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Analysis of *FANCB* and *FANCN/PALB2* Fanconi Anemia genes in *BRCA1/2*-negative Spanish breast cancer families

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Abstract Recent reports have shown that mutations in the *FANCB* and *FANCN/PALB2* Fanconi Anemia (FA) genes confer a moderate breast cancer risk. Discussion has been raised on the phenotypic characteristics of the *PALB2*-associated families and tumors. The role of *FANCB* in breast cancer susceptibility has not been tested to date. Likewise *PALB2* mutation frequency has not been studied in Spanish population. We analyzed the complete coding sequence and splicing sites of *FANCB* and *PALB2* in 95 index cases of *BRCA1/2*-negative Spanish breast cancer families. We also performed an exhaustive screening of three previously described rare but recurrent *PALB2* mutations in 725 additional probands. Pathogenic changes

were not detected in *FANCB*. We found a novel *PALB2* truncating mutation c.1056_1057delGA (p.K353IfsX7) in one of the 95 screened patients, accounting for a mutation frequency of 1% in our series. Further comprehensive screening of the novel mutation and of previously reported rare but recurrent *PALB2* mutations did not reveal any carrier patient. We report the first example of LOH occurring in a *PALB2*-associated tumor. Our results rule out a major contribution of *FANCB* to hereditary breast cancer. Our data are consistent with the notion of individually rare *PALB2* mutations, lack of mutational hot-spots in the gene and existence of between-population disease-allele heterogeneity. We show evidence that *PALB2* loss of function might also conform to the inactivation model of a classic tumor-suppressor gene and present data that adds to the clinically relevant discussion about the existence of a *PALB2*-breast cancer phenotype.

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Introduction

Fanconi Anemia (FA) is a rare recessive disorder (heterozygote frequency of around 1 in 250–300) characterized by chromosomal fragility, progressive bone marrow failure, congenital abnormalities and cancer predisposition including leukemias and solid tumors [1]. To date 13 FA complementation groups (FA-A, B, C, D1, D2, E, F, G, I, J, L, M, and N) have been described and with the recent identification of the gene responsible for subtype I [2] all 13 genes associated with the complementation groups are currently known. FA-A is the most common FA subtype accounting for approximately 60% of cases worldwide. The second most common groups are FA-C and FA-G with frequencies close to 9–10%, all remaining subtypes showing individual incidences lower than 4% [3]. However, these general frequencies may show variation in specific populations related to the existence of founder mutations as it has been described in Ashkenazy Jewish or Spanish Gypsies [4, 5]. Eight FA proteins, FANCA, B, C, E, F, G, L and M form a complex (FA nuclear core complex) that activates FANCD2 and, as recently shown, also the newly discovered FANCI protein through monoubiquitination [2, 6]. FANCD2 and FANCI interact with each other to form what it has been called the FA “ID” complex, both proteins being interdependent for their respective monoubiquitination [2]. Upon activation, the ID complex translocates to sites of DNA damage in chromatin, where interacts with the downstream FA proteins (acting downstream the ubiquitination step) and/or other proteins participating in recognition and repair of DNA damage [6].

In 2002 Howlett et al. discovered that the gene responsible for FA-D1 complementation group was *BRCA2*, which was the key fact that linked breast cancer susceptibility and the Fanconi Anemia-DNA repair pathway [7]. Subsequent works considered plausible that heterozygous mutation in FA genes other than *FANCD1/BRCA2* could be responsible for and increased susceptibility to breast cancer and evaluated such hypothesis. In this line of research, *FANCA*, *FANCC*, *FANCD2*, *FANCE*, *FANCF* and *FANCG* were screened for mutations in familial breast cancer patients negative for mutations in the *BRCA1* and *BRCA2* genes [8] but pathogenic sequence variants were not found. Similar analysis of *FANCF/BRIP1* was undertaken by several groups [9–12], the gene being recently reported as a breast cancer-moderate risk gene [13].

