Whole exome sequencing of a novel homozygous missense variant in *PALB2* gene leading to Fanconi anaemia complementation group

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Abstract. Partner and localiser of BRCA2 (*PALB2*), also known as *FANCN*, is a key tumour suppressor gene in maintaining genome integrity. Monoallelic mutations of *PALB2* are associated with breast and overian cancers, while bi-allelic mutations cause Fanconi anaemia (FA). In the present study, whole exome sequencing (WES) identified a novel homozygous missense variant, NM_024675.3: c.3296C>G (p.Thr1099Arg) in *PALB*2 gene (OMIM: 610355) that caused FA with mild pulmonary valve stenosis and dysmorphic and atypical features, including lymphangiectasia, non-immune hydrops fetalis and right-sided pleural effusion in a preterm female baby. WES results were further validated by Sanger sequencing. WES improves the screening and detection of novel and causative genetic variants to improve management of disease. To the best of our knowledge, the present study is the first reported FA case in a Saudi family with phenotypic atypical FA features. The results support the role of *PALB2* gene and pathogenic variants that may cause clinical presentation of FA. Furthermore, the present results may establish a disease database, providing a groundwork for understanding the key genomic regions to control diseases resulting from consanguinity.

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Introduction

Genomic variability is a key aspect of human genetics contributing to various types of diseases and cancer. One gene implicated in diseases and cancer predisposition is partner and localizer of BRCA2 (*PALB2*). *PALB2* gene, also known as *FANCN*, is located on chromosome 16 and has three structural domains: N-terminal, central and C-terminal. It serves a key role in tumour suppression through homologous recombination (HR) repair of DNA double-strand breaks. This is achieved by mediating the recruitment of polymerase (Pol η), BRCA2 and RAD51 recombinase to the site of DNA damage forming a D-loop intermediate, in addition to interaction with several other tumour suppressors, including BRCA1and KEAP1 (1). These tumour suppressors exert a synergistic effect on DNA repair through *PALB2*. For example, reactive oxygen species (ROS), natural byproducts of cellular metabolism, are highly reactive substances containing oxygen radicals; it accumilation leads to a detrimental effects, including DNA damage. KEAP1 causes downregulation of NRF2, a master transcriptional factor for antioxidant genes; PALB2 competes with NRF2 to bind KEAP1, thereby disturbing KEAP1-NRF2 complexes. *PALB2*, therefore, coordinates DNA repair via ROS detoxification (1).

PALB2 interacts with BRCA1 and BRCA2 to activate and maintain DNA damage G2/M checkpoints and protect active genes from genotoxic stress (2,3). To date, 604 different *PALB2* variants have been identified; only 140 variants are considered pathogenic, while ~400 have unclear significance (4). The presence of a heterozygous germline mutation in *PALB2* has been linked to the emergence of breast, pancreatic and ovarian cancer (5). A study in Saudi Arabia linked association of *PALB2* pathological variants (PVs) with familial cancer in patients with breast and colon cancer and relatives with a family history of cancer (5). Similarly, an international study (6) analysed >500 families from 21 countries with *PALB2* PVs and reported the link between *PALB2* PVs with breast, ovarian

and pancreatic cancer; however, prostate and colorectal cancer were independent of *PALB2*. Moreover, *PALB2* is included in the breast cancer gene panels based on guidelines of the UK Cancer Genetics Group (7).

Fanconi anemia (FA) is a rare autosomal and X-linked genetic condition characterised by bone marrow failure, physical abnormality, early onset cancer and premature death (3,8,9). Patients with FA have bi-allelic inheritance of pathogenic variants, either homozygous or compound heterozygous. A total of 22 genes are confirmed as FA genes, including *PALB2*/Fanconi Anemia, Complementation Group N (FANCN) (10). Among these genes, *FANCA*, *FANCC* and *FANCG* are the most frequently mutated genes in FA (11). FA genes encode proteins that participate in the FA pathway of interstrand crosslink (ICL) DNA repair and genome maintenance when cell DNA is damaged (12). An essential step in the FA pathway is monoubiquitination of upstream FA protein, FANCI and FANCD2, forming ID2 complex; this step depends on other FA proteins. Ubiquitinated ID2 complex orchestrates the actions of downstream repair proteins, such as BRCA2, BRCA1 Interacting Protein C-terminal Helicase 1(BRIP1), PALB2 (FANCN), RAD51C and FANCP, which are necessary for later stages of homologous recombination (12,13). FA proteins also stimulate stem cell function, inhibit inaccurate repair and prevent cancer (12). *PALB2*/*FANCN*-deficient cells exhibit reduced levels of *BRAC2* due to the role of PALB2 in stabilising BRAC2 (14,15). This indicates the key role of PALB2 for HR and tumour suppressive functions of BARC2. In a case series from Saudi Arabia, ten children were newly diagnosed with FA; two infants had homozygous *PALB2* mutation at c.3425del p.Leu1142Tyrfs*21, and one presented with early onset of cancer (16). In Turkey, 20 FA cases with PVs in PALB2/FANCN were found in 2.5% of the subjects with a paternal novel heterozygous gross deletion of exon 5-6 (17).

