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The *RAD51D* c.82G>A (p.Val28Met) variant disrupts normal splicing and is associated with hereditary ovarian cancer

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Abstract

Purpose—Mutations in *RAD51D* are associated with a predisposition to primary ovarian, fallopian tube, and peritoneal carcinoma. Beyond ovarian cancer, recent studies have also suggested that mutations in *RAD51D* could confer increased risk of triple negative breast cancer (TNBC). Our study aims to characterize a *RAD51D* missense variant in a hereditary breast and ovarian cancer (OC) family.

Methods—The effects of the *RAD51D* c.82G>A (p.Val28Met) variant on mRNA splicing were evaluated and characterized using RT-PCR, cloning and DNA sequencing.

Results—This variant completely disrupts normal splicing and results in the loss of 3'end of 5'UTR and the entire exon 1 (c.−86_c.82), which presumably leads to loss of the RAD51D protein. The *RAD51D* c.82G>A (p.Val28Met) variant is clinically significant and classified as likely pathogenic.

Conclusions—Our results indicate that the *RAD51D* c.82G>A (p.Val28Met) variant contributes to cancer predisposition through disruption of normal mRNA splicing. The identification of this variant in an individual affected with high grade serous fallopian tube cancer suggests that the *RAD51D* variant may contribute to predisposition to the ovarian cancer in this family.

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

Conflict of Interest: Z.K.S. reports that an immediate family member holds consulting/advisory roles with Allergan, Adverum, Genentech/Roche, Gyroscope Tx, Novartis, Neurogene, Optos Plc, Regeneron, Regenxbio. L.Z. reports honoraria (Future Technology Research LLC, BGI, Illumina); honoraria and Travel and accommodation expenses (Roche Diagnostics Asia Pacific). Family members hold leadership position and ownership interests of Shanghai Genome Center.

Keywords

Ovarian cancer; *RAD51D* ; Missense variant; Splicing

Introduction

RAD51D, one of the five RAD51 paralogs (*RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3*), encodes a protein required for homologous recombinational repair (HRR) pathway of double-stranded DNA breaks arising during DNA replication or induced by DNA-damaging agents [1–3]. RAD51D forms a complex with RAD51B, RAD51C and XRCC2 (BCDX2 complex), which binds to nicks in duplex DNA and is responsible for RAD51 recruitment or stabilization at DNA damage sites in the early step of the HRR pathway [4,5]. In addition, RAD51D plays an important role in preventing telomere shortening and chromosome fusions [6] as well as maintaining genome stability in response to spontaneous DNA damage [7]. RAD51D is essential for cell viability as *RAD51D*-deficient mice are embryonic lethal [8].

RAD51D germline mutations have been identified in women affected with familial primary ovarian, fallopian tube, and peritoneal carcinoma [9–14]. Germline loss of function variants in *RAD51D* confer an increased risk of ovarian cancer with a relative risk estimated to be 6.3 [9]. Several other studies have further demonstrated that *RAD51D* is an ovarian cancer predisposition gene [10,11,15,16]. A few *RAD51D* deleterious mutations have also been identified in patients affected with breast cancer, specifically triple negative breast cancer (TNBC) [9–12,17–24], indicating that *RAD51D* germline mutations might be associated with increased risk of both ovarian and breast cancers. The relative risk for breast cancer was estimated to be 3.07 [20]. These studies suggest that in addition to being a moderate-penetrance susceptibility gene for ovarian cancer, *RAD51D* may also be enriched in patients with breast cancer, specifically TNBC [9,18,20].

Inhibition of poly ADP ribose polymerase (PARP) is a potential synthetic lethal therapeutic strategy for patients with inherited mutations in tumor suppressor genes such as *BRCA1* and *BRCA2* that are involved in HRR pathways [25–27]. Recently, PARP inhibitors, Olaparib (AZD 2281) and Rucaparib (AG014699), have been approved for the treatment of HRR-deficient high-grade ovarian carcinomas in patients with germline or somatic *BRCA1* or *BRCA2* mutations [28]. Prolonged responses to Olaparib or Rucaparib were achieved in patients whose ovarian, breast, pancreatic, and prostate cancers harbor *BRCA1/2* loss of function mutations or had defects in DNA repair genes [25,29–36]. Similar to *BRCA1* and *BRCA2*-silenced cells, cells deficient for *RAD51D* exhibit a failure of HRR [8,37] and display sensitivity to inhibition of PARP [9]. Carriers of germline or somatic mutations in *RAD51D* have shown objective response to inhibition of PARP [38]. Therefore, determining germline *RAD51D* variant pathogenicity in patients with ovarian cancer and *RAD51D* related cancers is of significant clinical relevance, particularly for cancer surveillance, risk reducing decisions and possibly treatment selection.

