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PALB2 sequence variants in young South African breast cancer patients

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Abstract PALB2 (partner and localizer of BRCA2) is a recently identified breast cancer susceptibility gene, in which mutations confer doubling of breast cancer risk with moderate to low penetrance. Recent studies in various populations report that deleterious mutations in this gene account for approximately 1% of familial or early-onset breast cancer cases. This study aimed to determine the involvement of PALB2 mutations in a cohort of 48 young (29–45 years) South African breast cancer patients unselected for family history of breast cancer. The complete coding region and intron-exon boundaries of PALB2 were analyzed. A novel truncating mutation, c.697delG (V233fs) was identified in one patient. A missense variant (E211G), identified in another patient, appears to be segregating with the disease, but in silico analysis using SIFT, PolyPhen and A-GVGD, indicates that this variant is nonpathogenic. In addition, four other missense, one synonymous and three intronic variants were detected, all of which appear polymorphic. This represents the second study to analyze the role of PALB2 in early-onset breast cancer patients unselected for family history. The first study, of a Chinese population, established that PALB2 was responsible for 1.3% of early-onset breast cancer cases. Our study reports that deleterious mutations in PALB2 account for approximately 2% (1/48) of South African early-onset breast cancer.

Keywords Early-onset breast cancer · FANCN/PALB2 · Mutation · Sequence variation · South Africa

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Introduction

Breast cancer is the second leading cause of cancer mortality in women today (after lung cancer) and is the most commonly diagnosed non-dermatological cancer among women worldwide [1]. Analogously, it is the second most common cancer to afflict women in South Africa, and overall the life-time risk for developing cancer of the breast is 1 in 31 for South African women, ranging from 1 in 13 for white women to 1 in 57 for black women [2]. Five to ten percent of all breast cancers show a dominant pattern of inheritance. The two breast cancer susceptibility genes, *BRCA1* [3] and *BRCA2* [4] are clinically the most important genes associated with this type of cancer and are thought to be responsible for 0.7–29 and 1.5–25%, respectively for familial breast cancer cases. In South Africa, *BRCA1* and *BRCA2* disease-causing mutations are responsible for 19 and 47% of familial breast cancer [5, 6]. These data indicate that other susceptibility genes exist that play a role in the etiology of hereditary breast cancer. Various other lower penetrance breast cancer susceptibility genes have been identified, such as *CHEK2*, *p53* and *ATM*, but even so, a large portion of families with site-specific, early-onset breast cancer cannot be explained. The breast cancer susceptibility genes identified thus far, are all involved in a DNA damage response pathway [7, 8]. This suggests that impaired genome stability control may be a cause of familial breast cancer, and raises the possibility that germ line mutations in other genes involved in this pathway also predispose to breast cancer.

PALB2 (partner and localizer of BRCA2) is a recently identified *BRCA2*-interacting protein, which appears to be essential for the association of BRCA2 with chromatin and nuclear structures and for its functions in the DNA damage response pathway [9]. *BRCA2* (also known as *FANCD1*)

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and *PALB2* (also known as *FANCN*) are also Fanconi Anemia (FA) genes [10–12]. Biallelic mutations in either of these genes result in a FA disease phenotype in which affected children display typical FA symptoms such as early bone marrow failure, growth retardation, severely increased cancer risk and cellular hypersensitivity to DNA cross-linking agents [13].

Since biallelic mutations in both *BRCA2* and *PALB2* result in such similar phenotypes, and monoallelic mutations in *BRCA2* predispose to breast cancer, it was proposed that monoallelic *PALB2* mutations might also predispose to breast cancer [9, 11, 12]. Studies of familial breast cancer in Finnish [14], Spanish [15], British [16] and Canadian [17, 18] populations have confirmed that, like *BRCA2*, *PALB2* is indeed a breast cancer susceptibility gene. These studies have shown that *PALB2* is a moderate- to low-penetrance gene in which mutations are associated with doubling of breast cancer risk [19]. Most of these studies report that deleterious mutations in *PALB2* account for approximately 1% of familial breast cancer cases. Only one other study has considered the involvement of *PALB2* in early-onset breast cancer cases without a family history; these results describe a 1,3% frequency in the Chinese population [20].

