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Characterization of a novel germline PALB2 duplication in a hereditary breast and ovarian cancer family

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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* exon 13 duplication in a hereditary breast and ovarian cancer family.

Methods—The *PALB2* exon 13 duplication in this family was evaluated using Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT™) and confirmed by multiplex ligation-dependent probe amplification (MLPA). The duplication breakpoints were determined by long-range PCR and DNA sequencing. The effects of this mutation on mRNA splicing were characterized using RT-PCR, cloning, and DNA sequencing.

Results—The 5′ and 3′ breakpoints were mapped to intron 12 and downstream of 3′UTR. The tandem duplication is mediated by Alu elements in these regions. This duplication disrupts normal mRNA splicing and presumably leads to a frameshift and premature protein truncation. This duplication segregates with ovarian and breast cancer in multiple members in this family.

Conclusions—Our results indicate that the *PALB2* exon 13 duplication is a pathogenic variant. The presence of the *PALB2* duplication in the proband affected with high-grade serous ovarian cancer suggests that *PALB2* might be associated with a predisposition to ovarian cancer.

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Ciyu Yang and Angela G. Arnold have contributed equally to this work.

Compliance with ethical standards

Conflict of Interest David M. Hyman has received research funding from LOXO, PUMA, and AstraZeneca. The rest authors declare 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.

Keywords

Breast cancer; Ovarian cancer; PALB2; Duplication; Pathogenic

Introduction

PALB2 (partner and localizer of BRCA2) was originally identified as a BRCA2-interacting protein that stabilizes BRCA2 in key nuclear complexes, which are crucial for its chromatin localization and recruitment to DNA damage sites [1, 2]. PALB2 serves as a central core of the BRCA1-PALB2-BRCA2 complex, which is essential for homologous recombination [3]. It associates with BRCA1 through its N-terminal coiled-coil domain, and the COOH terminus containing four WD40 domains is required for its interaction with BRCA2 [3–5]. Deletion of part of the PALB2 C-terminus containing the last WD domain abolishes its interaction with BRCA2 [2]. Deletion of the last 32 amino acids disrupts the protein interaction between PALB2 and BRCA2, leading to defective homologous recombination [6].

Germline heterozygous mutations in *PALB2* have been linked to moderately increased risks for female breast cancer and pancreatic cancer [7]. *PALB2* mutations occur in about 1–2 % of individuals with familial breast cancer [8–11] and in 3–4 % in breast cancer patients without *BRCA1/2* mutations [12–14]. *PALB2* mutations are present in 3–4 % of patients with familial pancreatic cancer [15, 16]. Some studies also suggest that *PALB2* germline mutations confer increased risks of male breast cancer [8, 17], ovarian cancer [18, 19], and prostate cancer [20], although the spectrum of cancers and magnitude of cancer risks are still unclear.

Pennington KP et al. [21] hypothesize that patients with germline mutations in DNA homologous recombination genes will have a sensitivity to PARP inhibitors (PARPi). Inhibition of PARP is a potential synthetic lethal therapeutic approach to the treatment of patients with inherited mutations in genes such as BRCA1 and BRCA2 that are involved in DNA repair pathways [22–24]. Recently, Olaparib (AZD 2281), an oral PARP inhibitor, has been approved for the treatment of DNA repair-deficient high-grade ovarian tumors in *BRCA1* or *BRCA2* mutation carriers [25]. Prolonged responses to Olaparib were observed in patients harboring germline *BRCA1*/2 mutations with different tumor types including ovarian, breast, pancreatic, and prostate cancers [26, 27]. Like BRCA1 and BRCA2-deficient cells, cells with a genetic deficiency for PALB2 exhibit a defect in homologous repair [28] and display sensitivity to inhibition of PARP [28–31]. Since PALB2 protein participates in the same DNA repair pathway as BRCA2 [32], the synthetic lethal therapeutic strategy based on PARP inhibition could also be utilized for treatment of patients with germline mutations in *PALB2.* Determining *PALB2* variant pathogenicity in patients with ovarian and related cancers is thus of significant clinical relevance, particularly for guiding targeted therapy regimens.

