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PRECLINICAL STUDY

The prevalence of PALB2 germline mutations in BRCA1/BRCA2 negative Chinese women with early onset breast cancer or affected relatives

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Abstract PALB2 has been recently identified as breast cancer susceptibility gene in western populations. To investigate the contribution of PALB2 mutations to Chinese non-BRCA1/BRCA2 hereditary breast cancer, we screened all coding exons and intron-exon boundaries of PALB2 in 360 Chinese women with early-onset breast cancer or affected relatives from five breast disease clinical centers in China by utilizing PCR-DHPLC and DNA sequencing analysis. Some genetic variants identified in the cases were then studied in 864 normal controls with no personal or family history of breast cancer. Two protein-truncating PALB2 mutations, 751C>T and 1050_1051 delAAinsTCT, were identified in three separate families, and 751C>T was a recurrent mutation. Neither of them, however, were present in the controls ($P = 0.025$). All the truncating mutations occured in exon 4 of PALB2, and there were still three unclassified variants were detected in the same fragment. We found that exon 4 accounted for 44.1% (15/34) of the person-times carrying with any variant in our study. PALB2 mutations were responsible for approximately 1% of Chinese women with early-onset breast cancer and affected relatives. Our results suggested that a detection of exon 4 before the assay of the whole PALB2 gene might be a cost-effective approach to the screening of Chinese population.

Keywords Breast cancer · PALB2 · Sequence variation · DHPLC · Chinese

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Introduction

Mutations in known high penetrance genes such as BRCA1 and BRCA2 account for only about 11.8% of early-onset and familial breast cancer patients in Chinese population [1], which indicates that there still exists low penetrance susceptibility genes playing a very important role in the etiology of Chinese hereditary breast cancer. PALB2, a recently identified Fanconi anaemia (FA) gene, can bind to the extreme N terminus of BRCA2 and stabilize BRCA2 in key nuclear structures, allowing it to function in DNA repair and at the S phase checkpoint [2, 3]. Biallelic

mutations in PALB2 cause Fanconi anemia subtype FA-N and predispose to childhood cancer [4–6], and PALB2 also has been confirmed to be low-penetrance breast cancer susceptibility alleles in western populations [7]. We hypothesized that germline mutations in PALB2 may account for some of non-BRCA1/BRCA2 early-onset and familial breast cancer patients in Chinese population.

Materials and methods

Subjects

The patients in this study had come from five different medical centers in China, including Cancer Hospital of Fudan University, Shanghai; The Affiliated Xiangya Hospital of Zhongnan University, Changsha; The Affiliated Hospital of Qingdao University Medical College, Qingdao; Shandong Cancer Hospital and Institute, Jinan; and The Second Affiliated Hospital of Zhongshan University, Guangzhou. These five medical centers were located in the South and the North of China. The inclusion criteria were one or more of the following: (1) at least one first- or second-degree relative with breast cancer and/or ovarian cancer, regardless of age; (2) at least one first-degree relative with malignant tumor beyond breast cancer and ovarian cancer or (3) breast cancer diagnosed below 35 years of age. All of the cases included in our study had previously been shown to be BRCA1 and BRCA2 mutation-negative. Three hundred and sixty cases, who had received the standard treatment at one of the five centers between 2002 and 2007 were identified. The family histories were retrieved from the medical records and standard questionaires, ascertained by the families and/or the patients personally. All the cases came from independent families. A total of 864 women with no personal or family history of breast cancer were enrolled as normal control from Cancer Hospital of Fudan University during 2003–2007. This project has been approved by the Scientific and Ethical Committee of the Cancer Hospital of Fudan University. All the participants have been given informed consent.

Mutation analysis

Genomic DNA was isolated from peripheral blood leukocytes and stored in 10 mM Tris (pH8)–1 Mm EDTA at 4°C. The youngest patient who could be contacted in each family was analyzed for mutations.

Polymerase chain reaction (PCR) primer pairs as former publications were used to amplify exons and intron-exon boundaries from genomic DNA [5]. PCRs were performed in a final volume of 25 μl containing about 25–50 ng template DNA, 5 pmol of each primer, 100 μM of each dNTP, 2.5 μl of 10× PCR buffer (containing 15 mmol/l $MgCl_2$), different volume of 25 mM $MgCl_2$ according to different exon amplified, and 0.5U of *Taq* DNA polymerase (Transgenomic, USA). The cycling profile included: an initial denaturation at 94°C for 5 min, followed by 35 cycles consisting of a denaturation step of 94°C for 30 s, primer annealing for 30 s and an elongation step of 72°C for 30 s. In the cases of tounchdown-PCR (Td), the annealing temperature started at the indicated values and was decreased by 0.5 per cycle during the first 14 cycles. The final step at 72°C was extended to 5 min. Amplicons were checked by agarose gel electrophoresis prior to the mutation analyses to confirm that no contaminating bands were present in the PCR products.