Due to the involvement of *PALB2* in breast cancer in these populations and the fact that *PALB2* mutation frequencies in South African women is unknown, we screened for mutations in the exonic regions and splice junctions of *PALB2*, in a cohort of 48 white South African breast cancer patients (aged 29–45 years), unselected for family history.

Materials and methods

Patients and controls

A cohort of 48 white South African breast cancer patients attending the Oncology clinic at Steve Biko Academic Hospital, Pretoria were included in the study on the basis of having been diagnosed with breast cancer at or before the age of 45 years (Age range 29–45 years, mean 39.5 years). No selection based on family history was made. Of the patients selected, four (8%) reported a first-degree relative affected with breast cancer, ten (21%) a second-degree relative and three (6%) had both a first and a second-degree relative affected with breast cancer. The remaining patients did not report any family history of the disease. Controls consisted of 75 healthy individuals representative of the white South African population. Written informed consent was obtained from each patient for participation in the study that was approved by the Ethics Committee of the Faculty of Health Sciences, University of Pretoria.

Mutation analysis

Genomic DNA was extracted [21] from patient blood samples and the 13 *PALB2* exons amplified in 19 fragments, using both newly designed [22] (Table 1) and previously described primers [14, 17].

Exons 4 and 5 were bidirectionally sequenced using BigDye^{TM} Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and analysis performed on the ABI 3130 (Applied Biosystems, Foster City, CA, USA). All remaining fragments were screened using SSCP/HA (Single Strand Conformation Polymorphism/Heteroduplex Analysis) by means of polyacrylamide-based Mutation Detection Enhancement (MDE) gel (Lonza, Rockland, ME, USA) electrophoresis. Migrating fragments were visualized by silver staining. Samples displaying abnormal SSCP/HA patterns for particular fragments were sequenced. Sequences utilized for naming *PALB2* sequence variants were obtained from the NCBI RefSeq database [23] and were NG_007406.1 (genomic), NM_024675.3 (mRNA) and NP_078951.2 (Protein). Description of sequence variants are as recommended by the HGVS [24].

In silico analysis

Missense mutations were analyzed in silico using the following freely available web-based tools: PolyPhen [25, 26] (Polymorphism Phenotyping), SIFT [27, 28] (Sorting Intolerant from Tolerant) and Align-GVGD [29, 30] (Grantham Variance-Grantham difference). These classifier algorithms were specifically chosen since A-GVGD has better specificity than sensitivity (low risk of false positive pathogenic prediction), while SIFT and PolyPhen illustrate better sensitivity (low risk of false negative neutral prediction) [31]. Concordance between these methods will therefore be a strong predictor.

Table 1 List of newly designed primers

Primer name	Primer sequence	Amplicon size (bp)
2,3F*	gtgctactccctgcctcttg	278
2R	gagacaaaaacagccccaga	
3F	gccttcaggtaagtgaatcgta	312
2,3R*	cacactgtgggaaaaagaacaa	
13AF	tggatatgtaatctgaattatatcttc	298
13AR	gaggcccaatatatccagaaaa	
13BF	ggctggacaaaaagatggaa	216
13BR	atccaagatcagtggtgctac	
13CF	aaatgattgcttgtttatgtcc	250
13R*	tgcttctgcaaatgatcttga	

* Primers previously described by Tischkowitz et al. [17], used together with the newly designed primers [22] as a primer pair

Protein multiple sequence alignments (MSAs) for use with A-GVGD were constructed using EXPRESSO (3DCoffee)::Regular [32], a web-based program [33] specifically used to align multiple sequences, while taking protein structure into account in order to improve the alignment [32]. Ten sequences (obtained from *Ensembl* [34] were utilized for construction of the MSA and these included: *Homo sapiens* (human, ENSP00000261584); *Bos taurus* (bovine, ENSBTAP00000008398); *Equus caballus* (horse, ENSECAP00000006459); *Gallus gallus* (chicken, ENSGALP00000009796); *Macaca mulatto* (macaque, ENSMMUP00000022763); *Monodelphis demestica* (opossum, ENSMODP00000008251); *Mus musculus* (mouse, OTTMUSP00000025884); *Pan troglodytes* (chimpanzee, ENSPTRP00000013473); *Rattus norvegicus* (rat, ENSRNOP00000041446); and *Vicugna pacos* (alpaca, ENSVPAP00000005990).