Recent data indicates that heterozygous mutations in *PALB2* (“Partner and Localizer of *BRCA2*”) -the gene

found to be responsible for the most recently described FA-N complementation group [14, 15]- account for a proportion of around 1% of hereditary breast cancer non due to *BRCA1/2* mutations [16, 17]. The relative risk of breast cancer associated with this gene was estimated at 2.3 [16], value that increased to approximately 4-fold in a study carried out in Finish population where a founder mutation was detected [18]. Initial screening of the gene in breast cancer families from other specific ethnic backgrounds such as Ashkenazi Jewish or French Canadian population did not reveal any additional *PALB2* founder mutations [17]. However, a very recent extended study of French Canadian population allowed the identification of a novel *PALB2* founder mutation [19]. Finnish and Canadian studies included loss of heterozygosity (LOH) and immunohistochemical analysis of *PALB2*-associated breast tumors. Early reports shown that most *PALB2*-neoplasms were oestrogen and progesterone receptor-positive, sharing phenotypic characteristic of *BRCA2* tumors. In contrast, the latest Canadian study suggests that these initial phenotypic observations might not be as universal as formerly thought. None of the *PALB2* tumors reported to date have been found to present LOH, which has raised speculation on alternative mechanisms of *PALB2* functional inactivation.

In view of these antecedents we have analyzed the entire coding sequence and intron–exon boundaries of *FANCB* and *PALB2* in 95 *BRCA1/2*-negative Spanish breast cancer families. Implication of heterozygous mutations of *FANCB* in breast cancer predisposition has not been tested to date. Similarly, there is no data on *PALB2* mutation frequency in Spanish families of hereditary breast cancer. Our analysis has also encompassed a thorough screening of three previously reported rare but recurrent *PALB2* mutations, c.3113G>A (p. W1038X), c.3116delA (p. N1039IfsX2) and c.3549C>G (p. Y1183X) [16], in 725 additional *BRCA1/2*-negative patients.

Materials and methods

Patients and controls

Index cases from 95 *BRCA1/BRCA2* mutation-negative Spanish breast and ovarian cancer families were screened for mutations within the entire coding sequence and splicing sites of *FANCB* and *PALB2* genes. An additional set of 725 index cases from *BRCA1/BRCA2* mutation-negative families were specifically screened for the previously reported *PALB2* recurrent mutations c.3113G>A (p. W1038X), c.3116delA (p. N1039IfsX2) and c.3549C>G (p. Y1183X) [16] as well as for novel mutations and/or interesting sequence variants found in the initial set of 95 patients. Families were ascertained in different Spanish

hospitals and were selected for mutation analysis if they contained either (i) at least three cases of breast or ovarian cancer in the same family line; or (ii) at least two first-degree relatives diagnosed with breast cancer before age 50; or (iii) at least one case of breast cancer and one case of ovarian or bilateral breast cancer in the same family line; or (iv) at least one case of breast and ovarian cancer; or (v) at least one case of male breast cancer. Controls consisted of 760 healthy individuals representative of the Spanish population, mainly recruited from the Menopause Research Centre at the Instituto Palacios (Madrid, Spain) and from the College of Lawyers (Madrid, Spain). Details of this control series have been previously published [20]. The necessary ethics committee approval was obtained as well as informed consent from all participants in the study.

Analysis of *BRCA1* and *BRCA2*

All index cases had been previously screened for mutations in the *BRCA1/2* genes by Denaturing High Performance Liquid Chromatography (DHPLC) and/or other comprehensive approaches described in detail elsewhere [21–25] and found to be negative.

Mutation analysis of *FANCB* and *PALB2* genes

DNA from the index cases was subjected to Whole Genome Amplification (WGA) by using the GenomiPhi V2 Amplification Kit (GE Healthcare UK Limited, Buckinghamshire, UK) according to the manufacturer's instructions. Then genomic fragments covering *FANCB* and *PALB2* exons and splicing sites were PCR-amplified. Primers were designed to generate amplicons of size up to 350 bp to ensure optimal yield from WGA-DNA. In all, 17 and 24 PCR fragments were required to cover *FANCB* and *PALB2*, respectively. Seven primer pairs spanning exons 6–12 of *PALB2* were taken from a previous study [15], those reported but generating fragments larger than 350 bp being discarded and redesigned. All primer pairs are detailed in Supplementary Table 1. PCR-products were analyzed by DHPLC on the WAVE HT system (Transgenomic, Omaha, NE) using an acetonitrile gradient and scrutinized for aberrant profiles with the Navigator™ Software (Transgenomic, Omaha, NE). Samples representative of each DHPLC pattern were bi-directionally sequenced using the BigDyeTerminator Cycle sequencing kit and a 3730 automated sequencer (ABI Perkin Elmer). All non-described variants/mutations were confirmed by sequencing a fresh aliquot of the non-WGA stock DNA.

