappenschmidt, Barbara; University Hospital of Cologne, Department of Gynaecology and Obstetrics Schem, Christian; UKSH Campus Kiel, University of Kiel, Obstetrics and Gynaecology Deissler, Helmut; University of Ulm, Obstetrics and Gynaecology Ditsch, Nina; Ludwig-Maximilians-Universität, Obstetrics and Gynaecology Gress, Verena; Technische Universität München, Obstetrics and Gynaecology Kiechle, Marion; Technische Universität München, Obstetrics and Gynaecology Bartram, Claus; University of Heidelberg, Institute of Human Genetics Schmutzler, Rita; University Hospital of Cologne, Department of Gynaecology and Obstetrics Niederacher, Dieter; Heinrich Heine Universität, Obstetrics and Gynaecology Arnold, Norbert; UKSH Campus Kiel, University of Kiel, Obstetrics and Gynaecology Meindl, Alfons; Technische Universität München, Obstetrics and Gynaecology</p>
Key Words:	breast cancer, DNA repair, PALB2, population genetics

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Germline mutations in the *PALB2* gene are population specific and occur with low frequencies in familial breast cancer



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Contract grant sponsor: German Cancer Aid, grant number: Me 107352

Short Title: *PALB2* mutations in familial breast cancer cases

Communicated by <Please don't enter>

ABSTRACT: The Partner and Localizer of *BRCA2* (*PALB2*) protein has been linked to Fanconi anemia and breast cancer predisposition. Here we present data of a comprehensive mutation screening of the *PALB2* gene in 818 familial cases of breast cancer from Germany. By analysing the entire coding region of *PALB2*, we found seven truncating mutations (six of them novel) in families tested negative for *BRCA1/2*-mutations. In addition, two novel potentially disease causing missense mutations were found. Remarkably, only one mutation reported previously in other populations, was also identified in the German population. No *PALB2* mutation carriers were identified in 450 unaffected controls. Thus, our observations indicate a low prevalence of deleterious *PALB2* mutations and a specific mutation profile within the German population. As *PALB2*-deficient tumors were shown to be sensitive to Poly(ADP-ribose) Polymerase (PARP) inhibitors, our study has implications for newly developed, favourable treatment options in familial breast cancer. ©2011 Wiley-Liss, Inc.

KEY WORDS: Breast cancer, DNA repair, *PALB2*, Population Genetics

Received <date>; accepted revised manuscript <date>.

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INTRODUCTION

Screening of more than 5000 German breast and ovarian cancer families by PCR based techniques revealed that in high risk groups, mutation frequencies in the two known *BRCA* genes reach between 35-50% (Meindl, 2002). In contrast, mutations in other predisposing genes like *CHEK2* (Dufault et al., 2004), *ATM* (Dork et al., 2001) and *RAD51C* (Meindl et al., 2010) were detected with low prevalence. As a significant proportion of our families lacking mutations in the *BRCA1*, *BRCA2*, *RAD51C*, *CHEK2* or *ATM* genes, respectively, suggests a strong genetic background, we decided to screen for mutations in *PALB2*, an additional breast cancer predisposing gene described recently.

PALB2 encodes for a protein functioning as Partner and Localizer of BRCA2 (*PALB2* [MIM:610355]). It has been recently associated with recessive mutations in Fanconi anemia [MIM:227650] (Xia et al., 2007; Reid et al., 2007) and heterozygous mutations in familial breast cancer (Rahman et al., 2007). Moreover, a recurrent founder mutation has been reported for the Finnish population (Erkko et al., 2007). Interestingly, truncating *PALB2* mutations have also been found in familial pancreatic cancer cases (Jones et al., 2009).

The relative contribution of *PALB2* mutations to familial breast cancer cases for most populations, including the German one, is still largely unknown. Besides the Finnish founder mutation only one rare founder mutation has been described in a small study with French-Canadian breast cancer families (Foulkes et al., 2007). Complete analysis of the *PALB2* gene in 95 Spanish breast cancer families revealed only one novel mutation, which was not present in another 725 high risk breast cancer cases (Garcia et al., 2009). Moreover, sequencing of the entire *PALB2* gene in 360 Chinese women with early-onset breast cancer revealed only two novel truncating mutations present in three out of 360 families, which were further validated in a larger set of *BRCA1/2* negative Chinese samples (Cao et al., 2009). Finally, two novel aberrations were identified very recently in Italian families (Papi et al., 2009; Balia et al., 2010). Here we present the complete screening for mutations in the *PALB2* gene in 818 familial cases of breast cancer from Germany.