Results

PALB2 sequence variation

Sequence analysis revealed two novel mutations in exon 4; one missense mutation (E211G) and a frameshift mutation (c.697delG). Sequence analysis of 75 control samples did not reveal either of these variants. In addition to these, four previously reported missense variants, one synonymous change and three novel intronic variants were detected (Table 2).

The patient carrying the frameshift mutation c.697delG (Fig. 1) was of British descent and was diagnosed with infiltrating ductal carcinoma at the age of 44. The tumor was estrogen and progesterone negative, and tested negative for human epidermal growth factor receptor 2 (Her2/neu^{-}). No other breast cancer cases were reported in her family, but there is a history of pancreatic, colon and prostate cancers (Fig. 2). The c.697delG mutation occurs in codon 233 and should produce a truncated protein, by introducing a stop at codon 237. The mutant protein would consist of 232 native PALB2 residues, and four foreign residues. Since this truncation occurs well upstream (>50 bp) of the last splice site of the protein, the resulting mRNA will in all probability be degraded via nonsense mediated decay. However, should the mRNA evade this nonsense mediated decay, the resulting protein will be unable to bind to BRCA2 due to a loss of the WD-40 repeats, which are the protein–protein interacting motifs that regulate interaction of PALB2 with BRCA2.

The patient carrying the novel E211G missense variant was of Afrikaner descent and was diagnosed with breast cancer at the age of 42. This missense variant results from

Table 2 *PALB2* sequence variants detected in white South African breast cancer patients

Exon/intron	Nucleotide change[a]	Effect on protein[a]	Patient ethnicity[b]	Cases		Controls carrier frequency % (*n*/*N*)[e]	References[f]
				Carrier frequency, % (*n*/*N*)[c]	allele frequency % (*a*/*A*)[d]		
Exon							
4	c.632A > G	E211G	Afr	2.08 (1/48)	1.04 (1/96)	0 (0/75)	–
4	c.697delG	V233fs	Eng	2.08 (1/48)	1.04 (1/96)	0 (0/75)	–
4	c.925A > G	I309V	Afr	2.08 (1/48)	1.04 (1/96)	–	[16]
4	c.1676A > G	Q559R	Afr/Eng	14.58 (7/48)	7.29 (7/96)	–	[14–16], [20]
5	c.2014G > C	E672Q	Afr	6.25 (3/48)	3.13 (3/96)	–	[15], [16]
9	c.2993G > A	G998E	Afr	4.17 (2/48)	2.09 (2/96)	–	[15], [16], [20]
12	c.3300T > G	T1100T	Afr	6.25 (3/48)	3.13 (3/96)	–	[14–16]
Intron							
IVS3	c.212-58A > C	–	Afr	6.25 (3/48)	3.13 (3/96)	–	–
IVS5	c.2514 + 71delC	–	Afr	2.08 (1/48)	1.04 (1/96)	–	–
IVS10	c.3114-51T > A	–	Afr	2.08 (1/48)	1.04 (1/96)	–	–

[a] RefSeq *PALB2* sequences: NG_007406.1 (genomic DNA) and NP_078951.2 (protein), standardized nomenclature was utilized [24]

[b] *Afr* Afrikaner ancestry; *Eng* British ancestry

[c] Carrier frequency refers to the number of carriers (*n*) out of the number of screened samples (*N*)

[d] Allele frequency refers to the number of mutant alleles (*a*) out of the number of screened alleles (*A*). Since all carriers are heterozygous for the particular mutation, $a = n$ and $A = 2N$ in all cases

