Breast Cancer Research and Treatment (2019) 174:543–550
https://doi.org/10.1007/s10549-018-05094-8

BRIEF REPORT

Alternative transcript imbalance underlying breast cancer susceptibility in a family carrying *PALB2* c.3201+5G>T

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Received: 8 June 2018 / Accepted: 7 December 2018 / Published online: 14 December 2018

Abstract

Purpose Disruption of splicing motifs by genetic variants can affect the correct generation of mature mRNA molecules leading to aberrant transcripts. In some cases, variants may alter the physiological transcription profile composed of several transcripts, and an accurate in vitro evaluation is crucial to establish their pathogenicity. In this study, we have characterized a novel *PALB2* variant c.3201+5G>T identified in a breast cancer family.

Methods Peripheral blood RNA was analyzed in two carriers and ten controls by RT-PCR and Sanger sequencing. The splicing profile was also characterized by semi-quantitative capillary electrophoresis and quantitative PCR. RAD51 foci formation and *PALB2* LOH status were evaluated in primary breast tumor samples from the carriers.

Results *PALB2* c.3201+5G>T disrupts intron 11 donor splice site and modifies the abundance of several alternative transcripts (Δ11, Δ12, and Δ11,12), also present in control samples. All transcripts are predicted to encode for non-functional proteins. Semi-quantitative and quantitative analysis of *PALB2* full-length transcript indicated haploinsufficiency in carriers. One tumor exhibited *PALB2* LOH and RAD51 assay indicated homologous recombination deficiency in both tumors.

Conclusions Our results support a pathogenic classification for *PALB2* c.3201+5G>T, highlighting the impact of variants causing an imbalanced expression of natural RNA isoforms in cancer susceptibility.

Keywords Hereditary breast cancer · *PALB2* · Alternative splicing · RNA isoforms

Introduction

Genetic variants that disrupt splicing motifs can lead to RNA mis-splicing, contributing to human hereditary diseases. The most common type of variants that alter splicing are located in highly conserved GT and AG dinucleotides (positions + 1 and + 2 of the 5′donor site, and positions − 2 and − 1 of the 3′ acceptor site, respectively). Other exonic and intronic nucleotides surrounding these positions are also conserved and critical for a correct splice site selection [1, 2]. Yet, their potential effects on splicing are scarcely assayed in the clinical setting.

Routine splicing analysis in clinical diagnostics is usually performed by RT-PCR, agarose gel examination and Sanger sequencing. However, splicing profiles can be complex to interpret when several alternative transcripts are present, especially if these transcripts are in-frame events that might rescue gene functionality [3]. Moreover, the variant allele may still be able to produce full-length transcript [4], which

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10549-018-05094-8) contains supplementary material, which is available to authorized users.

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may affect the resulting cell phenotype. High-resolution electrophoresis and expression assays can provide a comprehensive qualitative and quantitative screening of the whole mRNA landscape [5].

PALB2 (Partner and Localizer of BRCA2) encodes for a nuclear protein of 1186 amino acids, that interacts with BRCA1, BRCA2, and other DNA repair proteins like RAD51 paralogs, to promote DNA double-strand break repair by homologous recombination (HR) [6–8]. *PALB2* is now unquestionably present in multi-gene panel testing for hereditary breast cancer (BC) individuals, since large studies of patients and controls found that *PALB2* pathogenic variants conferred high risk of developing BC [9, 10]. *PALB2* is also linked with male BC and pancreatic cancer [11, 12], and recent works also found an association with colorectal cancer [13], although larger epidemiological studies are still needed to definitely consider *PALB2* as a colorectal cancer susceptibility gene.

In this work, we have characterized at RNA level a novel *PALB2* c.3201+5G>T variant, located outside the canonical donor splice site from intron 11. Our study highlights the complexity to interpret the pathogenicity of variants causing an imbalanced expression of natural RNA isoforms.