In Saudi Arabia, the prevalence of *PALB2* mutations in breast and ovarian cancer is relatively low (0.65%), as found in a cohort of 918 patients (18). Prevalence of *PALB2* mutations is 5.2% among 879 Taiwanese patients with breast cancer (19) and 4.4% along with other PVs in ataxia-Telangiectasia Mutated, *BRCA1, BRCA2,* Checkpoint Kinase 2 *(CHEK2),* or *PALB2* in USA (20). These figures suggest that *PALB2* mutations are generally rare, especially in Saudi Arabia. FA may be underdiagnosed in Saudi Arabia due to unique mutation patterns observed in Saudi patients compared with Europe and North America (16). FA due to *PALB2* mutation is the least reported genotype (16). Furthermore, consanguinity plays a key role in the inheritance of diseases. Saudi Arabia, characterised by a high rate of consanguineous marriages across tribes, exhibits an increased prevalence of genetic disease (21). This highlights the need for further regional genetic studies to understand the full scope of *PALB2*-associated conditions, including its role in FA.

Materials and methods

Ethics approval and sample collection. The present study was approved by the local ethical committee (approval no. 013-CEGMR-02-ETH of the Center of Excellence in Genomic Medicine Research, King Abdulaziz University Jeddah (Jeddah, Saudi Arabia). Written informed consent for laboratory and genetic testing was obtained from the patient's legal guardians. A total of 2 ml of blood samples were collected from four family members, including the father 42 yeas old, mother 37 year, the 11 years old brother and the patient. The study was performed in accordance with the Declaration of Helsinki 2013. Blood samples were collected and DNA was extracted from blood stored in the EDTA tubes (Roche Life Science), as previously described (22). Nanodrop 2000/2000c spectrophotometer was used for DNA concentration and quality check.

Clinical report of the patient. The proband (IV-2) was a 2-month-old female who was admitted to the King Abdulaziz University hospital Jeddah Saudi Arabia in December 2019 with multiple physical abnormalities after birth. FA was diagnosed 2 months later based on mild pulmonary valve stenosis, dysmorphic features, lymphangiectasia, non-immune hydrops fetalis and right-sided pleural effusion. The prenatal course was complicated preterm; serious developmental concerns were noted as she was born at 32 weeks gestational age with non-immune hydrops fetalis, right-sided pleural effusion, mild pulmonary valve stenosis and lymphangiectasis and was underweight along with dysmorphic features.

The family had a history of three generations of consanguine marriages. The grandparents and parents were first cousins. Whole exome sequencing (WES) was performed for the affected proband at the age of 6 months. No previous history of the disease was reported in the other family members.

WES. Extracted DNA from the proband was enriched for the coding region and splice site junctions of genes. hg19, GRCH37/UCSC (https://genome.ucsc.edu/cgi-bin/hgTracks?db=hg19&chromInfoPage=) for reference sequences based on human genome build were used. Capillary sequencing was used to assess variants with clinical or uncertain significance. All sequence alterations were defined using the Human Genome Variation Society nomenclature guidelines (23). Data were analysed using gene-specific filtering. The products were sequenced on an Illumina NextSeq instrument with 2x76 paired-end reads, as previously described (24,25).