To date, most reported pathogenic *PALB2* mutations detected in patients are truncating mutations (nonsense and small indel mutations) and mutations affecting the canonical splicing sites. Only a few *PALB2* variants with large deletions or duplications have been

identified, namely the deletions of exons 1–10 [5], 7–8, 9–10 [13], 7–11 [17], and 12–13 [33]; the duplication of exons 9–11 [13]. Here, we report a novel Alu-mediated duplication of *PALB2* exon 13, which segregates with ovarian cancer and breast cancer in a large hereditary breast and ovarian cancer (HBOC) family. Our data indicate that this duplication disrupts normal splicing and leads to premature protein truncation. This study highlights the importance of functional studies in evaluating non-truncating variants including large duplications. It also indicates that *PALB2* germline mutations may contribute to the development of ovarian cancer, in addition to breast and pancreatic cancers.

Materials and methods

Subject

A 53-year-old female of Norwegian/German/Italian ancestry presented to the Clinical Genetics Service (CGS) at Memorial Sloan Kettering Cancer Center (MSKCC) following the diagnosis of stage IV, high-grade serous ovarian cancer involving bilateral ovaries and fallopian tubes. Given the high-grade serous pathology of her tumor and the reported maternal family history of breast and ovarian cancer, the patient was offered genetic testing for inherited mutations in known breast/ovarian cancer predisposition genes. A four-generation pedigree (Fig. 1) demonstrated that one of the proband's three sisters was diagnosed with breast cancer (ductal carcinoma in situ, DCIS) at 42 years of age. A maternal aunt was diagnosed with bilateral breast cancer at 54 and 56 years of age (of note, this aunt had been evaluated for inherited mutations in *BRCA1* and *BRCA2* via full gene sequencing and was not found to carry a mutation). The proband's maternal grandmother was diagnosed with colon cancer at 67 years of age. Her maternal great-aunt was diagnosed with ovarian cancer at her 50s (medical records were not available).

Given the strong family history of breast and ovarian cancer, the proband opted to proceed with testing via a commercially available hereditary cancer multi-gene panel (sequencing and large rearrangement analysis was performed for the following genes: APC, ATM, BARD1, BMPR1A, BRCA1, BRCA2, BRIP1, CDH1, CDK4, CDKN2A, CHEK2, EPCAM (large rearrangement only), MLH1, MSH2, MSH6, MUTYH, NBN, PALB2, PMS2, PTEN, RAD51C, RAD51D, SMAD4, STK11, and TP53). The patient provided written informed consent for genetic testing as part of a study approved by the Institutional Review Board of MSKCC (protocol #96-051 "Clinical Significance of Germline BRCA Mutations"). The proband was subsequently found to carry a duplication of the last exon of the *PALB2* gene (duplication exon 13). No other mutations or variants of uncertain clinical significance were identified in the remaining 24 genes analyzed. Additional studies performed at the commercial laboratory indicated that the *PALB2* rearrangement was a tandem head-to-tail duplication of exon 13. Given the potentially important clinical significance of this variant, the patient and her family agreed to provide additional blood samples to help further characterize the variant at the MSKCC. Peripheral blood samples were collected using the EDTA Blood tube and PAXgene Blood RNA tube and submitted to the Diagnostics Molecular Genetics Laboratory at MSKCC for further analysis. Control RNAs were extracted from unrelated individuals seen at MSKCC who do not carry the *PALB2* large duplication variant.

Duplication analysis

The *PALB2* gene copy number was determined for the proband's family members via the Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) test [34]. Duplication of *PALB2* exon 13 was confirmed by multiplex ligation-dependent probe amplification (MLPA) using the SALSA MLPA P260 PALB2-RAD50-RAD51C-RAD51D probe mix (MRC Holland, Amsterdam, The Netherlands).

Long-range PCR and breakpoint determination

Long-range (LR) PCR was performed on genomic DNA from the patient to confirm the large DNA duplication in *PALB2* detected by the commercial laboratory. LR-PCR was performed using a forward primer in intron 12 and a reverse primer in 3′-untranslated region (UTR) of *PALB2* and the TaKaRa LA PCR kit (TaKaRa, Clontech) following the manufacturer's instructions. The following thermal cycling conditions were used to perform LR-PCR: initial denaturation at 94 °C for 2 min, 30 cycles at 98 °C for 10 s, 68 °C for 6 min, and a final elongation at 68 °C for 10 min.