Materials and methods

The proband was diagnosed with a breast invasive ductal carcinoma (IDC) at age 45 and with a second primary lung cancer at age 62. Two first-degree relatives (brother and mother) were also affected with breast IDCs at the ages of 54 and 82, respectively. Hormonal status from the three IDCs was ER+, PR+, and HER2−. A paternal cousin was affected with BC at age 66, and two paternal uncles had stomach cancer (age unknown). The mother was also diagnosed with colorectal cancer at age 86, and the brother had colon polyposis at age 40 (Fig. 1a). Patients received genetic counselling and written informed consent was obtained for further genetic and research studies.

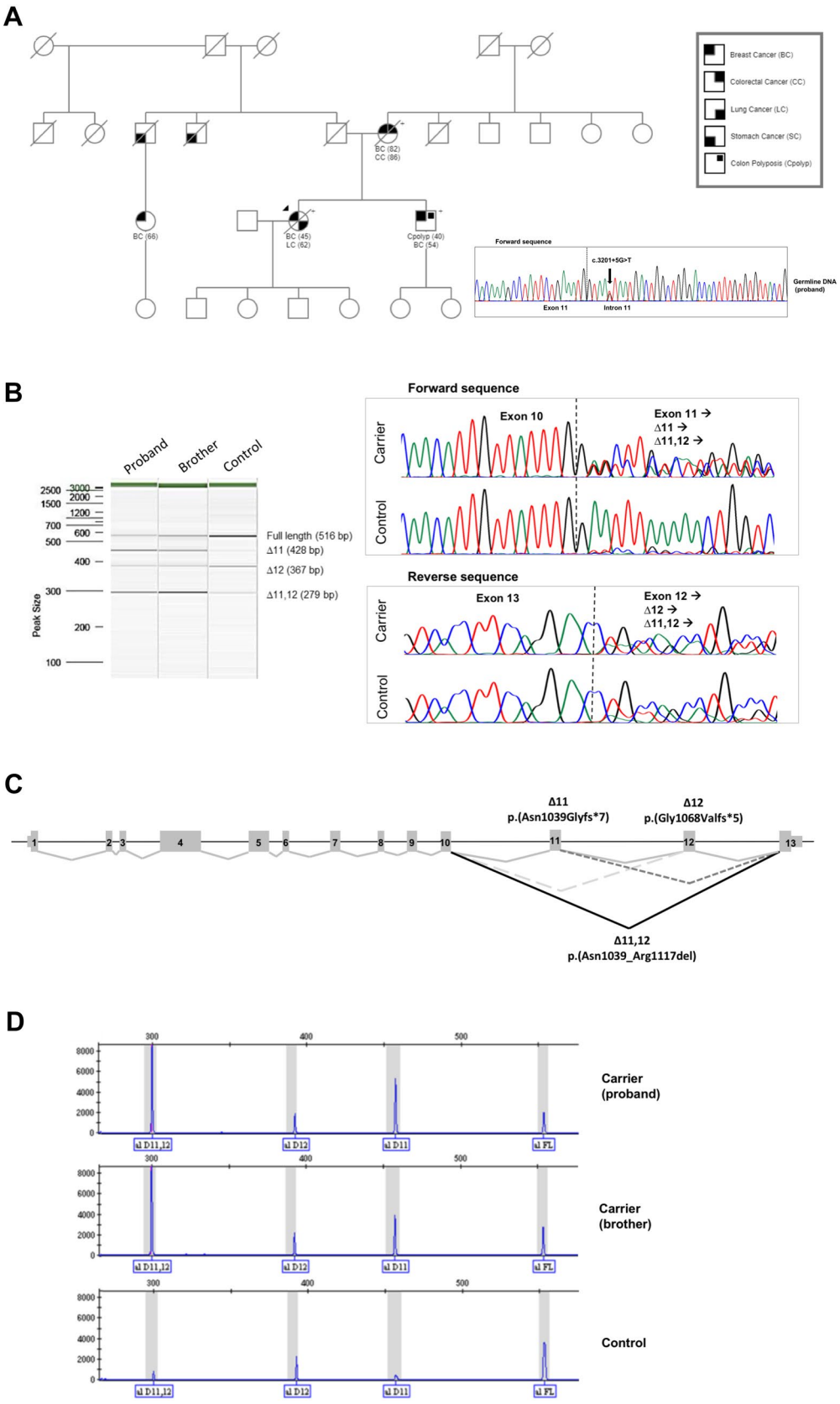
PALB2 c.3201+5G>T variant was identified by multi-gene panel testing as described in a previous work from our laboratory [14], and confirmed by Sanger sequencing. Protocols for germline DNA, RNA, and tumor DNA extraction, as well as in silico splicing analysis, RT-PCR experiments, sequencing methodologies and immunofluorescence for RAD51 foci detection, are extensively described in Supplementary Material. In brief, RT-PCR primers were designed to amplify the *PALB2* region comprised between exons 9 and 13 (Supplementary Table 1) in two carriers (proband and brother) and 10 healthy controls. RT-PCR products were qualitatively analyzed by capillary electrophoresis in a QIAxcel Advanced system (QIAGEN) and by Sanger sequencing. Semi-quantitative analysis was performed by capillary electrophoresis of FAM-labeled amplicons in a Genetic Analyzer ABI3130xl (Applied Biosystems); and *PALB2* expression was measured by quantitative real-time PCR (qPCR) using predesigned human-specific primers and TaqMan probes. *PALB2* loss of heterozygosity (LOH) was determined in primary breast tumors from the proband's mother and brother by Sanger sequencing and targeted gene sequencing. Homologous recombination repair activity was also assessed in the tumor specimens by immunofluorescence detection of RAD51 foci as previously described [15] (see Supplementary Material for further details).