Following WES, the generated files (FASTQ) were further converted to BAM and variant call format (vcf) files containing all identified variants. Moreover, obtained variants were used for identification of variants leading to the disease phenotype established through rare ulta rare common/novel (minor allele frequency) (MAF+0.01%) frequency, homozygous or heterozygous conditions along with structure and function [predicted damage by Polyphen-2 (v2.2.2) polyphen/sorting intolerant from tolerant; faculty.washington.edu/wjs18/GS561/cSNPs_lab.html], pathogenicity, genomic position, protein damaging effect and linkage with disease phenotype. Reference sequences were aligned using the GRCh37 database (ncbi.nlm.nih.gov/datasets/genome/GCF_000001405.13/. The list of obtained variants was filtered to determine the disease linked with the identified variants in public databases, such as 1000 genomes (internationalgenome.org/) and Genome Aggregation Database (gnomAD) for allele frequencies <5.0% (gnomad.broadinstitute.org/); frameshift, nonsense, and splice-site variants in disease-associated genes with a minor allele frequency ≤1.0% were observed in gnomAD. College of American Pathologists (26) were used for variant classification. Deleterious effects and abnormalities

Table I. Pathogenic mutations in partner and localiser of BRCA2 gene with Fanconi anaemia.

No.	First author, year	Mutation	Result	State	Origin	(Refs.)
1	Abdulkareem *et al*, 2024	c.3296C>G	p.Thr1099Arg	Homozygous	Saudi Arabian	Present study
2	Viakhireva *et al*, 2020	c.172_175delTTGT	p.Gln60Argfs	Homozygous	Russian	(40)
3	Byrd *et al*, 2016	c.1676_1677 delAAinsG	p.Gln559 ArgfsTer2	Homozygous	Serbian	(32)
4		c.2586 +1G>A	p.Thr839_ Lys862del	Homozygous		
5	Ghazwani *et al*, 2016	c.3425del	p.Leu1142 Tyrfs*21	Homozygous	Saudi Arabian	(16)
6	Serra *et al*, 2012	c.1676_c1677 delAAinsG	p.Gln526 ArgfsX1	Homozygous	German	(41)
7	Reid *et al*, 2007	395delT	V132fs	Biallelic (not specified)	Albanian	(9)
8		c.3113+5G>C	r.2835_ 3113del279/ A946fs		Moroccan	
9		757_758delCT	L253fs		German	
10		3294_3298 delGACGA	K1098fs		German	
11		2257C-T	R753X		Hispanic	
12		c.3549C>A	p.Tyr1183X		North American	
13		2393_2394insCT	T799fs		German	
14		3350+4A-G	r.3350insGCAG/ F1118fs		German	
15		2521delA	T841fs		North American	
16		3323delA	Y1108fs		African	
17		2962C-T	Q988X		British	
18		c.3549C>G	p.Tyr1183X		British North American	
19		3116delA	N1039fs		North American	

were identified using bionormatics tools such as Mutation Assessor version 2.0 (mutationassessor.org/r3/, Exome Aggregation Consortium Version 0.3.1 https://ngdc.cncb.ac.cn/databasecommons/database/id/3774, SIFT version 2.1 sans.org/tools/sift-workstation/, PolyP (phyloP46way_placental) epd.expasy.org/mga/hg19/phylop/phylop.html for identified variant leading to disease. Mutation Tester (mutationtaster.org/) also predicted the variant as a disease-causing variant.

Sanger sequencing. Sanger sequencing was performed for a novel homozygous missense variant, NM_024675.3: c.3296C>G (p.Thr1099Arg) in *PALB*2 gene (OMIM: 610355) based on WES results. Primers were designed using the online free software Primer 3 (https://bioinfo.ut.ee/primer3-0.4.0/) for PCR. Sanger sequencing was performed for all affected and normal family members. Thermocycling conditions were as follows: Initial denaturation at 95˚C for 15 min, followed by 30 cycles 58˚C for 30 sec and 1 min extention at 72˚C. Primer sequences were as follows: Forward, 5'AGC CTATCGGTCATTGCTTT3' and reverse, 5'AGGGAATCT GGGGTTTGACT3'. Sequencing files were obtained from the AB1 sequencing unit. The obtained files were aligned with the wild-type reference sequence using BioEdit version 7.2 (Informer Technologies, Inc.). Further validation was performed by using 100 normal control samples from Saudi Arabia to confirm that the identified variant is not present in the in the population.