The breakpoint was determined by LR-PCR with the primer located at the end of exon 13 and the reverse primer located at the beginning of exon 12. The duplication breakpoint was more specifically identified using a primer walking strategy with a series of forward primers in the 3′-UTR and reverse primers in intron 12. The duplication junction was amplified with a specific primer followed by direct sequencing to confirm the breakpoint. Sequencing reactions were performed with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) on an ABI 3730XL sequencer according to the manufacturer's instructions. The sequences were aligned against the wild-type *PALB2* nucleotide sequence (NM_024675; transcript ID, ENST00000261584) using LALIGN (ExPASy: SIB bioinformatics resource portal**).**

In silico analysis

The intron 12 sequence plus 2000 bps downstream of the 3′-UTR were analyzed using RepeatMasker (http://www.repeatmasker.org) to identify interspersed repeats. DNA sequence regions with Alu repeat signatures of interest were aligned using LALIGN to determine the similarity of the two Alu repeats.

cDNA analysis

The *PALB2* exon 13 duplication identified through commercial testing was confirmed prior to transcript analysis. Total RNA was extracted using the PAXgene BloodRNA 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* exon 13 duplication. 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, I12F: 5′-GTAAAACGACGGCCAG TCTGTGCCAAAGAGAGTGAGTC-3′; Reverse, 3′R: 5′-CAGGAAACAGCTATGAC CTGTCTGGACATAAA-CAAGCAA-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 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 I12F and 3′R primers covering cDNA regions of exon 13. The PCR products were visualized by QIAxcel (QIAGEN), purified by 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

Segregation analysis

The pattern of ovarian and breast cancers in the maternal kindred (Fig. 1) was consistent with autosomal dominant inheritance of a deleterious *PALB2* mutation. Given our suspicion based on family history, we wanted to identify whether the *PALB2* duplication segregated with the breast cancer in the family. Our family studies revealed that the proband's sister with breast DCIS and her maternal aunt with bilateral breast cancer also carry the *PALB2* duplication exon 13, as determined by the MSK-IMPACT assay (we reviewed *PALB2* copy number only) and confirmed by MLPA analysis (Fig. 2). Therefore, the *PALB2* duplication initially identified in the proband with high-grade serous ovarian cancer segregates with the breast cancers in this family.

Identification of duplication breakpoints

Given that the *PALB2* duplication was tracking with the cancers in the family, and the possibility of targeted therapeutic options becoming available for our patient should she be found to carry a deleterious mutation in a homologous recombination repair gene, we decided to investigate the biological effects of the exon 13 duplication. We first confirmed this rearrangement by amplifying exon 13 using LR-PCR. We observed an extra band of approximately 4–6 kb in size, which was absent in the negative control sample (Fig. 3a). One primer pair specific to the patient sample was designed to obtain adequate length of PCR product to determine the breakpoints. The 5′ breakpoint located in intron 12 was mapped using a few primers. The right breakpoint was determined by sequencing the LR-PCR product using three forward sequencing primers about 600 bp apart, specific to the downstream region of 3′-UTR. Combination of the forward and reverse sequencing primers amplified a fragment of approximately 300–400 bp size. A sequence analysis of this fragment revealed the duplication conjunctions located 1089 bp downstream of exon 12, in intron 12, and 989 bp downstream of the *PALB2* 3′-UTR (Fig. 4), resulting in a duplicated region of 4594 bp.

Analysis of intron 12 and the region downstream of the PALB2 3′-UTR by RepeatMasker revealed that there are eight Alu repeats in intron 12 and five Alu elements within the region 2000 bp downstream of the 3′-UTR. Both 5′ and 3′ breakpoints occurred within Alu elements, named AluSg in intron 12 and AluSc downstream of the 3′-UTR. These elements are characterized by a 32-bp region of perfect identity (Fig. 3c), implicating homologous recombination as the underlying mechanism of the tandem duplication (Fig. 4).

PALB2 exon 13 duplication disrupts normal splicing and presumably leads to premature protein truncation

The effect of *PALB2* exon 13 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 12 and the entire coding region of exon 13, which is likely affected by the duplication. An additional band was identified in the patient, patient's 48-year-old sister, aunt, and 52-year-old sister. It is absent in her 57-year-old sister (lane 5, Fig. 5a). This band represents an aberrant RNA splicing product attributable to the exon 13 duplication. Further RT-PCR, cloning, and sequencing results revealed that the duplication leads to a duplication of the first 53 bps at the beginning of exon 13 (Fig. 5b). This insertion causes a frameshift that creates a premature stop codon and leads to the loss of 41 amino acids within the WD domain of the C-terminus of the PALB2 protein, which is critical for its interaction with BRCA2 [6, 14, 35].

