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Functional characterization of BRCA2 variants to improve cancer risk assessment

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Skipping nonsense to maintain function: the paradigm of BRCA2 exon 12

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Abstract

Germline nonsense and canonical splice site variants identified in disease-causing genes are generally considered as loss-of-function (LoF) alleles and classified as pathogenic. However, a fraction of such variants could maintain function through their impact on RNA splicing. To test this hypothesis, we used the alternatively spliced *BRCA2* exon 12 (E12) as a model system since its in-frame skipping leads to a potentially functional protein. All E12 variants corresponding to putative LoF variants or predicted to alter splicing (n=40) were selected from human variation databases and characterized for their impact on splicing in minigene assays and, when available, in patient lymphoblastoid cell lines. Moreover, a selection of variants was analyzed in a mouse embryonic stem cell-based functional assay. Using these complementary approaches, we demonstrate that a subset of variants, including nonsense variants, induces in-frame E12 skipping through the modification of splice sites or regulatory elements and, consequently, leads to an internally deleted but partially functional protein. These data provide evidence for the first time in a cancer-predisposition gene, to our knowledge, that certain presumed null variants can retain function due to their impact on splicing. Further studies are required to estimate cancer-risk associated with these hypomorphic variants. More generally, our findings highlight the need to exercise caution in the interpretation of putative LoF variants susceptible to induce in-frame splicing modifications.

Significance

This study present evidence that certain presumed loss-of-function variants in a cancer-predisposition gene can retain function due to their direct impact on RNA splicing.

Keywords

Presumed loss-of-function variants; In-frame splicing alteration; Partial functional rescue; Variant reinterpretation; *BRCA2*

Introduction

Germline nonsense variants account for 20% of all Mendelian disease-causing single nucleotide variants (SNVs) reported within gene coding sequences (1). This type of variants, as well as frameshift deletions/insertions and SNVs located at the highly conserved intronic dinucleotides of splice sites (hereafter termed $IVS\pm1/2$), are presumed to lead to loss-of-function (LoF) since they are expected to introduce a premature termination codon (PTC). Based on these assumptions, these variants are generally regarded as null alleles and classified as pathogenic without further investigation. This is in particular the case for the iconic cancer predisposition genes, *BRCA1* and *BRCA2*, where mono-allelic germline dominant LoF variants confer high risks of hereditary breast and ovarian cancer (HBOC) (2).

Several consortia that provide guidelines for variant classification in the clinical setting, including the American College of Medical Genetics and Genomics (ACMG) (3) and the Evidence-based Network for the Interpretation of Germline Mutant Alleles (ENIGMA) (4,5), recommend caution with the systematic pathogenic classification of such presumed null variants. Indeed, possible rescue mechanisms have been described for (partial) function preservation of certain presumed LoF variants in *BRCA1* and *BRCA2* genes. In particular, the polymorphic *BRCA2* c.9976A>T (p.Lys3326*) nonsense variant results in a truncated protein with a minor loss in the C-terminal region that preserves functionality (6–8) and is associated with a modest increase in HBOC risks compared to typical LoF pathogenic *BRCA2* variants (9). A second rescue mechanism involves specific naturally occurring alternative splicing events, as recently illustrated by the characterization of *BRCA1* c.[594-2A>G; 641A>G] (5). This allele is not associated with high cancer risk despite its induced out-of-frame exon 10 skipping, potentially because the naturally occurring in-frame alternative transcript without exons 9 and 10 ($\Delta E9_E10$) can encode for a functioning BRCA1 protein. Naturally occurring alternative splicing can also enable survival of patients homozygous for certain *BRCA1* exon 11 nonsense variants, albeit with severe features of Fanconi anemia (10). Here, we raised the hypothesis that a subset of presumed LoF variants in HBOC susceptibility genes may potentially retain partial activity through their direct impact on splicing, leading eventually to questioning their clinical interpretation.

Virtually all variants may potentially induce a perturbation in RNA splicing, notably through the modification of either 3' and 5' splice sites (3'/5' ss) or exonic splicing regulatory elements (ESR) (11,12). The most frequently observed splicing anomaly is exon skipping that often leads to a frameshift, causing LoF. However, some specific presumed LoF variants could induce in-frame skipping of a non-essential exon, resulting in a functional protein, at least partially. Here, we envisioned that this rescue mechanism may be triggered by specific variants located in the exon 12 (E12) of *BRCA2*. This exon does not encode a known functional domain involved in the *BRCA2* tumor suppressor activity and its in-frame skipping leads to the internal deletion of a potentially dispensable 32-amino-acid sequence (13). In a mouse embryonic stem cell (mESC)-based functional assay, the E12-deleted *BRCA2* protein is able to complement

the loss of endogenous wild-type *Brca2* and exhibits the same functions with regard to (i) homologous recombination activity, (ii) radiation induced RAD51 foci formation and (iii) sensitivity to DNA-damaging agents (13).

An additional argument supporting the tumor suppressor functionality of the *BRCA2* E12-deleted protein came from the initial analysis of the clinical significance of a missense variant, c.6853A>G (p.Ile2285Val), that triggers E12 skipping. The family history likelihood ratio and the co-occurrence in *trans* with a pathogenic variant support the classification of this spliceogenic variant as neutral (13).

In this study, we selected from human variation databases a total of 40 variants in or near *BRCA2* E12, that were either presumed LoF variants or predicted to alter E12 splicing, and characterized their impact on splicing in minigene assays and patient biological material. Moreover, a selection of variants was tested in a mESC-based functional assay to assess their combined effects on RNA splicing and protein activity. To evaluate their association with cancer, clinical and family data were collected for spliceogenic *BRCA2* E12 variants. Altogether, these data provide evidence that presumed LoF variants, including nonsense SNVs, can induce splicing alterations that trigger in-frame deletion of a sequence not essential for *BRCA2* function. The existence of this potential rescue mechanism instigates the need to re-evaluate the interpretation of certain *BRCA2* variations currently considered as LoF.

Materials and Methods

Variant selection

All variants located in *BRCA2* E12 and its flanking intronic sequences were extracted from human variation databases (Figure S1). First, we selected the SNVs generally regarded as LoF variants, including: (i) IVS $\pm$ 1/2, (ii) nonsense and (iii) frameshift variants. Secondly, we extended this selection to all variants predicted to affect E12 splicing based on two types of bioinformatics approaches. Predictions of the variant effects on 3'/5' ss were obtained by using the MaxEntScan (MES) *in silico* tool (14), interrogated from the integrated software Alamut Visual 2.9 (Interactive Biosoftware). Predictions of variant impacts on ESR were generated by using a bioinformatics approach relying on Δ tESRseq values (total ESRseq score changes) (15–17), based on exonic hexamer scores. Variants that are associated with either a decrease by at least 15% of MES score (Δ MES $\leq$ -15%) or a Δ tESRseq score inferior to -0.7 (Δ tESRseq < -0.7) were retained for splicing minigene analysis.

Splicing minigene assay

In order to evaluate the impact of the selected variants on E12 splicing, we performed an *in cellulo* splicing assay based on the use of the two-exon minigene pCAS2 vector (17,18). Wild-type (WT) and mutant segments encompassing *BRCA2* E12 and its intronic flanking sequences were amplified from patient genomic DNA (gDNA) by PCR using specific primers (Table S1) and inserted into the intron of the pCAS2 minigene

using BamHI and MluI restriction sites, yielding pCAS2-*BRCA2*-E12 minigenes (Figure 1B). When patient gDNA was not available, the variant of interest was introduced by site-directed mutagenesis. WT and mutant pCAS2-*BRCA2*-E12 minigenes were transfected in parallel into HeLa cells using the FuGENE 6 transfection reagent (Roche Applied Science). Total RNA was extracted 24 h post-transfection using the NucleoSpin RNA kit (Macherey-Nagel). The splicing patterns were analyzed by semi-quantitative fluorescent RT-PCR (25 cycles) by using the OneStep RT-PCR kit (Qiagen), in a 25 μ L reaction volume, with 200 ng total RNA and specific minigene primers (Figure 1B and Table S1). RT-PCR products were separated by electrophoresis on 2% agarose gels. All RT-PCR DNA bands were gel-purified and sequenced. Because of the formation of heteroduplexes between the different RT-PCR products, the images of the gels were not used for quantification. For semi-quantitative analysis, fluorescent RT-PCR products were resolved under denaturing conditions by capillary electrophoresis on an automated 3500 Genetic Analyzer (Applied Biosystems). Electropherograms were then analyzed by using the GeneMapper v5.0 software (Applied Biosystems) and peak areas were used to quantify the relative levels of each transcript.

Analysis of E12 splicing patterns and allele-specific expression in lymphoblastoid cell lines

The EBV-immortalized lymphoblastoid cell lines (LCLs) of healthy donors and patients were collected in collaboration with French HBOC diagnostic laboratories within the Group “Genetic and Cancer” (GGC, Unicancer) (Supplemental note). These LCLs were cultured and treated or mock-treated with puromycin, as previously described (17). After extraction of total RNA, the E12 splicing patterns were analyzed by semi-quantitative fluorescent RT-PCR (26 cycles), as described above, with primers mapping to *BRCA2* exons 11 and 14 (Table S1).

In order to measure allele-specific expression (ASE) of E12-containing transcripts (+E12), a SNaPshot quantitative primer extension assay was performed, as described previously (17), with the same RT-PCR conditions as described above and using specific primers targeting the variants (Table S1). The ASE_{variant} was then calculated after normalization of the cDNA ratio to the corresponding gDNA ratio.

In order to extend these ASE analyses to both transcripts with (+E12) and without (Δ E12) E12, we set up a SNaPshot protocol targeting the c.7242A>G (p.Ser2414=) polymorphism located in *BRCA2* exon 14 (SNP rs1799955, minor allele frequency in gnomAD = 22.5%). Transcripts +E12 and Δ E12 were selectively amplified in parallel RT-PCR reactions (32 cycles) using a forward primer overlapping either exons 11/12 or 11/13, respectively, combined with a reverse primer in exon 15 (Table S1). The SNaPshot quantitative primer extension assay was then performed using a primer targeting the sequence immediately upstream the SNP. The ASE_{SNP} was then calculated as described above.

Mouse embryonic stem cell-based assay

The mESC-based assay (8,19) was used to assess the functional impact of *BRCA2* E12 variants. The mESC expressing the human *BRCA2* gene (*hBRCA2*) with specific variants were generated as previously described (20). After Cre-mediated deletion of the conditional mouse *Brca2* allele, cell survival, *BRCA2* protein expression and homology directed repair (HDR) activity were assessed, as previously described (20). For the drug sensitivity assays, mESC were seeded in quadruplicate at 10.000 cells per well on gelatin-coated 96-well plates to measure the sensitivity of *BRCA2* variants to the DNA-damaging agent cisplatin (Accord) and the PARP inhibitor, talazoparib (Axon Medchem). Cells were treated with the following concentrations of cisplatin: 0, 0.04, 0.08, 0.16, 0.31 and 0.63 μ M (in 1% phosphate buffered saline [PBS]). Applied concentrations of talazoparib were 0, 0.08, 0.16, 0.31, 0.63 and 1.25 nM (in 1% DMSO). Viable cells were counted using the Novocyte Flow Cytometer (ACEA Biosciences, Inc.) after 48h of treatment followed by 24h of additional culturing in normal culture medium without a DNA damaging compound. The E12 splicing patterns were analyzed by fluorescent RT-PCR (23 cycles) as described above for LCLs.

Patient and family data

Clinical, co-segregation and family data for patients carrying specific *BRCA2* E12 variants were collected in collaboration with the French GGC network and within the framework of the COsegregation of VARIants in the *BRCA1/2* and *PALB2* genes (COVAR) clinical trial (Supplemental note). These variants were previously detected in probands and relatives undergoing genetic counseling. The criteria for diagnostic variant screening of the *BRCA2* gene were applied according to the current French GGC recommendations and the French National Cancer Institute (INCa) guidelines.

Results

Variant selection

From a total of 133 *BRCA2* E12 variants collected from human variation databases, we selected all SNVs generally regarded as LoF variants ($n = 15$) encompassing 4 IVS $\pm$ 1/2, 5 nonsense and 6 frameshift variants (Figure 1A). In addition, all other intronic, missense or synonymous variants predicted to affect either 3'/5' ss (Δ MEs $\leq -15\%$, $n = 10$) or ESR (Δ tESRseq < -0.7 , $n = 14$) (Figure 2A) were retained for experimental analysis, as well as the previously described spliceogenic c.6853A>G variant (13). Altogether, this collection comprised 40 variants (Figure S1, Tables S2 and S3) that were assessed for their impact on E12 splicing by using an *in cellulo* minigene assay.

Splicing minigene analysis of presumed LoF variants

In this assay, the WT pCAS2-*BRCA2*-E12 minigene only produced +E12 transcripts, whereas, as expected, the 4 IVS $\pm$ 1/2 variants had a drastic impact on splicing (Figures 1C and S2). Indeed, c.6842-1G>T, c.6937+1G>A and c.6937+1G>T induced full E12 skipping (Figures 1C and S2), in agreement with the predicted destruction of the 3' or 5' ss (Figure S3). Surprisingly, despite bioinformatics predictions strictly identical to

c.6937+1G>A and c.6937+1G>T, c.6937+2del generated in addition to the transcript lacking E12 ($\Delta E12 = 51\%$), one RNA isoform with E12 plus retention of the 40 first nucleotides of intron 12 (+E12q(ins40) = 49%) (Figures 1C and S2). This latter isoform results from the activation of a cryptic intronic 5' ss predicted for all three variants but only used in the context of c.6937+2del (Figure S3).

