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PRECLINICAL STUDY

PALB2 analysis in *BRCA2*-like families

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Abstract BRCA2 and PALB2 function together in the Fanconi anemia (FA)–Breast Cancer (BRCA) pathway. Mono-allelic and bi-allelic *BRCA2* and *PALB2* mutation carriers share many clinical characteristics. Mono-allelic germline mutations of *BRCA2* and *PALB2* are risk alleles of female breast cancer and have also been reported in familial pancreatic cancer, and bi-allelic mutations cause a severe form of Fanconi anemia. In view of these similarities, we investigated whether the prevalence of *PALB2* mutations was increased in breast cancer families with the occurrence of *BRCA2* associated tumours other than female breast cancer. *PALB2* mutation analysis was performed in 110 non-*BRCA1/2* cancer patients: (a) 53 ovarian cancer patients from female breast-and/or ovarian cancer families; (b) 45 breast cancer patients with a first or second degree relative with pancreatic cancer; and (c) 12 male breast cancer patients from female breast cancer families. One truncating *PALB2* mutation, c.509_510delGA, resulting in p.Arg170X, was found in a male breast cancer patient. We conclude that germline mutations of *PALB2* do not significantly contribute to cancer risk in non-*BRCA1/2* cancer families with at least one patient with ovarian cancer, male breast cancer, and/or pancreatic cancer.

Keywords *PALB2* · *BRCA2* · Breast cancer · Fanconi anemia · Ovarian cancer · Pancreatic cancer

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Introduction

Breast cancer (BC) is a major cause of cancer-related deaths among women. At present, 20% of familial breast cancer is explained by germline mutations in known breast cancer predisposition genes. The high risk breast cancer susceptibility genes *BRCA1* and *BRCA2* account for the majority of these causative mutations [1]. Heterozygous *BRCA1* mutation carriers are at high risk of female breast cancer and ovarian cancer. Heterozygous *BRCA2* mutation carriers are at high risk of female breast cancer, and several other cancers. Estimated cumulative risks from several studies varies in *BRCA2* female carriers from 14–24% for ovarian cancer, and 3% for pancreatic cancer [2, 3]. In male *BRCA2* carriers, cancer risk is estimated around 6–7% for pancreatic cancer and male breast cancer [2, 3].

One of the pathways often involved in breast cancer susceptibility is the Fanconi anemia (FA)–Breast Cancer (BRCA) pathway [4]. At present, 13 FA genes have been identified. FA is an autosomal recessive or X-linked inherited disorder with hypersensitivity to DNA cross-linking agents and chromosomal instability at the cellular level. Clinically FA is characterized by congenital malformations, bone marrow failure and predisposition to cancer, primarily acute myeloid leukemia and squamous cell carcinoma.

BRCA2 and *PALB2* are two of the currently known FA genes. *PALB2* (*FANCN*), which is named to its function as a partner and localizer of *BRCA2* (*FANCD1*) [5–7]. PALB2 has been shown to enable the BRCA2 protein to function in DNA repair by stabilization in nuclear foci [8].

BRCA2 and *PALB2*, also share additional clinical features, namely an increased risk to childhood solid tumours in bi-allelic mutation carriers and an increased risk to female breast cancer (RR of 8–10 and 2, respectively) and

pancreatic cancer in mono-allelic mutation carriers [1, 7, 9, 10].

In view of the overlapping phenotype and function of BRCA2 and PALB2, we hypothesized that *PALB2* mutations may preferentially associate with *BRCA1/2* negative tested cancer families with a *BRCA2*-like cancer phenotype, namely families in which *BRCA2*-associated tumours other than female breast cancer are observed. We screened *PALB2* in a cohort of 110 cancer patients from non-*BRCA1/2* families selected for ovarian cancer, pancreatic cancer and male breast cancer.

The prevalence of *PALB2* mutations was low in our enriched non-*BRCA1/2* cohort since only one truncating mutation was found.