Extended screening of previously described *PALB2* recurrent mutations, new mutations, and interesting sequence variants in 725 additional index cases was performed by the KBiosciences (Herts, UK) fluorescence-based

competitive allele-specific PCR assay (KASPar) except for those changes consisting of deletions that were analyzed by DHPLC. Details of the KASPar methodology can be found at <http://www.kbioscience.co.uk/>. Analysis of the *PALB2* recurrent mutations c.3113G>A, c.3116delA and c.3549C>G included positive controls kindly provided by Prof. N. Rahman. Fisher exact test was used to test differences in allele frequencies when comparison with control individuals was performed (*PALB2* sequence variant c.629C>T, p.P210L).

GenBank Reference sequences used for naming sequence variants were NM_152633.2 and NT_011757.15 for *FANCB* and NM_024675.3 and NT_010393.15 for *PALB2*. Standardized nomenclature was reported considering the A of the ATG initiation codon of the coding DNA Reference Sequence as nucleotide position +1 [26, 27].

Loss of heterozygosity (LOH) analysis

Sections from paraffin-embedded tumor from *PALB2* positive patient were obtained following revision by a pathologist and subsequent macrodissection in order to enrich sample with pure cancer cell population. DNA was extracted by standard proteinase K protocol after desparaffination. *PALB2* sequence segment spanning the gene mutation was amplified by PCR (using a reduced number of cycles) and sequenced. DNA from peripheral blood of the patient was amplified and sequenced simultaneously along the procedure. LOH was scored by a significant reduction of at least 30% of peak height in the wild allele relative to normal sequence trace.

Results

FANCB mutation analysis

In order to establish the possible role of *FANCB* as a breast cancer susceptibility gene, 95 index cases from breast cancer families negative for *BRCA1* and *BRCA2* mutations were screened for sequence variants. Analysis of *FANCB* complete coding sequence and splicing regions did not reveal any pathogenic mutation. Four sequence variants were identified, three of them were non-coding (located at the 5' upstream region or introns) and one was exonic (Table 1). All had been previously reported except for one of the intronic variants (c.2165 + 8A > G) that did not involve a consensus splice site and was not additionally investigated.

PALB2 mutation analysis

We analyzed the complete coding sequence and splicing sites of *PALB2* in 95 probands from breast cancer families

Table 1 *FANCB* and *PALB2* sequence variants identified in Spanish familial breast cancer patients

Gene/location	Nucleotide change	Protein change	Heterozygote frequency % (n/N) ^a	Reference ^b
<i>FANCB</i>				
<i>Coding</i>				
Ex 3	c.1004G>A	p.G335E	17 (16/94)	rs.41309679
<i>Non-coding</i>				
5'flanking	c.-221C>A	—	43 (40/93)	rs.2285490
IVS5	c.1427 – 10T>C	—	46 (42/92)	rs. 2905223
IVS8	c.2165 + 8A>G	—	1 (1/89)	—
<i>PALB2</i>				
<i>Coding</i>				
Ex 4	c.629C>T	p.P210L	0.37 (3/794)	1
	c.765T>C	p.D255D	1 (1/93)	1
	c.999C>T	p.T333T	0.12 (1/797) ^d	—
	c.1010T>C	p.L337S	5 (42/797) ^d	1, 2
	c.1056_1057delGA ^c	p.K353IfsX7	0.12 (1/797)	1
	c.1194G>A	p.V398V	0.12 (1/797) ^d	1
	c.1572A>G	p.S524S	1 (1/95)	1
	c.1676A>G	p.Q559R	22 (21/95)	rs.152451
Ex 5	c.2014G>C	p.E672Q	5 (5/95)	1
Ex 7	c.2590C>T	p.P864S	5 (5/95)	1
Ex 8	c.2794G>A	p.V932M	1 (1/95)	1, 2
	c.2816T>G	p.L939W	1 (1/95)	1
Ex 9	c.2993G>A	p.G998E	3 (3/91)	1
Ex 12	c.3300T>G	p.T1100T	—	1, 2
<i>Non-Coding</i>				
5'UTR	c. –47G>A	—	4 (4/94)	rs.8053188
IVS3	c.212 – 58A>C	—	5 (5/95)	—
IVS4	c.1684 + 29A>G	—	2 (2/95)	—
IVS6	c.2586 + 31T>G	—	1 (1/95)	—
IVS6	c.2587 – 38C>G	—	1 (1/95)	—