[e] Controls were analyzed for the two novel sequence variants in exon 4

[f] Previous studies reporting the sequence variants: see reference list

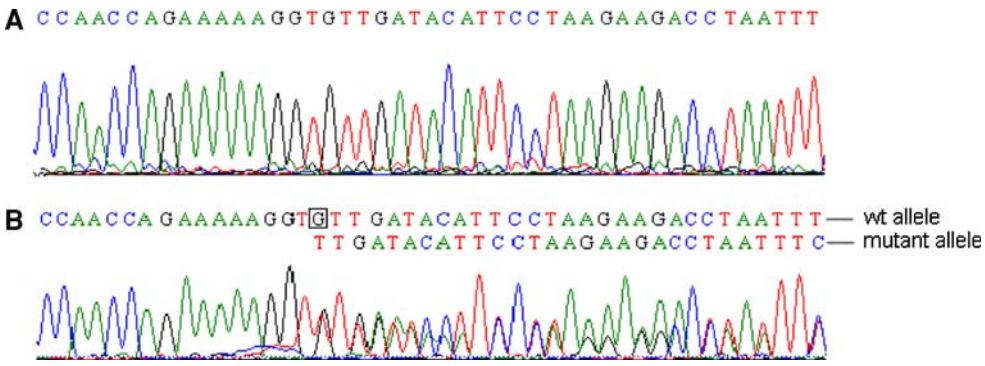

Fig. 1 Electropherogram for the sense strand of exon 4 of *PALB2*. **a** Wildtype sequence. **b** Carrier individual for the frameshift variant c.697delG. DNA sequence reveals a heterozygous G nucleotide deletion at nt697 (boxed), resulting in a frameshift (V233fs) and subsequent chain termination

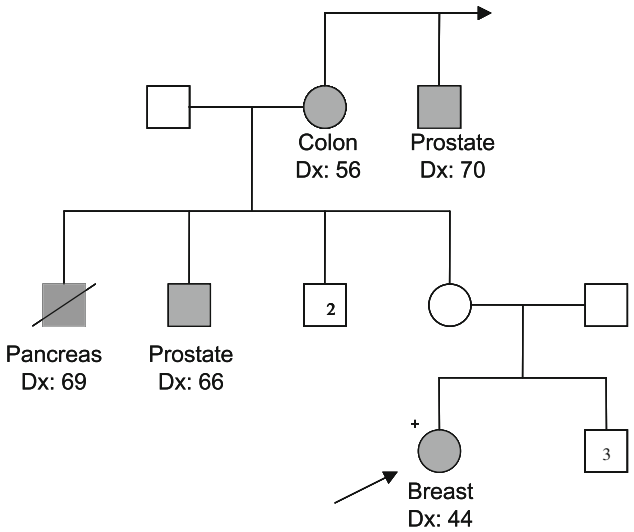

Fig. 2 Pedigree of family positive for V233fs. Individuals affected with cancer are indicated by shaded blocks/circles. The index case is indicated with an arrow. Dx: age at diagnosis

an A–G substitution in codon 211 (Fig. 3). It is a non-conservative substitution of an acidic glutamate for a polar uncharged glycine. The patient's mother (Fig. 4) was diagnosed with estrogen receptor positive breast cancer at the age of 67 years, and sequencing for this missense verified that she was indeed also a carrier. None of the 75 control samples sequenced for this variant were found to carry it.

In silico analysis

All missense substitutions detected in this study were analyzed with PolyPhen [26], SIFT [28] and Align-GVGD [30] (Table 3). Align-GVGD calculates the GV (Grantham Variation) for positions within a MSAs, which is essentially a measure of the amount of observed biochemical variation at a specific position. Align-GVGD then also calculates the GD (Grantham Difference) for particular missense mutations at those positions, thereby measuring the biochemical difference between the mutant and the observed variation at that position. These two scores are then combined to provide a classifier which ranges from C0 to C65, least likely deleterious, to most likely deleterious. MSAs were constructed using 3Dcoffee [32], and this data used for Align-GVGD analysis. MSAs, GV values and the Align-GVGD classifier for the G998E variant is shown in Fig. 5. The smaller the GV score is, the better the amino acid is conserved between the species for which the MSA was constructed. It is therefore clear from Fig. 5 that the amino acid at position 998 is conserved.