MATERIALS AND METHODS

Patients and Families

Index patients from 818 unrelated breast cancer families were recruited through a clinicogenetic counselling setting at the Familial Breast and Ovarian Cancer Centers in Cologne, Düsseldorf, Heidelberg, Kiel, Munich and Ulm. They had been categorized in different risk groups: Group A: three or more affected females with breast cancer, at least two of them diagnosed before the age of 50 ys (n = 330); Group B: three or more affected females independent of age (including families with one breast cancer case <50ys, n = 229); Group C: two cases of breast cancer diagnosed before the age of 50 ys (n = 68); Group E: at least one case of breast and one ovarian cancer, respectively (n = 143). All affected index patients had been screened for small nucleotide alterations in *BRCA1* and *BRCA2* by the PCR-based mutation detection techniques dHPLC and/or direct DNA-sequencing (Meindl, 2002) and tested negative for pathogenic mutations. DNAs from 450 control samples from healthy women aged older than 50 years were collected in Kiel (100), Düsseldorf (150) and Munich (200).

Direct sequencing

PCR and sequencing primers are listed in Supp. Table S1. DNAs were amplified using AmpliTaq Gold or Platinum Taq DNA polymerase (Invitrogen) or the Expand High Fidelity PCR system (Roche). Sequencing was carried out after PCR amplification of all 13 exons of the *PALB2* gene by using the same primers in the chain terminating reaction. Sequence reactions were electrophoresed on an ABI 3100 automated DNA sequencer (Applied Biosystems, Darmstadt, Germany). Sequences were analysed by using Sequence Pilot software (JSI-Medisys, Kippenheim, Germany) and by visual inspections of chromatograms. Reference sequence was obtained from NM_024675.3 using the first nucleotide of the ATG translation start as +1.

Denaturing high pressure liquid chromatography (dHPLC) and high resolution melting analysis (HRM)

The detection of sequence variants in exons 1, 2, 6, and 8-13 was carried out by dHPLC analysis as described elsewhere (Xiao and Oefner, 2001). In brief, the mutation analysis was done on a Wave DNA fragment analysis system (Transgenomic, Omaha, NE) under partially denaturing conditions. PCR products were heated up to 95°C

and slowly cooled down to generate heteroduplex molecules in the case of heterozygous DNA samples. The optimal system performance was checked by standard samples (Transgenomic) and positive controls generated by *in vitro* mutagenesis (see below).

Exons 3, 4, 5 and 7 were screened by HRM analysis on a Lightcycler 480 instrument (Roche, Penzberg, Germany) using the Gene scanning software module of the system and a sensitivity setting of 0.30. Melting differences were visualized by Difference plots.

***In vitro* mutagenesis**

To supply positive controls for a dHPLC/HRM screening, mutations were generated by *in vitro* mutagenesis (see asterisks in Fig. 1). We used the Quick change^R II Site-Directed Mutagenese kit (Stratagene, Heidelberg, Germany).

Prediction of pathogenicity of missense variants

Potential consequences of missense mutations were obtained by using the prediction softwares SIFT (<http://sift.jcvi.org/>), PolyPhen (<http://genetics.bwh.harvard.edu/pph/>), and Mutation Taster (<http://www.mutationtaster.org/>).

RESULTS

Mutation analysis of the *PALB2* gene in familial breast cancer cases

In order to determine *PALB2* mutation frequencies already identified in other populations (Fig. 1a) and/or to detect novel *PALB2* sequence variations in the German population, we decided to sequence in a first step all 13 coding exons of the entire *PALB2* gene in 108 familial breast cancer cases, **which were enriched for high risk families from group A and E**. Two novel truncating mutations in exon 4 (c.1240C>T, p.R414X) and exon 5 (c.2145_2146delTA, p.D715EfsX2) were found (Fig. 1b), whereas none of the published clearly pathogenic *PALB2* mutations were detected (Table 1). In addition, we identified a novel, probably disease causing missense variant in exon 13 (c.3428T>C; p.L1143P, see below). Additional 710 families were screened for *PALB2* mutations using the dHPLC and HRM high throughput techniques (see Fig. 1c). To establish the assay conditions, at least one positive mutational control was used. As for some of the exons mutated genomic DNAs were not available, we generated additional mutated templates by *in vitro* mutagenesis for an effective and reliable screening with respect to all melting domains of the amplicons (see Fig. 1, mutations with asterisks).

