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PRECLINICAL STUDY

A synonymous germline variant PALB2 c.18G>T (p.Gly6=) disrupts normal splicing in a family with pancreatic and breast cancers

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Abstract

Purpose Mutations in *PALB2* have been associated with a predisposition to breast and pancreatic cancers. This study aims to characterize a novel *PALB2* synonymous variant c.18G>T (p.Gly6=) identified in a family with pancreatic and breast cancers.

Methods The *PALB2* c.18G>T (p.Gly6=) variant in this family was identified using Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT™). RT-PCR and subsequent cloning were performed to investigate whether this variant affects normal splicing.

Results This variant completely disrupts normal splicing and leads to several abnormal transcripts, which presumably leads to premature protein truncation. The major abnormal transcript resulted in a deletion of 32 base pairs in exon 1 and frameshift.

Conclusions Our results indicate that the *PALB2* c.18G>T (p.Gly6=) variant is likely pathogenic. This study provided important laboratory evidence for classification of this variant and guided improved patient management.

Keywords Synonymous germline variant · PALB2 · Splicing

Introduction

The PALB2 (partner and localizer of BRCA2) gene encodes a protein that interacts and stabilizes BRCA2, which is crucial for its chromatin localization and recruitment to DNA damage sites [1, 2]. The PALB2 protein serves as a central core of the BRCA1–PALB2–BRCA2 complex which is critical for BRCA2-mediated RAD51 recruitment and homologous recombination that repairs double-strand DNA breaks [3, 4]. It associates with BRCA1 and BRCA2 independently through its NH_2 terminal coiled-coil and COOH terminal WD40 domains, respectively [3, 5, 6]. The ChAM domain is located at the center of the protein and is required for PALB2 chromatin localization [7]. The N-terminal coiled-coil domain of PALB2 also mediates its own dimerization or oligomerization [8, 9]. Deletion of even only the last 4 amino acids from the C terminus of PALB2 would result in a collapse of its structure and degradation of the protein [10, 11].

Biallelic inactivating germline variants in *PALB2* lead to Fanconi anemia and predispose to childhood cancer (Fanconi anemia type N) [11, 12], whereas germline monoallelic variants in *PALB2* are associated with hereditary predisposition to pancreatic cancer [13], female and male breast cancer [4, 14–17], and likely ovarian cancer [18], prostate cancer [19], gastric and colorectal cancer [20], as well as melanoma [21]. *PALB2* germline mutations are reported in 3–4% of patients with familial pancreatic cancer [22, 23] and in one of 615 unselected patients with exocrine pancreatic neoplasms [24] without including the case in this study. PALB2 germline mutations are related to an approximately 2.3 to sixfold increase in the risk of hereditary breast cancer, with a lower prevalence in unselected female breast cancer cases [15, 17, 25, 26]. It has also been demonstrated that the risk associated with breast cancer for at least some *PALB2* pathogenic variants is comparable to the average risk associated with *BRCA2* pathogenic variants, much higher than the expected rate [4].

To date, over 350 unique sequence variants in *PALB2* have been reported in different populations (http://databases.lovd.nl/shared/variants/PALB2/unique). A considerable

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number of these variants are exonic point substitutions that are categorized into missense, synonymous, or nonsense mutations. Synonymous variants are generally considered benign. However, recent studies have shown that some exonic substitution variants may severely affect gene function through disruption of pre-mRNA splicing [27, 28]. These mutations may strengthen or weaken critical splicing regulatory elements like exonic/intronic splicing enhancers or silencers, or create or destroy splice sites [28–30]. Alterations in pre-mRNA splicing can result in exon skipping, intron inclusion and activation of cryptic sites or creation of ectopic splice sites, and can subsequently lead to production of aberrant spliced transcripts with premature termination codons that trigger nonsense-mediated mRNA decay, and this has been associated with the pathogenesis of a diverse set of human diseases [28, 29].

Here, we report a rare synonymous *PALB2* substitution variant, c.18G>T (p.Gly6=) in a breast/ovarian cancer family. We demonstrate that this mutation creates a novel 5′ splicing site and completely disrupts normal splicing of *PALB2*. This mutation led to a deletion of 32 bp in exon 1, which presumably leads to a frameshift and truncated protein. In addition, c.18G>T does not generate any normal transcript, which supports the pathogenicity of this variant. Our results indicated that, instead of being a silent and benign variant, this variant likely contributes to cancer predisposition through disruption of normal splicing.