In silico analysis of the novel E211G variant revealed that all three classifier algorithms were in agreement that it is neutral. For the variant E672Q, PolyPhen and A-GVGD predict it to be neutral, while SIFT contradicts this prediction, but with a very low confidence. The E672Q variant has, however, been detected with an allele frequency of 3%

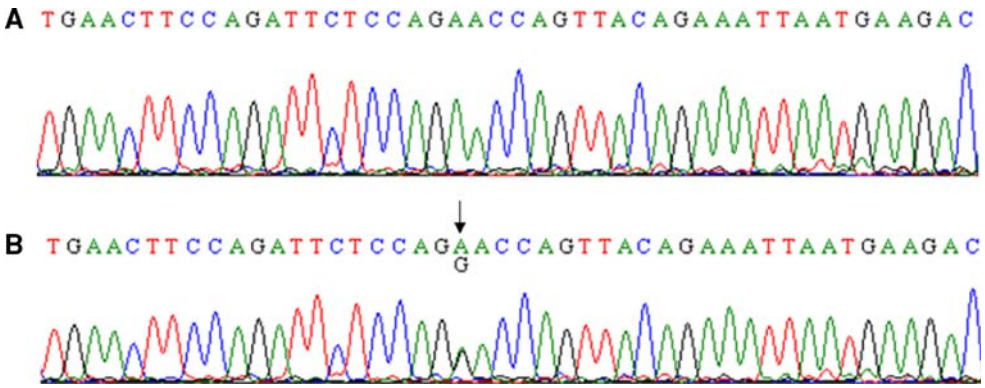

Fig. 3 Electropherograms for sense strand of exon 4 of *PALB2*. **a** Wildtype sequence. **b** Carrier individual heterozygous for the missense variant E211G. DNA sequence reveals an *A* > G substitution at nt632 (arrow)

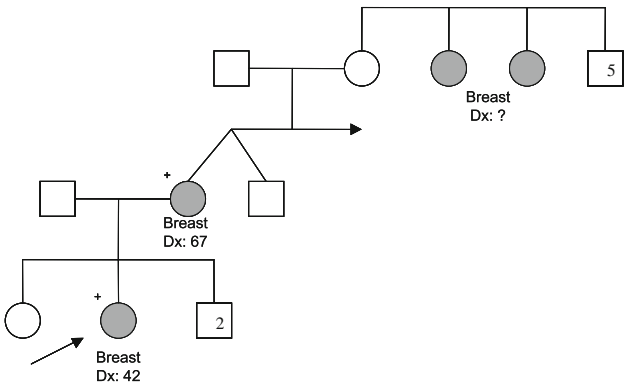

Fig. 4 Pedigree of family positive for E211G. Shaded circles indicate individuals affected with breast cancer. The index case is indicated with an arrow. Dx: age at diagnosis

(65/2168) in a control population studied by Rahman et al. [16]. Together, this evidence suggests that E672Q is not damaging. The G998E variant is predicted by all three in silico programs as deleterious, but SIFT makes this prediction with a very low confidence. In disagreement with this finding, G998E was found to occur in 44 of 2,168 alleles (2%) studied in a control population by Rahman et al. [16], reducing the likelihood of this variant being deleterious. The G998E variant was detected in two patients in the current study, where both were also found to carry two other missense variants, Q559R, and E672Q, a synonymous (T1100T) and an intronic variant (c.212-58A > C). With the exception of the G998E variant, a third patient was found to harbour all of these alleles. Each of the five variants detected in these different combinations (excluding the intronic variant) has been previously detected in the British population [16], while all excepting I309V were found in the Spanish population [15]. In addition, G998E was found in Chinese patients [20] and Q559R was detected in samples from the UK [16], Finland [14], Spain [15] and China [20].