Prescreening for PALB2 mutations was carried out by denaturing high-performance liquid chromatography (DHPLC) analysis (3500HT Wave® DNA Fragment Analysis System, Transgenomic, Omaha, USA) as previously reported. In brief, crude PCR products were subjected to an additional 95°C denaturing step for 5 min followed by gradual reannealing from 95°C to 65°C over a period of 30 min prior to analysis to facilitate heteroduplex formation, and then the PCR samples were injected into a temperature-equilibrated DNA® cartridge (transgenomic) and eluted with a linear gradient of acetonitrile in 0.1 M triethylammonium acetate (TEAA) at a flow rate of 0.9 ml/min, finally detected by online ultraviolet (UV) absorbance monitoring at 254 nm. The DHPLC gradients and temperatures were determined by use of WAVEmaker or Navigator software and properly adjusted.

DNA sequencing was applied to analyze abnormally migrating fragments on DHPLC. Abnormally migrating fragments were re-amplified and gel purified, Both strands were sequenced by an automated ABI PRISM® 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. GenBank reference sequences used for naming PALB2 sequence variants were NC_000016.8 (genomic DNA) and NM_024675.3 (mRNA and protein).

Except the synonymous and intronic variants or the rare variants which had been observed $<1\%$ of the cases, the identified variants of PALB2 sequence were also analyzed in the normal controls.

Statistical analysis

PALB2 mutation frequency across groups was compared using Chi square tests or Fisher's exact test. SPSS 11.5 version for windows was used. The cutoff value for statistical significance was $P = 0.05$.

Table 1 PALB2 sequence variants in our study

Nucleotide change[a]	Effect on protein	Segment	Number of heterozygous		Reported in SNP per[b]
			Cases	Controls	
−47 G>A		5′UTR	13/360	13/379	Yes
751C>T	Q251X	Exon4	2/360	0/864	No
1050_1051delAAinsTCT	Q350 fs	Exon4	1/360	0/864	No
1273A>G	V425 M	Exon4	1/360	ND	No
1656G>A	Q552Q	Exon4	9/360	ND	No
1676A>G	Q559R	Exon4	2/360	ND	Yes
IVS4+19delGAT		Intron4	1/360	ND	No
2438T>C	I813T	Exon5	2/360	ND	No
2993G>A	G998E	Exon9	1/360	ND	Yes
3122A>C	K1041T	Exon11	1/360	ND	No
3449T>C	L1150P	Exon13	1/360	ND	Yes

[a] GenBank accession number: NC_000016.8 (genomic DNA) and NM_024675.3 (mRNA and protein). [b]Variants have been previously reported in the SNP database SNPper [21]. Fs, Frameshift; ND, not done

Results

Patients' characteristics

Three hundred and sixty patients fulfilled the inclusion criteria, 167 patients had breast cancer diagnosed before 35, 210 had at least one affected relative, 17 had both early-onset breast cancer and affected relatives. Of them, 269 came from the southern China, 91 came from the northern China. All of them were detected for PALB2 successfully. In all of the 360 patients, 47 of them reported family history of other malignancies, including stomach cancer, lung cancer, leukemia, esophagus cancer, liver cancer, pancreatic cancer, colorectal cancer, uterus cancer, and bladder cancer.

PALB2 sequence variants

In all the studied patients, two novel truncating mutations were detected in three unrelated families (Table 1), compared with none in the 864 controls ($P = 0.025$). 751C>T (Fig. 1) as a recurrent mutation generates a truncated protein which retains only 250 residues of PALB2 sequence, and the other pathogenic mutation, 1050_1051delAAinsTCT (Fig. 2), results in a little longer fusion protein (Q350 fs). As both the two exonic mutations occur before the WD-40 repeats, which are common protein–protein interacting motifs of PALB2 with BRCA2, the two truncated proteins have lost their functional domain along the whole sequence of PAlB2. Moreover, we found a frameshift mutation (IVS4 + 19delGAT) in intron 4 of PALB2, but it did not result in protein truncating. The data reduces the likelihood that truncating mutations in PALB2 are responsible for a significant fraction of non-BRCA1/BRCA2 breast cancer in Chinese population.