Materials and methods

Cohort

A total of 110 cases from independent families referred to the clinical genetics department of the VU Medical Center in Amsterdam between 1994 and 2008 were analysed for *PALB2* mutations and large deletions. All cases were excluded for mutations in *BRCA1* and *BRCA2* genes. Individuals with a *BRCA2* associated tumour were selected for this study on the basis of being affected with: (a) ovarian cancer ($n = 53$) with a family history of female breast-and/or ovarian cancer (age at diagnosis of ovarian cancer ranged from 22–76 years, average 54 years), (b) female breast cancer with a first or second degree relative with pancreatic cancer ($n = 45$, age at diagnosis of breast cancer ranged from 23–74 years, average 43 years) and (c) male breast cancer with a family history of female breast cancer ($n = 12$, age at diagnosis of male breast cancer ranged from 32–77 years, average 54 years).

Of the 53 women with ovarian cancer, 16 had a personal history of both ovarian cancer and breast cancer. Of the 45 breast cancer cases, eight were bilaterally affected.

Mutation carrier probability in our cohort was scored with two manual *BRCA1/2* risk assessment models. Of our selected cases, 60 and 39% scored a $\geq$15% and $\geq$20% probability of being carrier of a *BRCA1/2* mutation according to Myriad II (Frank model), respectively (www.myriadtests.com). Since this model does not account for relatives with breast cancer above 50 and/or relatives with pancreatic cancer, we also scored the families with the Manchester model, which appears to be more predictive for *BRCA2* mutations [11]. The total Manchester score was $\geq$15 or $\geq$20 in 72 and 43% of the selected families, respectively.

All cases gave informed consent to search for new cancer susceptibility genes.

Sequencing and MLPA

The presence of germline mutations in *PALB2* was evaluated by direct sequencing of the entire coding region and intron–exon boundaries on genomic DNA isolated from whole blood. Primer pairs that were used have been previously described [6].

To determine the segregation of the c.509_510delGA mutation in the family, a nested PCR within exon 4 of *PALB2* was performed using the following forward (F) and reverse (R) primer sequences surrounding the mutation; F 5′-GAA GCA GCA GAA GAG GAC AT-3′ and R 5′-TTC AGT TAC TGG TGA TCT AGC-3′. DNA was isolated from whole blood from one sister, and DNA was isolated from paraffin embedded normal and tumour tissue from the deceased sister (Fig. 1).

The presence of large deletions in the *PALB2* gene was analyzed by Multiplex Ligation-dependent Probe Amplification (MLPA) using the MLPA P057 kit of MRC-Holland as previously described [12]. As a positive control, genomic DNA from the previously described *PALB2* FA patient EUFA1341 was included in the analysis [6].

All coding exons (including intron/exon boundaries) of *BRCA1* and *BRCA2* were screened for mutations by a combination of denaturing gradient gel electrophoresis and DNA sequencing. In addition, *BRCA1* was tested for exon deletions and duplications by MLPA.

Results

Sequence analysis of the *PALB2* gene in a selected cohort of 110 non-*BRCA*1/2 cases (for selection see Materials and Methods) revealed a frameshift mutation in exon 4, c.509_510delGA. The mutation is predicted to result in p.Arg170X at the protein level and was identified in one of the male breast cancer cases (Fig. 1). The male patient had breast cancer and a melanoma in situ, both at age 62 years. The breast cancer was a grade 3 invasive ductal carcinoma, which was positive for Estrogen (ER) and Progesteron (PR), and negative for Her2. He died at the age of 64 years. The melanoma in situ was located on his back.

The *PALB2* mutation was not present in DNA isolated from paraffin-embedded tumour and normal breast tissue from the deceased sister with lobular breast cancer (ER negative, PR negative and Her2 positive). DNA of the mother and one of the affected sisters with breast cancer was not available. Although not considered as a *BRCA*-associated tumour, we did test whole blood DNA from the sister with uterine carcinoma at age 77 years. She did not carry the mutation either.