Discussion

Previous studies have established *PALB2* as one of ten known genes associated with hereditary breast cancer [14–18, 20], in which mutations confer doubling of breast cancer risks [19]. The prevalence of *PALB2* variants in the South African population has not yet been established. It is for this reason that we assessed the role/impact of *PALB2* sequence variants in white South African breast cancer patients.

Recent studies have shown that deleterious mutations in this gene account for a moderate (0.83–1.5%) proportion of hereditary breast cancer cases in Canadian, British and Spanish populations [15–18]. This proportion is much higher (2.7%) in the Finnish population, where a founder mutation was identified [14]. Cao and colleagues [20] analyzed 360 Chinese patients, of which 150 were early-onset (<35 years) cases without family history. In total, three of these patients were identified with a truncating *PALB2* mutation, two of whom were in the early-onset patient group with no family history of breast cancer, thus accounting for a carrier frequency of 1,3% (2/150) in that group. Our study revealed one pathogenic variant from 48 patients, accounting for a carrier frequency of 2% in this white South African cohort with relatively early-onset breast cancer.

Table 3 Pathogenicity prediction of missense variants using three in silico methods

Variant	In silico prediction		
	PolyPhen	SIFT	A-GVGD
E211G	Benign	Tolerated	Neutral
I309V	Benign	Tolerated	Neutral
Q559R	Benign	Tolerated	Neutral
E672Q	Benign	Affect protein function	Neutral
G998E	Probably damaging	Affect protein function	Most likely deleterious

Fig. 5 A Section of PALB2 alignment from human residue A968. The mutant residue for the human missense substitution G998E is positioned above the alignment, together with the GV score and A-GVGD classifier

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                                  G998E
                                  GV=0, C65
Human     968  AVLGLTKRRLVSSSGTLSDQQVEVMTFAEDGGGKENQFLMPPEETILTFAEVQGMQEALL
Bovine         AVLGLAKRRLVSSSRTLCDQQVEMMTVAEDGGSKEKQLLMPPEETILTFAEVQGMQEALL
Horse          AVLGLTQRRLVTSSGTLCDQQVEIMTFAEDGGSKEKQFLMPPEETILTFAEVQGMQEALL
Chicken        AVLGLRDGKLISSSRAMQEQQVEIVSLSETGRCKDRQTLMPPEETVLAFAEVEGVRDALV
Macaque        AVLGLTKRRLVSSSGTLSDQQVEVMTFAEDGGGKENQFLMPPEETILTFAEVQGMQEALL
Opossum        AVLGLRQKRLVCSNGTLHDHRVDLMTFSESGRSKEKQTLMSPEETILAFAEIQGMQDALL
Mouse          AMLGLTKRRLVSSTGTFCNQQIQIMTFADDGSSKDEQLLMPPDETVLTFAEVQGTQEALL
Chimpanzee     AVLGLTKRRLVSSSGTLSDQQVEVMTFAEDGGGKENQFLMPPEETILTFAEVQGMQEALL
Rat            AMLGLTKRRLVSSTGTFCNQQIQIMTFAEDGSSKDEQLLMPPDETILTFAEVHGMQEALL
Alpaca         TVLGLTKRRLVSSSGTLCDQQVEIMTFAEDGGSKEKQFLMPPEETILTFAEVQGMQEALL
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The pathogenic variant identified in this study is a novel truncating mutation, c.697delG (V233fs), which is the second most 5′-deleterious mutation described to date. The most proximal pathogenic variant, c.229delT (C77fs), that was described by Tischkowitz et al. [17] would generate a protein that consists of 76 of the 1,186 native PALB2 residues, and 99 non-native residues. In contrast, V233fs would generate a protein with 232 native residues and four non-native residues, also rendering it the second shortest pathogenic variant described so far. Interestingly, there is a history of prostate, pancreatic as well as colon cancer in the family identified with the V233fs mutation (Fig. 2). This supports the finding by Erkko et al. [14] that all mutation positive families in their cohort had other forms of cancer. Erkko also found that the Finnish founder mutation segregated in one family with four cases of prostate cancer, leaving us to wonder whether this gene also increases the risk for prostate cancer. However, the age at diagnosis for prostate cancer in our family is very close to the average age of diagnosis for sporadic prostate cancer [35]. Nevertheless, it would be interesting to screen the members of this family for the *PALB2* variant.

