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Bypass of Premature Stop Codons and Generation of Functional BRCA2 by Exon Skipping

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Abstract

A pathogenic mutation in *BRCA2* significantly increases the risk of breast and ovarian cancers making it imperative to examine the functional consequences of variants of uncertain clinical significance. Variants that are predicted to result in a truncated protein are unambiguously classified as pathogenic. We have previously shown how a pathogenic splice site variant known to generate a premature termination codon (PTC) in exon9 and a nonsense mutation at exon7, can generate functional BRCA2 by skipping exons4–7 and restoring the reading frame. Using a well-established mouse embryonic stem-based assay, we functionally characterize here one splice site mutation and 11 pathogenic *BRCA2* variants that are either nonsense mutation or generate PTC in different exons ranging from exons 4 to 7. Our study shows that 5 variants can restore the open reading frame by exon skipping and generate functional protein. This suggests further need to exercise prudence when classifying clearly pathogenic variants.

Keywords

BRCA2; variants; splicing; PTC bypass; functional assay

Mutations in breast cancer susceptibility gene, *BRCA2*, contribute to increased risk of early onset familial breast and ovarian cancers (1). Sequencing based genetic tests are now being used to identify individuals at risk of developing the disease (2). However, determining the disease risk associated with a *BRCA2* variant is challenging, especially for variants that do not clearly disrupt the gene product. Such variants are called variants of uncertain clinical significance (VUS) (3). Given the increased risk of breast, ovarian and other cancers in *BRCA2* mutation carriers, functional evaluation of VUS is clinically very relevant. One of the consortiums that is dedicated to functional classification of BRCA2 VUS is Evidence based Network for the Interpretation of Germline Mutant Alleles (ENIGMA, <https://enigmaconsortium.org/>) (4). BRCA exchange is another global repository focused on improving our understanding of BRCA variants (5).

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Conflict of interest statement

On behalf of all authors, the corresponding author states that there is no conflict of interest.

While most functional studies are focused on determining the pathogenicity of VUS, functional interpretation of some variants that are predicted to be pathogenic may not also be clear-cut. A splice site variant of *BRCA2* in intron 7, NM_000059.3(*BRCA2*):c.631+2T>G (IVS7+2T>G), is predicted to cause skipping of exon 7 and generate a PTC in exon 9 (6). We uncovered that in addition to skipping of exon 7, the variant also generates transcripts that lack exons 4–7. Skipping of exons 4–7 restores the open reading frame (ORF) and results in a full-length protein with an internal deletion of 105 amino acids (7). Another pathogenic variant NM_000059.3(*BRCA2*):c.581G>A (p.Trp194Ter) which results in a premature stop codon in exon 7, also restores the ORF by skipping exons 4–7 (7).

Using a mouse embryonic stem cell (mESC)-based assay, we demonstrated that these 105-amino acids are dispensable for the DNA repair function of *BRCA2*. Furthermore, we have generated mice lacking exons 4–7 and shown that they are viable, fertile and do not exhibit any increase in tumor predisposition (8). We describe here one splice site mutation and four pathogenic variants that bypass the PTC and generate a functional protein by skipping different exons in this region (exons 4–7). Our findings highlight the need to thoroughly characterize all potentially pathogenic (frame-shift, nonsense and splice site) variants.

To examine other pathogenic variants that may generate partially functional protein, we selected 12 potential pathogenic mutations (NM_000059.3:c.396T>A (p.Cys132Ter); NM_000059.3:c.407delA (p.Asn136fs); NM_000059.3:c.462_463delAA (p.Arg155_Asp156insTer); NM_000059.3:c.470_474delAGTCA (p.Lys157fs); NM_000059.3:c.489_490insG (p.Leu164fs), NM_000059.3:c.518delG (p.Gly173Valfs), NM_000059.3:c.538_539delAT (p.Ile180fs); NM_000059.3:c.539_540insAT (p.Ser181fs); NM_000059.3:c.541delT (p.Ser181fs); NM_000059.3:c.574_575delAT (p.Met192fs), NM_000059.3:c.610delC (p.Ser205fs), NM_000059.3:c.632–2A>G) that map to exon/ introns 4–7 (Table 1). We hypothesized that these mutations may affect the splicing of *BRCA2* mRNA. We examined the impact of these variants on splicing regulatory sequences using the Human Splicing Finder (<http://www.umd.be/HSF/>), an *in silico* tool. We found several variants to result in a number of potential changes such as activation of cryptic donor site, creation of exonic splicing silencer (ESS) sites, alteration of exonic splicing enhancer (ESE) sites as summarized in Table 1. To directly assess the impact of these alterations on splicing that may generate a functional protein, we tested their ability to support the viability of *Brca2*^{KO/KO} ES cells(9). We generated these mutations in human *BRCA2* cloned in a bacterial artificial chromosome (BAC) and electroporated individual BACs into PL2F7 mouse ES cells (Figure 1A). PL2F7 ES cells contain a knockout allele (*KO*) of *Brca2* and a conditional allele (*CKO*) where the two loxP sites are flanked by two halves of the human *HPRT* minigene(9). Cre-mediated deletion of the conditional allele generates a functional *HPRT* minigene that can be selected for in hypoxanthine-aminopterin thymidine (HAT) media (Figure 1A). In the absence of functional *BRCA2*, HAT resistant *Brca2*^{KO/KO} mESCs fail to survive. However, cell lethality is rescued when a BAC containing WT *BRCA2* or a neutral variant is expressed in the cells. We found 5 variants (c.407delA, c.470_474delAGTCA, c.518delG, c.610delC, c.632–2A>G) to support cell viability, albeit at a frequency that was much reduced than the cell viability conferred by WT *BRCA2* (Table 1). We confirmed the genotype of the viable clones by Southern blot analysis which showed the loss of the conditional allele in some of the HAT resistant colonies (Supplementary