Interpretation of non-truncating variants can be challenging because it is not known whether these variants cause functional changes sufficient to predispose to cancer development, and

thus the ambiguity complicates cancer risk assessment and therapeutic treatment for patients who carry these variants of uncertain significance. Classification of substitutions at the last nucleotide of an exon is more complicated since such alterations may disrupt normal splicing and cause exon skipping [39–41], result in a missense mutation [42], or generate either a missense transcript or cause exon skipping in a patient-specific manner as reported in XSCID patients with the same 868G to A pathogenic variant of the gamma c gene [43]. Here, we report a rare heterozygous *RAD51D* substitution variant, c.82G>A (p.Val28Met), located at the last nucleotide of exon 1, in a breast/ovarian cancer family. This variant is absent from large population databases (gnomAD, 1000 Genomes), and has not been previously reported in the literature. We demonstrate that this variant completely disrupts the normal splice donor site at the beginning of intron 1 and activates a cryptic splice donor site at the c.−86G position, resulting in the loss of 3'end of 5'UTR and the entire exon 1 (c.−86_c.82). It presumably leads to an absent protein. Our results indicated that this variant likely contributes to cancer predisposition through disruption of normal splicing and activation of a cryptic splice site upstream of ATG translation initiation codon.

Materials and Methods

Subject

The proband was diagnosed with high grade serous fallopian tube cancer (HGSFT) at 54 years of age. She subsequently underwent debulking surgery including bilateral salpingo-oophorectomy (BSO). She subsequently completed adjuvant IV/IP cisplatin/Taxol chemotherapy, and, as of time of publication, the patient has remained without evidence of recurrence. A two-generation pedigree (Fig. 1) shows that one of the proband's two sisters was confirmed to have triple negative breast cancer at age 60 years and died of disease at age 60 years. This patient had negative *BRCA1/2* genetic testing at a commercial laboratory via sequence analysis; testing for large rearrangements was not performed. The patient's father and mother are both alive and, to our best knowledge, are not affected with cancer.

The proband consented to genetic testing as part of IRB approved Memorial Sloan Kettering Cancer Center (MSKCC) protocol "Clinical Significance of Germline BRCA Mutations" and subsequently had genetic testing performed via a reference laboratory with a hereditary cancer panel. The patient was found to carry the following three variants of uncertain significance (VUS): *BARD1* c.2171C>T (p.Ala724Val) [NM_000465]; *RAD51D* c.82G>A (p.Val28Met) [NM_002878]; and *RAD51D* c.629C>A (p.Ala210Glu) [NM_002878].

The patient's unaffected living sister, had already undergone a risk-reducing bilateral salpingo-oophorectomy (RR-BSO) prior to this study. She underwent predictive genetic testing for the *RAD51D* c.82G>A (p.Val28Met) variant and was positive for the variant.

cDNA analysis

Research blood samples were obtained for additional studies performed at MSKCC Diagnostic Molecular Genetics Laboratory (DMG). The *RAD51D* c.82G>A variant identified through commercial testing was confirmed prior to RNA 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 *RAD51D* c.82G>A 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'- GTA AAA CGA CGG CCA GT TCCTCCTCTCTCCTTTCTCC -3'; Reverse, e2R: 5'- CAG GAA ACA GCT ATG AC GGTCTGCAGAAACCAGGTC -3'). Each PCR reaction contained 12.5 μl 2× JumpStart REDTaq Ready Mix, 2 μl 10μM primers (1μl for each), 2μl cDNA (16 ng) 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 5min (1×).