Remarkably, in minigene assays, 4 out of the 5 selected nonsense variants affected splicing (Figures 1C and S2). More precisely, c.6898C>T and c.6920C>A induced weak exon skipping ($\Delta E12 = 12\%$ and 13%), whereas c.6844G>T and c.6901G>T led to major exon skipping ($\Delta E12 = 73\%$ and 72%) (Figures 1C and S2). In contrast, none of the 6 frameshift variants affected E12 splicing (Figures 1C and S2).

Splicing minigene analysis of variants with a predicted splicing effect

We extended our analysis to all SNVs located outside $IVS\pm 1/2$ and predicted to alter the E12 3'/5' ss ($\Delta MES \leq -15\%$) (Figure S5). All ten variants from this group had a splicing impact in the minigene assay, albeit to different extents (Figures 2B and S4A, Table S3). Of note, additional transcripts deleted for either the first (+E12p(del3)) or the last (+E12q(del3)) three nucleotides of E12 were detected in the context of c.6842G>A and c.6935A>G, respectively (Figures 2B and S4A). Finally, the impact on E12 splicing of the variants predicted to modify ESR ($\Delta tESRseq < -0.7$) was experimentally assessed. In this study, *BRCA2* c.6853A>G was used as a positive spliceogenic control because this variant has been previously shown to promote E12 exclusion in patient LCLs, most likely through the alteration of ESR (13). In the minigene assay, we confirmed that this variant induces E12 skipping, albeit moderately ($\Delta E12 = 16\%$) (Figures 2C and S4B, Table S3). Of note, the $\Delta tESRseq$ score associated with this variant ($\Delta tESRseq = -0.3$) didn't correctly predict its splicing impact when taking into account a $\Delta tESRseq$ value inferior to -0.7 as a positive indicator. In parallel to this variant, we assessed in the minigene assay the splicing effect of fourteen E12 variants predicted to affect ESR ($\Delta tESRseq < -0.7$) (Figures 2C and S4B, Table S3). By comparison, among these selected variants, 2 variants induced a major or near-total exon skipping ($\Delta E12 = 58\%$ and 95%), whereas 7 variants exhibited a minor or moderate effect ($3\% \leq \Delta E12 \leq 17\%$) (Figures 2C and S4B, Table S3). The remaining 6 selected variants did not cause splicing defect despite predictions of ESR alteration ($\Delta tESRseq < -0.7$). Altogether, these data not only confirmed initial findings but, notably, uncovered new spliceogenic variants potentially altering ESR. They also showed that the approach relying on $\Delta tESRseq$ score is not fully reliable for predicting variant-induced E12 splicing effects.

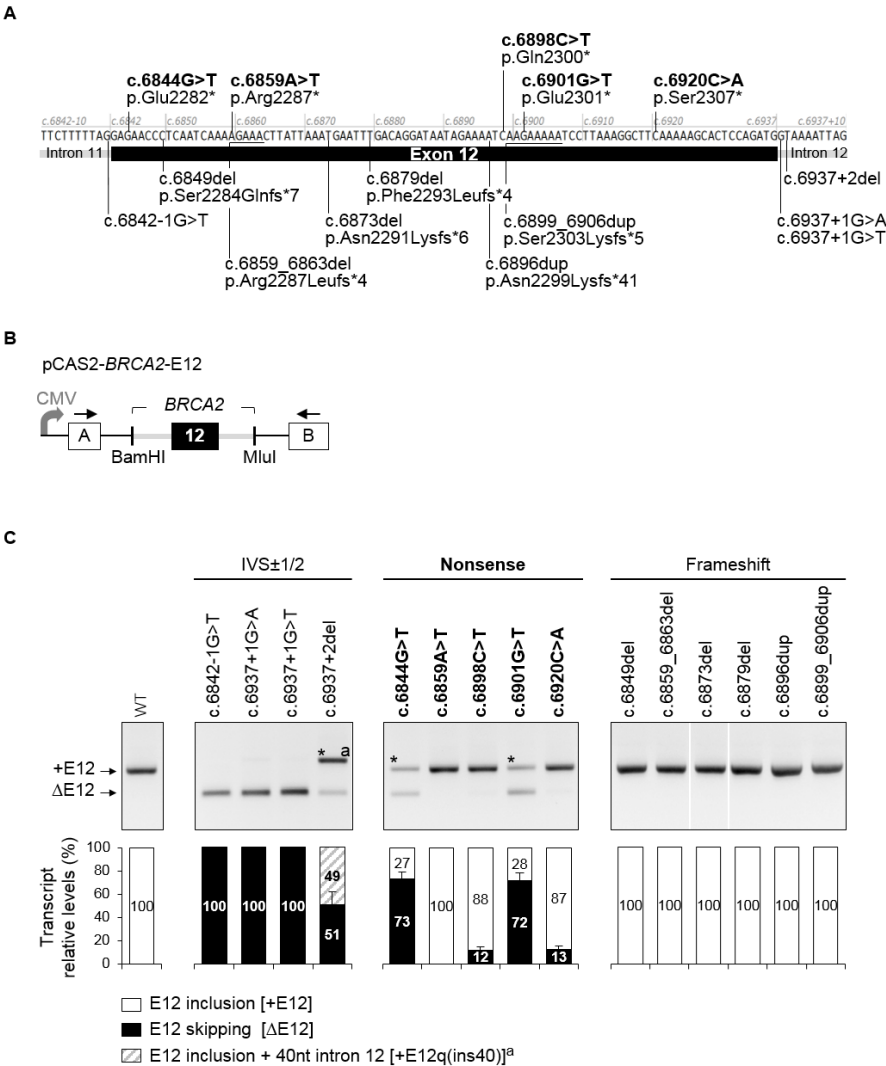


Figure 1. Identification in minigene assays of *BRCA2* exon 12 spliceogenic variants among presumed LoF SNVs

(A) Distribution of 15 presumed LoF variants (IVS±1/2; nonsense; frameshift) in *BRCA2* exon 12. The figure shows the nucleotide sequence of *BRCA2* exon 12 and part of its flanking intronic regions as well as the positions of the 15 selected variants, including 4 intronic variants located at position IVS±1/2 (below the sequence), 5 nonsense (above the sequence, in bold) and 6 frameshift (below the sequence) variants. **(B) Schematic representation of the pCAS2-*BRCA2*-E12 minigene used in the splicing reporter assay.** Boxes indicate exons and lines in between represent introns. The pCAS2-*BRCA2*-E12 minigenes were generated by inserting a genomic fragment encompassing *BRCA2* exon 12 into the intron of pCAS2, by using BamHI and MluI restriction sites. Arrows above the exons A and B indicate the position of primers

used in RT-PCR analysis. CMV, CMV promoter. **(C) Impact of the 15 presumed LoF variants on *BRCA2* exon 12 splicing.** Wild-type (WT) and mutant pCAS2-*BRCA2*-E12 minigene constructs were transiently expressed in HeLa cells. The splicing patterns of the RNA produced from the different minigenes were analyzed by RT-PCR, as described in Materials and Methods. Images show agarose gel electrophoresis of the RT-PCR products. The identities of the two major RT-PCR products, with (+E12) or without (Δ E12) exon 12 are indicated on the left of the gel. The other isoform identified (a) corresponds to the inclusion of exon 12 plus the retention of the first 40 nucleotides of intron 12 (+E12q(ins40)). Here, it is important to note that the gel images were not used for the quantification of the relative transcript levels because of the formation of heteroduplex between the different RNA isoforms (noted by the star symbol on the gel images), as revealed by band sequencing. Instead, the relative quantifications of the different RNA isoforms were determined by capillary electrophoresis of the fluorescent RT-PCR products (see Figure S2), as indicated in Materials and Methods. The data shown represent the average of at least three independent experiments. Error bars indicate standard deviation values.

***BRCA2* E12 splicing patterns in patient lymphoblastoid cell lines**

In LCLs of three control individuals, we detected an alternative splicing pattern of *BRCA2* E12 (average Δ E12 = 10.3%) (Figures 3A, 3B and S6), in agreement with published data (21,22). In a LCL derived from a patient heterozygous for a genomic deletion of *BRCA2* E12 (DelE12), a major increase in the relative levels of Δ E12 transcripts (Δ E12 = 64%) was observed (Figures 3B and S6), as expected. A comparable level of exon exclusion (Δ E12 = 61%) was measured for a patient carrying c.6937+1G>A (Figures 3B and S6), congruent with this variant being responsible for complete E12 skipping. Remarkably, in LCL of a patient carrying the nonsense c.6844G>T variant, the relative levels of Δ E12 transcripts were greatly increased (Δ E12 = 57%) (Figures 3B and S6). Similar results were obtained for LCLs of two patients carrying the nonsense c.6901G>T variant (Δ E12 = 52% and 46%, respectively) (Figures 3B and S6). Treatment of these LCLs with puromycin did not affect significantly transcript proportions (Figure S7), suggesting that the increase of Δ E12 levels does not result from nonsense-mediated mRNA decay of the PTC-containing +E12 RNA. In contrast, in LCLs with c.6935A>T or c.6853A>G, a weak or moderate increase in exon skipping was observed (Figures 3B and S6).

Altogether, the results obtained in patient LCLs are in agreement with minigene data. Still, it is important to note that the WT minigene didn't reproduce the alternative splicing of *BRCA2* E12 observed in control LCLs, most probably because of different cellular and/or genomic contexts. As a consequence, the minigene assay may underestimate the level of variant-induced exon skipping. It is also possible that some variants without an effect in the minigene assay exhibit a weak splicing impact in patient LCLs. Despite these limitations, the minigene assay represents a powerful approach for screening spliceogenic *BRCA2* E12 variants, as all the variant-induced effects detected by this method were confirmed in available patient LCLs.

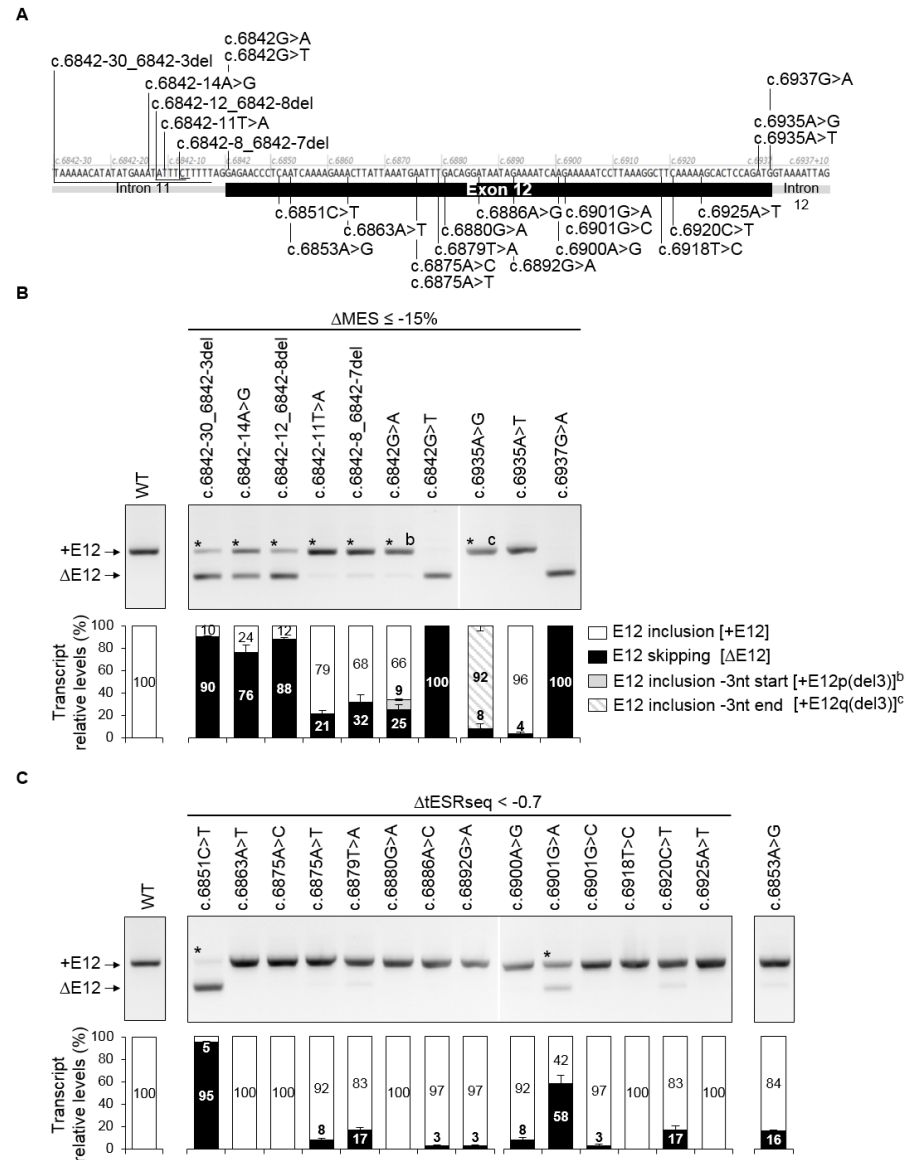


Figure 2. Identification in minigene assays of *BRCA2* exon 12 spliceogenic variants among SNVs predicted to affect splicing

(A) Distribution of 24 variants predicted to affect *BRCA2* exon 12 splicing. The figure displays the nucleotide sequence of *BRCA2* exon 12 and part of its flanking intronic regions. The relative positions of the 10 selected variants predicted to affect *BRCA2* exon 12 splice sites ($\Delta\text{MES} \leq -15\%$) are indicated above the sequence, whereas the 14 selected variants that may impact *BRCA2* exon 12 splicing regulation based on ESR predictions ($\Delta\text{ESRseq} < -0.7$) are shown under

the sequence. The position of the control spliceogenic variant, c.6853A>G, is also indicated.