None of the known published truncating mutations (Erkko et al., 2007; Cao et al., 2009; Garcia et al., 2009; Rahman et al., 2007; Foulkes et al., 2007; Sluiter et al., 2009; Adank et al., 2010; Dansonka-Mieszkowska et al., 2010; Balia et al., 2010), except one single nonsense mutation in exon 5, c.2257C>T (p.R753X) (Papi et al., 2009), as well as none of our two novel mutations and the one putative pathogenic missense variation identified by direct sequencing, could be found in the 710 additional familial cases. However, we could identify one novel splice mutation in exon 1 (c.48 +1G>C), one novel frameshift mutation in exon 3 (c.172_175delTTGT, p.Q60RfsX7), and two further novel nonsense mutations in exon 4 (c.503C>A), and exon 9 (c.2962C>T) causing direct introduction of premature stop codons at the positions p.S168X and p.Q988X, respectively. The family history and histopathology of the tumor tissues of our index patients is indicated in Table 1.

Moreover, we identified by the dHPLC and HRM method additional 11 novel missense variants (Supp. Table S2). The application of three different prediction programs and conservation profiles indicated that only one of them might be disease associated (c.3128G>C; p.G1043A).

Several observations support the classification of the missense mutations L1143P and G1043A as pathogenic: a) not present in 450 healthy controls, b) highly evolutionary conserved, and c) all the three prediction algorithms PolyPhen, SIFT and Mutation Taster predicted a damaging effect on the protein. Nevertheless, they still have to be considered as unclassified variants and functional analyses are warranted. **This also applies for the missense mutations G998E and L939W**, which are both present in patients and controls, however, overrepresentation of both changes in patients cannot be ruled out currently. Finally, one complex change in the 5'UTR, c.-214_236delins10, was found both once in patients and controls, but RNA analysis is required for a final characterization. This might also be indicated for two intronic aberrations (c.49-15t>c; c.109-12t>a), which were found each once in patients, but not in controls.

Patients' and family characteristics of identified mutation carriers

In total, we identified seven truncating mutations and two putative pathogenic missense mutations in the 818 familial cases (Table 1). As an extensive segregation analysis was available only in two families (e. g. Fig. 2), calculation of penetrance for our families revealed to be difficult. However, based on the obtained and partially documented family data, it can be considered as high, supporting the observation of higher ORs of clearly pathogenic *PALB2* mutations within a familial context (Byrnes et al., 2008); Southey et al., 60th Annual meeting of the ASHG, Washington DC, 2010). The appearance of a *PALB2* mutation might not be associated with breast cancer only, but also with colon/ovarian cancer. Concerning histology, *PALB2* mutations can cause either hormone receptor positive, but equally hormone receptor negative breast cancer. As previously observed (Tischkowitz and Xia, 2010), estrogen/progesterone receptor negative carcinomas appear therefore to be slightly overrepresented in *PALB2*-related tumors compared to all breast cancers.