Cloning, DNA gel extraction and sequencing

The RT-PCR products (4 μl) 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 E2R primers covering RNA regions of 5'UTR, exons 1 and 2. The rest RT-PCR products were run on 2% agarose gel at 90 Volts for 45 min. DNA bands were cut from the agarose gel and DNA was extracted using QIAquick Gel Extraction Kit, following the manufacturer's procedures (QIAGEN). Extracted DNA was amplified using the 5'utrF and E2R primers covering cDNA regions of 5' UTR, and exons 1 and 2. The PCR products from gel extraction or from colonies 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

RAD51D c.82G>A disrupts normal splicing and presumably leads to an absent protein

We first confirmed the presence of the *RAD51D* c.82G>A variant by Sanger sequencing using the patient's genomic DNA and found no other variants present in the 5'UTR and exon 1 (Fig. 2a). To evaluate the potential effects of the variant on splicing, we used the Alamut software, which incorporates four tools to predict the potential effects of *RAD51D* c.82G>A on mRNA splicing. Three out of four tools predicted a loss of or a weakening effect on the canonical donor site at c.82 position (Fig. 2b, 2c). The effect of the variant c.82G>A on RNA splicing was evaluated by amplifying regions of *RAD51D* from cDNA derived from the patient. PCR was designed to generate a fragment that spanned part of 5'UTR and exon 2 and the entire coding region of exon 1, which is likely affected by the substitution. An additional band was detected in the patient, but not in the negative control (Fig.3a). This band represents an aberrant RNA splicing product attributable to the variant. Further RT-PCR and sequencing results revealed that the lower band lacks the 3'end of 5'UTR and the entire exon 1 (c.−86_c.82) (Fig. 3b). As shown in Fig.5, the c.82G>A variant activates a cryptic 5' splice donor site at the c.−86G position within 5' UTR. This splice site is predicted to be a cryptic donor site with higher prediction score and confidence by Alternative Splicing Site Predictor (ASSP) http://wangcomputing.com/assp/index.html. The recognition and utilization of this cryptic splice site by the splicing machinery results in

deletion of 168 bp (c.−86_c.82) and presumably leads to the loss of protein due to deletion of the start codon (Fig. 5).

The *RAD51D* c.82G>A variant completely disrupts normal splicing in the mutant allele

To determine whether the *RAD51D* c.82G>A mutant allele completely disrupts normal splicing (i.e., whether the mutant allele is able to generate the *RAD51D* wild type transcript), we extracted the wild type and the mutant bands from the RT-PCR products in agarose gels. Sequencing results showed that the wildtype transcript contains only G at the 82 position, indicating that the mutant allele was unable to generate any normal transcript (Fig. 4a). These results suggest that this variant completely abolishes normal mRNA splicing in the mutant alleles. To rule out the possibility that a small portion of wildtype transcript containing the mutant A at the 82 position was not visible by Sanger sequencing because of the relatively low sensitivity, we cloned the RT-PCR products into the TOPO sequencing vector and then sequenced 109 colonies. Fifty-two (52) clones from the patient contained the full-length transcript and all of them contained the G at the c.82 position, indicating that the mutant allele was unable to generate any normal transcript (Fig. 4a). Fifty-seven (57) clones contained the mutant transcript with deletion of 3'end of 5'UTR and the entire exon 1 (Fig. 4b). These results indicate that the mutant A allele at the c.82 position completely abolishes normal splicing.

Discussion

Our data demonstrate that the c.82G>A (p.Val28Met) variant located at the last nucleotide of exon 1 completely abolishes the normal splicing of the mutant allele and activates a cryptic splice donor site at the c.−86G position, resulting in the deletion of 3'end of 5'UTR and the entire exon 1 (c.−86_c.82), which may lead to an absent RAD51D protein. Although we were unable to test the proband's deceased sister or her children, she had a 50% chance to carry the variant. If she did, it is possible that the variant may have contributed to her diagnosis of TNBC.