Figure 1). Survival of *Brca2*^{KO/KO} mESC suggests that these variants are likely to encode a partially functional protein (Table 1).

We examined the BRCA2 protein expression in the rescued clones by Western blotting. In spite of the potential to generate a PTC in every case, we detected BRCA2 protein that was apparently similar in size to the full-length protein (Fig. 1B). The level of BRCA2 protein in those variants were low compared to the cells that express WT BRCA2 (Fig. 1B). To uncover how these variants are able to bypass the PTC, we performed RT-PCR analysis of total RNA from the viable clones followed by Sanger sequence analysis (Figure 1C, D Supplementary Table 1). Among various transcripts that can restore ORF, we identified the transcripts lacking exons 4–7 in c.407delA, c.518delG, c.610delC and c.470_474delAGTCA, (Figure 1C, D, Supplementary Table 1). A novel transcript lacking 3' 39 bp of exon 6 and entire exons 7 and 8 was detected in c.407delA and in c.632–2A>G (Fig. 1C, D and Supplementary Table 1). This transcript is predicted to encode protein with an internal deletion of 68 amino acids in c.632–2A>G whereas in c.407delA the transcript has PTC in exon 5. A transcript lacking exon 5 was observed in c.518delG and in c.610delC (Fig. 1C, D and Supplementary Table 1). Although exon 5 deletion is predicted to generate PTC in exon 7, deletion of an additional base in exon 7 due to the c.518delG mutation will restore the ORF and generate a protein lacking 17 amino acids. In c.610delC, exon 5 transcripts have the PTC before the one bp deletion, resulting in a truncated protein. A transcript lacking exons 5, 6 and 7 was observed in c.407delA (Fig. 1C, D and Supplementary Table 1). Deletion of exons 5, 6 and 7 generates a PTC in exon 8 but this transcript in c.407delA also has single base deletion in exon 4, which restores the ORF and generates a protein with an internal deletion of 69 amino acids.

We next examined if the protein expressed by these alternatively spliced transcripts is proficient in DNA repair functions by challenging the *Brca2*^{KO/KO} ES clones with five different DNA damaging agents. We selected genotoxins that affect essential DNA metabolic processes such as strand separation, replication or repair using various mechanisms (cisplatin and mitomycin C (MMC) are DNA inter-strand cross-linking agent, methyl methane-sulfonate (MMS) is an alkylating agent, camptothecin is a topoisomerase inhibitor, and olaparib is a poly ADP-ribose polymerase (PARP) inhibitor). A defect in BRCA2 function results in hypersensitivity to these agents (10–12). Cells expressing c.610delC exhibited no or mild sensitivity to these agents including a PARP inhibitor, suggesting the presence of a functional BRCA2 (Table 1). In these cells, we detected the expression of transcripts lacking exons 4–7 (exon 4–7), which we have shown before to encode a functional protein (Figure 1C and Supplementary table 1). In contrast, we found *Brca2*^{KO/KO} ESC rescued by c.470_474delAGTCA, c.407delA and c.518delG and c.632–2A>G variants to be hypersensitive to these DNA damaging suggesting the BRCA2 is not fully functional. This is surprising based on our previous finding that exons 4–7 are dispensable for the DNA repair function of BRCA2. In addition, although the expression level of BRCA2 is reduced compared to the WT BRCA2 levels, it is similar in all the clones except one of the clones expressing c.632–2A>G (Figure 1B). While we do not precisely understand the cause of hypersensitivity of these cells, we speculate that this may be attributed to expression of other alternatively spliced transcripts that may restore the reading frame but may not generate a functional protein such as the transcripts lacking exons 3–7 (

exon 3–7) (Figure 1B and C, Supplementary Table 1). Exon3, which encodes the PALB2 binding domain of BRCA2, has been shown to be essential for BRCA2 function (12, 13). This is further supported by the finding that a *BRCA2* variant that results in complete skipping of exon 3, c.316+5G>C, confers high risk of breast and ovarian cancer (14). Therefore, it is possible that although Western analysis showed the levels of BRCA2 to be comparable between different variants, the levels of functional BRCA2 may be low in *Brca2*^{KO/KO} ESC rescued by c.470_474delAGTCA, c.407delA and c.518delG and c.632–2A>G variants.