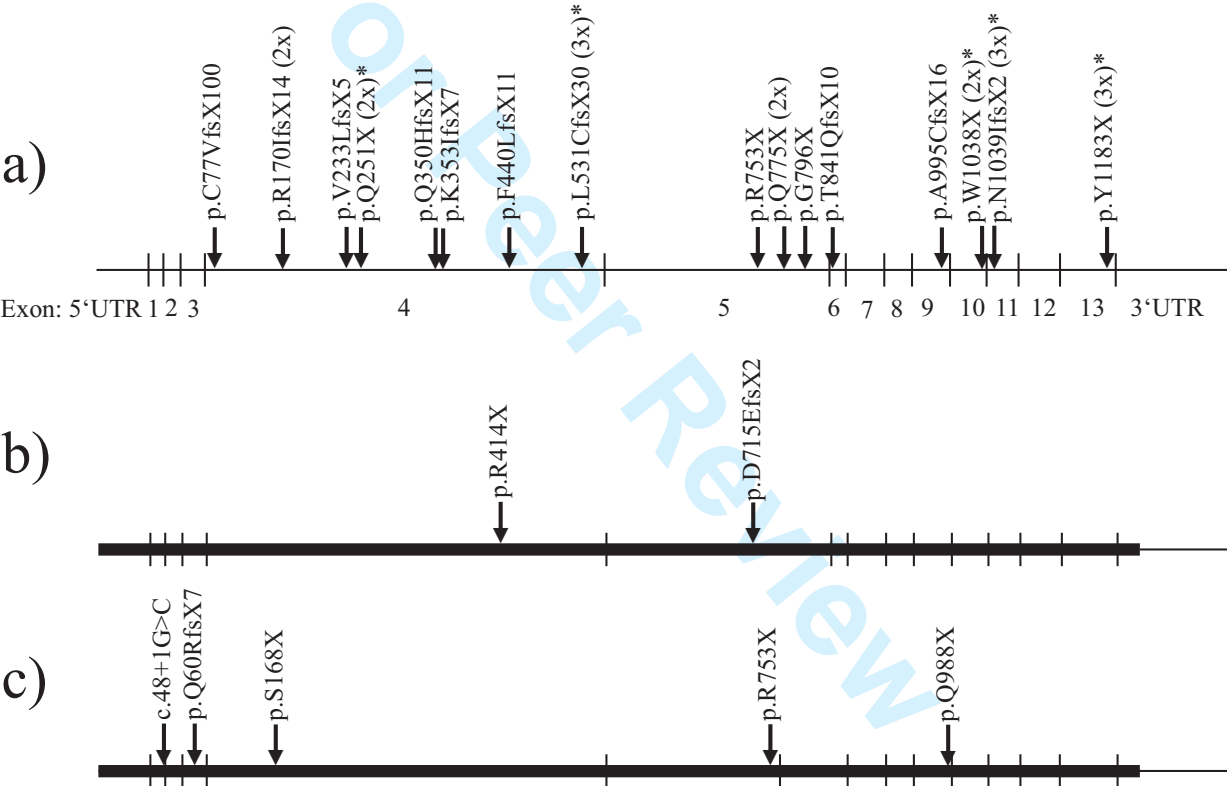


Figure 1: Truncating mutations in the *PALB2* gene. a) Localization of the previously reported deleterious mutations in the *PALB2* gene identified in breast cancer families; published mutations indicated by an asterisk were additionally prepared by *in vitro* mutagenesis for the purpose of using them as positive controls for the dHPLC screening (see 1c). b) Depiction of novel mutations found by direct sequencing in 108 familial cases tested negative for *BRCA1/2* mutations; c) Mutation sites in the *PALB2* gene found in 710 familial cases analysed by dHPLC and HRM analysis.

Table 1. Truncating and putative deleterious missense mutations in the *PALB2* gene in 818 familial breast cancer cases

Exon:	Nucleotide change:	Protein change:	Family history	Tumor histology of index patient
1	c.48+1G>C	Splice defect	3 x BC with late onset > 60ys	HR+, HER2neu-
3	c.172_175delTTGT	p.Q60RfsX7	1 BC, 1 OC + colon cancer	Invasive ductal carcinoma at age 46, HR+/HER2neu-
4	c.503C>A	p.S168X	2 x BC	Ductal carcinoma in situ at 39ys, HR-
4	c.1240C>T	p.R414X	4 x BC (one bilateral case with subsequent onset of OC or uterine cancer),	Invasive intraductal BC at 43ys, HR-
5	c.2145_2146delTA	p.D715EfsX2	3 x BC, 1 putative OC	Invasive ductal carcinoma at 44ys, HR+
5	c.2257C>T	p.R753X	3 x BC	BC at age 33ys, HR-
9	c.2962C>T	p.Q988X	4 x BC (one bilateral case), see also Fig. 2	Ductal carcinoma in situ of one sister, moderately HR+; grade 3 tumor of other sister HR-unknown
11	c.3128G>C;	p.G1043A	2 x BC (two affected sisters, but only one mutation carrier)	
13	c.3428T>C;	p.L1143P	2 x BC (one bilateral case)	Bilateral BC at age 40 and 45 of unknown histology

BC, breast cancer; OC, ovarian cancer; HR, hormone receptor. Nucleotide numbering is based on cDNA

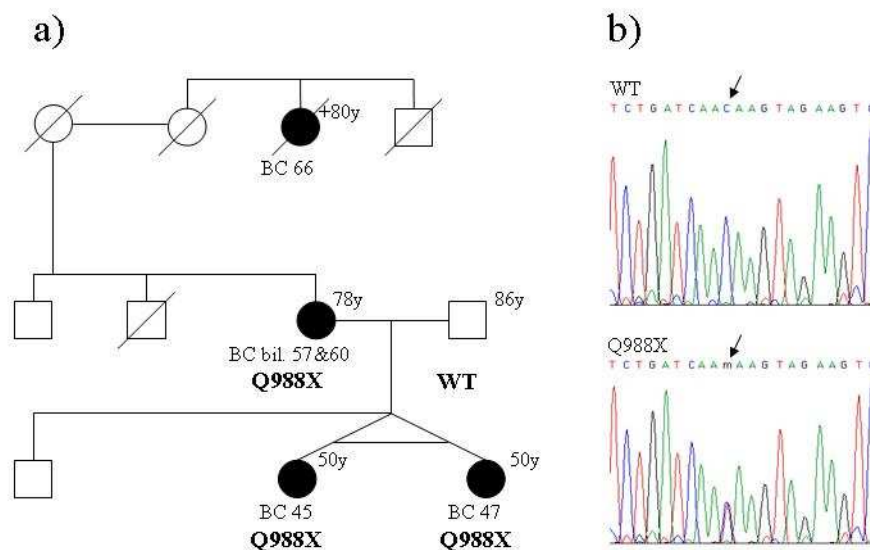


Figure. 2: Segregation of the nonsense mutation p.Q988X in a breast cancer family. a) Pedigree of the family. b) Corresponding sequence alteration c.2962C>T (p.Q988X) detected in exon 9 of the *PALB2* gene. Additional pedigrees of the *PALB2* mutation carriers are available in Supp. Figure S1.