The patient carries three variants previously classified as VUS. We have performed splicing analysis for BARD1 c.2171C>T (p.Ala724Val) and demonstrated that this variant does not affect splicing (data not shown) and remains VUS classification as its impact on protein function is still unknown. As of today, the other variant RAD51D c.629C>A (p.Ala210Glu) remains VUS classification in Clinvar. Determination of the clinical significance of the RAD51D c.82G>A variant allows the proband and other affected carriers in the family to be potential candidates for treatment with drugs that target the specific DNA repair pathways such as a PARP inhibitor [38]. The reclassification of this variant gave the proband, her sister, and their treating physicians more definitive information which allowed them to make more informed decisions about ongoing breast surveillance. This is also relevant to other individuals (within or outside this family) who carry the RAD51D variant but, most importantly, for individuals who carry this variant and have intact ovaries and fallopian tubes, this has significant implications for management of ovarian cancer risk and recommendations for RR-BSO. It is worth noting that, due to the revised classification of the *RAD51D* variant and the family history of TNBC, both the patient and the patient's

living sister were recommended to undergo enhanced breast cancer surveillance with annual breast MRI and contrast enhanced mammography (CESM) as part of MSKCC RISE (Risk Assessment, Imaging, Surveillance, and Education) Program (NCCN v. 1.2020). This also means anyone else testing positive for this *RAD51D* variant in the family or in other families will be able to alter their clinical cancer prevention and risk reduction strategies as appropriate.

Loss of function of *RAD51D* is associated with increased risk for primary ovarian, fallopian tube, and peritoneal carcinoma [11,14,44] and may confer a moderately increased breast cancer risk [18]. Based on its demonstrated impact on splicing in our patient, the c.82G>A variant is now classified as likely pathogenic. Such information is valuable for unaffected family members who would benefit from predictive testing for this likely pathogenic variant. Any individual who is found to carry this *RAD51D* variant would be eligible for relevant cancer risk reduction and prevention measures as outlined in the current NCCN Clinical Practice Guidelines [45] specifically RR-BSO. The reclassification of this *RAD51D* variant may allow for additional treatment options/ clinical trial eligibility related to PARP inhibitors. Our findings demonstrated that it is necessary to perform additional studies to further evaluate the consequences of certain missense variants in known moderate to high penetrance cancer predisposition genes, as they may affect protein function through a completely different mechanism, and thereby affect the clinical care of individuals with such genetic variants.

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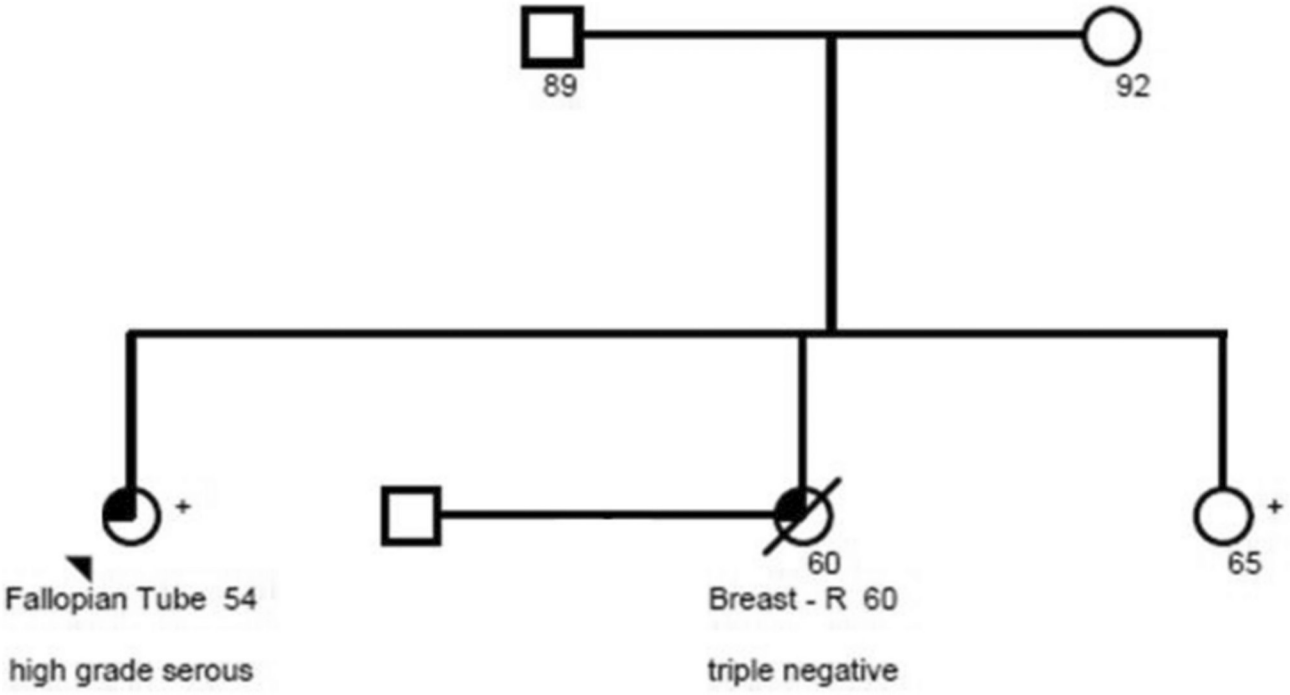