In conclusion, we have demonstrated that alternatively spliced transcripts can restore the function of variants that are predicted to result in PTC. Previously, we have confirmed the expression of *BRCA2* transcript skipping exons 4–7 that encodes fully functional protein in human EBV-lymphoblastoid cells with c.581G>A (p.Trp194Ter) variant (7). Such alternatively spliced transcripts may explain the lack of genetic data to support the pathogenicity of some truncating variants identified in *BRCA2* (c.9699_9702del (p.Cys3233Trpfs)) and also in *BRCA1* and *MSH2* (15). Although we cannot rule out the differences in the DNA damage response between mouse and human cells as reported previously by Biechonski and Milyavsky (2013), our findings further bolster the need to carefully examine all BRCA2 variants, including those that are apparently functionally null and considered pathogenic (16).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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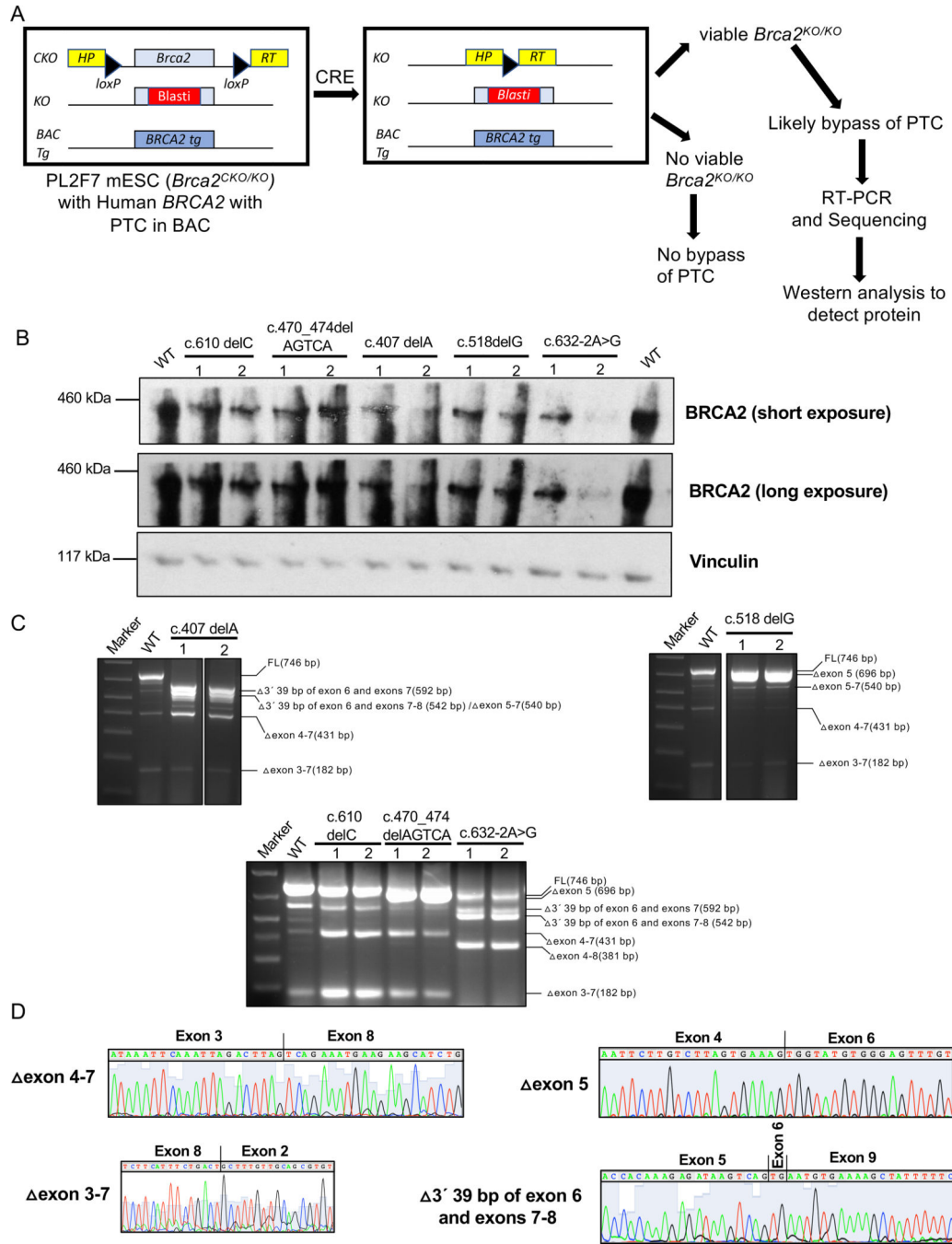