Fig. 1 Family pedigree and qualitative analysis of the RNA effect caused by *PALB2* c.3201+5G>T variant. **a** The index case is indicated with an arrow head and ages of cancer onset are specified between brackets. Confirmed *PALB2* c.3201+5G>T carriers are marked with a + symbol. Sanger electropherogram confirming the presence of the variant in proband's germline DNA is shown. **b** Capillary electrophoresis in a QIAxcel instrument of RT-PCR products from two carriers (proband and brother) and one control showing different abundance of *PALB2* isoforms. Forward and reverse electopherograms show cDNA sequences of *PALB2* c.3201+5G>T carrier and a healthy control. Isoforms lacking exon 11, exon 12 and both 11 and 12 were confirmed. **c** Diagram showing the different *PALB2* isoforms detected: reference full-length isoform (solid gray lines), Δ11 (discontinuous line), Δ12 (spotted line) and Δ11,12 (solid black line). **d** Fluorescence fragment profiles of the two carriers and one control showing the presence of Δ11, Δ12 and Δ11,12 in all samples but at different expression levels

Results

PALB2 variant c.3201+5G>T was identified in a breast cancer family by multi-gene panel testing. This variant was confirmed by Sanger sequencing in blood DNA from the proband and her brother, also affected with BC (Fig. 1a). To our knowledge, this variant is not present in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), LOVD (Leiden Open Variation Database, http://www.lovd.nl/3.0/home), gnomAD (Genome Aggregation Database, http://gnomad.broadinstitute.org/), FLOSSIES (https://whi.color.com/) and HGMD (The Human Gene Mutation Database, http://www.hgmd.cf.ac.uk/ac/index.php) databases, and it is not reported in the literature. In silico analysis showed a reduction of the natural donor splice site, indicating a potential splicing alteration (Supplementary Table 2).