Fig. 1.

Patient pedigree. The patient described here is a 60-year-old female who was diagnosed with high grade serous fallopian tube cancer (HGSFT) cancer at 54 years of age. One of the patient's two sisters was diagnosed with TNBC cancer at age 60.

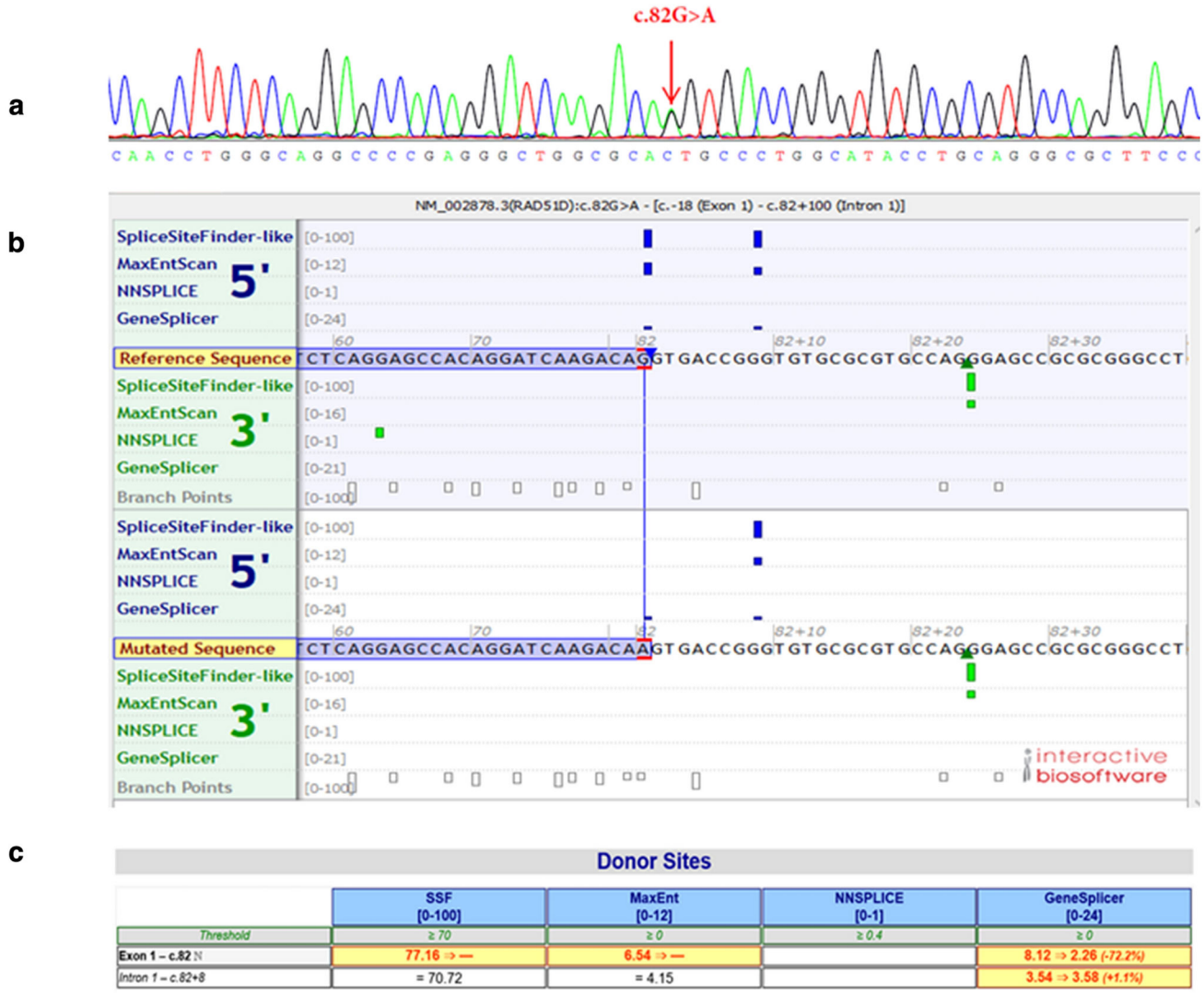

Fig. 2.

In silico predictions of the *RAD51D* c.82G>A variant. The Alamut software was used to evaluate the potential effects of the variant on splicing. Three tools predicted that the variant may disrupt the 5' donor splice site of intron 1.