(B) Impact on *BRCA2* exon 12 splicing of 10 variants predicted to alter splice sites ($\Delta\text{MES} \leq -15\%$). Wild-type (WT) and mutant pCAS2-*BRCA2*-E12 minigene constructs were transiently expressed in HeLa cells. The splicing patterns of the RNA produced from these different minigenes were analyzed by RT-PCR, as described in Materials and Methods. The image shows the agarose gel electrophoresis of the RT-PCR products. The identities of the two major RT-PCR products, with (+E12) or without (ΔE12) exon 12 are indicated on the left of the gel. The other isoforms identified, b and c, correspond to the inclusion of exon 12 deleted either for the first (+E12p(del3)) or the last (+E12q(del3)) three nucleotides. Here, it is important to note that the gel images were not used for the quantification of the relative transcript levels because of the formation of heteroduplex between the different RNA isoforms (noted by the star symbol on the gel images), as revealed by band sequencing. Instead, the relative quantifications of the different RNA isoforms were determined by capillary electrophoresis of the fluorescent RT-PCR products (see Figure S4), as indicated in Materials and Methods. The data shown represent the average of at least three independent experiments. Error bars indicate standard deviation values. **(C) Impact on *BRCA2* exon 12 splicing of 14 variants predicted to modify regulatory elements ($\Delta\text{tESRseq} < -0.7$).** The effects on splicing of the variants selected based on predictions of ESR alterations were assessed using the minigene reporter assay, as described in (B). In parallel, we analyzed the previously described spliceogenic c.6853A>G variant (13). The image shows agarose gel electrophoresis of the RT-PCR products. For each minigene, the relative quantifications of the different RNA isoforms were determined as described in (B). The data represent the average of at least three independent experiments. Error bars indicate standard deviation values.

Allele-specific expression of *BRCA2* transcripts in patient lymphoblastoid cell lines

We took advantage of the quantitative SNaPshot assay to measure ASE in +E12 RNA by directly interrogating the presence of exonic variants. From the LCLs with the nonsense variants c.6844G>T or c.6901G>T, we observed an important allelic imbalance (AI) in +E12 transcripts in favor of the WT allele (Figure 3C). In contrast, a moderate AI was detected in +E12 isoforms expressed in two LCLs with c.6935A>T and in three LCLs with c.6853A>G. Next, ASE were measured in both +E12 and ΔE12 transcripts by using the c.7242A>G polymorphism located in *BRCA2* E14 (Figure 4). As expected, in control LCL, we observed, a nearly equal contribution of the two alleles in the expression of both RNA isoforms. In contrast, in LCL with c.6937+1G>A, a complete imbalance was observed in +E12 transcripts (Figure 4B). Congruently, a strong AI was detected for ΔE12 transcripts, confirming that this isoform is predominantly produced from the minor allele which, by deduction, also carries c.6937+1G>A (Figure 4C).

In LCLs of two patients heterozygous for both the c.6901G>T nonsense variant and the E14 SNP, we confirmed that +E12 transcripts are predominantly produced from the major/WT allele (Figure 4B), whereas ΔE12 transcripts are predominantly produced from the minor/variant allele (Figure 4C). These results strongly suggest that the c.6901G>T nonsense variant is responsible for major E12 skipping in patient samples,

in good agreement with the minigene data.

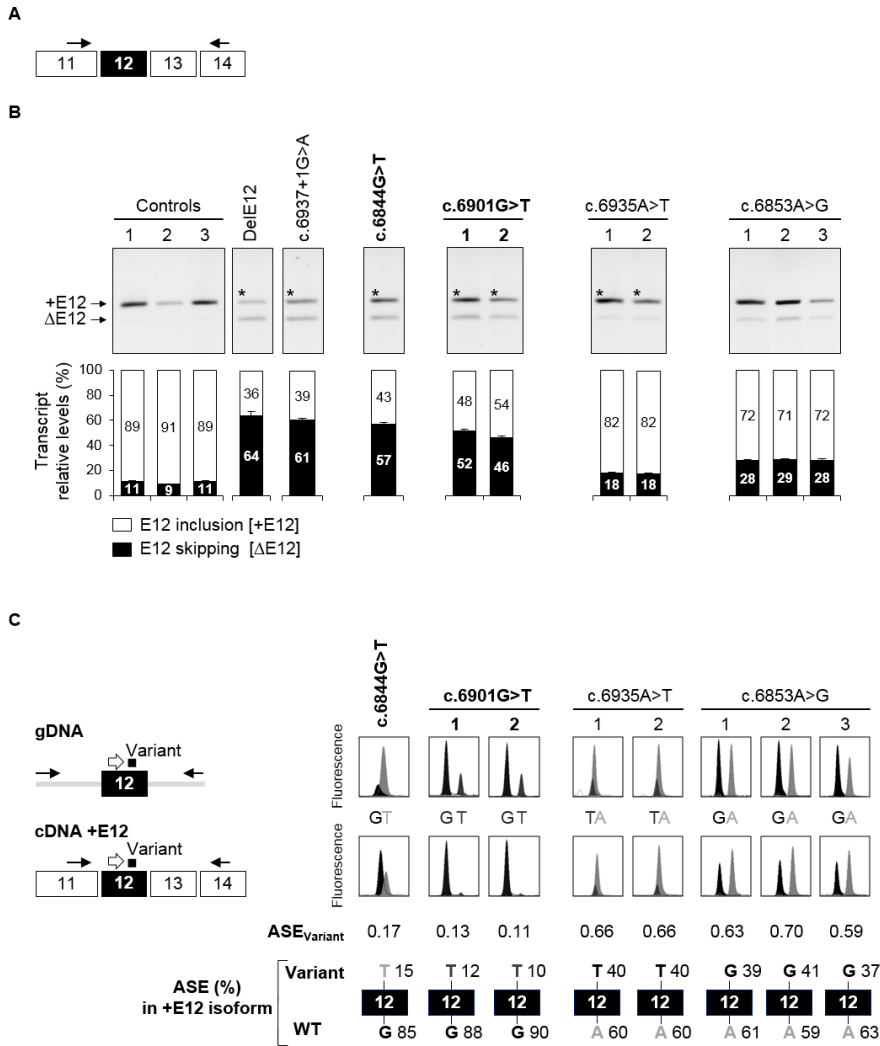


Figure 3. *BRCA2* exon 12 splicing patterns and allele-specific expression in control and patient lymphoblastoid cell lines

(A) Location of the primers used for RT-PCR analysis of *BRCA2* exon 12 splicing patterns. Boxes indicate *BRCA2* exons 11 to 14 and arrows above indicate the position of primers used in RT-PCR analysis. **(B)** RT-PCR analysis of *BRCA2* exon 12 splicing patterns. The *BRCA2* exon 12 splicing profiles were assessed by RT-PCR in LCLs of 3 healthy donors (controls, 1 to 3) and of patients carrying selected variants. For the nonsense c.6844G>T and c.6901G>T (bold), the missense c.6935A>T and c.6853A>G variants, the analyses were conducted in LCLs of one, two or three unrelated patients, as indicated. The image shows the agarose gel electrophoresis of the RT-PCR

products. The identities of the two RT-PCR products, with (+E12) or without (Δ E12) exon 12, are indicated on the left of the gel. Here, it is important to note that the gel images were not used for the quantification of the relative transcript levels because of the formation of heteroduplex between the different RNA isoforms (noted by the star symbol on the gel images), as revealed by band sequencing. Instead, for each LCL, the relative quantifications of these RNA isoforms were determined by capillary electrophoresis of the fluorescent RT-PCR products (see Figure S6), as indicated in Materials and Methods. The data shown represent the average of at least three independent experiments. Error bars indicate standard deviation values. **(C) Allele-specific expression of transcripts including *BRCA2* exon 12 assessed by SNaPshot analysis.** In order to measure allele-specific expression (ASE) of transcripts with exon 12 (+E12) in patient LCLs, a SNaPshot quantitative primer extension assay was performed, as described in Materials and Methods. As shown in the left-hand panel, genomic segment (gDNA) and complementary DNA (cDNA) containing *BRCA2* exon 12 (+E12) were amplified in parallel using specific primers (black arrows). The SNaPshot quantitative primer extension assay was then performed with a primer (white arrow) targeting the sequence immediately upstream the *BRCA2* exon 12 variant (?). Results from gDNA (top graphs) and cDNA (bottom graphs) are shown on the right-hand panel. ASE measured using the variant (ASE_{Variant}) was then calculated after normalization of the cDNA peak area ratio (Variant/WT) to the corresponding gDNA peak area ratio. Based on these data, a schematic representation of the ASE, expressed as percentage of the variant and WT alleles, is also presented. Results are representative of three independent experiments.

Functional characterization of *BRCA2* variants in mESC

For determination of the functional consequences of variant-induced enhanced production of the Δ E12 isoform, we employed the mESC-based *BRCA2* functional assay (8,19,20). We tested one missense (c.6842G>T), one IVS $\pm$ 1/2 (c.6937+1G>A) and three nonsense (c.6844G>T, c.6859A>T, c.6901G>T) variants selected based on their splicing effects in minigene assays. First, we showed that all five selected variants were able to rescue the cell-lethal phenotype induced after Cre-mediated deletion of the conditional mouse *Brca2* allele, as well as the 2 controls DelE12 and c.6853A>G (Figure 5).

As previously described (13), the WT *BRCA2* gene expressed in this system reproduced E12 alternative splicing (Δ E12 = 18%), whereas the DelE12 variant produced solely Δ E12 transcripts (Figures 6B and S8). The analysis of mESC expressing c.6842G>T and c.6937+1G>A revealed almost complete or full E12 skipping, respectively (Figures 6B and S8). Importantly, the impact on E12 splicing of the two nonsense variants, c.6844G>T and c.6901G>T, were confirmed in mESC as they caused nearly full E12 skipping (Figures 6B and S8). In contrast, the third nonsense variant tested, c.6859A>T, exhibited a slight increase in the relative levels of Δ E12 transcripts, as compared to WT (Figures 6B and S8). For the known spliceogenic c.6853A>G variant (13), a more extensive increase of E12 skipping levels was observed.

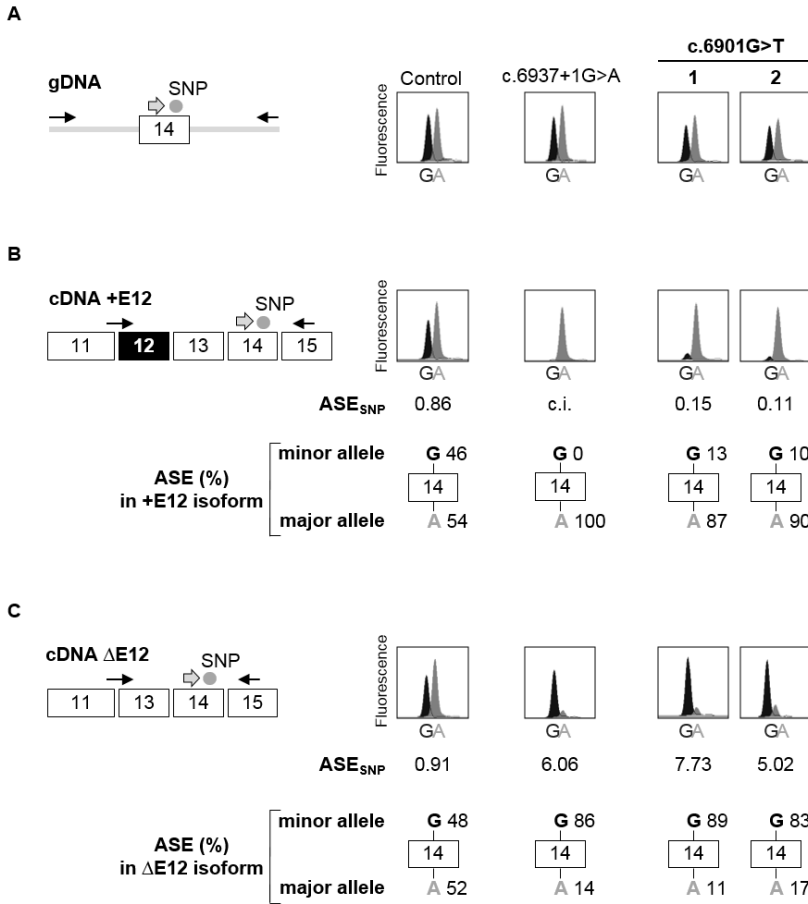


Figure 4. Allele-specific expression of *BRCA2* transcripts with and without exon 12 in control and patient lymphoblastoid cell lines

(A) SNaPshot results obtained from genomic DNA. As indicated in the left-hand panel, the genomic segment encompassing *BRCA2* exon 14 was PCR amplified from gDNA of a control individual and patients carrying the heterozygous polymorphism located in *BRCA2* exon 14, c.7242A>G (SNP, ·), by using specific primers (black arrows). The SNaPshot quantitative primer extension assay was then performed with a primer (grey arrow) targeting the sequence immediately upstream the SNP. Results shown on the right-hand panel are representative of 3 independent experiments. **(B) Allele-specific expression of transcripts containing *BRCA2* exon 12 (+E12).** As shown in the left-hand panel, +E12 cDNA was RT-PCR amplified from LCL RNA using specific primers (black arrows). The SNaPshot assay was next performed as described in (A). Results are presented in the right-hand panel. ASE measured in +E12 cDNA using the SNP (ASE_{SNP}) was calculated after normalization of the cDNA peak area ratio (minor allele/major allele) to the corresponding gDNA peak area ratio. Complete imbalance (c.i.) is indicated when only one allele was detected. A schematic representation of ASE_{SNP}, expressed as percentage of the minor and major alleles, is also presented. Results are representative of three independent experiments. **(C) Allele-specific expression of transcripts lacking *BRCA2* exon 12 (ΔE12).** As

indicated in the left-hand panel, $\Delta E12$ cDNA was RT-PCR amplified from LCL RNA using specific primers (black arrows). The SNaPshot quantitative primer extension assay was performed as described in (A). Results are presented in right-hand panel. ASE measured in $\Delta E12$ cDNA using the SNP (ASE_{SNP}) was calculated after normalization of the cDNA peak area ratio (minor allele/major allele) to the corresponding gDNA peak area ratio. A schematic representation of ASE_{SNP} , expressed as percentage of the minor and major alleles, is also presented. Results are representative of three independent experiments.