GenBank Reference sequences used were NM_152633.2 and NT_011757.15 for *FANCB* and NM_024675.3 and NT_010393.15 for *PALB2*. Nucleotide position +1 corresponds to the A of the ATG translation initiation site of the coding DNA Reference Sequences. ^a Frequency refers to number of carriers (n) out of successfully screened samples (N). Screened samples were 95 for all fragments except for that containing the novel mutation and sequence variant c.629C>T where 725 additional cases were tested. ^b dbSNP identifier or previous studies reporting the sequence variants are indicated: 1, Rahman et al. 2007 [16]; Erkkö et al. 2007 [18]. ^c Novel mutation. ^d Frequency of sequence variants contained within same PCR-fragment as the novel mutation

negative for mutations in *BRCA1* and *BRCA2* genes aiming to define the mutation frequency of the gene in Spanish population. Sequencing of one index case from our families revealed a non-previously reported frameshift mutation, c.1056_1057delGA (p.K353IfsX7), which is predicted to generate a translation-stop seven codons downstream from the first affected amino acid. Extended analysis of this novel mutation by DHPLC in 725 additional *BRCA1/2*-negative probands, 702 of them successfully screened, did not show any other carrier of the mutation. The pedigree of the family of the *PALB2*-mutation carrier is presented in Fig. 1. Interestingly, the pedigree corresponds to a female and male breast cancer family. Index case developed breast cancer at age 54, her father and one of her two sisters

having also developed breast cancer at ages 78 and 49, respectively. Unfortunately we could not assess the mutation carrier status of any of the proband's relatives with breast cancer to evaluate penetrance. However, paraffin-embedded tissue from the proband's tumor was available and LOH and immunohistochemical analysis were performed (shown below).

In addition to the c.1056_1057delGA (p.K353IfsX7) truncating mutation we identified 18 *PALB2* sequence variants (Table 1). Five of the variants were non-coding and thirteen coding. Of the non-coding variants one was located at the 5' UTR region and had already been reported and four corresponded to non-previously described intronic variants distant from the intron–exon boundaries not likely

Fig. 1 Pedigree of the breast cancer family carrying the c.1056_1057delGA (p.K353IfsX7) *PALB2* mutation. The proband screened for *PALB2* mutations is indicated by an arrow. Segregation analysis was not possible because of lack of DNA from family members other than the index case. Individuals with breast cancer are marked with a black circle/square. Age at diagnosis for cancer patients and at monitoring for healthy individuals is shown when known. A slashed circle/square indicates a deceased individual

Fig. 2 Sequence chromatograms corresponding to the c.1056_1057delGA (p.K353IfsX7) *PALB2* mutation carrier. Wt: Wild-type allele strand. Mut: Mutant allele strand. Asterisk indicates deleted GA and frameshift starting point. (a) Sequence from the patient's peripheral blood. Overlapping peaks of wild type and mutant strand show similar signal intensity. (b) Chromatogram the patient's macrodissected paraffin-embedded tumor. Wild-type strand shows a clearly diminished signal compared to that of the mutant strand indicating LOH of the wild-type allele. DNA from peripheral blood and tumor were amplified simultaneously under same PCR conditions

to be pathogenic. Of the coding variants five were synonymous and eight non-synonymous, all of them previously reported [16, 18] except for the synonymous variant c.999C>T (p.T333T). The missense variant c.629C>T (p.P210L) was detected in 2 out of 95 index cases (2%), while none out of 923 probands and 1 out of 1084 controls previously analyzed by Rahman et al. presented it [16].