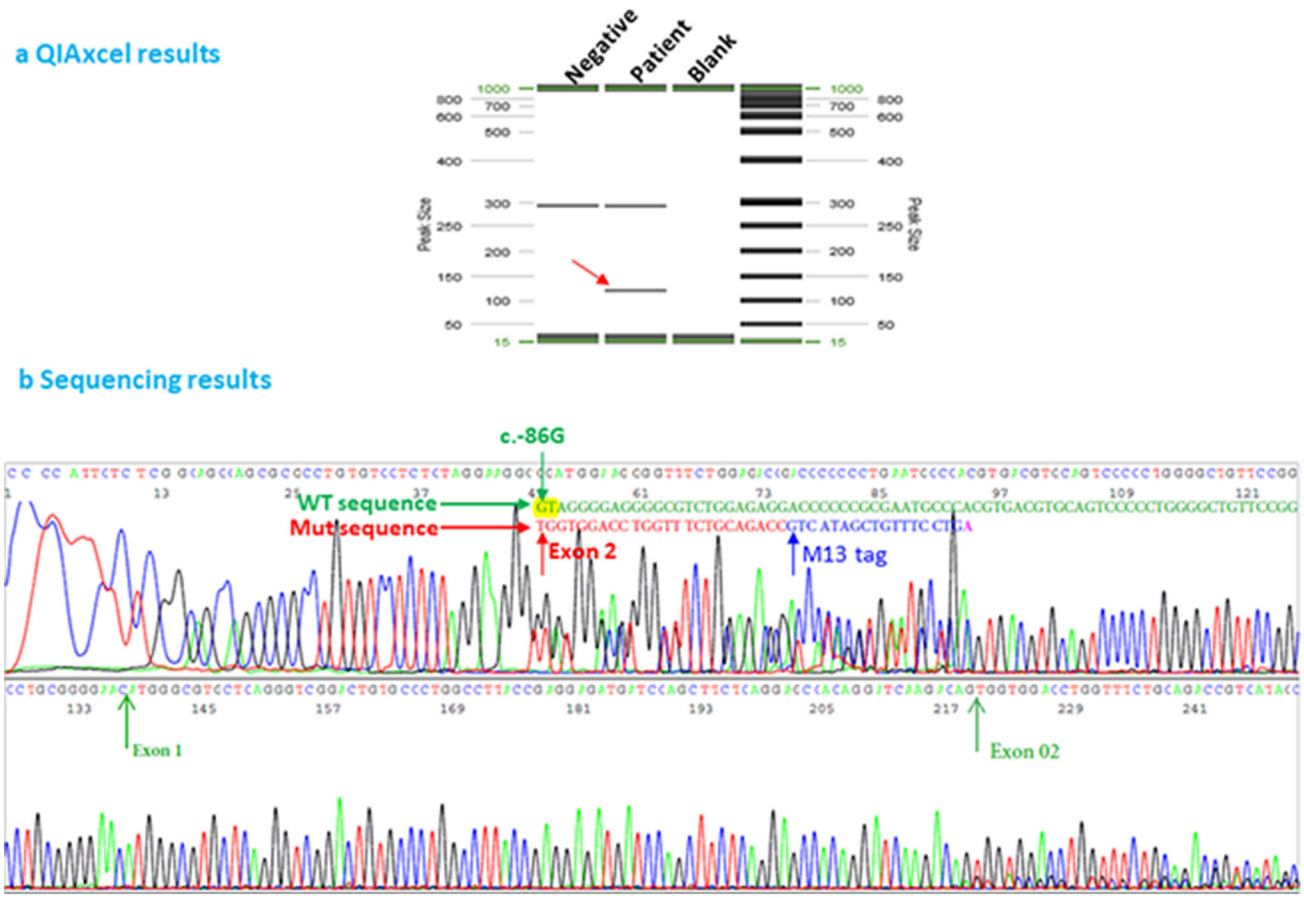

Fig. 3.

RT-PCR analysis demonstrates *RAD51D* c.82G>A leads to a deletion of 3'end of 5'UTR and the entire exon 1 (c.−86_c.82). a. RT-PCR products run on QIAxcel. One additional