Qualitative cDNA study of the two carriers and 10 controls by QIAxcel capillary electrophoresis revealed in all samples the presence of four transcripts corresponding to the reference full-length transcript (516 bp), Δ11 (428 bp), Δ12 (367 bp), and Δ11,12 (279 bp) (Fig. 1b, c). All transcripts were confirmed by Sanger sequencing (Fig. 1b). Capillary electrophoresis of FAM-labeled amplicons revealed a variation in the proportion of the alternative transcripts


A
Breast Cancer (BC)
Colorectal Cancer (CC)
Lung Cancer (LC)
Stomach Cancer (SC)
Colon Polyposis (Cpolyp)
BC (82)
CC (86)
BC (66)
BC (45)
LC (62)
Cpolyp (40)
BC (54)
Forward sequence
c.3201+5G>T
Exon 11
Intron 11
Germline DNA (proband)
B
Proband
Brother
Control
Peak Size
2500
1500
700
500
400
300
200
100
3000
2000
1200
600
Full length (516 bp)
Δ11 (428 bp)
Δ12 (367 bp)
Δ11,12 (279 bp)
Forward sequence
Carrier
Control
Exon 10
Exon 11 →
Δ11 →
Δ11,12 →
Reverse sequence
Exon 13
Exon 12 →
Δ12 →
Δ11,12 →
C
Δ11
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Control
300
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2000
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aJ D11,12
aJ D12
aJ D11
aJ FL

Δ11, Δ11,12, and Δ12 in variant carriers compared to controls (Fig. 1d). Semi-quantitative data obtained from QIAxcel electrophoresis showed a > 0.5 reduction of full-length transcript levels in both carriers compared to controls, suggesting that the variant allele is not producing normal transcripts (Fig. 2a). Splicing fraction estimation obtained from capillary electrophoresis of FAM-labeled amplicons indicated that isoform Δ11,12 substantially contributes to the total splicing fraction in carriers: this isoform was present at higher proportion in both carriers (48.50%) compared to controls (11.94%) (Fig. 2b), and it causes an in-frame deletion resulting in a PALB2 protein lacking 79 aa (p.Asn1039_Arg1117del) that are part of the WD40 domain (Supplementary Fig. 1). Isoform Δ11 was also increased in carriers (24.14%) compared to controls (5.27%), and it is predicted to introduce a premature stop codon (p.Asn1039Glyfs*7). Finally, the proportion of isoform Δ12, also predicted to introduce a premature stop codon (p.Gly1068Valfs*5), diminishes in carriers (11.88%) compared to controls (24.83%) (Fig. 2b). Quantitative measurement of *PALB2* global expression and *PALB2* full-length expression using Taqman assays targeting exon 5–6 and exon 11–12 junctions, respectively, showed a significant reduction in carriers compared to controls: carriers = 0.67 ± 0.18 vs. controls = 1.42 ± 0.11, $p = 0.0175$; carriers = 0.36 ± 0.07 vs. controls = 1.90 ± 0.19, $p = 0.0059$, respectively (Mean ± 95% CI, *t-test* of unpaired samples) (Fig. 2c).

We also examined *PALB2* LOH status in breast tumor samples from the proband's mother and brother. Targeted gene sequencing and Sanger sequencing revealed the loss of the wild-type allele in the mother's tumor (80% cellularity; Variant Allele Frequency (VAF) = 81.82%), whereas the brother did not exhibit LOH (70% cellularity; VAF = 56.52%) (Fig. 3a). Immunofluorescence assay did not show RAD51 foci in breast tumor-FFPE from carriers (see Fig. 3b for mother's tumor; refer to [16] for brother's tumor), indicating homologous recombination deficiency in both carriers.