Western blot analysis confirmed the expression of BRCA2 protein for all variants after Cre-mediated deletion of the conditional *mBrca2* allele (Figures 6C and S9). It is important to note that the full-length and $\Delta E12$ isoforms cannot be distinguished by western blot due to the small difference in size. A truncated (TC) BRCA2 protein at the expected size (~260 kDa) was only detected for the nonsense variant c.6859A>T (p.Arg2287*) that expressed substantial amount of PTC-containing +E12 transcript. In contrast, no TC protein isoforms were detected for the two other nonsense variants, c.6844G>T (p.Glu2282*) and c.6901G>T (p.Glu2301*), suggesting that these nonsense variants only produce BRCA2 $\Delta E12$ protein, in good agreement with the RNA data (Table S3).

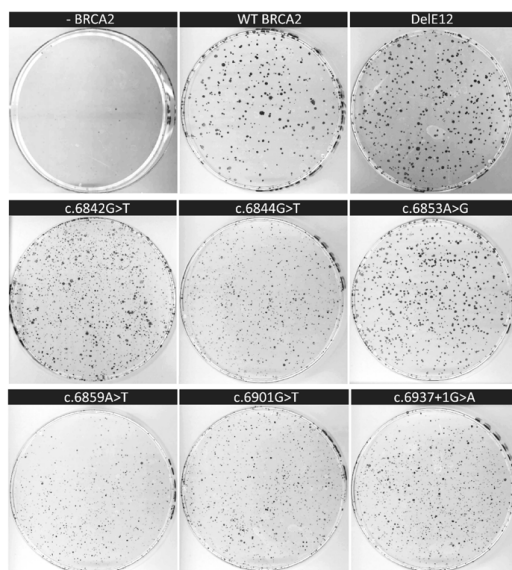
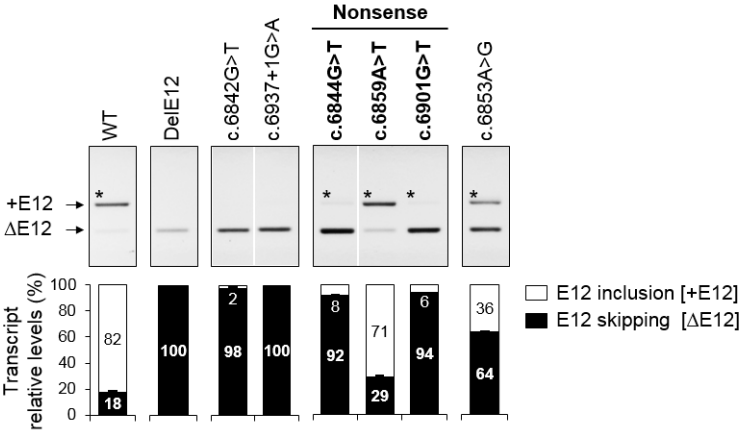


Figure 5. Complementation phenotypes of *BRCA2* variants expressed in mESC *Brca2* $^{-/loxP}$; *Pim1DR-GFP/WT* cells without *BRCA2* (-*BRCA2*), or expressing *BRCA2* WT or variant were transfected with a Cre-GFP expression plasmid to induce loss of the conditional mouse *Brca2* allele. Upon Cre-recombinase expression cells become *Brca2* deficient, which is lethal unless complemented by the expression of a (partially) functional *BRCA2* variant. Thirteen days post Cre-GFP transfection, culture dishes were stained with methylene blue.

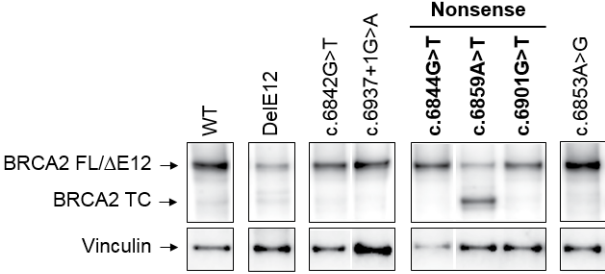
A



B



C



D

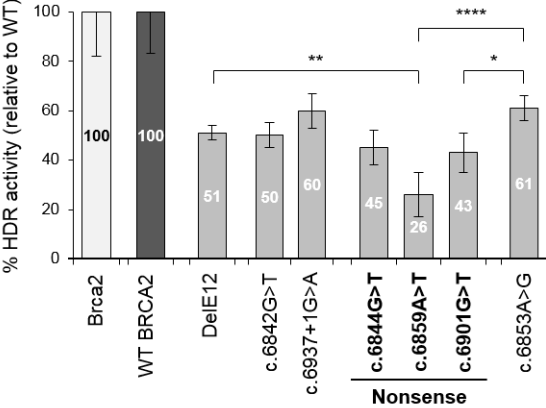


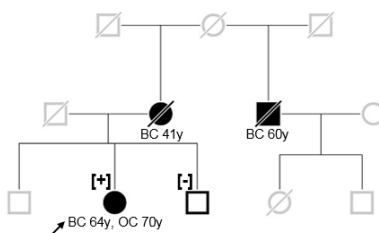
Figure 6. Functional analysis by using a mESC-based assay of *BRCA2* variants that affect exon 12 splicing

(A) Location of the primers used for RT-PCR analysis of *BRCA2* exon 12 splicing patterns. Boxes indicate *BRCA2* exons 11 to 14 and arrows represent primers used in RT-PCR analysis. **(B) *BRCA2* exon 12 splicing patterns.** The *BRCA2* exon 12 splicing profiles were assessed by RT-PCR in mESC expressing either WT or mutant *BRCA2* from a genomic copy of the human gene. The identities of the two RT-PCR products, with (+E12) or without (Δ E12) exon 12, are indicated on the left of the gel. Here, it is important to note that the gel images were not used for the quantification of the relative transcript levels because of the formation of heteroduplex (noted by the star symbol on the gel images) between the different RNA isoforms, as revealed by band sequencing. Instead, the relative quantifications of different RNA isoforms were determined by capillary electrophoresis of the fluorescent RT-PCR products (see Figure S8). Data represent the average of at least three independent experiments. Error bars indicate standard deviation values. **(C) *BRCA2* protein expression.** Expression of *BRCA2* protein either full length (FL), or deleted for exon 12 (Δ E12) or truncated (TC) was detected by western blot analysis. Vinculin was used as loading control. **(D) HDR activity.** GFP signal was measured by flow cytometry in I-Sce1 expressing cells two days post transfection. HDR activity is expressed as the percentage observed in *BRCA2* variant-expressing cells relative to *BRCA2* WT-expressing cells. *Brca2* represents the conditional *Brca2*^{-floxP}*Pim1*^{DR-GFP/WT} cell line expressing endogenous mouse *Brca2*. Error bars indicate the SD of six independent GFP measurements per variant as represented by the dots. Tukey's multiple comparisons test was applied to calculate p-values comparing Δ E12 or c.6853A>G with exon 12 variants. *, p < 0.05; **, p < 0.01; **** p < 0.0001.

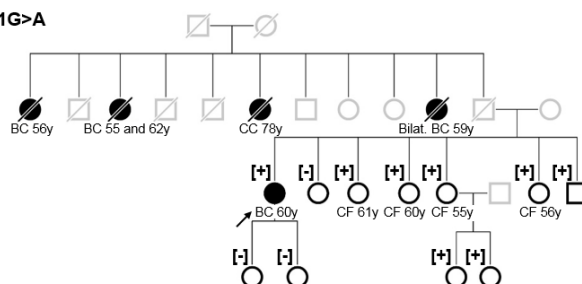
For functional characterization of the variants, we assessed their impact on HDR activity, the most prominent *BRCA2* tumor suppressor function (Figure 6D). In a previous validation study, HDR ranges were defined to allow classification of variants in clinically relevant categories. *BRCA2* variants with >50% HDR activity are considered to be (likely) benign, while variants with <30% HDR activity are (likely) pathogenic (20). The Δ E12 variant displayed a 50% reduction in HDR activity as compared to WT. A similar reduction in HDR activity was observed for the previously described spliceogenic c.6853A>G missense variant (13), as well as for c.6842G>T, c.6937+1G>A and the two nonsense variants, c.6844G>T and c.6901G>T. In contrast, the c.6859A>T nonsense variant displayed a more severe functional impact with a reduction of 70% in HDR activity and is the only variant in this set that is associated with a statistically significant reduction in HDR activity as compared to the Δ E12 variant (Tukey's multiple comparisons test comparing Δ E12 with c.6859A>T, p < 0.01). The extent of functional impairment is in line with the observation that this nonsense variant displayed the lowest relative levels of the "functional" Δ E12 transcript.

Attenuation of the HDR pathway sensitizes cells to cisplatin and PARP inhibitors (20). Congruent with their substantial residual capacity to perform HDR (*i.e.* 43% $\leq$ HDR $\leq$ 61%), the major spliceogenic E12 variants displayed a relatively mild sensitivity to cisplatin and PARP inhibitor (talazoparib) whereas the c.6859A>T nonsense variant with a minor splicing effect and a more severe functional impact (*i.e.* HDR = 26%) is associated with the highest drug sensitivity (Figure S10).

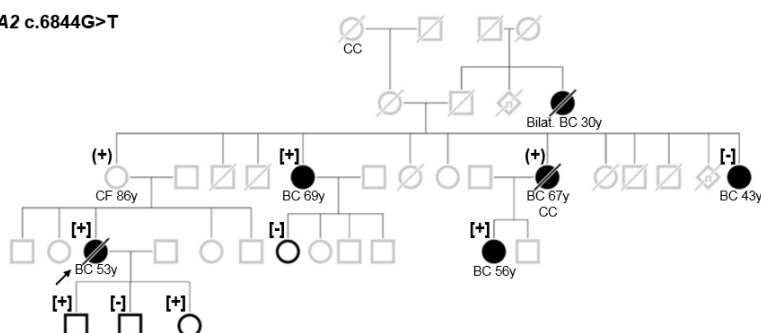
A *BRCA2* c.6842G>T
+ c.9195_9196delinsAT
(P2)



B *BRCA2* c.6937+1G>A
(P3)



C *BRCA2* c.6844G>T
(P5)



D *BRCA2* c.6901G>T
(P10)

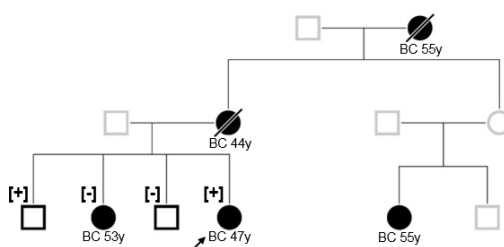


Figure 7. Pedigrees of patients carrying *BRCA2* exon 12 spliceogenic variants.

Here, the most informative pedigrees are presented. Proband's referred as P2, P3, P5 and P10, respectively carriers of *BRCA2* c.6842G>T (p.Gly2281Val) (A), c.6937+1G>A (B), c.6844G>T (p.Glu2282*) (C) and c.6901G>T (p.Glu2301*) (D), are indicated by an arrow. Note that, in patient P2, both *BRCA2* exon 12 variant (c.6842G>T) and *BRCA2* exon 24 pathogenic frameshift variant

(c.9195_9196delinsAT) were identified. Filled symbols represent patients with cancer (BC, breast cancer; Bilat., bilateral; OC, ovary cancer; CC, colon cancer; PO, prostate cancer). Ages at cancer diagnosis are indicated. For some individuals, prophylactic salpingo-oophorectomy (PSO) was performed. Heterozygote carriers for the variant are indicated by the symbol [+], obligate carriers by the symbol (+) and non-carriers by the symbol [-].

Clinical and family data of patients carrying E12 spliceogenic variants

We focused on families of 10 probands (P) that carry either variants inducing complete E12 skipping (DelE12 [P1], c.6842G>T [P2], c.6937+1G>A [P3, P4]) or nonsense variants that trigger major E12 skipping (c.6844G>T [P5, P6], c.6901G>T [P7 to P10]) (Table S4 and Figure 7). When taking into account women over 45 years of age, we observed a partial co-segregation of the variants with breast cancer (BC). Indeed, besides probands, we identified: 3 carriers with BC, 5 cancer-free carriers and 3 non-carriers with BC. Of note, co-occurrence of c.6842G>T with a pathogenic *BRCA2* exon 24 variant, c.9195_9196delinsAT (p.Phe3065_Gln3066delinsLeu*), was observed in P2. Yet, the chromosome phasing of these two variants remains unknown.