band was observed in the patient which is not in the negative control. b. Electropherogram showing that the variant causes partial 5'UTR and whole exon 1 deletion.

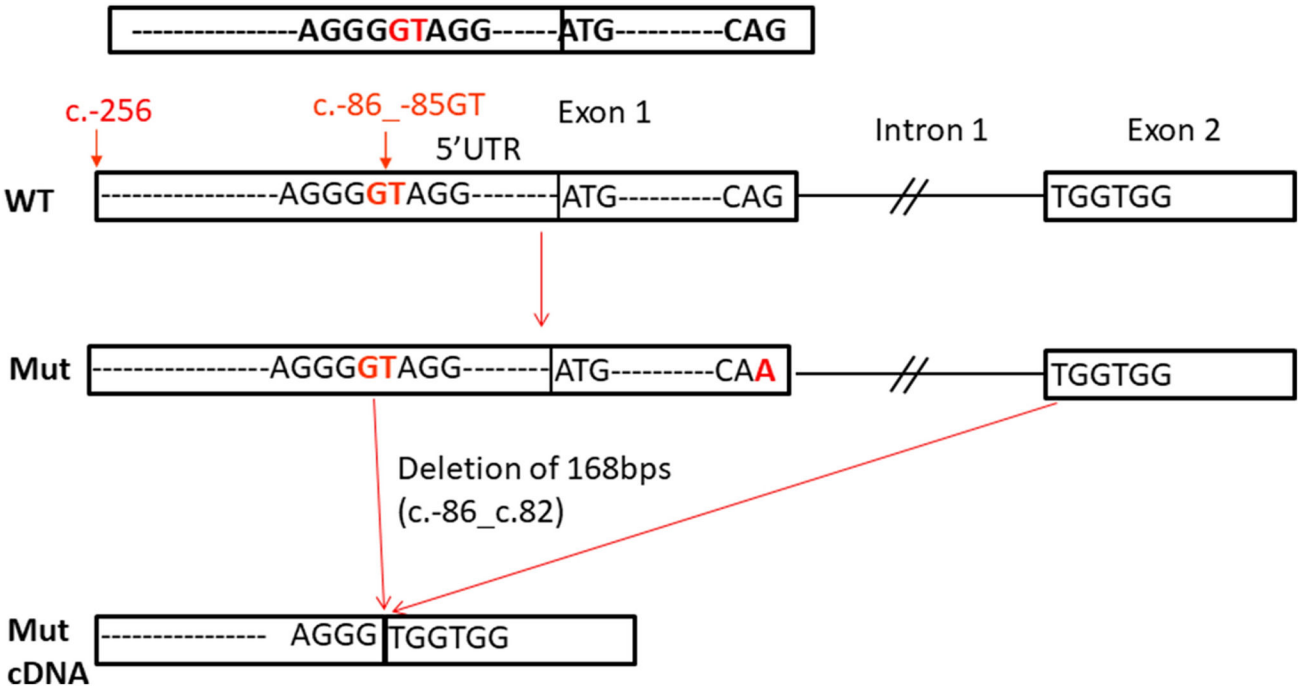

Fig. 4.