Materials and methods

Subject

The proband is a 56-year-old female diagnosed with pancreatic and thyroid cancers at 55 and 25 years old, respectively. A three-generation pedigree (Fig. 1) demonstrated that her father was diagnosed with lymphoma in his 50s. A paternal uncle was diagnosed with pancreatic cancer at 52 years old and two paternal aunts were diagnosed with breast cancer in their 60s. One of her three sisters was diagnosed with breast cancer at 48 years of age and other sister had total abdominal hysterectomy with bilateral salpingo-oophorectomy at her 40s. The patient provided written informed consent for genetic testing as part of a study approved by the Institutional Review Board of Memorial Sloan Kettering Cancer Center.

cDNA analysis

The *PALB2* c.18G>T variant identified through commercial testing was confirmed prior to transcript analysis. Total RNA from the patient was extracted using the PAXgene Blood RNA Kit (PreAnalytiX, Qiagen, Valencia, CA) and was subsequently used for cDNA synthesis (Superscript III First-Strand Synthesis SuperMix, Invitrogen Life Technologies, Carlsbad, CA). Control RNA was extracted from another individual who did not carry the *PALB2* c.18G>T

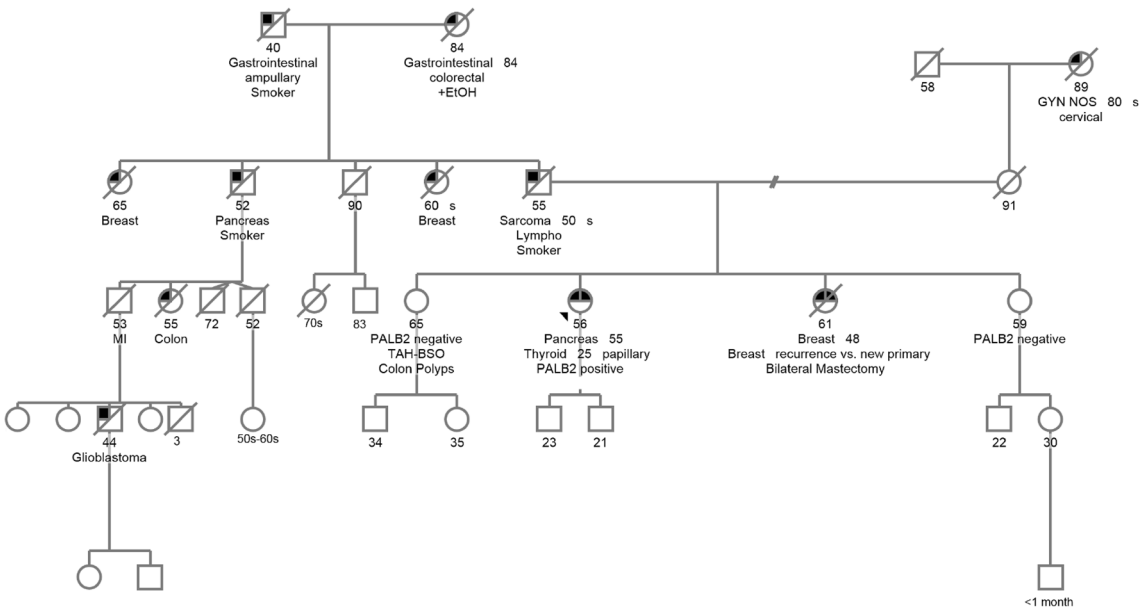


Fig. 1 Patient pedigree. The patient described here is a 56-year-old female who was diagnosed with pancreatic and thyroid cancer at 55 and 25 years old, respectively. The patient has one sister and two paternal aunts affected with breast cancer in their 60s. Her paternal uncle was affected with pancreatic cancer at 52 years old