Discussion

We have characterized a novel *PALB2* variant c.3201+5G>T identified in a family with breast cancer history. The variant is located at +5 position from intron 11 donor splice site (CAG/GTAA**G**TAT) and in silico analysis predict the disruption of the splice site (Supplementary Table 2). Results obtained from RNA analysis confirmed a splicing alteration consisting of an imbalanced expression of several *PALB2* alternative RNA isoforms. The variant up-regulates isoforms Δ11,12 (in-frame) and Δ11 (frameshift), and down-regulates isoform Δ12 (frameshift). The splicing profiles detected in peripheral blood from control samples are consistent with RNAseq results obtained from lymphoblastoid cell lines [17], i.e., Δ12 isoform is predominant over Δ11 and Δ11,12 (Fig. 2b). Isoform Δ11,12 presumably contributes to variant pathogenicity by encoding a PALB2 protein lacking 79aa of the WD40 domain. Protein alignment of the deleted region in 10 reference model species, shows high conservation of this region with ≈ 50% of the residues conserved across species (Supplementary Fig. 1). WD40 domain mediates direct interactions between PALB2 and key proteins involved in homologous recombination (HR), such as BRCA2 and RAD51 [18], RAD51C and XRCC3 [8], as well as the binding to POLH which mediates recombination-associated DNA synthesis [19]. Hence, the loss of amino acids 1039 to 1117 from WD40, comprising the PALB2 key residues (Leu1046, Lys1047, Leu1070, Pro1097, and Lys1098) that provide interaction with BRCA2 [18], is probably related to HR repair impairment. In fact, functional studies evaluating interactions between PALB2 WD40 domain and HR proteins found that breast cancer-associated missense variants (L939W, T1030I, L1143P) gave rise to unstable PALB2 proteins that altered the binding to BRCA2, RAD51C, and RAD51 [20]. In addition, WD40 domain contains a nuclear export sequence (NES) (amino acids 852–987) that would be exposed to an export protein if a premature stop codon is present after aa 987, leading to an unusual cytoplasmic localization and aberrant function of PALB2 [21]. Therefore, isoforms Δ11 and Δ12 would also contribute to variant pathogenicity by producing truncated proteins (p.Asn1039Glyfs*7 and p.Gly1068Valfs*5, respectively) exposing NES and in consequence inducing a cytoplasmic mislocalization of PALB2 along with its interacting proteins, preventing their access to sites of DNA damage [21].

Semi-quantitative analysis showed a drastic reduction of full-length transcript levels in carriers. Unfortunately, none of the carriers had informative heterozygous exonic variants to perform allele-specific assays and formally exclude the possibility that the variant allele produces a certain amount of full-length transcript. Alternatively, specific amplification and measurement of full-length transcript by qPCR (exons 11–12 probe), showed a significant reduction in carriers compared to controls, supporting that the variant allele is not transcribing full-length transcripts (Fig. 2c).

The loss of the wild-type allele in one tumor sample also supports a potential causality of the variant. However, the second tumor did not exhibit LOH and massive sequencing of *PALB2* whole coding region ruled out the presence of somatic deleterious mutations. In this regard, some *PALB2* heterozygous tumors with no LOH and high HR deficiency scores have been described, suggesting alternative mechanisms of PALB2 functional loss [22] or a dominant negative effect of PALB2 mutated proteins [23].