Family expansion testing

Prior to meeting with the CGS, all three of the proband's sisters had opted to undergo risk-reducing bilateral salpingo-oophorectomy based on the family history of ovarian cancer. Prior to genetic testing, all three sisters expressed that they would not regret having had risk-reducing surgery should they be shown to not share the familial *PALB2* duplication that is expected to be responsible for the ovarian cancer, particularly since the proband was not found to carry another mutation that could explain her personal history of cancer. Subsequent to the molecular studies being performed to determine the pathogenicity of the *PALB2* duplication in this family, we offered genetic testing to the proband's unaffected sisters through MSK-IMPACT and confirmed by both RT-PCR (Fig. 5a, 52-year-old and 57-year-old sisters) and MLPA analysis (data not shown). One of the unaffected sisters was found to carry the *PALB2* duplication and the other unaffected sister does not carry the familial duplication. Since we have confirmed that the *PALB2* duplication is pathogenic, we have spoken to the maternal aunt informing her of the amended results and recommended that her three sons, all of whom have daughters, meet with a local genetic counselor for an individualized risk assessment and their own genetic testing. At the time of submission, genetic testing results were pending for these family members.

Discussion

This study identified the exact breakpoints of the *PALB2* exon 13 duplication and demonstrated a large duplication involving AluSg in intron 12 and AluSc8 in the downstream region of the *PALB2* 3′-UTR through a mechanism of Alu-mediated non-allelic homologous recombination (NAHR). Alu-mediated HR inversions, duplications, deletions,

and other alterations have been implicated not only in copy number and structural variations within the human genome but also in numerous human genetic disorders [36, 37]. Similar to the *BRCA1* gene known to bear Alu-mediated rearrangements [38, 39], analysis of *PALB2* also shows a high density of Alu elements which favor the occurrence of non-allelic homologous recombination. Therefore, Alu-mediated NAHR could be responsible for other exonic deletions or duplications in *PALB2*. To date, six large deletions and duplications have been found in the *PALB2* gene: three of which were mediated by Alu sequences and the rest were not yet characterized [5, 13, 17, 33].

We need to be cautious when interpreting truncating variants downstream of the most 3′ truncating variants established as pathogenic in the literature [40]. In our case, the large duplication disrupts normal splicing, introduces a premature stop codon at amino acid position 1151, and putatively produces a truncated PALB2 protein of 1150 amino acids instead of 1186 amino acids. *PALB2* pathogenic truncating mutations affecting the very C-terminus of the protein have been reported to be associated with breast cancer and/or Fanconi anemia [14, 41]. Since the premature truncation caused by the reported *PALB2* exon 13 duplication presumably removes the 36 amino acids at the C-terminus of the PALB2 protein, which is critical for its normal function, we speculate that the duplication would contribute to the ovarian cancer in the proband and the breast cancers that occurred in the family. Our co-segregation data further supports a role for this duplication in cancer predisposition in this family.

To date, *PALB2* gene mutations have not been extensively studied in patients with ovarian cancer and its role in ovarian cancer predisposition is not well-established. Two out of 339 patients with ovarian cancer (0.6 %) were found to carry a *PALB2* gene mutation (c. 509_510delGA) in a study from central Poland [42]. One pathogenic *PALB2* mutation (c. 172_175delTTGT) was identified in 1/253 (0.4 %) ovarian cancer patients from the Volga-Ural region [43]. *PALB2* promoter hypermethylation was detected in 4 of 53 sporadic ovarian tumor cell lines [44]. In our study, the proband who carries this *PALB2* duplication was diagnosed with stage IV high-grade serous ovarian cancer. Her maternal aunt and one sister carrying the same alternation were diagnosed with breast cancer. It is known that germline loss-of-function mutations in *PALB2* confer a hereditary predisposition to breast cancer [12, 45, 46] and pancreatic cancer [16, 33, 47]. Our results suggest that *PALB2* deleterious mutations may also predispose to ovarian cancer. The presented results support the recently published suggestion that genetic screening for *PALB2* protein-truncating alterations and large genome rearrangement mutations in the clinical setting may be considered for hereditary breast/ovarian cancer families [12, 32]. It is worthy to note that the proband's mother, who was 80 years of age and cancer-free during these analyses, is an obligate carrier. This fits in with our understanding that the *PALB2* gene is a moderate risk gene with reduced penetrance compared to that of a *BRCA1/2* mutation carrier.