Previous studies have reported certain trends in the hormone receptor status of tumors with deleterious mutations in *PALB2*. It appears as if PALB2 tumors have similar phenotypic properties as BRCA2 tumors, in that they are most commonly estrogen and progesterone receptor positive [14, 17, 20]. There is, however, currently no consensus on human epidermal growth factor receptor (Her2/neu) status. Interestingly, our results are in contrast with previous findings, since the individual carrying the frameshift mutation V233fs had estrogen- and progesterone receptor negative tumor status. This is, however, similar to the findings of a recent study involving French-Canadian women, in which the authors report ER negative tumor status in two of three *PALB2* mutation carriers [18]. Clearly the hormone receptor status of *PALB2*-related breast tumors is still controversial, and further studies will be needed to obtain a better understanding of the phenotypic properties of these tumors.

As far as the novel missense variant (E211G) is concerned, it was not predicted to have a damaging effect on the protein when analyzed by PolyPhen, SIFT and A-GVGD prediction programs. However, its non-conservative nature, the fact that it occurs in a region of PALB2 that is evolutionarily conserved among primates, and the finding that the patient's mother (diagnosed with breast cancer at the age of 67) was also a carrier (Fig. 4.) of the E211G missense variant strengthen the possibility that this variant may possibly increase breast cancer risk. Additionally, it was not detected in any of the 75 control individuals. Admittedly, the control group is too small to be convincingly informative. At this stage, this variant cannot be ruled out as a non-disease causing mutation but further studies are required to determine whether or not it increases breast cancer risk.

Of the remaining missense variants found, in silico analysis showed possibly damaging effects for only two. SIFT predicted with a low confidence, a possibly damaging effect on protein function for the missense mutations E672Q and G998E, while PolyPhen and A-GVGD predicted a damaging effect for only G998E. Despite these predictions, previous studies have detected high frequencies of both variants in control populations [14, 16], increasing the likelihood of these variants being non-pathogenic. Interestingly though, three particular individuals in this study were found to carry a combination of a number of mutations including the missense variants Q559R and E672Q, the synonymous mutation T1100T, and the intron 3 variant. In addition to these, two of the three patients also carried the missense G998E. Although each of these mutations is probably individually neutral, it is tempting to speculate that there may be a link between this combination of variants and breast cancer susceptibility. Nevertheless, the pathogenic significance of the missense and synonymous variants detected cannot be confirmed until functional studies have been completed.

Since the majority of the gene was screened using SSCP/HA (only exons 4 and 5 were directly sequenced), it is possible that a number of mutations were missed, since SSCP/HA is expected to only have a sensitivity of approximately 80%. Therefore, the frequency of deleterious mutations detected in this study (2%) may be an underestimate. On the other hand, this estimate could be biased due to the small sample size (48 samples).

From the results obtained in this study, it appears as if mutations in *PALB2* account for approximately 2% of relatively early-onset ($\leq$45 years) breast cancer cases in South African women. In addition, approximately half of the *PALB2* variants comprise mutations in exon 4, confirming the observation found in other populations [14, 15, 20]. This study represents the first to analyze the involvement of *PALB2* in South African breast cancer susceptibility, and only the second study worldwide to consider the involvement of this gene in a cohort of early-onset breast cancer patients unselected for family history. It emphasizes the need for further studies to uncover the role of *PALB2* in breast cancer in the South African population. In addition, further investigations will be needed to determine the phenotypic impact of particular mutations, or combinations of mutations, in order to draw meaningful conclusions regarding the clinical implications for carriers of these *PALB2* variants.

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