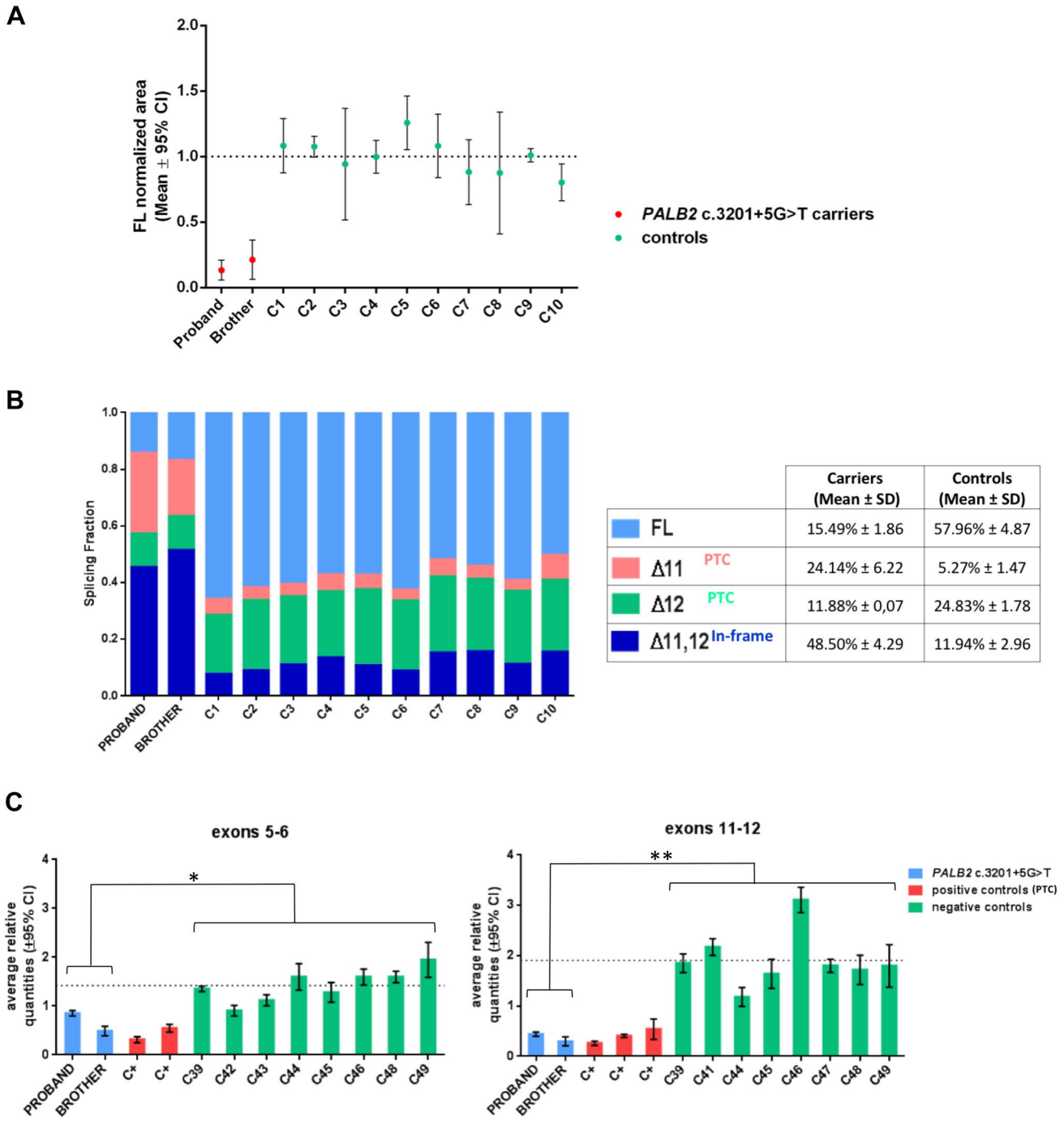

Fig. 2 Semi-quantitaive and quantitative evaluation of the effect on RNA yielded by *PALB2* c.3201+5G>T. **a** Full-length relative amounts measured in the two carriers (red dots) and 10 non-carrier controls (green dots). Normalized full-length mean obtained from controls is indicated in dotted line. Error bars indicate 95% confidence interval. **b** Splicing fraction measured by capillary electrophoresis analysis of FAM-labeled fragments. Bar graphs show the splicing fraction mean of each transcript, which is indicated by different colors: full-length (FL) in light blue, Δ11 in orange, Δ12 in green and Δ11,12 in dark blue. **c** *PALB2* expression measured by qPCR in peripheral blood from *PALB2* c.3201+5G>T carriers (blue) and non-carrier controls (green). Carriers of *PALB2* frameshift variants were used as positive controls (in red). *B2M* and *GAPDH* were used as reference genes for data normalization. Mean ±95% CI of technical replicates is denoted. Unpaired *t-test* in carriers vs. controls (*$p < 0.05$; **$p < 0.01$)