For the proband, identification of a pathologic germline *PALB2* mutation identified her as a potential candidate for treatment with a DNA damage repair inhibitor such as a PARP inhibitor [48]. Had this variant not been identified (since she was *BRCA*-negative), PARPi may not have been a therapeutic option for treatment of her ovarian cancer. For the proband's unaffected sister and any other female family members who do not carry the

PALB2 duplication, these results provide reassurance regarding their breast cancer risk. Since the *PALB2* gene has been shown to be associated with increased risks of developing breast cancer and the duplication segregates with the breast cancer diagnoses in this family, this is convincing data that the duplication accounts for the family history of breast cancer. Thus, unaffected relatives who do not carry the *PALB2* duplication can follow general population guidelines for breast cancer surveillance. Had a cancer predisposition marker not been identified; this sister and other unaffected female family members would be subjected to unnecessary breast surveillance by way of annual magnetic resonance imaging (MRI) based on an unexplained significant family history of breast cancer. In addition, these negative results provide reassurance for any non-carrier'schildren in terms of future cancer risks. For the proband's unaffected sister and any other female relatives who are found to carry the *PALB2* duplication, knowing this result provides them with information so they can proceed with appropriate cancer surveillance. Various groups, including the National Comprehensive Cancer Network, recommend that females with *PALB2* mutations undergo increased breast surveillance, including annual mammograms and breast MRI, with some suggesting that surveillance begin at age 30 [7]. Based on currently available data, risk-reducing BSO is not typically recommended for *PALB2* mutation carriers, although given this family's history of ovarian cancer, surgery could be supported. Further analyses of a larger population of *PALB2* mutation carriers, including classification of novel *PALB2* variants of unclear clinical significance, are highly desirable for precise evaluation of ovarian cancer penetrance to improve clinical management in individuals and families with *PALB2* mutations.

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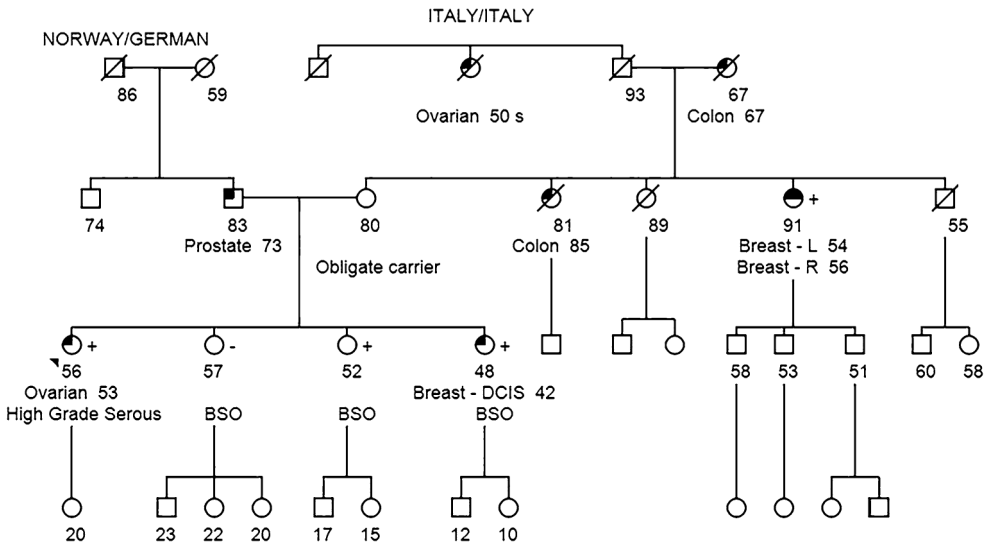

Fig. 1.