Results

WES identified a novel homozygous missense variant, NM_024675.3: c.3296C>G in *PALB*2 gene. The patient was homozygous for the *PALB*2 variant c.3296C>G, where p.Thr1099Arg was a change in the protein. Both parents were identified to have heterozygous missense variant, NM_024675.3: c.3296C>G (p.Thr1099Arg) in *PALB*2 gene, while a male sibling was healthy without *PALB2* gene

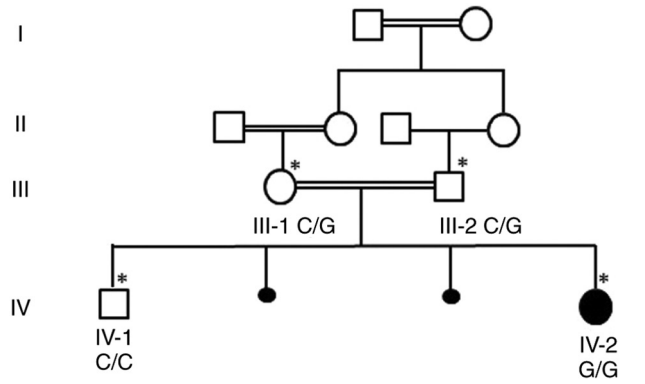

Figure 1. Pedigree of the family. IV-2 is the affected patient. * indicates subjects included in the present study.

mutation (Table I). The parents were heterozygous carriers who were first cousins, (Fig. 1). Sanger sequencing chromatogram showed that both the parents III-1 and III-2 were heterozygous carriers having G/C on both alleles; sibling IV-1 was wild-type C/C and IV-2 was homozygous G/G (Fig. 2). Table I lists known variants in homozygous/heterozygous state of *PALB2* gene.

Moreover, the variant was not reported in gnomAD exomes and 1000 genomes. There was a moderate physiochemical difference between thiamin and arginine acid. Furthermore, *in silico* tools used to aid in interpreting sequence variants identified the variant as disease-causing. Moreover, protein alignment showed highly conserved amino acids between different species (Fig. 3).

Discussion

FA is a rare genetic disease characterised by bone marrow failure and several physical anomalies. The estimated worldwide incidence is 1 in 360,000 live births, while the prevalence of carriers is estimated to be 1 in 181 (8). FA was first described in 1927 by the Swiss paediatrician Guido Fanconi when 3/5 brothers died from severe conditions resembling pernicious anaemia associated with physical birth defects (27). A total of 22 genes responsible for FA have been identified and labelled *FANCA-W* (10). The most commonly affected gene is *FANCA,* followed by *FANC-C*, *-G,-D2* and *-P* (11,26). The major function of the FA genes pathway is to orchestrate DNA repair ICL. During the final phase of ICL, seven downstream FA proteins, including PALB2, combine to repair broken DNA via HR (8). Therefore, mutations in the FA pathway lead to defective HR, increasing dependence on error-prone non-homologous DNA end-joining repair pathway (28).

Bi-allelic mutations in *PALB2* account for FA, which is a rare autosomal and X-linked disorder characterised by severe bone marrow failure, developmental and physical abnormality and susceptibility to early-onset cancers. A total of >20 distinct genes have been reported to cause FA. ≈The present study reports a case of a preterm female baby with FA associated with mild pulmonary valve stenosis, dysmorphic features, lymphangiectasia, non-immune hydrops fetalis and right-sided pleural effusion. To the best of our knowledge, the present study is the first to reported FA with lymphangiectasia, a dilation of lymphatic blood vessels leading to oedema (29). The typical reported clinical features of FA are distinctive for each FA gene; they generally include growth retardation, facial abnormality, radial ray defects, hyper-hypo pigmentation, renal malformation and microcephaly (9,30). For example, in a recent Chinese study of 148 patients with FA, finger deformities and café au lait spots were associated with *FANCA,* while *FANC,-D2, G, I* and *-P* presented mostly with cardiovascular deformity (31). Fiesco-Roa *et al* (30) reported that *PALB2* abnormality is associated with anal congenetal abnormalities due to a defect in the downstream DNA repair pathway. Ghazwani *et al* (16) reported 10 cases of FA, two with *PALB2* genotype, one of which exhibited a novel mutation c.3425del (p.Leu1142Tyrfs*21) with multiple congenital anomalies, metastatic Wilms tumour and stage I neuroblastoma in the first year of life and one had café au lait spots and mosaic duplication of the long arm of chromosome 17 (band q21.2). The present patient exhibited two typical features of FA (cardiovascular abnormality and facial deformity); to the best of our knowedge, however, lymphangiectasia, non-immune hydrops fetalis and right-sided pleural effusion have not been reported before. Similarly, a previous study reported atypical features of FA with cancer (32) in two sisters (aged 12.0 and 3.5 years at time of FA diagnosis) with *PALB2* mutations, one truncating, c.1676_1677delAAinsG; (p.Gln559ArgfsTer2), and c.2586 +1G>A; p.Thr839_Lys862del causing frame skip of exon 6 (24 amino acids), resulting in B cell non-Hodgkin lymphoma and atypical features of FA including learning difficulty and dyslexia without developmental defect. Similarly, an *in silico* study linked *PALB2* genotype with tumours of embryonal origin, including medulloblastoma, Wilms tumour and neuroblastoma (11). Notably, *PALB2* mutations differ from other subtypes of FA owing to their association with high-risk paediatric malignancy similar to those induced by BRCA1 and BRCA2 (10,33). This highlights the key interaction of PALB2 in the localisation and stability of BRCA2, which facilitates BRCA2 functions in DNA repair, and interaction with several other tumour suppressors, facilitating the role of *PALB2* as a typical cancer suppressor gene (3).