variant. RT-PCR was performed using the JumpStart REDTaq Ready Mix (Sigma), with control cDNA or the patient's cDNA in the presence of M13-tagged forward and reverse primers (Forward, 5′utrF: 5′-GTAAAACGACGGCCAGTG GCTCCCC ATTCCTTCCT-3′; Reverse, e3R: 5′-CAG GAA ACA GCT ATG AC TTAGCTGCGGTGAGAGATCC-3′). Each PCR reaction contained 12.5 µl 2×JumpStart REDTaq Ready Mix, 2 µl 10 µM primers (1 µl for each), 2 µl cDNA, and water to make a final volume of 25 µl. Cycling conditions used in this study were 96 °C for 5 min, 94 °C for 30 s (35×), 58 °C for 45 s (35×) , and 72 °C for 60 s (35×) with a final extension at 72 °C for 5 min (1×).

Cloning

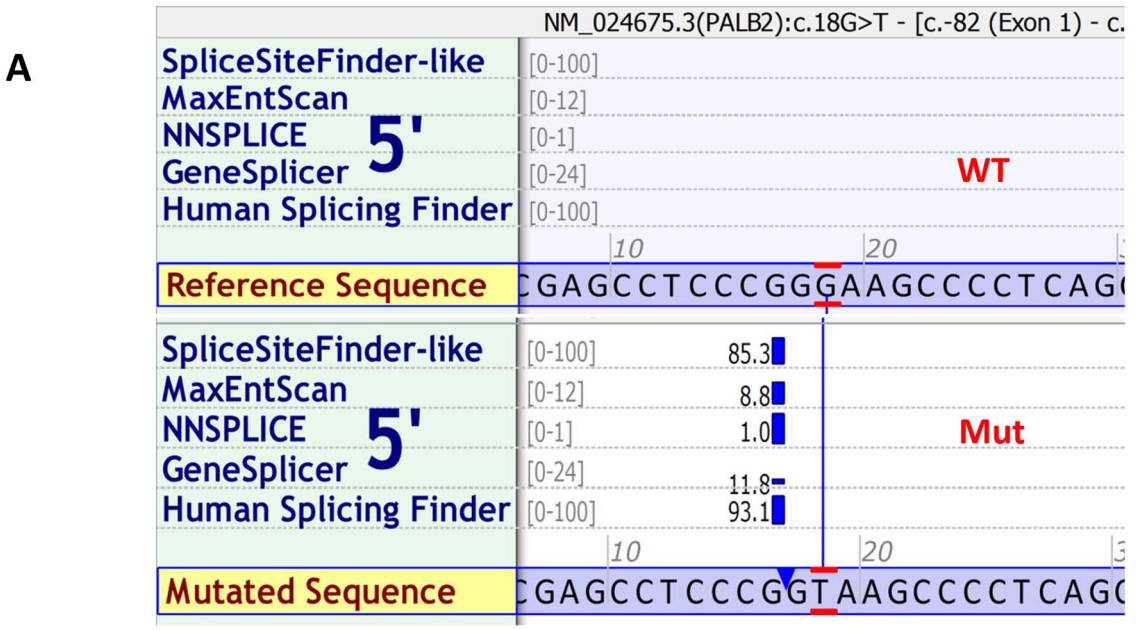
The RT-PCR products were cloned into pCR4 TOPO vectors (Invitrogen, Carlsbad, CA), following the manufacturer's procedures (Invitrogen, Carlsbad, CA). DNA from colonies was amplified using the 5′utrF and e3R primers covering cDNA regions of exons 1 and 2. The PCR products were visualized by QIAxcel (QIAGEN), purified using ExoSAP-IT (Affymetrix), and then subjected to direct DNA sequencing analysis using primers M13F and M13R (BigDye Terminator v3.1 Cycle Sequencing Kit and 3730 DNA Analyzer, Applied Biosystems, Foster City, CA).

Results

The variant c.18G>T disrupts normal splicing and presumably leads to premature protein truncation

To evaluate the potential effects of the variant on splicing, we used Alamut software, which incorporates five tools to predict the potential effects of *PALB2* c.18G>T on normal mRNA splicing. All five tools predicted that the variant create a novel 5′ donor splice site (Fig. 2A, B).