Patient pedigree. The patient (indicated with the *arrow*) is a 56-year-old Norwegian/German/Italian woman who was diagnosed with stage IV, high-grade serous ovarian cancer involving bilateral ovaries and fallopian tubes. One of the proband's three sisters was diagnosed with breast cancer (ductal carcinoma in situ, DCIS) at 42 years of age. A maternal aunt was diagnosed with bilateral breast cancer at 54 and 56 years of age. The proband's paternal great-aunt was diagnosed with ovarian cancer (medical records were not available)

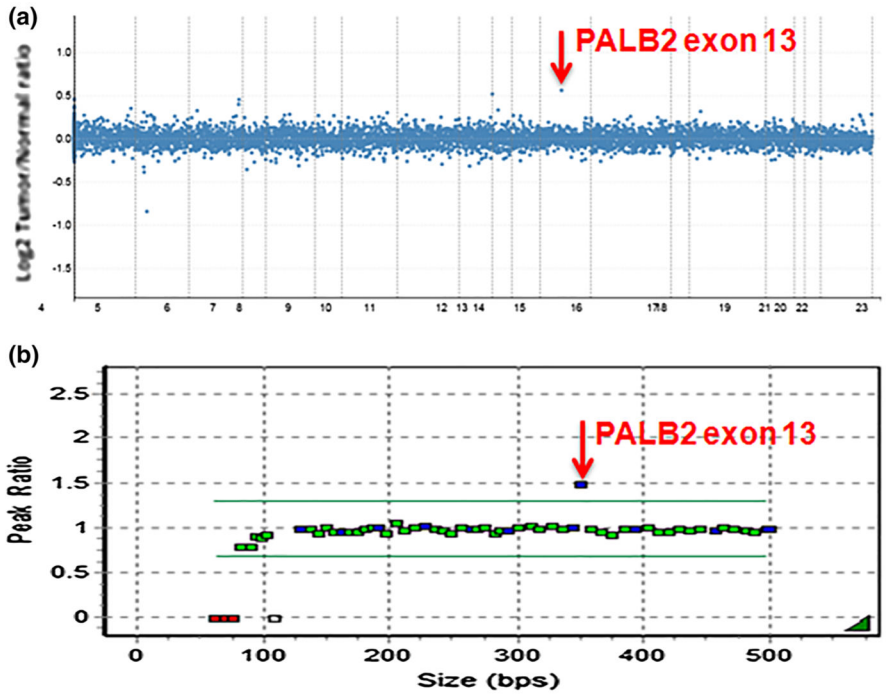

Fig. 2.

PALB2 Exon 13 duplication testing. **a** *PALB2* Exon 13 duplication detected by the MSK-IMPACT. **b** *PALB2* Exon 13 confirmation by MLPA

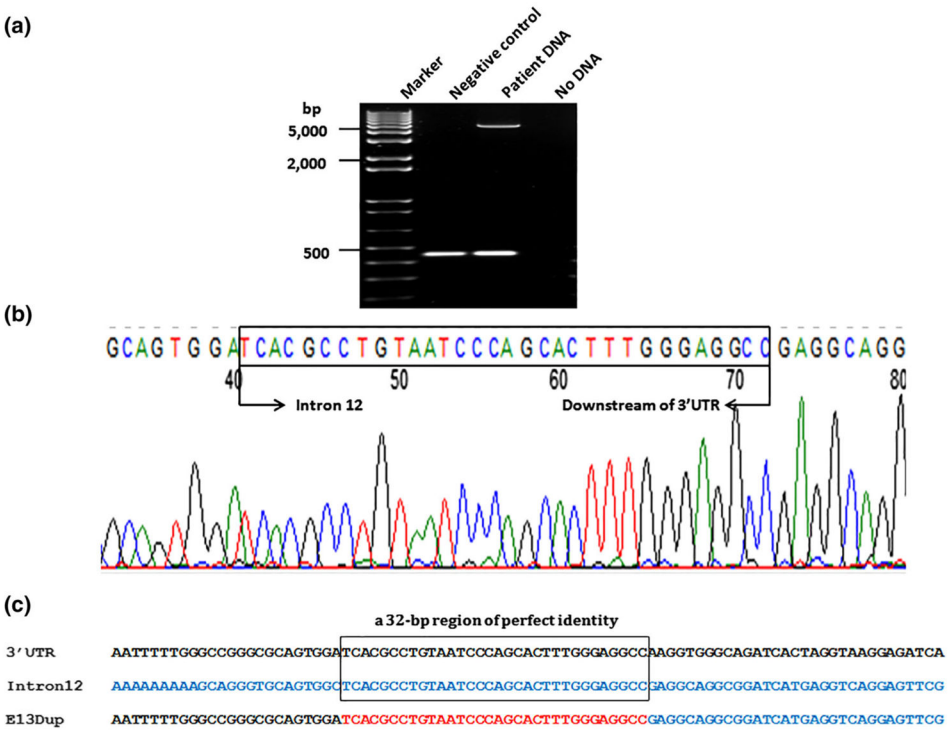

Fig. 3.