DISCUSSION

The PALB2 gene product is one of approximately ten proteins which directly complex with or bind to BRCA1 or BRCA2. The PALB2 protein interacts at the site of four WD40 domains with the very N-terminus of BRCA2 and at the very N-terminal site with a heptad coiled coil stretch in BRCA1, thus stabilizing its nuclear functions in DNA repair (Xia et al., 2006; Sy et al., 2009b; Zhang et al., 2009; Zhang et al., 2009). Patients lacking a functional PALB2 protein on the basis of inactivating mutations either develop Fanconi anemia (FANCN) or familial breast cancer. Moreover, truncating mutations have also been reported for familial pancreatic cancer (Jones et al., 2009). More than 20 distinct clearly pathogenic mutations have been reported in the literature (most of them summarized in ref.(Jones et al., 2009); quite novel ones in ref.(Dansonka-Mieszkowska et al., 2010; Papi et al., 2009; Balia et al., 2010) or in international databases, e.g. HGMD, so far. However, only 16 of them have been identified in breast cancer families (see Fig. 1a). Based on these findings we were prompted to study 818 unrelated German familial breast cancer patients tested negative for *BRCA1/2*, together with 450 unaffected controls. In addition to the recently and quite actually published data (Papi et al., 2009; Dansonka-Mieszkowska et al., 2010; Balia et al., 2010), we could add six novel truncating mutations and two novel putative disease contributing missense variants.

Population and ethnicity specific mutation profiles for the *PALB2* gene

Our data convincingly support the evidence of population-specific mutational profiles for the *PALB2* gene. Only one out of the truncating mutations described here has been found before in other breast cancer families from different geographic or ethnic origin, nor were we able to find any of the published mutations in our population. Likewise, in addition, we could exclude the occurrence of the recurrent Finnish founder mutation (Erkko et al., 2007), the four distinct truncating *PALB2* mutations found in nine British breast cancer families of mixed ethnical background (Rahman et al., 2007), and the recurrent mutation (c.2323C>T) in the French-Canadian population (Foulkes et al., 2007), in our 818 familial cases. The nonsense mutation c.2257C>T, found in a breast cancer family from Italy, had been published after finding this mutation in our cohort.

So far, the identified mutations in the *PALB2* gene are scattered among the entire gene and seem to be of very low frequency and distinct for different populations. The lack of recurrent founder mutations for most of the Caucasian populations or other ethnicities seems therefore to require initial comprehensive mutation screenings for the different populations or ethnicities. However, large genomic rearrangements in the *PALB2* gene do not appear to contribute to the hereditary breast cancer susceptibility as reported by two independent publications (Ameziane et al., 2009; Pylkas et al., 2008).

Unclassified variants in the *PALB2* protein

The prevalence of truncating mutations in the *PALB2* gene is in the range of about 1.0% (Fig. 1 and ref. (Turnbull and Rahman, 2008)). The importance of *PALB2* as a predisposing gene for familial breast cancer might on the other hand be increased by the detection and characterization of additional protein affecting missense mutations. For example, it has been reported that the PALB2 protein directly interacts with the very 5'end of the BRCA2 protein (Xia et al., 2006; Sy et al., 2009b; Zhang et al., 2009), and recently it has been shown that the aminoterminal part of the PALB2 protein is required for binding a coiled-coiled domain of BRCA1 (Sy et al., 2009a; Zhang et al., 2009). Obviously, amino acid substitutions in these binding domains might be pathogenic. In agreement with these observations, we could find two missense mutations in this study, which are most likely pathogenic (p.T1030I and p.L1143P) and located in a domain of the PALB2 protein, which is interacting with BRCA2 (Sy et al., 2009a). Functional studies are underway to systematically investigate these putative pathogenic missense mutations by complementation assays (Takata et al., 2001). On the other hand, we identified four novel aberrations in the 5'UTR and intronic part of the gene, which might cause either aberrant expression or splicing of the gene. RNA analysis experiments are underway. Two missense mutations (L939W, G998E) were found in patients and controls. Both seemed to be slightly overrepresented in patients, both are highly conserved and both were predicted to be deleterious by three algorithms. Their putative character as low risk variants should be clarified by the application of an extensive case-control study.