The effect of the variant c.18G>T on RNA splicing was evaluated by amplifying regions of *PALB2* from cDNA derived from the patient. PCR was designed to generate a fragment that spanned part of exon 1 and the entire coding



B

Donor Sites

	SSF [0-100]	MaxEnt [0-12]	NNSPLICE [0-1]	GeneSplicer [0-24]	HSF [0-100]
Threshold	*≥ 70*	*≥ 0*	*≥ 0.4*	*≥ 0*	*≥ 65*
Exon 1 – c.16	— ⇒ 85.31	— ⇒ 8.80	— ⇒ 1.00	— ⇒ 11.82	— ⇒ 93.07

Fig. 2 In silico predictions of the PALB2 c.18G>T variant. The Alamut software was used to evaluate the potential effects of the variant on splicing. All five tools predicted that the variant creates a new 5′ donor splicing site (**A** WT and Mut; **B** Splicing report showing the creation of a new splice donor site at the c.16 position)

region of exon 2, which is likely affected by the substitution. An additional band was detected in the patient, but not in the 13 controls (Fig. 3A). This band represents an aberrant RNA splicing product attributable to the variant. Further RT-PCR, cloning, and sequencing results revealed that the alteration leads to a 32 nucleotide deletion at the end of exon 1 (Fig. 3B). As shown in Fig. 4B, the c.18G>T mutation introduced a new 5′ splice site (GU) within exon 1 that is 32 bps away from the canonical splice donor site at the 5′ end of intron 1. The recognition and utilization of this newly created splicing site by the splicing machinery results in the deletion of 32 base pairs in the mRNA transcript and presumably leads to a stop codon at amino acid position 93. The mutation presumably produced a truncated PALB2 protein of 30 amino acids instead of 1186, which is nonfunctional.

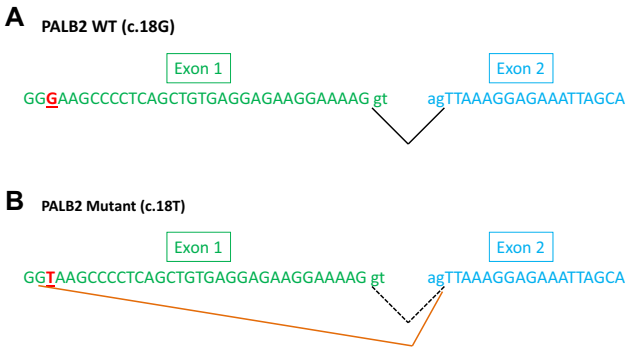


Fig. 4 PALB2 c.18G>T mutant creates a novel 5′ splice site that results in a 32 nucleotide deletion of the 3′ end of exon 1. **A** Wild-type sequence adjacent to the PALB2 c.18 position. **B** the PALB2 c.18G>T mutant sequence. The splicing sites for the wild type and mutant sequences are indicated

The PALB2 c.18G>T variant completely disrupts normal splicing in the mutant allele

To determine whether the *PALB2* c.18G>T mutant allele completely disrupts normal splicing, i.e., whether the mutant allele is able to generate any *PALB2* normal transcripts with the p.Gly6 synonymous mutation, we sought other heterozygous variants within this region. The heterozygous variant (c.−47G>A) in the 5′UTR allowed us to determine whether the mutant allele generates any *PALB2* wild-type full-length transcripts. We first determined which allele at the c.−47 position is in cis with the mutant allele at the c.18 position by cloning the exon 1 PCR product using genomic DNA as a template followed by DNA sequencing. The sequencing results indicate that c.−47A