Taking into account that in silico predictions suggest that this change is likely to affect the protein function [16], we decided to further characterize this change in our population in a case–control study. KASPar assay detected one carrier of the variant both, in our set of additional *BRCA1/2*-negative patients ($n = 699$ successfully genotyped) and in our group of controls ($n = 735$ successfully genotyped). Our results taken together with those of Rahman et al. render global frequencies of 0.17% in cases (3/1717) and 0.10% in controls (2/1819) ($P = 0.7$), which supports a neutral role for this missense variant.

Rahman et al. reported three *PALB2* mutations that appeared in more than one proband out of 923 screened-hereditary breast cancer families, the probands with identical mutations being unrelated and from different parts of the UK. None of these previously reported rare but recurrent mutations, c.3113G>A (2/923), c.3116delA (3/923) and c.3549C>G (3/923), were detected either in our initial set of 95 *BRCA1/2*-negative cases or in the subsequently screened 725 additional index cases. Positive controls included along with our samples allowed us to ensure the adequacy of our screening methods to discriminate these mutations.

PALB2 tumor features

In order to obtain information about the clinicopathological characteristics of the *PALB2* tumor and to assess whether loss of heterozygosity had occurred we requested the Pathology report and paraffin block of the breast tumor from the mutation-positive patient. The tumor was a grade III-invasive ductal carcinoma with areas of apocrine carcinoma in situ negative for oestrogen and progesterone receptors. We also assessed HER2 protein expression by the DAKO HercepTest™ and score protocol system (DAKO A/S, Glostrup, Denmark) that indicated a negative status. P53 staining was low (<10%) while proliferative index measured by Ki-67 showed a value of 30%. To test whether LOH had occurred in the *PALB2*-associated tumor we PCR-amplified and sequenced the segment spanning the c.1056_1057delGA (p.K353IfsX7) mutation from DNA obtained from the paraffin-embedded tissue (along with DNA from the peripheral blood of the patient as control). Chromatogram of the tumor showed a height reduction greater than 30% in the peaks from the wild type-strand relative to the sequence trace of the mutant allele indicating LOH (Fig. 2).

Discussion

FANCD1/BRCA2 is one of the two currently known high-penetrance breast cancer susceptibility genes [7]. Two

other FA genes, *FANCN/PALB2* and *FANCI/BRIP1*, have been recently shown to confer a moderate risk to develop breast cancer [13, 16, 28]. These recent findings have further strengthened the link between the FA proteins and breast cancer but while most FA genes have been tested for their association with breast cancer [8], there are still a few whose implications in breast cancer susceptibility remains unknown. *FANCB* is one of these genes and in order to elucidate its possible association with breast cancer risk we have looked for heterozygous mutations along its entire coding sequence and exon–intron boundaries in 95 *BRCA1/2*-negative index cases from Spanish breast cancer families. We did not detect any pathogenic sequence change, which would indicate that *FANCB* does not confer a high risk of breast cancer or makes a major contribution to hereditary breast cancer. This is consistent with the overall lack of involvement of FA core complex genes in this type of cancer. However, larger-scale studies are required in order to rule out the existence of rare pathogenic *FANCB* alleles that might confer a modest effect. In this sense, a recent epidemiologic study assessing whether FA heterozygotes are at increased risk for cancer has shown evidences that *FANCC* mutations are possibly low-risk breast cancer susceptibility alleles [29], a major contribution of *FANCC* to familial breast cancer having been ruled out in a previous report [8].