Discussion

It is now well established that mis-splicing triggered by specific SNVs is a common cause of Mendelian diseases (23). As a shift of paradigm, the present study provides evidence for the first time in a cancer susceptibility gene, as far as we know, that variant-induced perturbation of splicing can rescue complete LoF of presumed pathogenic variants, at least partially.

For this demonstration, we used *BRCA2* E12 as a model system and took advantage of complementary experimental approaches to demonstrate that (i) a subset of presumed LoF IVS±1/2 variants as well as nonsense SNVs enhance in-frame skipping of *BRCA2* E12 and, as a consequence, (ii) lead to the production of an internally deleted but partially functional *BRCA2* protein. Altogether, these data call into question the clinical interpretation of specific spliceogenic hypomorphic IVS±1/2 and nonsense variants.

Whereas the severe impact of IVS±1/2 variants on *BRCA2* E12 splicing was expected given the disruption of the highly conserved 3' or 5' canonical ss, the effects of the five nonsense variants on splicing, albeit to different extents, were not anticipated and are most probably due to the modification of ESR, via either the destruction of exonic splicing enhancers (ESE) and/or the creation of exonic splicing silencers (ESS) (11,24). Interestingly, the two major spliceogenic nonsense variants described here, *BRCA2* c.6844G>T (p.Glu2282*) and c.6901G>T (p.Glu2301*), lie within purine-rich 5'-AGAA-3' motifs resembling those described as binding sites for the splicing activator protein Tra2-β1 (25). Most of the selected nonsense variants (4/5) promoted E12 skipping, albeit to different degrees, whereas none of the 6 frameshift variants tested had a splicing effect in minigene assays. Considering the relative limited number of variants

in these two groups, the apparent specific association between nonsense mutations and E12 skipping might be coincidental as, in theory, small insertions or deletions within an exon could also fortuitously disrupt critical ESEs and/or create putative ESSs, both mechanisms potentially promoting exon skipping. Still, the association between nonsense mutations and E12 skipping could also reflect the fact that putative stop codon trinucleotides are enriched in ESS hexamers (26–28).

Nonsense-associated altered splicing has been previously described in cancer susceptible genes (11). In particular, it has been shown that *BRCA1* c.5080G>T (p.Glu1694*) causes in-frame skipping of exon 18 resulting into non-functional protein, given the disruption of its first BRCT domain (29,30). In contrast, for *BRCA2* E12 spliceogenic nonsense variants, the in-frame skipping of the PTC-containing exon not only circumvents protein truncation, but also increases the production of an internally deleted partially functional isoform, as revealed in the mESC assay.

Recently, it has been shown that the mESC assay is a reliable method to predict pathogenicity of missense *BRCA2* variants (20). The complementation phenotype, the intermediate HDR activity and the mild effect on cisplatin and PARP inhibitor sensitivity measured for the two major spliceogenic *BRCA2* E12 nonsense variants (c.6844G>T, c.6901G>T) suggest that these initially presumed null variants do not lead to complete LoF. The hypomorphic activity observed for these variants might confer moderate or low risks of breast cancer, as shown for previously characterized hypomorphic missense *BRCA1* and *BRCA2* variants (31–33). In contrast, the nonsense variant with the lowest level of E12 skipping (c.6859A>T) displays a significant decrease in HDR activity comparable to a previously reported pathogenic missense variant (20). Consistent with the impact on HDR, this variant shows hypersensitivity to cisplatin-induced DNA damage and to PARP inhibition.

Besides the 15 presumed LoF variants, we extended the screening of *BRCA2* E12 spliceogenic variants to 25 additional SNVs (intronic, missense and synonymous variants). Up to this date, only 3 missense variants have been reported to increase the level of alternative splicing of *BRCA2* E12 (13,34,35). The present study not only confirmed those initial findings but, notably, uncovered 17 new spliceogenic variants affecting either 3'/5' ss or ESR.

In this study, we confirmed that the variant c.6853A>G promotes E12 skipping, as previously shown (13). However, this effect is partial: the mutant allele also significantly contributes to the production of the full-length +E12 transcript encoding the *BRCA2* p.Ile2285Val protein. This observation does not call into question the non-pathogenic status of this variant. However, it prevents (i) from considering the neutral classification of this variant as an additional argument in favor of the functionality of the *BRCA2* E12-deleted protein, as initially reported (13) and (ii) from extending this classification to all other *BRCA2* E12 spliceogenic variants.

To apprehend the clinical relevance of *BRCA2* E12 spliceogenic variants with respect to cancer risk, we analyzed patient clinical data and family history available within the French diagnostic network. Several observations (*i.e.* identification of both cancer-free carriers and non-carriers with breast cancer and co-occurrence with a pathogenic *BRCA2* variant) could be in line with the hypomorphic activity associated with these variants. Still, the interpretation of these spliceogenic variants cannot be inferred from these limited patient and family information and further larger-scale co-segregation and case-control studies will be needed to accurately evaluate cancer risks conferred by *BRCA2* E12 spliceogenic variants.

To our knowledge, the existence of a rescue mechanism for nonsense variants through their direct impact on in-frame splicing has never been described for cancer susceptibility genes. Yet, it has been documented in genes involved in other Mendelian diseases. For instance in the *DMD* gene, several truncating SNVs induce in-frame skipping of the PTC-containing exon, resulting into an internally deleted dystrophin which exhibits partial function and consequently leads to a milder phenotype known as Becker muscular dystrophy (36–38). It is quite possible that presumed LoF *BRCA2* E12 variants would confer severe, moderate or minimal reduction in function, depending of their impact on splicing (*i.e.* levels of Δ E12 transcript), and would hence be associated with variable degrees of cancer susceptibility.

Of note, for certain SNVs, the *BRCA2* Δ E12 RNA levels were somewhat variable in the three complementary approaches used in this study (*i.e.* minigene, patient LCL and mESC assays), most probably because of different cellular and genomic contexts. Ideally, E12 splicing patterns should also be quantified in the patient relevant tissues (normal and tumoral breast tissues). Intriguingly, it has been previously reported that *BRCA2* E12 alternative splicing is increased in some breast tumors as compared with their matched normal tissues (39) and up-regulated in several mammary and ovarian cancer cell lines (40). Some of these cell lines could carry specific spliceogenic variants and provide attractive cellular model systems for future investigations on *BRCA2* E12-deleted protein function. Importantly, during the preparation of this manuscript, it has been shown that the highly conserved region encoded by E12 may be involved in the reproductive rather than the tumor-suppressing function of *BRCA2* (41,42). Indeed, the E12-encoded region (a.a. 2281-2313) is part of a binding domain for MEILB2/HSF2BP. This testis-expressed protein recruits *BRCA2* to DNA double-strand breaks to ensure meiotic homologous recombination during spermatogenesis. Altogether, these data strongly suggest that *BRCA2* E12 is essential to the successful completion of meiotic homologous recombination but not for the role of *BRCA2* in mitotic homologous recombination, as a cancer suppressor.

In conclusion, this study provides for the first time experimental arguments to reevaluate the clinical interpretation of a subset of *BRCA2* IVS $\pm$ 1/2 and nonsense spliceogenic variants that enhance in-frame skipping of a potentially non-essential exon. Based on these findings and following the ACMG (3) and ENIGMA (4) recommendations, we propose to reclassify these SNVs as variants of unknown significance (VUS) until further

genetic analyses establish the cancer risks associated with these variants. This study emphasizes the requirement of complementary RNA and protein analyses to ensure comprehensive variant interpretation, with particular caution taken for presumed LoF variants located in in-frame dispensable exons. In the NGS era, the main challenges for medical geneticists are not only the classification of the variants detected in patients but also the quantification of the associated risk, especially for variants retaining partial function. Indeed, our results highlight the need to move forwards a more accurate interpretation of variants in terms of both pathogenicity and penetrance.

Supplementary data

Supplementary data include 10 figures, 1 supplemental note and 4 tables.

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Supplementary Data

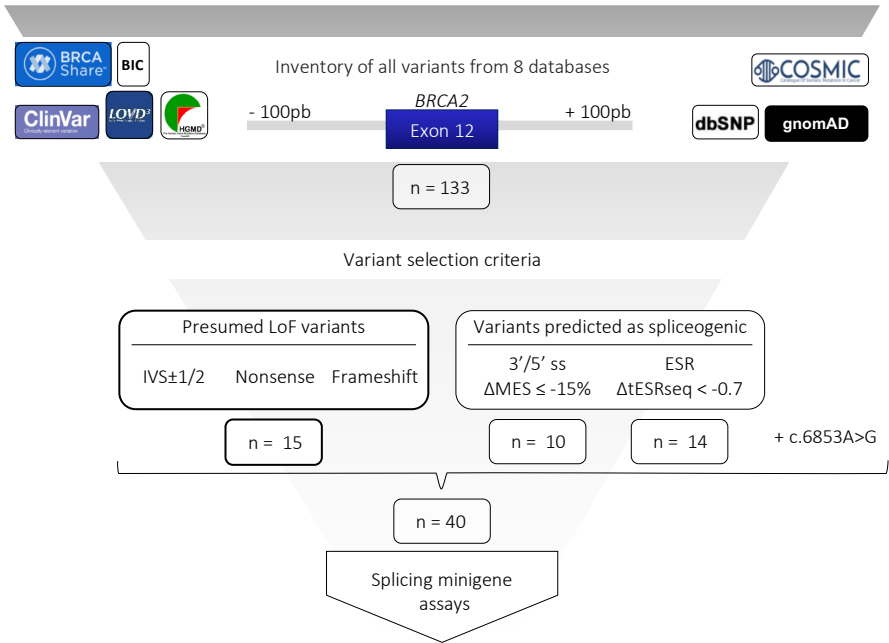


Figure S1. Variant selection from human variation databases

All variants located in *BRCA2* E12 and its 100 bp long flanking intronic sequences were extracted from the eight following human variation databases (last accessed: 05 September 2017): BRCA Share™, BIC (Breast Cancer Information Core), ClinVar, LOVD (Leiden Open Variation Database), HGMD (Human Gene Mutation Database), COSMIC (Catalogue of Somatic Mutations in Cancer), dbSNP (Single Nucleotide Polymorphism database) and gnomAD (genome Aggregation Database). A total of 133 variants were then collected. Firstly, all presumed LoF variants were selected, including intronic variants affecting the canonical intronic dinucleotides at 3'/5' ss (IVS±1/2) as well as nonsense and frameshift variants. In parallel, all other variants predicted to alter either 3'/5' ss or ESR, according respectively to the MaxEntScan algorithm ($\Delta\text{MES} \leq -15\%$) or the ESRseq scores ($\Delta\text{tESRseq} < -0.7$), were retained. In addition, c.6853A>G was included into the selection because it has been previously described as a *BRCA2* exon 12 spliceogenic variant (1). Altogether, this selection comprised 40 variants that were assessed for their impact on *BRCA2* exon 12 splicing in splicing minigene assays.

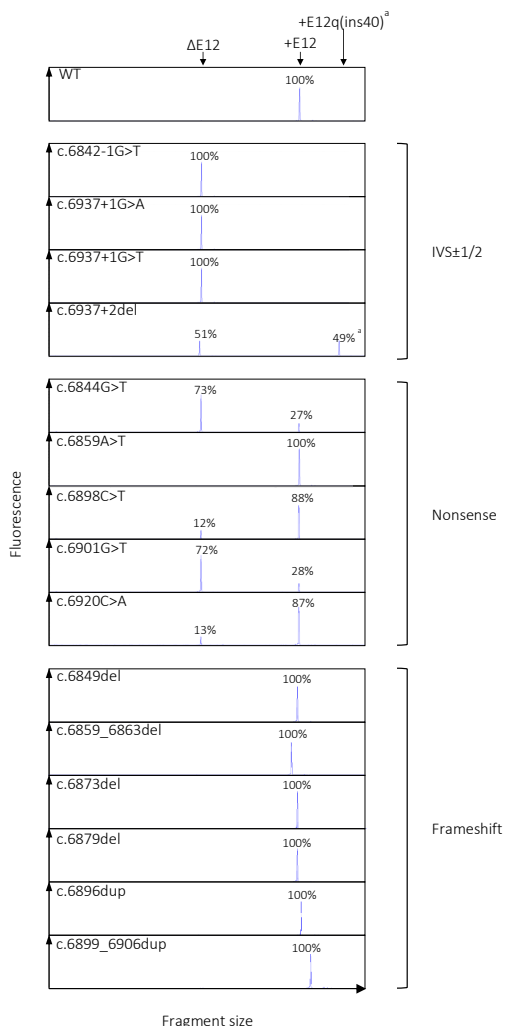


Figure S2. Capillary electrophoresis analyses of *BRCA2* exon 12 splicing patterns in minigene assays of presumed LoF variants

The *BRCA2* exon 12 splicing patterns of WT and mutant (IVS±1/2; nonsense; frameshift) minigenes expressed in HeLa cells were assessed by fluorescent RT-PCR, as described in Materials and Methods. In order to quantify the relative levels of each RNA isoform, the fluorescent RT-PCR products were resolved by capillary electrophoresis on an automated genetic analyzer. For each variant, one representative electropherogram is shown. The different fluorescence peaks correspond to transcripts either without (ΔE12) or with (+E12) exon 12. An additional isoform (a) with the retention of the first 40 nucleotides of intron 12 (+E12q(ins40)) was detected in the context of c.6937+2del. Peak areas were used to quantify the relative levels of each transcript, expressed as percentage, and correspond to the average of at least three independent experiments (see Figure 1).