Seven different amino acid substitutions were found. All are known SNP's and six have been previously described to

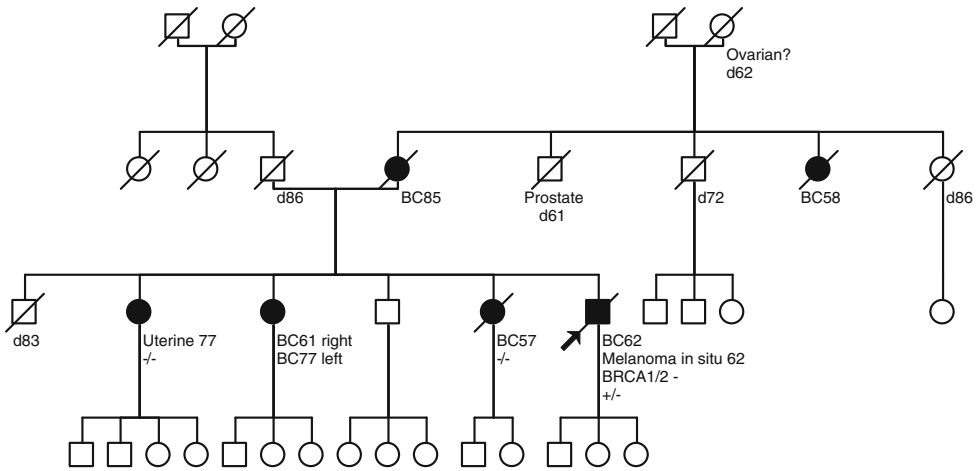

Fig. 1 Pedigree of the family with the c.509_510delGA *PALB2* mutation. In *black* are the individuals affected with cancer; *arrow* indicates the patient with the c.509_510delGA mutation. The mutation carrier was *BRCA*1/2 negative tested. Cancer type (*BC* breast cancer), side and age of cancer diagnosis or if unknown age of death (*d*) is mentioned below the individuals. Tested individuals were marked with −/− for wild type (WT) or +/− for mono-allelic mutation carrier

occur frequently and in equal percentages in affected breast cancer cases and controls [13]. One of these variants, the L939W, is predicted (although with low confidence) to possibly affect the protein function in three in silico tests (PolyPhen, SIFT and align GVGD). However, this variant was found in three previous studies including the large breast cancer case control study by Rahman et al. [13–15]. Therefore all missense variants are unlikely to have a significant pathogenic effect (Table 1). Intronic and synonymous variants which are not predicted to affect splicing are unlikely to have any clinical relevance (Table 1).

The cohort was also analyzed for larger deletions using MLPA in *PALB2*. No alterations in the *PALB2* gene were identified by MLPA in any of the 110 cases.

Discussion

The chance of finding a cancer predisposing allele generally increases when families harbour increasing numbers of patients, or multiple phenotypes associated with the risk allele. For example the mutation rate in the high risk breast cancer predisposing genes *BRCA1* and *BRCA2* increases dramatically in an individual when multiple cases of early onset breast cancer, or additionally ovarian cancer occurs within the family (www.myriadtests.com). Likewise, for the low risk breast cancer predisposing allele *CHEK2**1100-delC the chance of finding this allele increases from 1, 3, to 5–6% in the Dutch population, in sporadic breast cancer, and in familial breast cancer or in bilateral breast cancer [16, 17]. Statistical modelling showed that this was best explained by a polygenic model in which multiple low risk alleles increased cancer risk in a multiplicative way [16].

We therefore hypothesized that the frequency of mutations in the moderate risk breast cancer predisposing gene *PALB2* may increase in case additional cancer phenotypes are present within the family.

We screened a cohort of 110 non-*BRCA1/2* cancer patients, including 53 ovarian cancer patients, 45 female breast cancer patients with a first or second degree relative with pancreatic cancer and 12 male breast cancer patients, for the presence of truncating mutations in the *PALB2* gene by sequencing and MLPA analysis. Direct sequencing revealed one truncating mutation (p.Arg170X) in a male breast cancer patient, which was not present in DNA from one sister with breast cancer and in the sister with uterine cancer (Fig. 1).