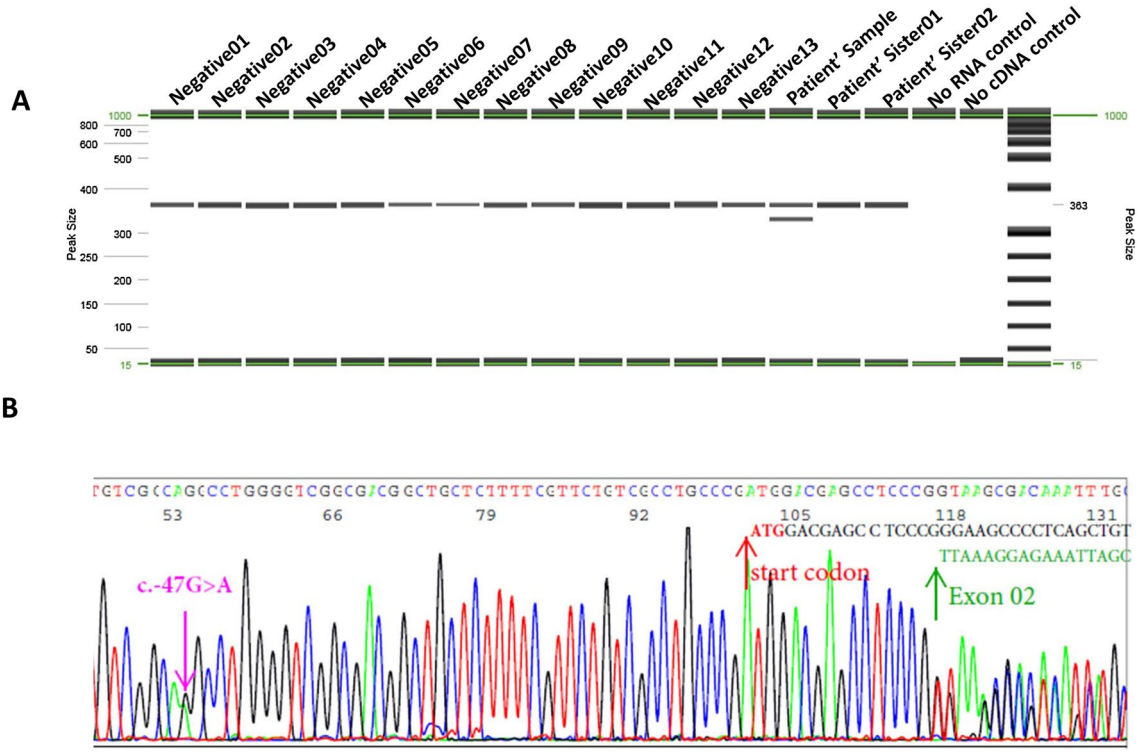


Fig. 3 RT-PCR analysis demonstrates PALB2 c.18G>T leads to deletion of 32 bp in exon 1. **A** RT-PCR products run on QIAxcel. Two extra bands were observed in the patient, but not in the negative controls ($n=13$). **B** Electropherogram showing that the variant causes exon partial deletion. The boundary of exons is marked by red (exon 1) and green (exon 2) arrow

is in cis with the wild-type c.18G allele and c.−47G is in cis with the mutant c.18T allele in the patient.

To investigate whether the c.−47G is present in the wild-type transcript, we cloned the RT-PCR products into the TOPO sequencing vector and then sequenced 126 colonies. Thirty-eight (38) clones from the patient contained the full-length transcript, all of which contained the A at the c.−47 position, indicating that the mutant allele was unable to generate any normal transcript (Fig. 5A). Seventy-seven (77) clones with the exon 1 3′ 32 bp deletion contained the G allele at the c.−47 position (Fig. 5B). These results indicate that the aberrant splicing caused by this mutation is quite efficient as the mutant T allele at the c.18 position completely abolishes normal splicing. In addition, a variety of alternative splicing products that led to different retention of intron 1 or deletion of exon 2 were detected in different clones (Fig. 6). Interestingly, in additional to these two major transcripts, we also observed 2 clones containing 463 bp retention of intron 1 (the 809 bp RT-PCR product), 6 clones containing 134 bp retention of intron 1 (the 480 bp product), and one clone containing 134 bp retention but lack of exon 2 (the 420 bp product) with the A allele at the c.−47 position. We also observed 2 clones containing 134 bp retention of intron 1 with the G allele at the c.−47 position (the