Chapter 4. Skipping nonsense to maintain function: the paradigm of *BRCA2* exon 12

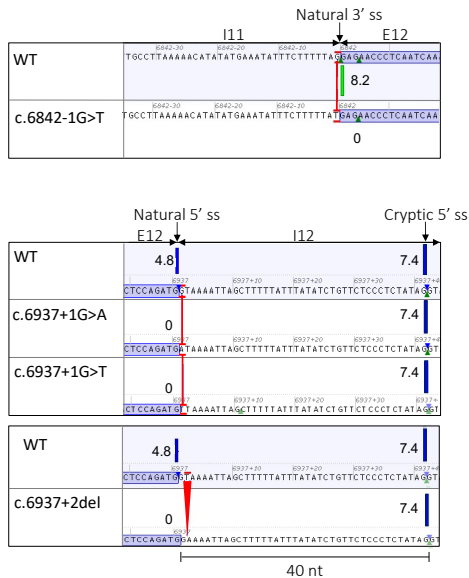


Figure S3. Bioinformatics predictions of 3'/5' ss alterations for variants located at position IVS±1/2 of *BRCA2* exon 12

Bioinformatics predictions of the variant effects on 3'/5' ss were based on the algorithm MaxEntScan (MES) (2), interrogated by using the integrated software Alamut Visual 2.9 (Interactive Biosoftware). The MES score of the 3' and 5' ss are indicated in the wild-type (WT) and variant contexts.

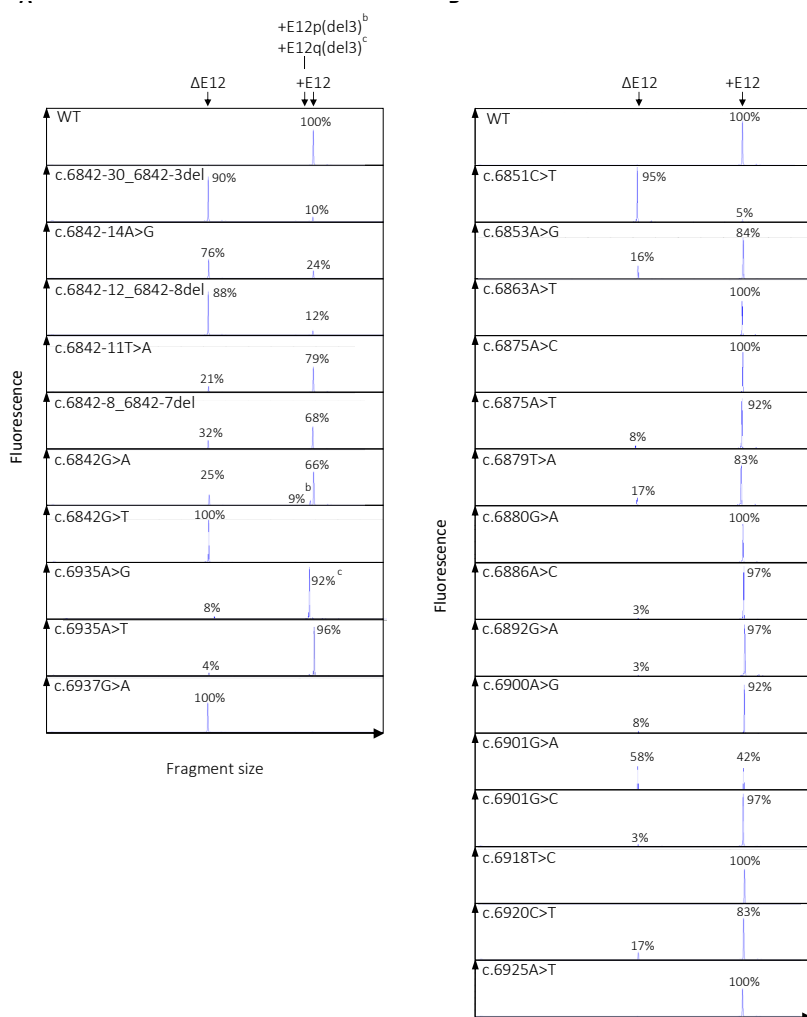


Figure S4. Capillary electrophoresis analyses of *BRCA2* exon 12 splicing patterns in minigene assays of variants predicted to alter 3'/5' ss (A) or ESR (B)

The *BRCA2* exon 12 splicing patterns of WT and mutant minigenes expressed in HeLa cells were assessed by fluorescent RT-PCR, as described in Materials and Methods. In order to quantify the relative levels of each RNA isoform, the fluorescent RT-PCR products were resolved by capillary electrophoresis on an automated genetic analyzer. For each variant, one representative electropherogram is shown. The different fluorescence peaks correspond to transcripts either without (Δ E12) or with (+E12) exon 12. Additional isoforms, b and c, deleted either for the first (+E12p(del3)) or the last (+E12q(del3)) 3 nucleotides of exon 12 were detected for c.6842G>A and c.6935A>G, respectively. In parallel, the previously described spliceogenic c.6853A>G variant (1) was analyzed. Peak areas were used to quantify the relative levels of each transcript, expressed as percentage and correspond to the average of at least three independent experiments (see Figure 2).

Chapter 4. Skipping nonsense to maintain function: the paradigm of *BRCA2* exon 12

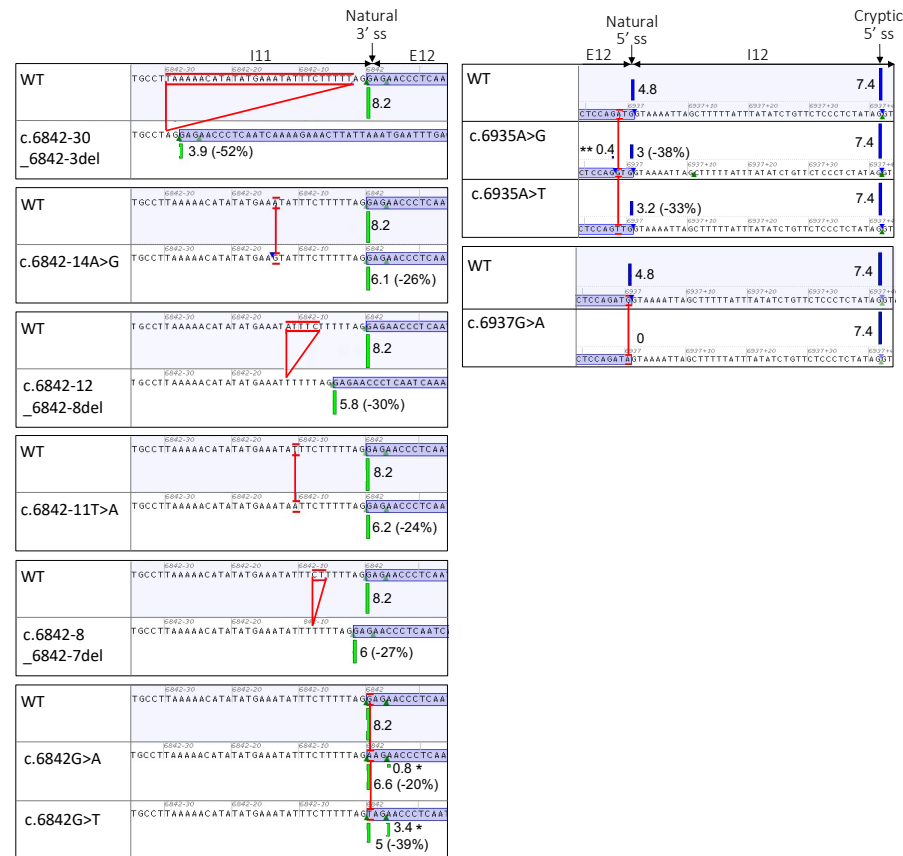


Figure S5. Bioinformatics analysis of variants predicted to alter 3'/5' ss of *BRCA2* exon 12 (Δ MES $\leq$ -15%)

Bioinformatics predictions of the variant effects on 3' or 5' ss were performed based on the algorithm MaxEntScan (MES) (2), interrogated by using the integrated software Alamut Visual 2.9 (Interactive Biosoftware). For each variant, the MES score differences (Δ MES) between wild-type (WT) and variant contexts are indicated between brackets for 3'/5' natural ss. All these variants were selected for experimental analysis based on a Δ MES value inferior to -15%. In addition, the MES scores of new predicted 3' (*) or 5' (**) ss are indicated.

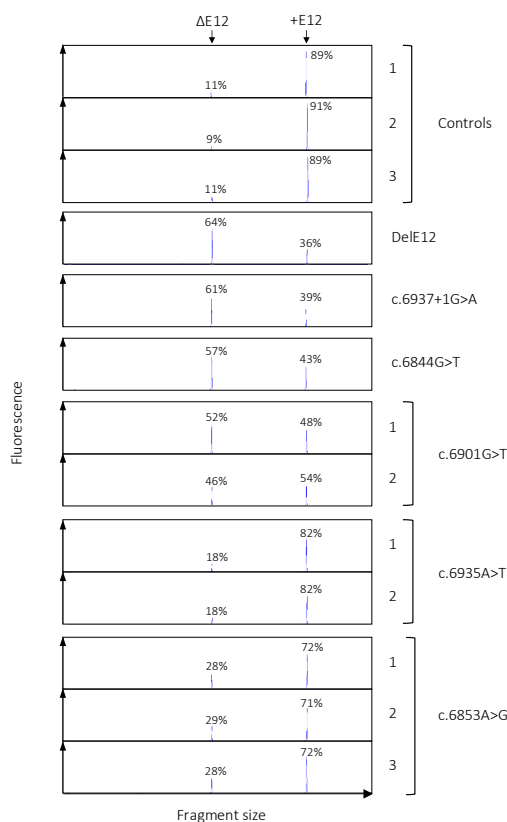


Figure S6. Capillary electrophoresis analyses of *BRCA2* exon 12 splicing patterns in control and patient lymphoblastoid cell lines

The *BRCA2* exon 12 splicing patterns were assessed by RT-PCR in LCLs of 3 healthy donors (controls, 1 to 3) and of patients carrying selected variants. For the two nonsense, **c.6844G>T** and **c.6901G>T** (bold), the missense **c.6935A>T** and **c.6853A>G** variants, the analyses were conducted in LCLs of one, two or three unrelated carriers, as indicated. In order to quantify the relative levels of the different isoforms, the fluorescent RT-PCR products were resolved by capillary electrophoresis on an automated genetic analyzer. For each LCL, one representative electropherogram is shown. The different fluorescence peaks correspond to transcripts either without (Δ E12) or with (+E12) exon 12. Peak areas were used to quantify the relative levels of each transcript, expressed as percentage, and correspond to the average of at least three independent experiments (see Figure 3).

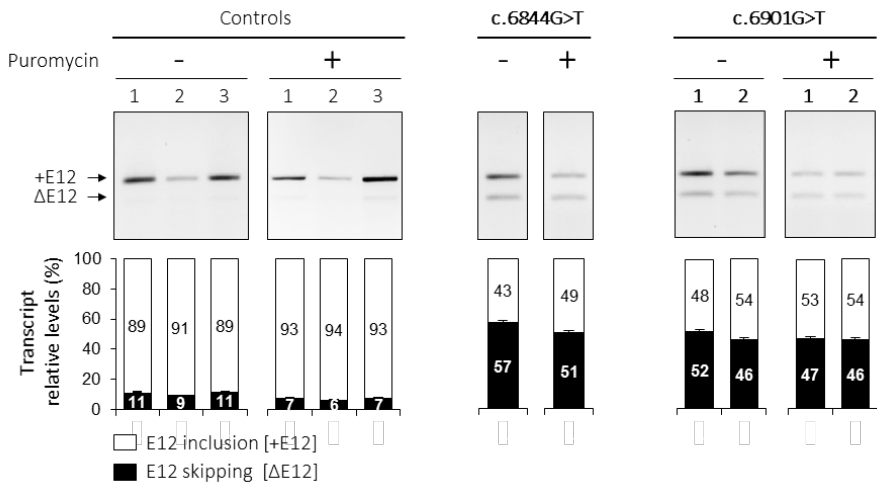


Figure S7. RT-PCR analysis of *BRCA2* exon 12 splicing patterns in puromycin- or mock-treated lymphoblastoid cell lines of control individuals and patients carrying the c.6844G>T or c.6901G>T nonsense variants

LCLs of three healthy donors (controls) and of a patient carrying the c.6844G>T nonsense variant as well as two unrelated patients carrying the c.6901G>T nonsense variant were cultured and treated (+) or mock-treated (-) with puromycin, as previously described (3). In these biological samples, the *BRCA2* exon 12 splicing patterns were assessed by RT-PCR, as described in Materials and Methods. The images show the agarose gel electrophoresis of the RT-PCR products. The identities of the two RT-PCR products, with (+E12) or without (ΔE12) exon 12, are indicated on the left of the gel. For each LCL, the relative abundances of these two RNA isoforms were determined by capillary electrophoresis of the fluorescent RT-PCR products, as indicated in Materials and Methods. The data shown under the images are expressed as percentage of transcript levels relative to all RT-PCR products and represent the average of at least three independent experiments. Error bars indicate standard deviation values.