Impact on therapy and genetic counseling

As BRCA1- and BRCA2-deficient cells have been found to be highly sensitive to poly(ADP-ribose) polymerase (PARP) inhibition, it has been assumed that this would be true as well for tumors lacking PALB2. Indeed, it could be shown recently that PARP inhibitor AZD2281 was cytotoxic to PALB2-mutant lymphoblasts (Buisson et al., 2010). Hence, regarding the favorable treatment options by PARP inhibitors observed in familial BRCA1/2-associated breast cancer cases (Fong et al., 2009), the presence of PALB2 mutations should be of clinical relevance for providing tailored therapy regimens to the respective patients.

Acknowledgements

We are indebted to Dr. D. Schäfer, Institute of Human Genetics, University of Frankfurt for providing one family. We thank Stefanie Engert, Jochen Meyer, Farnoosh Fathali-Zadeh, Maike Kreikemeier, Doris Karow, Birgit Teegen and Regina Grunewald for expert technical assistance.

Web Resources

The Human Gene Mutation Database at the Institute of Medical Genetics in Cardiff: <http://www.hgmd.org/>
Online Mendelian Inheritance in Man (OMIM): <http://www.ncbi.nlm.nih.gov/Omim/>
Mutation Taster: <http://www.mutationtaster.org/>
SIFT: <http://sift.jcvi.org/>
PolyPhen: <http://genetics.bwh.harvard.edu/pph>

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Table S1. Primer sequences covering the coding region of the *PALB2* gene