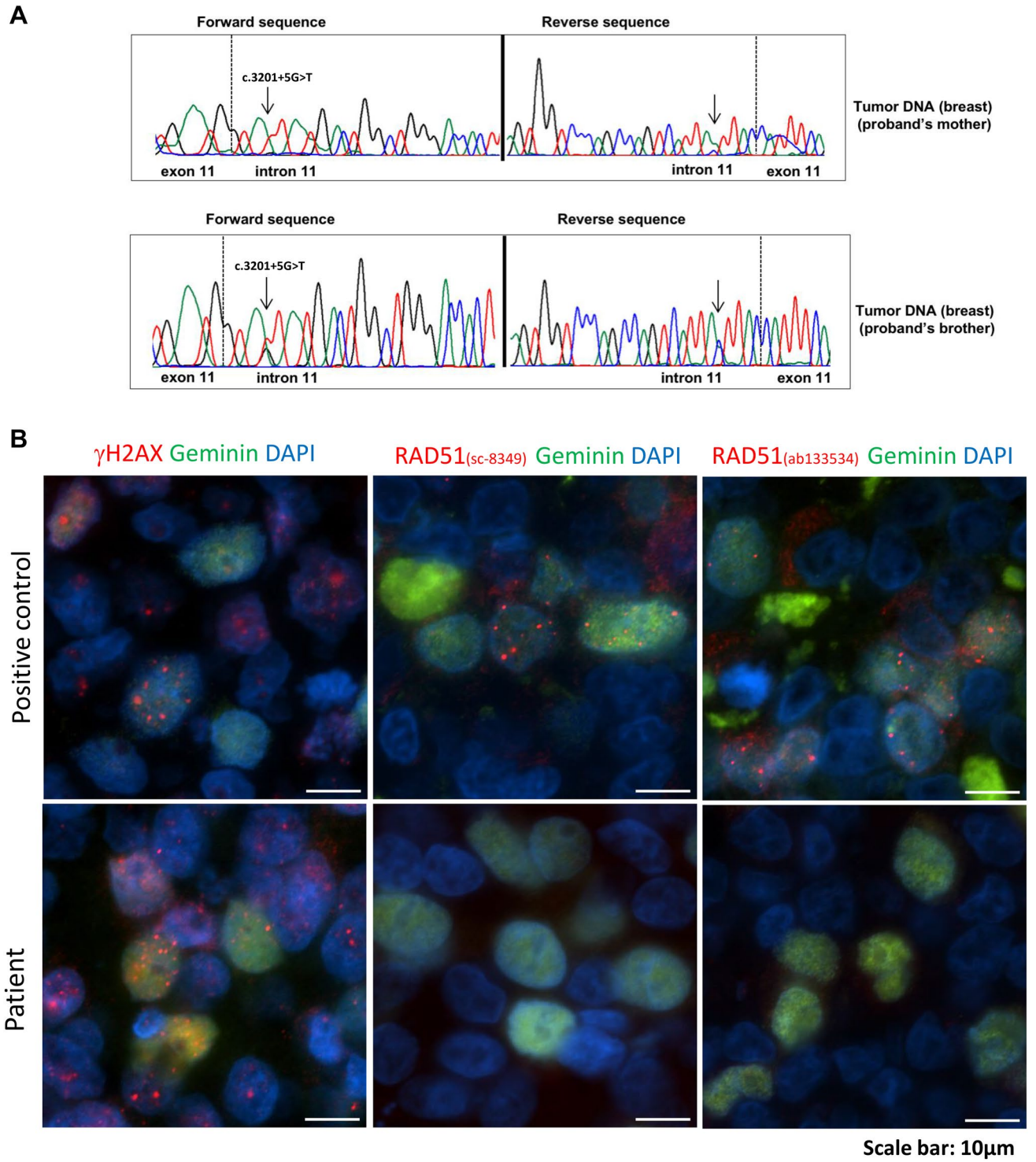

Fig. 3 LOH analysis and RAD51 assay in tumor samples. **a** Sanger analysis in primary breast tumor samples detected LOH in one tumor (mother). **b** Detection of γH2AX and RAD51 foci (red) in S/G2-phase cells (geminin-positive; green) by immunofluorescence. RAD51 foci were not detected in mother's tumor (patient). Nuclei were visualized with DAPI (blue). A *PALB2* wild-type patient-derived xenograft model of a breast tumor sample was used as positive control