The mutant allele does not generate any wild type transcript. *RAD51D* c.82G>A mutant creates a novel 5' splice site that results in a 168 bp deletion including 3'end of 5'UTR and the entire exon 1. a. Wild type sequence adjacent to the RAD51D c.82G>A position. All clones (n=52) with the full-length transcript contained the G at the c.82 position. b. Sequence of mutant transcript with a deletion of 3'end of 5'UTR and the entire exon 1.

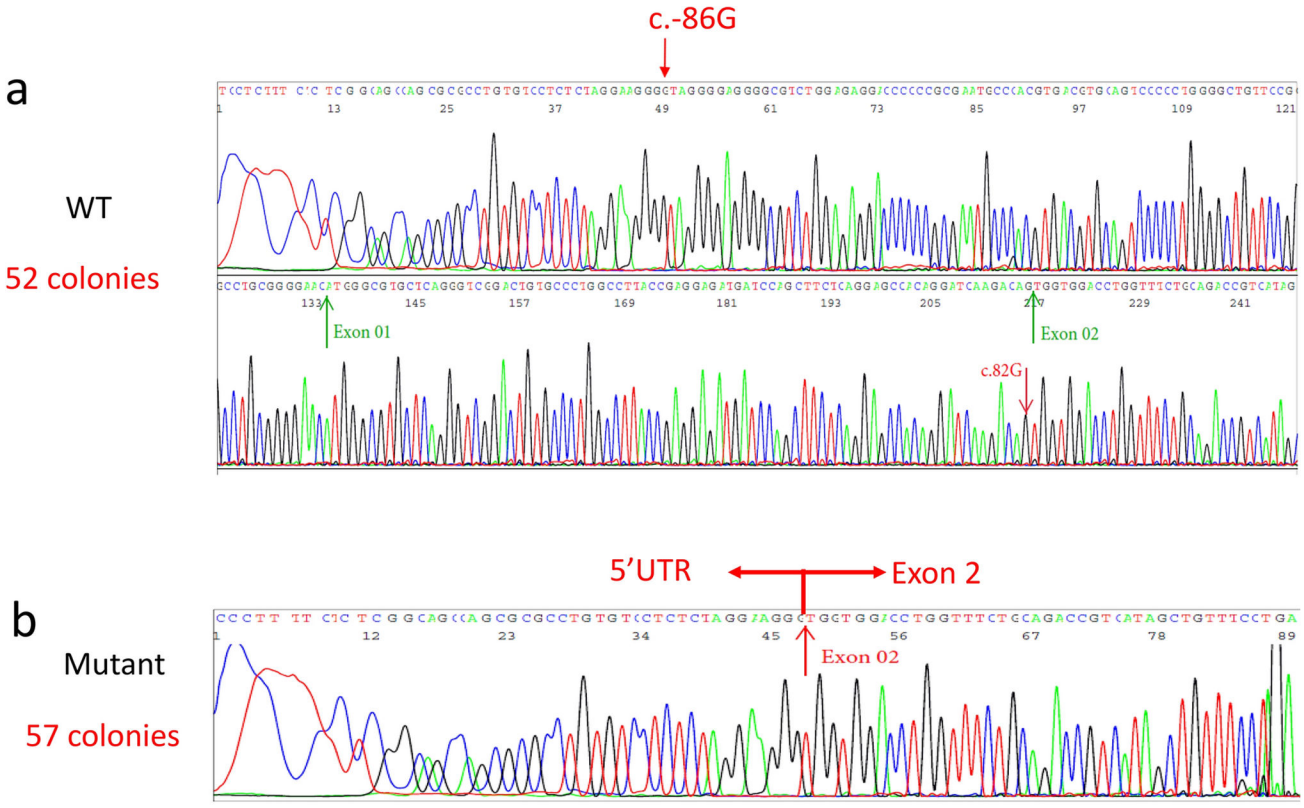

Fig. 5.

Schematic view of the RAD51D c.82G>A mutant leading to deletion of 3'end of 5'UTR and the entire exon 1.