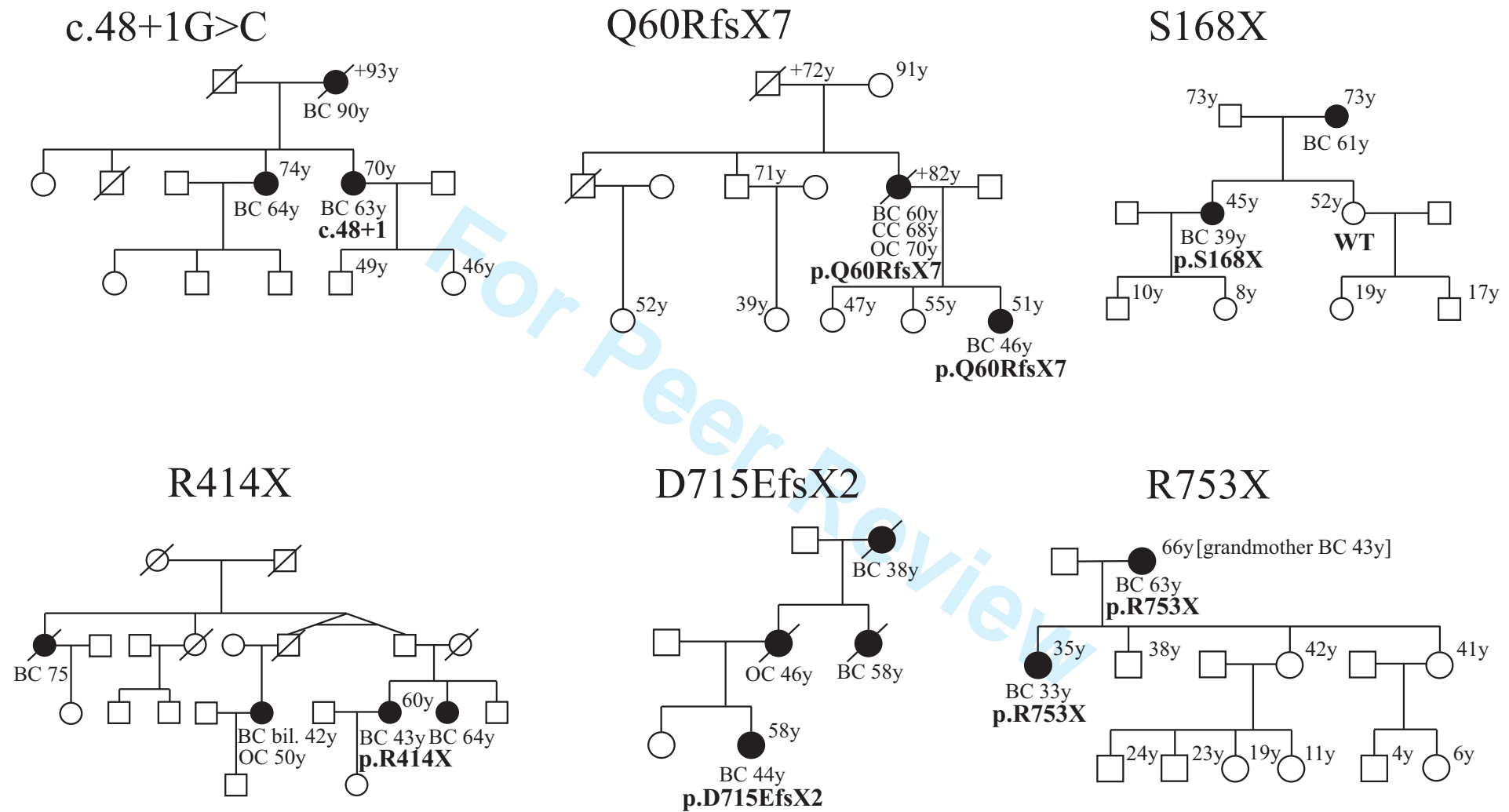
Exon	Forward Primer	Reverse Primer
1	5' - ACTGGGACTCATCGACAGC - 3'	5' - TGGTCAGATGATACTGCTGC - 3'
2	5' - TTATGGACCAGTGCTACTCC - 3'	5' - AGCTCTTTGGGCACGCTAG - 3'
3	5' - GCCTTCAGGTAAGTGAATCG - 3'	5' - TCTTCACACTGTGGGAAAAAG - 3'
4a	5' - CATCTGCCTGAATGAAATGTC - 3'	5' - GGGGAAAATGTTCTTGGGTG - 3'
4b	5' - CCCAGGAGGATTACCTATAC - 3'	5' - GGCAGTTGGTGGAATTAATAC - 3'
4c	5' - AATCTGAACTTCCAGATTCTC - 3'	5' - TAGAACTTGTGGGCAGTTGG - 3'
4d	5' - GACTGTCTCTACAGATAACC - 3'	5' - CTTTCCTCTGGCAATTGGAC - 3'
4e	5' - GGCCTTCTGTTTCCTGCAG - 3'	5' - CTTGGACCTGTTAACAATCG - 3'
4f	5' - TGA CT TGTCTAGGAAGGCAG - 3'	5' - AGGAAGTGCCAGGCAAATAG - 3'
5a	5' - GGAGATCCTATTCTCTTTGTC - 3'	5' - TTTGACTCAAAGGGCTCCAC - 3'
5b	5' - AAGACTTTGGACCTCTTAAGC - 3'	5' - TTTCATAGGAGCCTTGAGGG - 3'
5c	5' - TCCTTTAAATACGGTTGCGC - 3'	5' - TGCGCAAGCAAGTCATGCTG - 3'
6	5' - TGCATTTGGAGCTTTGCTGC - 3'	5' - AGAACAAGAAGCTATATGACTG - 3'
7	5' - TTCTTTTCACCTGCATAAGAC - 3'	5' - CCAATTTTGGTAAGCTGCCC - 3'
8	5' - ATCCTTGTACAGTGAGAATAC - 3'	5' - TTTCAAGACTCAAGCCTAGG - 3'
9	5' - AGGTTACTCCTCACATCACC - 3'	5' - AAACGAGATCCTAGTTACCC - 3'
10	5' - GCAGTTCAACAATGCGGAGAAG - 3'	5' - GAGGTATATCCTCATACTACAG - 3'
11	5' - CCCACAGTTCTACTTTTACC - 3'	5' - GGGGAAGGTTTGTTCATTAC - 3'
12	5' - TTTGGTTTTTGTCTCTGCCAG - 3'	5' - TCAGAATGTCCCACCCATAG - 3'
13	5' - TTGTTTTTTGGATATGTAATCTG - 3'	5' - AAGTGTCATTCAGATATTCTCC - 3'