MLPA did not detect any deletion in our cohort. Previously we already described that exon deletions and duplications in *PALB2* were not observed in a large cohort of Dutch familial breast cancer patients [12].

The *PALB2* mutation frequency in this enriched cohort with *BRCA2* associated tumours was not increased when compared to the described prevalence of approximately 1% in familial non-*BRCA1/2* breast cancer cohorts [13, 14, 18].

Currently, there is evidence that *PALB2* is involved in predisposition to breast cancer and familial pancreatic cancer, but this is lacking for other cancers, in particular male breast cancer and ovarian cancer.

In total, 77 *PALB2* mutation-positive cases have been identified mainly in familial or early onset breast cancer cohorts from different populations [13, 15, 18–25]. Of the 77 described *PALB2* mutation cases, 52 are carrier of the 1592delT Finnish founder mutation, which could influence the currently known phenotype [19, 24, 26]. The selection criteria of these previous breast cancer studies varied, but most studies included *BRCA1/2* mutation negative early onset breast cancer patients with diagnosis under 50 years or breast cancer patients from families with first or second degree relatives affected with breast-or ovarian cancer in unknown numbers.

Ovarian cancer is the most prevalent cancer type in *BRCA2* mutated families next to breast cancer. Our cohort included 53 familial ovarian cancer patients, but no mutation was found in *PALB2* in any of these cases.

Table 1 Variants identified in *PALB2/FANCN*

PALB2	cDNA change	Protein change	SNP	Number found	Prediction programs		
					Polyphen	SIFT	AGVGD
m1	c.509_510delGA	p.Arg170X		1			
as1	c.194C>T	p.Pro65Leu	rs62625272	1	Possibly damaging	Tolerated	Less likely (C0)
as2	c.1010T>C	p.Leu337Ser	rs45494092	6	Possibly damaging	Affect protein function	Less likely (C0)
as3	c.1676A>G	p.Gln559Arg	rs152451	26	Benign	Tolerated	Less likely (C0)
as4	c.2014G>C	p.Glu672Gln	rs45532440	9	Benign	Affect protein function	Less likely (C0)
as5	c.2590C>T	p.Pro864Ser	rs45568339	1	Probably damaging	Tolerated	Less likely (C0)
as6	c.2816T>G	p.Leu939Trp	rs45478192	1	Possibly damaging	Affect protein function	Likely (C55)
as7	c.2993G>A	p.Gly998Glu	rs45551636	6	Probably damaging	Affect protein function	Less likely (C0)
syn1	c.1470C>T	p.=	rs45612837	1			
syn2	c.1572A>G	p.=	rs45472400	3			
syn3	c.3300T>G	p.=	rs45516100	8			
Non-coding							
ip1	c.−47 G>A		rs8053188	5			
ip2	c.3114−51T>A		rs249936	110			
ip3	c.3201+101A>G		rs249935	26			
ip4	c.2586+58C>T		rs249954	51			
ip5	c.2996+264T>C		rs420259	56			
iv1	c.−145 G>C			1			
iv2	c.2749−18C>T			2			
iv3	c.3201+95C>T			8			
iv4	c.212−58A>C			4			
iv5	c.1684+37_1684+39del			1			
iv6	c.2996+17T>C			1			

m mutation, *as* amino acid substitution, *syn* synonymous variant, *ip* intronic polymorphism, *iv* intronic variant, *SNP* is a single nucleotide polymorphism. Nomenclature according to the Human Genome Variation Society (HGVS) recommendations (www.hgvs.org). Pathogeneity of missense variants was predicted using 3 in silico tests; Polymorphism Phenotyping (PolyPhen) prediction (http://genetics.bwh.harvard.edu/pph); Sorting Intolerant From Tolerant (SIFT) (http://blocks.fhcrc.org/sift/SIFT.html) and align GVGD (http://agvgd.iarc.fr). According to the splice site prediction algorithms incorporated in Alamut version 1.5 (SpliceSiteFinder-like, MaxEntScan, NNSPLICE and GeneSplicer) no evidence was found for splice defects caused by the non-coding intronic variants