Figure 1: Functional evaluation of BRCA2 variants in mouse ES cells

A. Overview of the ES cell-based functional assay where mutations are expressed in mESC containing a null allele (KO) and a conditional allele (CKO) of *Brca2*, which is flanked by two *loxP* sites containing the 5' and 3' halves of human *HPRT* minigene. Upon Cre expression the conditional allele is deleted and generates a functional *HPRT* minigene. Recombinant clones are selected in the presence of hypoxanthine-aminopterin-thymidine (HAT). In the absence of a functional *BRCA2*, such cells are not viable. Viable clones were further evaluated by RT-PCR and Western blot analysis.

- B.** Western blot analysis of *Brca2*^{KO/KO} mESC showing expression of full-length protein by variants with a premature stop codon.
- C.** RT-PCR analysis to identify transcripts responsible for viability of *Brca2*^{KO/KO} mESC expressing *BRCA2* mutant alleles predicted to result in premature protein truncation. Primers used for RT-PCR are 5'GACACGCTGCAACAAAGCA3' (exon 2) and 5'CATGACTTGCAGCTTCTCTTTGA3' (exon 9).
- D.** Chromatogram of alternatively spliced transcripts that are predicted to encode functional BRCA2.

Table 1.

Summary of *BRCA2* variants with PTC examined and their impact on ES cell survival, sensitivity to DNA damaging agents

VARIANT Nucleotide change (HGVS)	Protein	Exon/Intron	Distance from splice site	PTC in exon	In-silico prediction on splicing	ES cell rescue (%)			Drug sensitivity					
						WT	Clone 1	Clone 2	Mitomycin C	Methyl Methane sulfonate	Camptothecin	Olaparib	Cisplatin	
														0
c.396T>A	p.Cys132Ter	Ex4	-30	4		0	0	0	NA	NA	NA	NA	NA	NA
c.407delA	p.Asn136fs	Ex4	-19	5		7	1.02	1.62	Yes	Yes	Yes	Yes	Yes	Yes
c.462_463delAA	p.Asp156Ter	Ex5	-13	5	Activation of cryptic donor site, Creation of ESS site, Alteration of ESE site		0	0	NA	NA	NA	NA	NA	NA
c.470_474delAG TCA	p.Lys157fs	Ex5	-2	6	Alteration of WT splice donor site, Creation of ESS site, Alteration of ESE site	9	0.87	1.16	Mild	Yes	Yes	Yes	Yes	Yes
c.489_490insG	p.Leu164fs	Ex6	14	7	Creation of ESS site		0	0	NA	NA	NA	NA	NA	NA
c.518delG	p.Gly173fs	Ex7	2	7	Alteration of ESE site	21	0.85	1.8	Yes	Yes	Mild	Yes	Yes	Yes
c.538_539delAT	p.Ile180fs	Ex7	22	7	Alteration of ESE site		0	0	NA	NA	NA	NA	NA	NA
c.539_540insAT	p.Ser181fs	Ex 7	23	7	Creation of ESS site, Alteration of ESE site		0	0	NA	NA	NA	NA	NA	NA
c.541delT	p.Ser181fs	Ex 7	25	7	Alteration of ESE site		0	0	NA	NA	NA	NA	NA	NA
c.574_575delAT	p.Met192fs	Ex 7	-57	7	Activation of cryptic acceptor site, Creation of ESS site, Alteration of ESE site		0	0	NA	NA	NA	NA	NA	NA
c.610delC	p.Ser205fs	Ex 7	-22	7	Creation of ESS site, Alteration of ESE site	9	2.3	4.8	Mild	No	Mild	No	Mild	Mild
c.632-2A>G		Int7	-2		Alteration of WT splice acceptor site	9	0.4	0.4	Yes	Yes	Yes	Yes	Yes	Yes

In-silico splicing prediction was performed using the tool Human Splicing Finder-Version 3.1 (<http://www.umd.be/HSF/>). ESE: Exonic splicing enhancer; ESS: Exonic splicing silencer. ES cell rescue percentage was calculated by comparing the cells that lost conditional *BRCA2* (confirmed by Southern analysis) to the total number of cells plated on M15 medium.