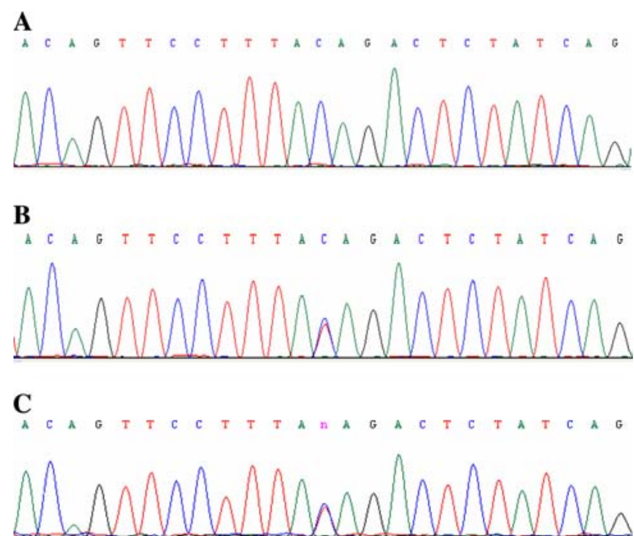

Fig. 1 Mutation detection in exon 4 of PALB2 gene. (**a**) Non-carrier individual. (**b**, **c**) DNA sequencing reveals a nucleotide C>T substitution at nt751, which results in chain termination at codon 251

In our study, we also detected nine unclassified variants (Table 1), seven of which were never reported in breast cancer. Except −47G>A and 1656G>A, the other variants were observed in <1.0% of the cases, most of which were missense variants, −47G>A was located in 5′UTR. The likelihood of carrying −47G>A was also detected in the controlled individuals, but we did not find the significant difference ($P = 0.894$).

Clinical features of mutation carriers

We analyzed the clinical features of PALB2 truncating mutation carriers in the current study (Table 2). Three

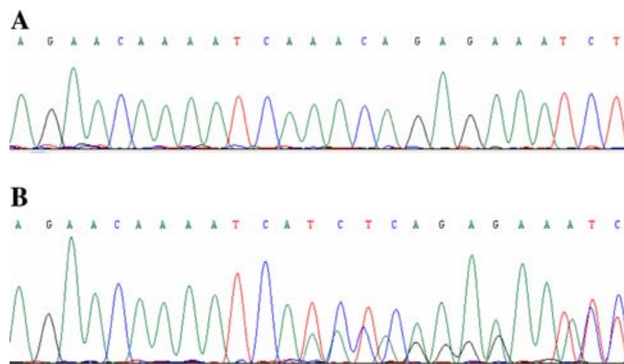

Fig. 2 Mutation detection in exon 4 of PALB2 gene. (**a**) Non-carrier individual. (**b**) DNA sequencing reveals a AA deletion and TCT insertion at nt1050_1051, which generates a fusion protein (Q350 fs) that retains only 349 residues of native PALB2 sequence

PALB2 mutation carriers included two early-onset breast cancer patients who both carried the mutation 751C>T. Another frameshift mutation carrier was diagnosed at age of 44 with familial breast cancer history of first-degree relatives, the proband's mother was diagnosed breast cancer at the same age. We further sequenced exon 4 of PALB2 from DNA sample of the proband's mother, and verified that she carried the same mutation (data not shown). All of PALB2 truncating mutation carriers reported no family history of other malignancies beyond breast cancer. The median age at diagnosis of individuals with PALB2 mutations in our study was 36.7 years (variation 32–44 years), compared with a median age at diagnosis of 48.2 years (variation 25–81 years) in individuals with breast cancer without PALB2 mutations.

Pathology of mutation-samples

The breast specimens from four women with PALB2 truncating mutation were also studied. As shown in Table 3, cancer type comprised infiltrating ductal and lobular carcinoma (IDC and ILC) in PALB2-related tumors. Histological grade and nodal involvement could only be available from three carriers, and higher grade and axillary lymph nodes metastasis were found to be predominant. Immunohistochemistry was also performed on the above tumor tissue, which showed the expression of estrogen receptor (ER) or progesterone receptor (PR), as well as moderate expression of human epidermal growth factor receptor 2 (HER2/neu) in two of them.

Discussion

Germline mutations in 10 different genes in pathways critical to genomic integrity are associated with inherited breast cancer**,** and the prevalence of deleterious mutations in such genes may be variable among different populations owing to the environmental and genetic factors [8], Previous studies have identified different mutation sites in PALB2 [9–13], and protein-truncated mutations of PALB2 have been confirmed to confer doubling of breast cancer risks in western populations [7].

Up to now, there is no investigation about the prevalence of PALB2 mutations in Chinese population. In our multi-hospital-based study, we screened 360 women with early-onset or familial breast cancers for PALB2 mutations. To our knowledge, this is also the first study about the germline mutations of some low penetrance genes of breast cancer in Chinese population.