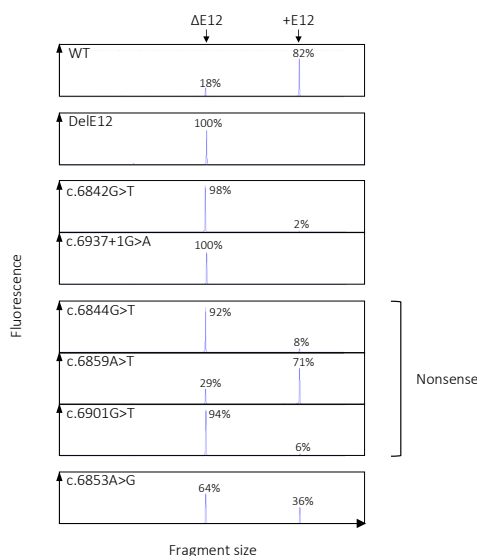


Figure S8. Capillary electrophoresis analyses of *BRCA2* exon 12 splicing patterns in variant-expressing mESC. The exon 12 splicing patterns of WT and mutant *hBRCA2* BAC constructs expressed in mESC were assessed by fluorescent RT-PCR, as described in Materials and Methods. In order to quantify the relative levels of each isoform, the fluorescent RT-PCR products were resolved by capillary electrophoresis on an automated genetic analyzer. For each variant-expressing mESC, one representative electropherogram is shown. The different fluorescence peaks correspond to transcripts either without (Δ E12) or with (+E12) exon 12. Peak areas were used to quantify the relative levels of each transcript, expressed as percentage and corresponding to the average of at least three independent experiments.

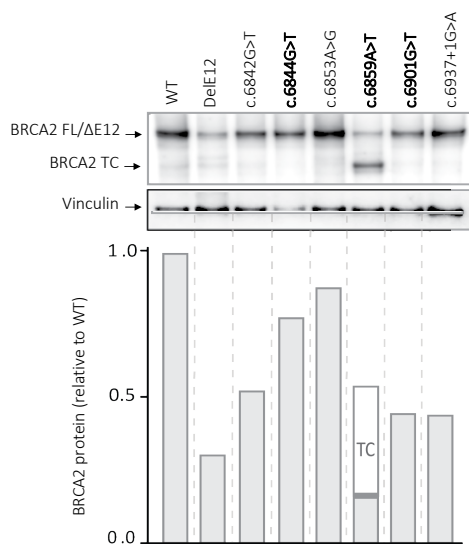


Figure S9. Quantitation of *BRCA2* protein expression in mESC

Expression of *BRCA2* full length (FL), without exon 12 (Δ E12) and truncated (TC) protein isoforms were detected by western blot analysis. Due to the small difference in size between the full length *BRCA2* protein (*BRCA2* FL protein isoform, 3418 aa) and the *BRCA2* protein deleted for E12 (*BRCA2* Δ E12 protein isoform, 3386 aa), these two protein isoforms are indistinguishable by western blot analysis. Vinculin was used as loading control. Chemiluminescent signals were captured by the Amersham Imager 600 (GE Healthcare Life Sciences) and band intensity quantification was performed on non-saturated images using ImageQuant TL software. The abundance of *BRCA2* protein was normalized to the amount of vinculin in each lane (loading control). *BRCA2* protein expression is presented relative to WT *BRCA2* protein level.

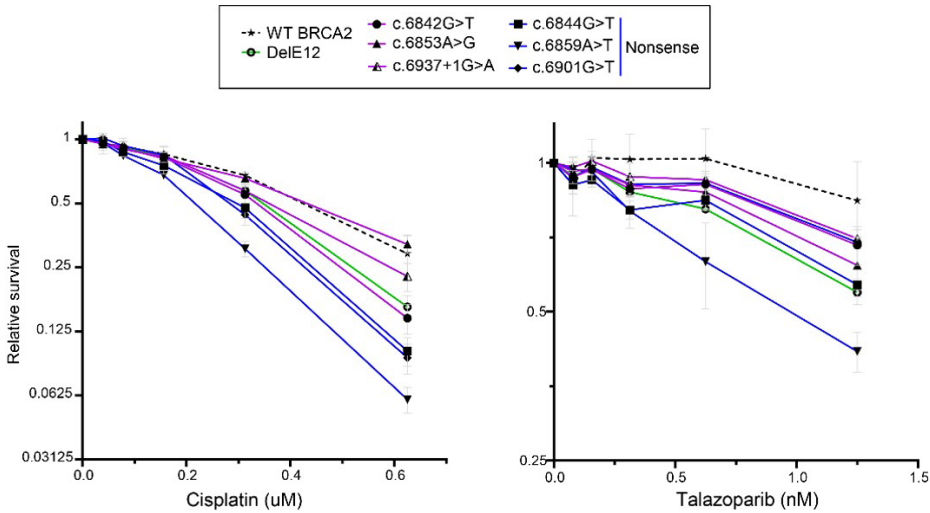


Figure S10. Sensitivity of *BRCA2* variants to cisplatin and PARP inhibitors

Mouse ESC expressing *BRCA2* WT or variants were treated with cisplatin or PARP inhibitor (Talazoparib) and relative cell survival compared to untreated cells was determined as described in Materials and Methods. Data represent the average of four experiments per variant. Error bars indicate the standard error.

Supplemental references

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Supplemental Note

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(<https://clinicaltrials.gov/ct2/show/NCT01689584>)

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Chapter 4. Skipping nonsense to maintain function: the paradigm of *BRCA2* exon 12

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Table S1. Description of primers used in this study

Primer's name ¹	Location	Sequence ²	Use
B2I11BamHI-F	BRCA2 I11	GACCGGA <u>lcc</u> CTGTAAATTGACATTGAAGACTGACTTTAC	Genomic DNA PCR amplification for minigene cloning
B2I12MluI-R	I12	GACCACGCGTGAGTATAAAGAGGTCCTTGATTAGGCA	Site directed mutagenesis pCAS2 : PCR ³
B2E12 c.6842-30_6842-3del-F	I11/E12	ATTATTTGCCTAGGAGAACCC	
B2E12 c.6842-30_6842-3del-R	I11/E12	GGGTCTCTCTAGGCAATAAT	
B2E12 c.6842-14A>G-F	I11/E12	CATATATGAAGTATTCTCTTTTAGGA	
B2E12 c.6842-12_6842-8del-F	I11/E12	ATATATGAAATTTTTTTTAGGAGAACCC	
B2E12 c.6842-12_6842-8del-R	I11/E12	GGTTCTCTTAAAAAATTTTCATATAT	
B2E12 c.6842-11T>A-R	I11/E12	TCCTAAAAAGAAATATTTTCATATATG	
B2E12 c.6842-8_6842-7del-F	I11/E12	TATGAAATATTTTTTTTAGGAGAAC	
B2E12 c.6842-1G>T-R	I11/E12	GTTCTCATAAAAAGAAATATTTTCATA	
B2E12 c.6842G>A-F	I11/E12	ATTCTTTTTTAGAAGAACCCCTCAAT	
B2E12 c.6849del-R	I11/E12	ATTGAGGTTCTCTTAAAAAGAAAT	
B2E12 c.6851C>T-R	I11/E12	CTTTTGATTAAAGGGTTCTCTAA	
B2E12 c.6859_6863del-F	E12	CCCTCAATCAAACTTATTAAATGA	
B2E12 c.6859_6863del-R	E12	TCATTTAATAAGTTTGATTGAGGG	
B2E12 c.6863A>T-F	E12	AAAAGAACTCTATTAAATGAATTGA	
B2E12 c.6873del-F	E12	AAACTTATTAAAGAATTTGACAGG	
B2E12 c.6873del-R	E12	CCTGTCAAATCTTTAATAAGTTT	
B2E12 c.6875A>T-F	E12	CTTATTAAATGTATTTGACAGG	
B2E12 c.6875A>C-R	E12	TCAAATGCATTTAATAAGTTTCTTT	
B2E12 c.6879del-F	E12	TATTAAATGAATTGACAGGATAATAG	
B2E12 c.6879T>A-F	E12	TAAATGAATTAGACAGGATAATA	

table continues

Primer's name ¹	Location	Sequence ²	Use
B2E12 c.6880G>A-R	E12	CTATTATCCTGTTAAATTCATTTAATA	Site directed mutagenesis pCAS2 (PCR1 and PCR11) and sequencing Analysis of <i>BRCA2</i> E12 splicing patterns by RT-PCR and sequencing
B2E12 c.6886A>C-R	E12	TATTAGCCTGTGCAAAATTCATTTAA	
B2E12 c.6892G>A-F	E12	CAGGATAATAAAAAATCAAGA	
B2E12 c.6896dup-F	E12	GGATAATAGAAAAATCAAGAAAAATC	
B2E12 c.6896dup-R	E12	GATTTTCTTGATTTTCTATATCC	
B2E12 c.6898C>T-F	E12	GAAAAATTAAGAAAAATCCTTAAAGGC	
B2E12 c.6899_6906dup-F	E12	TAGAAATCAAGAAAAAGAAAAATCCTTAAAG	
B2E12 c.6899_6906dup-R	E12	CTTTAAGGATTTTTCTTTTTCTTGATTTTCTA	
B2E12 c.6900A>G-F	E12	ATAGAAAAATCAGGAAAAATCCTTA	
B2E12 c.6900A>G-R	E12	TAAGGATTTTCTCTGATTTTCTAT	
B2E12 c.6901G>A-R	E12	GCCTTAAAGGATTTTTTTTGATTTTC	
B2E12 c.6901G>C-R	E12	GAAATCAAAGAAAAATCCTTAAAGGC	
B2E12 c.6918T>C-F	E12	CCTTAAAGGCCTCAAAAAGCAC	
B2E12 c.6918T>C-R	E12	GTGCTTTTGAAGGCCTTTAAGG	
B2E12 c.6920C>A-R	E12	CATCTGGAGTGCTTTTAAAGCCTTAAAGG	
B2E12 c.6920C>T-F	E12	TAAAGCCTTAAAGGCACTC	
B2E12 c.6925A>T-R	E12	GAGTGCATTTGAAGCCTTTA	
B2E12 c.6935A>G-F	E12/I12	CACTCCAGGTGGTAAAAATTAGCT	
B2E12 c.6937G>A-F	E12/I12	CACTCCAGATAGTAAAAATTAGC	
B2E12 c.6937+1G>T-R	E12/I12	AGCTAAATTTTAAACATCTGGAGTG	
B2E12 c.6937+2del-R	E12/I12	GCTAATTTTCCATCTGGAGTG	
pCASseq-F	pCAS2	GGGGTCAATAGCAGTGAGAG	pCAS2 minigene
pCASseq-R	IB	GCTCCATTTACAGGTAGAGA	
pCASKO1-F*	EA	TGACGTGCGCCCATCAC	pCAS2-R
pCAS2-R	EB	ATTGGTTGTTGAGTTGGTTGTC	

table continues

Primer's name ¹	Location	Sequence ²	Use
B2E11/12-F	<i>BRCA2</i>	CCCTTATCTAGTGG GAGAACCTCTCA	Analysis of <i>BRCA2</i> E12 splicing patterns by RT-PCR and sequencing
B2E11/13-F		ATCTTAGTGG GCACAATAAAAG	
B2E15-R		AAGAAGAAACAAAGGCAACG	
B2I11BamHI-F	I11	GACCGAICCTGTAATTGACATTGAAGACTGACTTTAC	Genomic DNA PCR amplification and sequencing
B2I12MluI-R	I12	GACCAGCGTGGAGTATAAAGAGGTCCTTGATTAGGCA	
B2I13-F	I13	AGCAAAATGAGGGTCTGCAAC	Genomic DNA PCR amplification
B2I14-R	I14	GCAAAAATTCATCACACAAATTGTC	
B2E12-Snap-c.6844GT-R	E12	GTTTCTTTTGATTGAGGGTT	Analysis of allele-specific expression by SNaPShot
B2E12-Snap-c.6853AG-R	E12	ATTTAATAAGTTTCTTTTGA	
B2E12-Snap-c.6901GT-F	E12	ACAGGATAATAGAAAATCAA	
B2E12-Snap-c.6901GT-R	E12	TGAAGCCTTTAAGGATTTT	
B2E12-Snap-c.6935AT-F	E12	GGCTTCAAAAAGCACTCCAG	
B2E14-Snap-c.7242AG-F	E14	CCACCTTTTAAAACTAAATC	
B2E12-SDMc.6842G>T-F	I11/E12	GAAATATTTCTTTTAGTAGAAACCTCAATCAAAAG	
B2E12-SDMc.6842G>T-R	I11/E12	CTTTTGATTGAGGGTTCTCTAAAGAAATATTTTC	Site directed mutagenesis pUC19
B2E12-SDMc.6844G>T-F	I11/E12	GAAATATTTCTTTAGGATTAACCTCAATCAAAAGAAAC	
B2E12-SDMc.6844G>T-R	I11/E12	GTTTCTTTTGATTGAGGGTTATCTTAAAGAAATATTTTC	
B2E12-SDMc.6853A>G-F	I11/E12	GGAGACCTCTAGTCAAAAGAAACTATTAAATG	
B2E12-SDMc.6853A>G-R	I11/E12	CATTATAAGTTTCTTTTGACTGAGGGTTCTCC	
B2E12-SDMc.6859A>T-F	E12	GAACCTCAATCAATGAAGAACTATTAAATG	
B2E12-SDMc.6859A>T-R	E12	CATTATAAGTTTCTTTTGATTGAGGGTTTC	
B2E12-SDMc.6901G>T-F	E12	GGATAATAGAAATCAATAAAATCCTTAAAGGC	
B2E12-SDMc.6901G>T-R	E12	GCCTTTAAGGATTTTATTGATTCTTCTATTATCC	
B2E12-SDMc.6937+1G>A-F	E12/I12	AAGCACTCCAGATGATATAAATTAGCTTT	

table continues

Primer's name ¹	Location	Sequence ²	Use
B2E12-SDMc.6937+1G>A-R	E12/I12	AAAGCTAATTTTATCATCTGGAGTGCTT	Transgene DNA PCR amplification and sequencing
B2I11 BamHI-F	I11	GACCGGATCCTGTAATTGACATTGAAGACTGACTTTAC	
B2I12 MluI-R	I12	GACCACGCGTIGAGTATAAAGAGTCTTGTATTAGGCA	
B2E11-F*	BRCA2	CCAAGTCATGCCACACATTC	Analysis of <i>BRCA2</i> E12 splicing patterns
B2E14-R	E14	ATTCTTGACCAGGTGCGGTA	

¹ F, forward; R, reverse; *, fluorescent primer

² Intronic and exonic sequences are indicated in grey and black, respectively.