In the present study, WES results validated by Sanger sequencing identified a homozygous missense variant, NM_024675.3: c.3296C>G (p.Thr1099Arg) in *PALB*2 gene in a preterm female baby. The parents were first cousins and heterozygous carriers and the brother was healthy. *In silico*, a total of 84 *PALB2* variants with uncertain significance, including the variant identified in the present study, were assessed; four variants (p.L24S,c.71T>C; p.L35P,c.104T>C; pI944N,c.2831T>A and p.L1070P,c.3209T>C) were associated with disrupted PALB2-mediated homology-directed DNA repair (34). Although the aforementioned study did not list the present variant as a PV, the present study confirms the pathological features of the variant.

FA is an autosomal recessive genetic disease with chromosomal instability. The present patient was a product of a consanguineous marriage (three generations). Consanguinity is a practice in several tribes in Saudi Arabia and other countries, resulting in disease in the offspring of carrier families (35,36). WES is a diagnostic tool to identify

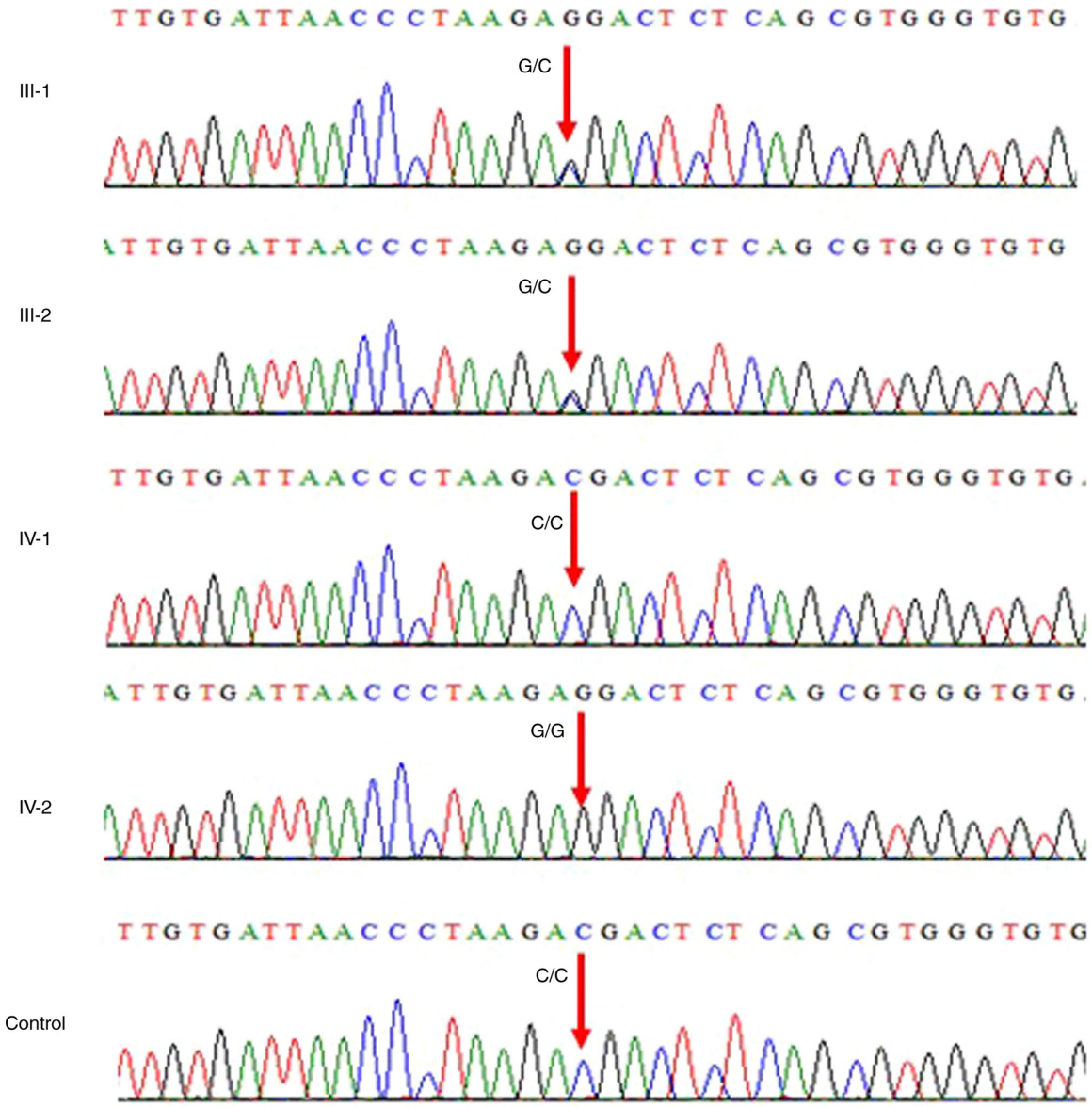

Figure 2. *PALB2* gene variants. Sanger sequencing showed that parents III-1 and III-2 were heterozygous carries with C/G on both alleles. Sibling IV-1 exhibited wild-type homozygous C/C, whereas IV-2 exhibited a novel homozygous missense variant c.3296C>G (p.Thr1099Arg) in *PALB2* gene. Normal control samples were used from the local population. PALB2, Partner and localiser of BRCA2.

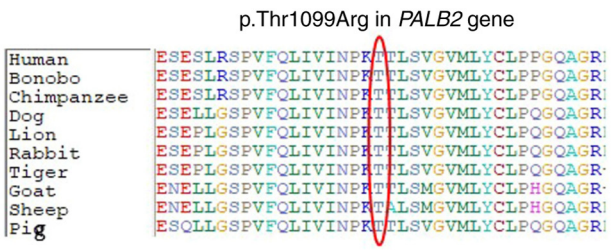

Figure 3. Conserved amino acid p.Thr1099Arg in *PALB2* across species. PALB2, partner and localiser of BRCA2.

molecular defects in patients with suspected genetic disorders (37-39). Identifying deleterious PVs facilitates genetic counselling, prenatal testing and development of therapeutic strategies.