Two distinct heterozygous truncating mutations were observed in PALB2, both resulted in amino acid changes (Q251X and Q350 fs) in the helicase domain of the protein, and 751C>T is a recurrent mutation which occurred in two separate patients. In the current study, all deleterious

Table 2 Clinical features of PALB2 truncating mutation

Individual	Mutation	Age at diagnosis	Family history	
			Breast cancer	Other malignancies
1	751C>T	34	Nil	Nil
2	751C>T	32	Nil	Nil
3	1050_1051delAAinsTCT	44	Mother	Nil

Table 3 Pathology of the breast specimens from PALB2 truncating mutation carriers

Individual	Mutation	Cancer type	Histological grade	Nodal involvement	IHC
1	751C>T	IDC	II	4/18	ER++, PR+, HER2+
2	751C>T	IDC	II	0/13	ER+, PR++, HER2+
3[a]	1050_1051delAAinsTCT	ILC	III	2/8	ER-, PR+, HER2-
4[a]	1050_1051delAAinsTCT	IDC	N/A	N/A	N/A

[a] Two carriers in the same family, 3-the proband, 4-the proband's mother; IHC, immunohistochemistry; IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma; N/A, not available

mutations were found in affected women. Consistent with the data described previously in other ethnic groups [9–13], the likelihood of carrying PALB2 truncating mutations is approximately 1% (3/360) in Chinese population.

As the allele frequencies of these variants in the controls were too low (0/864) to generate a robust estimate of the relative risk, we were unable to estimate the penetrance for PALB2 in our population. The average age of disease onset for PALB2 mutation-positive individuals was 36.7 years, which seems slightly younger than that in BRCA2 mutation carriers in our former study (42.7 years).

Six studies have reported ten distinct truncating mutations in PALB2 associated with an increased risk for breast cancer [9–13]. Among these mutations, 2323C>T (Q775X) has been recently identified as a founder mutation in French Canadians, which was found in about 0.5% (2/356) of unselected French-Canadian women with early-onset (age <50) breast cancer [11]. Similarly, 1592delT (L531 fs) is considered to be a founder mutation in Finland and has not been seen outside of Finland [12]. In Chinese inherited breast cancers, two recurrent mutations of BRCA1 have been identified in our former study, which account for about 34.8% of the deleterious mutations of BRCA1 in our population. In the current study, we also revealed a recurrent mutation 751C>T of PALB2 in two separate early-onset breast cancer individuals, both of them showed no family history of breast and ovarian cancer or other malignant tumors. In view of the fact that the carriers came from two adjacent provinces of southern China, and they were absent in samples from the northern Chinese cases and controls, we cannot rule out the possibility that this recurrent mutation is a 'hot-spot' in a defined subpopulation of China. Therefore an additional testing about this mutation in more samples is warranted.

A frameshift mutation, 1050_1051delAAinsTCT, was detected in a family with breast cancer history, the genetic testing about the proband's mother indicated that this mutation was inherited from the maternal side. Coincidentally, this mutation also occurs in exon 4 of PALB2, then we further detected three unclassified variants in this fragment, We found that exon 4 accounted for 44.1% (15/34) of the person-times carrying with any variant of PALB2 in our study. Even though exon 4 possesses the longest length among all the coding fragments of PALB2, we still infer exon 4 should be a common mutation fragment in Chinese population.

In addition, none of the six missense variants within the PALB2 coding sequence detected in our study had an allele frequency greater than 1%, so they were not tested in the controls, and the differences of each allele frequency between the cases and controls were unknown.

Data about the pathologic features of BRCA2-related tumors are usually controversial. Some investigators have reported that higher histological grade and ER or PR positive are for the most part [14–16]. When taken PALB2-related tumors into account, ER and PR positivity also show to be the main certain characteristics, but there is not yet a common consensus on the over-expression of HER2/neu [11–13]. In the three PALB2 mutation carriers reported here (Table 3), we have observed the same manifestation by the analysis of immunohistochemistry. Combined with the results of other studies, we speculate that PALB2-related tumors may share the resembled phenotypic properties with BRCA2-related tumors.

In spite of the similar prevalence to other ethnic groups observed in Chinese population, we analyzed only the youngest patient in each family, and as the testing method used in our study, DHPLC has its limitations in detection rate compared with the direct sequencing [17, 18], accordingly there still exits the possibility that we are unfortunate to miss the deleterious mutations. Besides, a considerable proportion of early-onset patients without affected relatives and some familial patients affected with only one-second-degree relative entered into our study, this somewhat different inclusion criteria from other studies may probably reduce the possibility of detecting genetic variants.

In our series of studies, we sought to investigate the mutation spectrum of some known predisposing genes of breast cancer in Chinese high-risk families, which will help us establish the genetic testing strategy in Chinese population in the long run. The former studies conducted have provided us with the detailed data about the prevalence of BRCA1/2 mutation in our population [1, 19, 20]. At present, we focused on the prevalence of the low-penetrance gene PALB2, and the results indicated that PALB2 mutations might be responsible for about 1% of Chinese women affected with early-onset and familial breast cancer, and exon 4 appeared to be the common fragment accounting for PAlB2 mutations, so we concluded that a detection of exon 4 before the assay of the whole gene suggested to be a cost-effective approach to the screening of Chinese population.

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