Since the initial description of *PALB2* as a breast cancer susceptibility gene [16], three more studies have been published that screened the gene in *BRCA1/2*-negative breast cancer families and/or women diagnosed with early-onset breast cancer from different ethnic backgrounds such as Finish, French Canadian or Ashkenazy Jewish populations [17–19]. Founder mutations were found in Finish and, recently, in French Canadian population accounting for 1% of all breast cancer in Finland and about 0.5% of breast cancer cases affecting young French Canadian women [18, 19]. Data from these studies suggested that the relative risk for breast cancer associated with *PALB2* mutations might be greater than 2, which has emphasized the need for further *PALB2* analysis in different populations so that solid conclusions on the associated breast cancer relative risk can be established. Mutations in the gene seem to be individually rare, suggesting that there are not mutational hot-spots along its sequence and that the disease-alleles are heterogeneous. However, identification and characterization of more mutations is required. Similarly, attention has been drawn towards the identification of more *PALB2* tumors in order to evaluate the existence of a *PALB2*-tumour distinctive phenotype.

Our search for *PALB2* germline mutations in 95 probands from Spanish *BRCA1/2*-negative breast cancer families revealed only one pathogenic sequence variant accounting for a frequency of 1% in our series. Our results

indicate that frequency of *PALB2* mutations in Spanish hereditary breast cancer families is similar to that described in other populations where a founder effect was not observed [16]. The pathogenic change consisted of a non-previously described truncating mutation, c.1056_1057delGA (p.K353IfsX7). This mutation is the second most 5′deleterious mutation described so far. In contrast to the most proximal one described by Tischkowitz et al. c.229delT (p.C77VfsX100) that generates a fusion protein consisting of 76 residues of the *PALB2* native protein and a long non-native tail of 99 residues [17], c.1056_1057delGA is predicted to generate a stop 7 codons downstream the first amino acid affected by the frameshift. Tischkowitz et al. suggested that such a premature truncation of the native protein might be related to the strong breast cancer history observed in the family harboring the c.229delT mutation and proposed that there might be a genotype–phenotype correlation. In this respect the pedigree of the family carrying c.1056_1057delGA (p.K353IfsX7) did not show particularly aggressive characteristics but it is worth noting that it corresponded to a male and female breast cancer family. In a previous study, 6.7% (1/15) of such families carried *PALB2* mutations compared to 1% (9/908) of families with female breast cancer only [16]. In our series the c.1056_1057delGA mutation affected to 1 out of 11 (9%) families with male and female breast cancer. The small size of our series does not allow us to be more conclusive, but our results are consistent with the notion that *PALB2* might confer a higher relative risk of breast cancer in men than in women, which seems plausible for a *BRCA2*-related gene.

Comprehensive screening of the previously described recurrent *PALB2* mutations c.3113G>A (p. W1038X), c.3116delA (p. N1039IfsX2) and c.3549C>G (p.Y1183X) [16] in nearly 800 *BRCA1/2*-negative Spanish breast cancer families did not reveal any positive case. Similarly, extended analysis of the novel c.1056_1057delGA (p.K353IfsX7) mutation did not identify any other carrier of this change additional to that found in the initial set of 95 *BRCA1/2*-negative patients. These results confirm that, except for founder mutations, *PALB2* alterations seem to be individually rare with no common or prevalent pathogenic alleles that might show different frequencies in different populations.

Analysis of the breast tumor from the c.1056_1057delGA (p.K353IfsX7) mutation carrier determined the existence of LOH. This is the first example of LOH occurring in a *PALB2*-associated tumor out of 11 analyzed to date [17, 18]. Different mechanisms have been proposed to explain *PALB2* functional inactivation such as haploinsufficiency or a dominant-negative effect of the truncated proteins [17, 18]. Here we present evidence that *PALB2* loss of function might also conform to the inactivation

model of a classic tumor-suppressor gene. Our *PALB2* tumor did not express oestrogen and progesterone receptors, which is also in contrast with prior observations that suggested that *PALB2*-associated tumors share phenotypic characteristic with *BRCA2* tumors [17, 18]. In this respect and in agreement with more recent findings [19] our data indicate that such observation might not be widely applicable to *PALB2*-tumors. This heterogeneity emphasizes the value of *PALB2* mutation reports and the need for further characterization of *PALB2*-associated tumors and families. Accumulation of data in that respect will help to draw meaningful conclusions on genotypic-phenotypic correlations and to properly evaluate the indication of germinal analysis of this gene in a clinical context.

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