Recently, a Polish study tested a set of sporadic 70 ovarian carcinomas for *PALB2* mutations [27]. Remarkably they found the same mutation (c.509_510delGA) as we did. After this finding, they analyzed additional cohorts for the prevalence of the c.509_510delGA mutation: a cohort of 339 unselected tumour fragments of ovarian carcinomas; a cohort of 982 breast cancer patients, of which 648 familial cases; and 1310 controls. They identified this mutation in 2 out of 339 ovarian cancers (0.6%), 4 out of 982 breast cancers (0.4%; all familial cases), and 1 out of 1,310 controls (0.08%). One of the ovarian cancer patients appeared to carry a pathogenic *BRCA*2 mutation. Most of the cancer cases included in this study were not or only partially analyzed for mutations of *BRCA1* and *BRCA2*. Since the c.509_510delGA mutation was recurrently found in the Polish population, it was suggested to be a possible *PALB2* founder mutation in central Poland. Till now all mutations, except the Finnish *PALB2* founder mutation, were unique. We identified the c.509_510delGA mutation in a male breast cancer patient from Dutch ancestry. The

c.509_510delGA mutation may therefore either represent a recurrent mutation or a founder mutation with a much larger geographical distribution than central Poland.

The data of this Polish study and our data suggest that *PALB2* has only a marginal role in ovarian cancer predisposition.

Pancreatic cancer risk related to *PALB2* was evaluated in familial ($n = 197$) and in sporadic pancreatic cancer cases ($n = 114$) [10, 28]. In total, five *PALB2* mutation carriers with pancreatic cancer were identified. All but one of these five families were also affected with breast cancer and the only two female *PALB2* mutation carriers both suffered from pancreatic cancer and breast cancer. We analyzed 45 breast cancer patients with a first or second degree relative with pancreatic cancer. However, no truncating mutation was found.

The only truncating mutation (p.Arg170X) in our cohort was found in one out of 12 male breast cancers patients. This confirms the results of a previous breast cancer study in which one out of 15 female breast cancer patients with male breast cancer in the family contained a mutation (6.7%) [13]. Three previous studies have reported on the frequency of *PALB2* mutations in male breast cancer. In 141 unselected Finnish male patients, the Finnish *PALB2* founder mutation was not found [19]. The whole coding sequence of *PALB2* was analyzed in 25 Australian male breast cancer cases from breast cancer families, and in 97 *BRCA1/2* negative male breast cancer cases from Italy, of which 26% had a positive family history of breast and/or ovarian cancer in a first degree relative. No truncating mutations were found in either study [29, 30].

Overall, these data do not suggest a significant relation of *PALB2* mutations and male breast cancer.

With respect to various other cancers, the Finnish *PALB2* founder mutation was tested in two independent studies including in total 476 unselected colorectal cancer cases, 1223 unselected prostate cancer cases, and 342 familial prostate cancer cases [19, 26]. Two familial and two unselected prostate cancer cases carried the founder mutation (0.6 and 0.16% respectively). Of note, the frequency of the Finnish *PALB2* founder mutation is 0.2% in the general (Finnish) population. Tischkowitz et al. sequenced *PALB2* in 95 prostate cancer families from American descent and found no truncating mutations [31]. Overall, these data suggest that these cancer types are not significantly associated with mutations in *PALB2*. Cancer types that are still disputed to be associated with *BRCA2*, like prostate cancer, colonic cancer, melanoma, and stomach cancer were left out of our cohort.

To conclude there is no enrichment for the prevalence of germline heterozygous *PALB2* mutations in familial non-*BRCA1/2* ovarian cancer patients, breast cancer patients with a familial history of pancreatic cancer, and familial non-*BRCA1/2* male breast cancer patients. Therefore diagnostic testing of *PALB2* in non-*BRCA1/2* families is not indicated in these settings.

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Conflict of interest statement None declared by any of the authors.

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