Breakpoint determination. **a** *PALB2* exon 13 duplication confirmation by long-range PCR. An extra band of approximately 4–6 kb in size was detected in the proband's blood DNA, which is absent in the negative control sample. **b** Localization of breakpoint. Electropherogram showing the breakpoint sequence. **c** Sequence alignment in the breakpoint region showing the 32-bp perfect identity between the intron 12 and 3′-UTR of *PALB2*

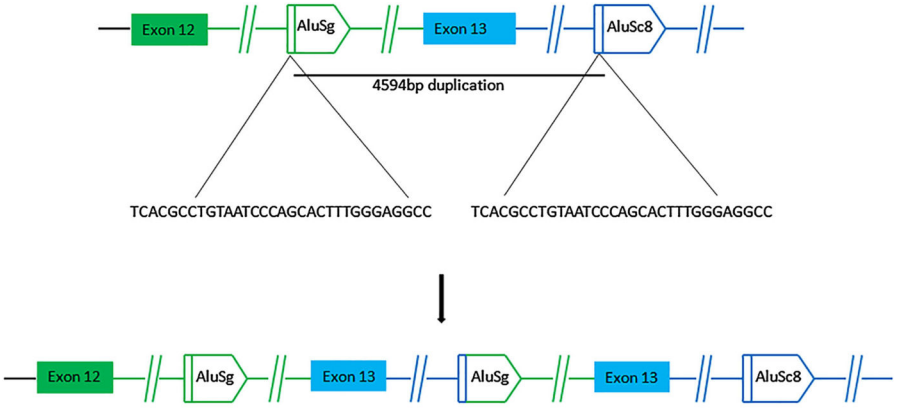

Fig. 4.

An Alu-mediated mechanism appears to be responsible for exon 13 duplication. A sequence analysis of the LR-PCR product allowed us to identify the duplication conjunctions which are located 1089 bp downstream of exon 12, in intron 12, and 989 bp downstream of the *PALB2* 3′-UTR

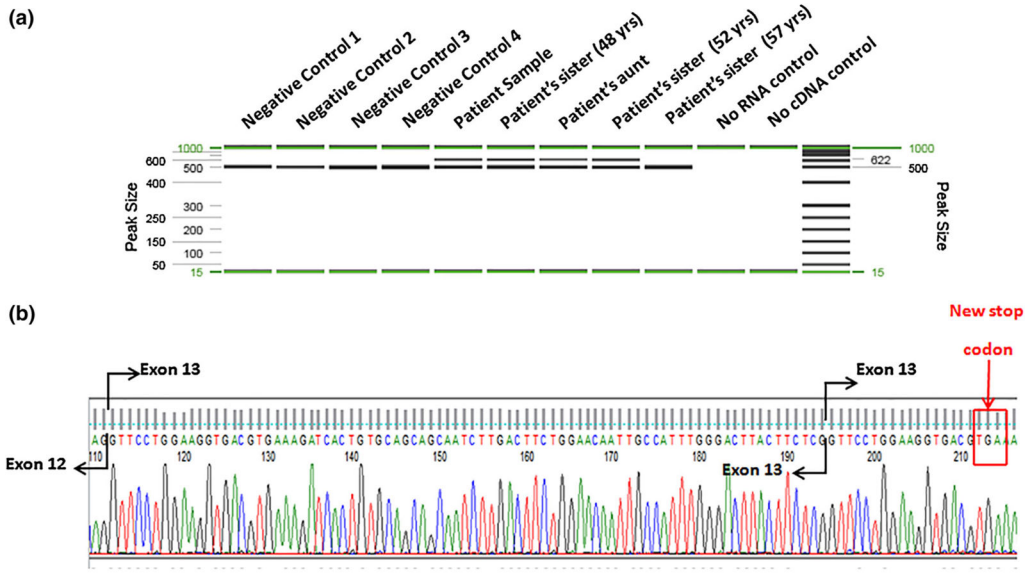

Fig. 5.

PALB2 Exon 13 duplication disrupts normal splicing and leads to frameshift. **a** RT-PCR products run on QIAxcel. An extra band was observed in the patient, patient's 48-year-old sister, aunt, and 52-year-old sister. **b** Electropherogram showing the inserted sequence from the upper band in the patient of figure (**a**). The insertion leads to a new stop codon as indicated by the *red box*