The present study reported a Saudi family with *PALB2* with a novel homozygous missense variant, c.3296C>G (p.Thr1099Arg). The present results contribute to the mutation spectrum of *PALB2*-associated diseases, which will help to manage this disease in the future. To the best of our knowledge, the present study is the first report of this rare genetic variant related to the *PALB2* gene in Saudi Arabia with other symptoms that have not been reported previously. *PALB2* variant carries important clinical implications, including an increased risk for certain types of cancer including breast and pancreatic cancers (6). This necessitates monitoring and potential adjustments in patient management strategies. Moreover, it underscores the need for thorough genetic counselling for affected individuals and their family members, given the potential hereditary nature of the risk associated with this variant. WES testing of patients of consanguineous marriages with a strong family history of genetic disease is advisable.

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Availability of data and materials

The data generated in the present study are not publicly available due to refusal of consent by the parents of the patient but may be requested from the corresponding author.

Authors' contributions

MIN and AAA designed and performed the experiments. BHS and HAB designed experiments and collected clinical details and samples. MIN, AH and AAA analysed the data. MIN, BHS and HAB wrote the manuscript. AAA, HAB, AH, BHS and MIN revised the manuscript. MIN, HAB and BHS confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Ethics approval was obtained from the Ethical Committee (approval no. 013-CEGMR-02-ETH) of the Center of Excellence in Genomic Medicine Research, King Abdulaziz University Jeddah (Jeddah, Saudi Arabia). Written informed consent was obtained from the parents.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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