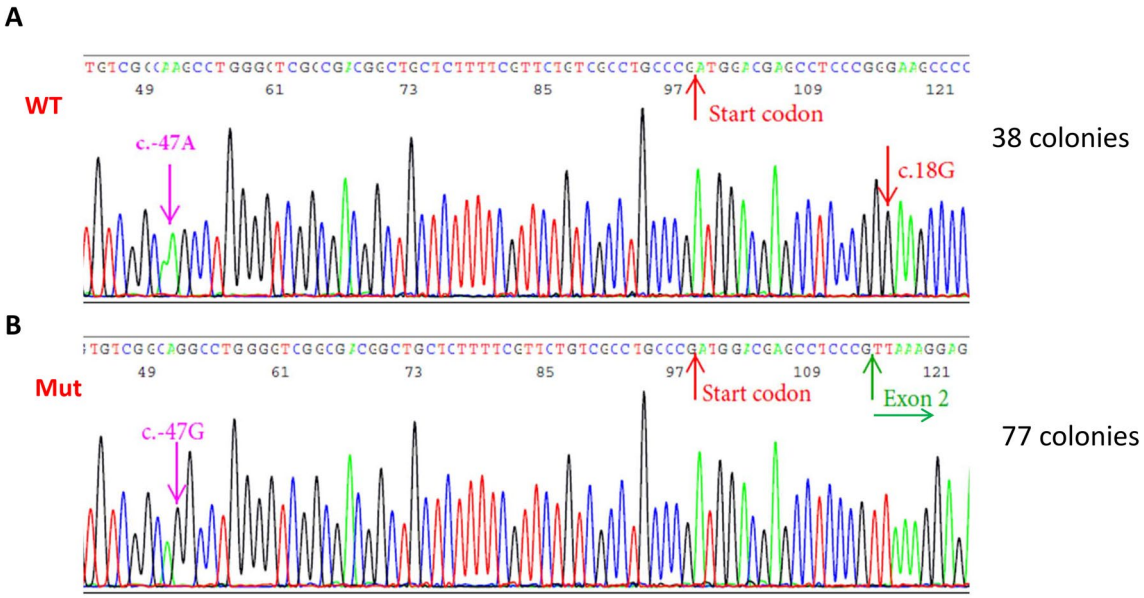


Fig. 5 SNP tagging demonstrates that the mutant allele does not generate any wild-type transcript. **A** The sequencing result revealed that c.−47A is in *cis* with the wild-type allele. **B** c.−47G is in *cis* with the mutant allele in the patient

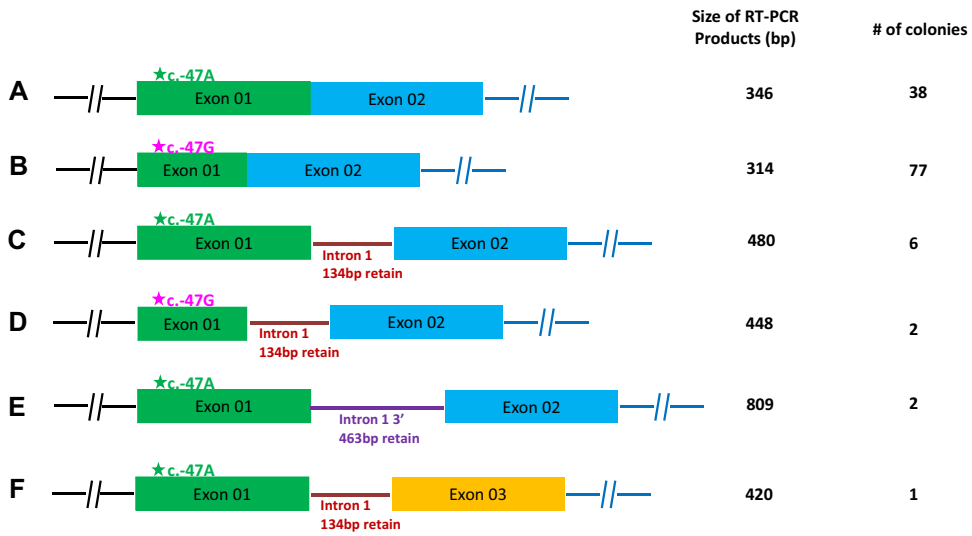


Fig. 6 Summary of splicing variants from the RT-PCR products. **A** Wild type; **B** 32 bp deletion at the end of exon1; **C** 134 bp Retention of intron 1; **D** Retention of intron 1 and 32 bp deletion at the end of exon1; **E** 463 bp retention of intron 1; **F** 134 bp Retention of intron 1 and exon2 deletion. Star indicates the SNP at the c.−47 position

480 bp product). The partial intronic retentions of intron 1 and exon 2 deletion are caused by activation or inactivation of alternative 3′ splice acceptor sites (AG) in the intron and represent the naturally occurring alternative transcripts affecting this region. Taken together, we did not detect any correctly spliced mRNA transcripts from the mutant allele. These results suggest that this mutation completely abolishes normal mRNA splicing in the mutant alleles.