Previous works have proposed RAD51 foci formation as predictive biomarker of response to PARP inhibitors (PARPi) in homologous recombination (HR) deficient tumors [15, 24] and demonstrated that RAD51 foci detection is feasible in formalin-fixed paraffin-embedded breast cancer samples to accurately detect HR activity [15].

Interestingly, tumor samples from carriers did not display RAD51 foci, supporting that HR-deficiency is due to non-functional PALB2 proteins generated from *PALB2* c.3201+5G>T alleles.

PALB2 has been defined as a crucial mediator of HR in human cells, and PALB2-deficient cells have been shown to be sensitive to PARPi [25]. In this regard, the promising clinical applicability of PARP inhibitors in HR-deficient tumors is likely to be feasible in the short term for carriers of germline *PALB2* pathogenic variants [26, 27].

According to ACMG (American College of Medical Genetics and Genomics) guidelines for variant interpretation [28], *PALB2* c.3201+5G>T variant should be classified as likely pathogenic: there are well-established in vitro functional studies supporting a damaging effect (PS3), the variant is present in affected individuals and absent in gnomAD controls (PM2), it cosegregates with disease in multiple-affected family members (PP1) and there is computational evidence supporting a deleterious effect (PP3).

Other *PALB2* variants affecting intron 11 donor site (intronic + 1,+2 positions, or beyond) have been described in breast cancer families and received different types of classification. Some variants were predicted to alter splicing, but to our knowledge, no experimental characterization has been performed (Supplementary Table 2).

The lack of studies evaluating the functionality of proteins generated from in-frame RNA isoforms, questions whether *PALB2* isoform Δ11,12 could retain some functionality and modulate c.3201+5G>T cancer risk. However, our study supports a pathogenic role for the variant based on: (i) segregation in three relatives affected with BC (Fig. 1a); (ii) reduction of *PALB2* global expression in carriers (exons 5–6 probe; Fig. 2c) which would indirectly indicate less amount of PALB2 proteins; (iii) reduction of *PALB2* full-length transcript levels in carriers (exons 11–12 probe; Fig. 2c); (iv) detection of isoforms Δ11,12, Δ11 and Δ12 that encode proteins lacking totally or partially an important functional domain (WD40); (v) absence of RAD51 foci in tumor samples from the variant carriers, indicating homologous recombination deficiency (Fig. 3b).

In all, our study shows how an imbalanced expression of natural occurring *PALB2* RNA isoforms can predispose to breast cancer disease, and highlights the use of accurate qualitative, quantitative and functional assays as a key procedure to correctly interpret genetic variants that generate complex splicing landscapes.

Acknowledgements The authors thank Cristina Cruz and Violeta Serra from the Experimental Therapeutics Group at VHIO for kindly providing immunofluorescence protocols and helpful discussions. The authors also acknowledge the Cellex Foundation for providing research facilities and equipment, and Leo Judkins for English language editing.

Funding This work was supported by Spanish Instituto de Salud Carlos III (ISCIII) funding, an initiative of the Spanish Ministry of Economy and Innovation partially supported by European Regional Development FEDER Funds: FIS PI12/02585 and PI15/00355 (to O Diez), PI13/01711 and PI16/01218 (to S. Gutiérrez-Enríquez). S. Gutiérrez-Enríquez is supported by a Miguel Servet contract (CP10/00617). M. Castroviejo-Bermejo is awarded with a Junta Provincial de Barcelona, Fundación Científica Asociación Española Contra el Cáncer (AECC) fellowship. S. Bonache is recipient of an Asociación Española Contra el Cáncer (AECC) contract.

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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