Table S2. 5'UTR - and non-truncating variants of the PALB2 gene

818 cases	No. of alleles	Exon	Nucleotide change ¹	Protein change	in dbSNP	conservation	PolyPhen:	SIFT:	Mutation Taster:
fam	1	1	c.-214_236delins10		no	-	-	-	-
fam	4	1	c.-158G>C		no	high	-	-	-
fam	12	1	c.-47G>A		rs8053188	medium	-	-	-
fam	1	2	c.49-15T>C		no	high	-	-	-
fam	1	3	c.109-12T>A		no	high	-	-	-
fam	1	3	c.136C>T	H46Y	no	low	probably damaging	affect Protein Function	polymorphism
fam	1	4	c.656A>G	D219G	rs45594034	no	benign	tolerated	polymorphism
fam	1	4	c.939C>T	G313G	no	silent mutation	-	tolerated	polymorphism
fam	1	4	c.1001A>G	Y334C	no	high	benign	tolerated	polymorphism
fam	15	4	c.1010T>C	L337S	rs45494092	no	possibly damaging	tolerated	polymorphism
fam	3	4	c.1194G>A	V398V	rs61755173	silent mutation	-	tolerated	polymorphism
fam	1	4	c.1273G>A	V425M	no	high	benign	tolerated	polymorphism
fam	1	4	c.1419A>C	P473P	rs62625275	silent mutation	-	tolerated	polymorphism
fam	1	4	c.1471G>A	A491T	no	not at all	benign	tolerated	polymorphism
fam	1	4	c.1563C>T	T521T	no	silent mutation	-	tolerated	polymorphism
fam	2	4	c.1572A>G	S524S	rs45472400	silent mutation	-	tolerated	polymorphism
fam	127	4	c.1676A>G	Q559R	rs152451	no	benign	tolerated	polymorphism
fam	1	5	c.1935G>A	E645E	no	silent mutation	-	tolerated	polymorphism
fam	48	5	c.2014G>C	E672Q	rs45532440	no	benign	tolerated	polymorphism
fam	1	5	c.2135C>T	A712V	no	low	benign	tolerated	polymorphism
fam	1	5	c.2182T>C	F728L	no	very high	probably damaging	tolerated	disease causing
fam	9	7	c.2590C>T	P864S	rs45468339	high	probably damaging	tolerated	polymorphism
fam	1	8	c.2750T>C	V917A	no	high	benign	affect Protein Function	polymorphism
fam	11	8	c.2794G>A	V932M	rs45624036	very high	benign	tolerated	polymorphism
fam	6	8	c.2816T>G	L939W	rs45478192	very high	probably damaging	affect Protein Function	disease causing
fam	31	9	c.2993G>A	G998E	rs45551636	very high	probably damaging	affect Protein Function	disease causing
fam	1	10	c.3057C>T	V1019V	no	silent mutation	-	tolerated	polymorphism
fam	1	10	c.3073G>A	A1025T	no	high	benign	affect Protein Function	disease causing
fam	1	11	c.3128G>C	G1043A	no	very high	possibly damaging	affect Protein Function	disease causing
fam	1	12	c.3223A>G	S1075G	no	high	possibly damaging	tolerated	polymorphism
fam	47	12	c.3300T>G	T1100T	rs45516100	silent mutation	-	tolerated	polymorphism
fam	1	12	c.3314T>C	V1105A	no	very high	possibly damaging	tolerated	polymorphism
fam	1	12	c.3342G>C	Q1114H	no	medium	benign	tolerated	polymorphism
fam	1	13	c.3428T>C	L1143P	no	high	probably damaging	affect Protein Function	disease causing

450 controls	No. of alleles	Exon	Nucleotide change	Protein change	in dbSNP	conservation:	PolyPhen:	SIFT:	Mutation Taster:
con	1	1	c.-214_236delins10	-	no	-	-	-	-
con	2	1	c.-158G>C	-	no	high	-	-	-
con	1	1	c.-109delG	-	no	-	-	-	-
con	6	1	c.-47G>A	-	rs8053188	medium	-	-	-
con	15	4	c.1010T>C	L337S	rs45494092	no	possibly damaging	tolerated	polymorphism
con	1	4	c.1194G>A	V398V	rs61755173	silent mutation	-	tolerated	polymorphism
con	1	4	c.1241G>A	R414Q	no	very high	possibly damaging	tolerated	polymorphism
con	1	4	c.1544A>G	K515R	no	high-very high	benign	tolerated	polymorphism
con	6	4	c.1572A>G	S524S	rs45472400	silent mutation	-	tolerated	polymorphism
con	67	4	c.1676A>G	Q559R	rs152451	no	benign	tolerated	polymorphism
con	16	5	c.2014G>C	E672Q	rs45532440	no	benign	tolerated	polymorphism
con	4	7	c.2590C>T	P864S	rs45468339	high	probably damaging	tolerated	polymorphism
con	5	8	c.2794G>A	V932M	rs45624036	very high	benign	tolerated	polymorphism
con	2	8	c.2816T>G	L939W	rs45478192	very high	probably damaging	affect Protein Function	disease causing
con	1	9	c.2896 A-G	I 966V	no	very high	benign	tolerated	polymorphism
con	12	9	c.2993G>A	G998E	rs45551636	very high	probably damaging	affect Protein Function	disease causing
con	1	12	c.3297G>A	T1099T	rs45565738	silent mutation	-	tolerated	polymorphism
con	17	12	c.3300T>G	T1100T	rs45516100	silent mutation	-	tolerated	polymorphism
con	2	13	c.3495G>A	S1165S	rs45439097	silent mutation	-	tolerated	polymorphism
con	2	13	c.3508C>T	H1170Y	no	high	probably damaging	tolerated	polymorphism

¹ possibly protein damaging unclassified variants in bold, nucleotide numbering is based on cDNA starting with the first nucleotide of the ATG translation initiation codon



Supp. Figure. S1: Pedigrees of the breast cancer families harbouring *PALB2* mutations