We also tested other family members in this family. The proband's two living sisters unaffected with breast or pancreatic cancer do not carry this variant. Unfortunately, specimens from the deceased sister and other family members were unavailable for testing.

Discussion

Clinical laboratories detect many novel and rare synonymous variants in the course of sequencing patient samples for a panel of genes. Unless they have been previously reported to segregate in affected individuals or impact splicing, their clinical significance remains uncertain or is assumed to be likely benign, since they do not alter the amino acid at that position. However, synonymous variants have the potential to create new splice sites and cause abnormal splicing, leading to protein truncation, loss of expression due to nonsense-mediated decay, or in-frame deletion of a portion of the protein. All of these consequences likely cause absent or altered protein function and would be pathogenic, if loss of the gene's function is associated with disease.

Several software tools that predict the generation or loss of splice sites are available [31]. Although most splice site prediction tools use similar types of data as the basis of their predictions, different programs often give varying scores for the likelihood of a splicing impact by a particular variant. Despite the variability in their predictions, they have been reported to have high sensitivity to predict splice sites, although their specificity may be low [32, 33]. ACMG and AMP guidelines for variant interpretation recommend using multiple in silico tools and combining their predictions to use as a single piece of supporting evidence for variant interpretation [34]. Although they cannot be used as the sole source of evidence for variant classification, they can suggest further analysis of a variant, such as investigating RNA from patient cells, using in vitro splicing assays, or testing additional family members when available, to help clarify its clinical significance.

The synonymous c.18G>T variant in PALB2 gene has been classified as a variant of uncertain significance by three clinical laboratories in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/variation/142432/, accessed August 24, 2018). As this variant has not previously been reported in the literature and is absent from large population databases (gnomAD, 1000 Genomes), without further analysis our initial classification would also be uncertain significance. However, this variant was predicted to create a new 5′ splice site by five different software programs, which prompted us to further investigate its clinical significance using patient's RNA. Our results demonstrate that the c.18G>T variant indeed created a new 5′ splice site at position c.17, causing abnormal splicing including deletion of the downstream portion of exon 1 as well as several other abnormal transcripts. The major abnormal transcript alters the reading frame and introduces a premature stop codon 26 amino acids downstream (p.Gly6Valfs*26). Based on these results, the impact of this variant on the PALB2 protein is not a synonymous change, but is in fact loss of function of the protein either through nonsense-mediated decay (predicted to occur based on the position of the variant) or truncation.

Interestingly, two other synonymous variants in *PALB2* have also been reported to cause altered splicing. One of them is a c.48G>A (p.Lys16=) variant that occurs at the last base of exon 1, which was reported in a patient with breast cancer and was demonstrated to impact splicing, leading to a frameshift and introducing a premature termination codon 20 amino acids downstream [35]. The other is a c.2559C>T (p.Gly853=) variant that has been reported in a patient with breast cancer and was shown to alter splicing, leading to a frameshift in exon 6 [36]. Both of these variants were predicted to create new splice sites by five different computational tools, as well.

Loss of function of *PALB2* is associated with increased risk for breast cancer [15, 37, 38] and with autosomal recessive Fanconi anemia, type N [6, 11]. Based on its demonstrated impact on splicing in patient cells, the c.18G>T variant is now considered to be likely pathogenic for these diseases. The improved classification of this variant has significant impact on the patient's medical management and for counseling of the patient and family members regarding disease risk and reproductive planning. It is worth mentioning that, based on the classification of this variant as likely pathogenic, we were able to treat our patient with a PARP inhibitor and she had ongoing treatment response. This finding highlights the importance of interpreting synonymous variants with caution, particularly when they are predicted to alter splicing by multiple in silico tools. Unfortunately, further analysis of variants for their splicing impact by additional assays is often not feasible for most clinical laboratories. However, due to their significant consequences for patient management and counseling, laboratories should make effort to pursue such analyses when possible.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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