The position of the variant is underlined. The double underlined sequences correspond to restriction sites for BamHI and MluI.

³ The forward primers listed were used with the reverse primer pCAS-seq-R and the reverse primers listed were used with the forward primer pCAS-seq-F for mutagenesis.

Table S2. Variants selected in *BRCA2* exon 12 and its flanking intronic regions

Selection criteria	Variant	Predicted amino acid change	Databases ²												
			BRCA-Share	BIC	ClinVar	LOVD	HGMD	COSMIC	dbSNP	gnomAD					
Presumed LoF variants n = 15	IVS±1/2 n = 4	c.6842-1G>T	5		4				4	✓					
		c.6937+1G>A			5				5						
		c.6937+1G>T			3										
		c.6937+2del					DM								
	Nonsense n = 5	c.6844G>T	5												
		c.6859A>T	5												
		c.6898C>T	5												
		c.6901G>T	5								5				
		c.6920C>A	5												
		c.6849del									4				✓
		c.6859_6863del									5		DM		5
	Frameshift n = 6	c.6873del			✓										
		c.6879del									5				5
		c.6896dup									5		DM		5
		c.6899_6906dup									5				5

table continues

Selection criteria	Variant	Predicted amino acid change	Databases ²							
			BRCA-Share	BIC	ClinVar	LOVD	HGMD	COSMIC	dbSNP	gnomAD
Variants predicted as spliceogenic	c.6842-30_6842-3del	p.?		✓	3					3
	c.6842-14A>G	p.?		✓	2					2
	c.6842-12_6842-8del	p.?								
	c.6842-11T>A	p.?		✓	2					3
	c.6842-8_6842-7del	p.?	3							
	c.6842G>A	p.Gly2281Glu								
	c.6842G>T	p.Gly2281Val	3	✓	3		DM?		3	3
	c.6935A>G	p.Asp2312Gly								
	c.6935A>T	p.Asp2312Val	1	✓	c.i.	✓	DM?		c.i.	
	c.6937G>A	p.Gly2313Ser						✓		
n = 24	c.6851C>T	p.Ser2284Leu						✓		
	c.6863A>T	p.Asn2288Ile			3				3	
	c.6875A>C	p.Glu2292Ala								
	c.6875A>T	p.Glu2292Val	3						3	
	c.6879T>A	p.Phe2293Leu					FP			
	c.6880G>A	p.Asp2294Asn	3							
	c.6886A>C	p.Ile2296Leu			3				3	
										✓

table continues

Selection criteria	Variant	Predicted amino acid change	Databases ²							
			BRCA-Share	BIC	ClinVar	LOVD	HGMD	COSMIC	dbSNP	gnomAD
Control ¹	c.6892G>A	p.Glu2298Lys		V	3				3	
	c.6900A>G	p.= (p.Gln2300=)								V
	c.6901G>A	p.Glu2301Lys		V	3				3	
	c.6901G>C	p.Glu2301Gln			3				3	
	c.6918T>C	p.= (p.Ala2306=)			2				2	
	c.6920C>T	p.Ser2307Leu						V		
	c.6925A>T	p.Ser2309Cys						V		
	c.6853A>G	p.Ile2285Val	2	V	1	V	FP		1	V

¹ Variant previously described as spliceogenic

² Variant classification reported in BRCA-Share, ClinVar and dbSNP are indicated: 1, benign; 2, likely benign; 3, uncertain significance; 4, likely pathogenic; 5, pathogenic; c.i., conflicting interpretation = class 1, 2 or 3. For HGMD, the variant classification is indicated: FP, functional polymorphism; DM, disease causing mutation; DM?, disease causing mutation? In other databases: V, variant reported without classification.

Table S3. Overview of bioinformatics predictions and experimental data obtained for the 40 selected *BRCA2* exon 12 variants

Selection criteria	Variant	Bioinformatics predictions	Splicing outcomes			mESC assay	
			LCL (biallelic) ²	Minigene assay (monoallelic)	mESC assay (monoallelic)	Protein expression ³	HDR activity
Presumed LoF variants n = 15	Wild-type		ΔE12 = 11% ; 9% ; 11%	ΔE12 = 0%	ΔE12 = 18%	BRCA2 FL	100%
	c.6842-1G>T	ΔMES 3' ss = -100%		ΔE12 = 100%			
	c.6937+1G>A	ΔMES 5' ss = -100%	ΔE12 = 61%	ΔE12 = 100%	ΔE12 = 100%	BRCA2 ΔE12	60%
	c.6937+1G>T	ΔMES 5' ss = -100%		ΔE12 = 100%			
	c.6937+2del	ΔMES 5' ss = -100%		ΔE12 = 51% ; +E12q(ins40) = 49%			
Nonsense n = 5	c.6844G>T	ΔtESRseq = -1.22	ΔE12 = 57%	ΔE12 = 73%	ΔE12 = 92%	BRCA2 ΔE12	45%
	c.6859A>T	ΔtESRseq = -1.20		ΔE12 = 0%	ΔE12 = 29%	BRCA2 TC (BRCA2 ΔE12)	26%
	c.6898C>T	ΔtESRseq = -2.38		ΔE12 = 12%			
	c.6901G>T	ΔtESRseq = -3.72	ΔE12 = 51% ; 46%	ΔE12 = 72%			
	c.6920C>A	ΔtESRseq = -2.40		ΔE12 = 13%	ΔE12 = 94%	BRCA2 ΔE12	43%
Frameshift n = 6	c.6849del	ΔtESRseq = 0.56		ΔE12 = 0%			
	c.6859_6863del	ΔtESRseq = -2.20		ΔE12 = 0%			
	c.6873del	ΔtESRseq = -1.50		ΔE12 = 0%			
	c.6879del	ΔtESRseq = 0.23		ΔE12 = 0%			
	c.6896dup	ΔtESRseq = -0.59		ΔE12 = 0%			
Variants predicted as spliceogenic	c.6899_6906dup	ΔtESRseq = -1.60		ΔE12 = 0%			
	c.6842-30_6842-3del	ΔMES 3' ss = -52%		ΔE12 = 90%			
	c.6842-14A>G	ΔMES 3' ss = -26%		ΔE12 = 76%			
	c.6842-12_6842-8del	ΔMES 3' ss = -30%		ΔE12 = 88%			
n = 24	ΔMES ≤ -15%	ΔMES 3' ss = -24%		ΔE12 = 21%			
	n = 10						
		ΔMES 3' ss = -27%		ΔE12 = 32%			
		ΔMES 3' ss = -20%	ΔE12 = 25% ; +E12p(del3) = 9%				
		ΔMES 3' ss = -39%	ΔE12 = 100%		ΔE12 = 98%	BRCA2 ΔE12	50%

table continues

Selection criteria	Variant	Bioinformatics predictions	LCL (biallelic) ²	Splicing outcomes		mESC assay	
				Minigene assay (monoallelic)	mESC assay (monoallelic)	Protein expression ³	HDR activity
In silico predictions of ESR alteration	c.6935A>G	AMES 5' ss = -39%	$\Delta E12 = 18\% ; 18\%$	$\Delta E12 = 8\% ; +E12q(\text{del}3) = 92\%$			
	c.6935A>T	AMES 5' ss = -39%					
	c.6937G>A	AMES 5' ss = -100%					
	c.6851C>T	$\Delta tESRseq = -2.23$					
	c.6863A>T	$\Delta tESRseq = -0.82$					
	c.6875A>C	$\Delta tESRseq = -1.22$					
	c.6875A>T	$\Delta tESRseq = -1.32$					
	c.6879T>A	$\Delta tESRseq = -1.41$					
	c.6880G>A	$\Delta tESRseq = -1.01$					
	c.6886A>C	$\Delta tESRseq = -1.40$					
	c.6892G>A	$\Delta tESRseq = -0.98$					
	c.6900A>G	$\Delta tESRseq = -2.44$					
	c.6901G>A	$\Delta tESRseq = -2.44$					
	c.6901G>C	$\Delta tESRseq = -2.17$					
	c.6918T>C	$\Delta tESRseq = -0.74$					
	c.6920C>T	$\Delta tESRseq = -3.16$					
	c.6925A>T	$\Delta tESRseq = -1.06$					
Control ¹	c.6853A>G	$\Delta tESRseq = -0.30$	$\Delta E12 = 28\% ; 29\% ; 28\%$	$\Delta E12 = 16\%$	$\Delta E12 = 64\%$	BRCA2 FL, BRCA2 $\Delta E12$	61%

¹ Variant previously described as spliceogenic

² When available, the data for unrelated controls or patients are indicated.

³ BRCA2 FL, full-length protein; BRCA2 $\Delta E12$, protein deleted for E12; BRCA2 TC, truncated protein (p.Arg2287*). The minor protein isoform is indicated in parenthesis.

Table S4. Clinical and family data of patients carrying *BRCA2* exon 12 spliceogenic variants

Effect on splicing	Variant	Proband	Patient clinical and pathology data	Family data	Co-segregation data ¹	
					Carrier (Y) or non-carrier (N)	Breast cancer (BC), or other cancer (CC, PC) or cancer-free (Cf)
Complete skipping of <i>BRCA2</i> E12	c.6842-?_6937+7del (p.?) c.6842G>T (p.Gly2281Val) + c.9195_9196delinsAT (p.Phe3065_Gln3066delinsLeu*)	P1	BC (67y) (SBR I, RE+, RP+, Her2-)	Sister 1	n.d.	BC (57y)
				Sister 2	n.d.	BC (58y)
				Maternal aunt 1	n.d.	BC (?) y
				Maternal aunt 2	n.d.	BC (80y)
				Mother	n.d.	BC (41y)
		P2	BC (64y) (SBR I, RE+, RP-) OC (70y)	Half maternal uncle	n.d.	BC (60y)
				Brother	N ²	CF (65y)
				Paternal aunt 1	n.d.	BC (56y)
				Paternal aunt 2	n.d.	BC (55y, 62y)
				Paternal aunt 3	n.d.	Bilat. BC (59y)
	c.6937+1G>A (p.?)	P3	BC (60y) (SBR II, RE+, RP-, Her2+)	Paternal aunt 4	n.d.	CC (78y)
				Sister 1	N	CF (66y)
				Sister 2	Y	CF (61y)
				Sister 3	Y	CF (60y)
				Sister 4	Y	CF (55y)
				Sister 5	Y	CF (56y)
				Brother	Y	CF (51y)
				2 daughters	N	CF (41y, 39y)
				Niece 1, daughter of sister 4	Y	CF (38y)
				Niece 2, daughter of sister 4	Y	CF (33y)
	P4		BC (58y) (SBR II, RE+, RP+, Her2-)	Sister	N	BC (63y)
				Mother	n.d.	BC (87y)
				Maternal aunt	n.d.	BC (70y)
				Maternal grandmother	n.d.	BC (69y)

table continues

Effect on splicing	Variant	Proband	Patient clinical and pathology data	Family data	Co-segregation data ¹		
					Carrier (Y) or non-carrier (N)	Breast cancer (BC), or other cancer (CC, PC) or cancer-free (CF)	
Major increase of <i>BRCA2</i> ΔE12	c.6844G>T (p.Glu2282*)	P5	BC (53y)	Son	Y	CF (40y)	
				Daughter	Y	CF (30y)	
				Mother	Y ^o	CF (86y)	
				Maternal aunt 1	Y	BC (69y)	
				Maternal aunt 2	Y ^o	BC (67y), CC	
				Maternal cousin (daughter of maternal aunt 2)	Y	BC (56y)	
	c.6901G>T (p.Glu2301*)	P6	BC (67y) (RE-, RP-, Her2-)	Maternal aunt	N	BC (43y)	
				Paternal great aunt	n.d.	Bilat. BC (30y)	
				Sister	n.d.	BC (65y)	
				Mother	n.d.	BC (50y)	
				Sister	n.d.	BC (45y)	
				Maternal cousin 1 (RE+, RP+, Her2-)	n.d.	BC (77y)	
				Maternal cousin 2	n.d.	BC (53y)	
				Maternal grandmother	n.d.	BC (55-60y)	
				Mother	n.d.	BC (56y, 64y)	
				Maternal uncle 1	n.d.	PC	
				Maternal uncle 2	n.d.	PC	
		P8	BC (61y)	Son	Y	CF (45y)	
				Daughter	Y	CF (33y)	
				Maternal grandmother	n.d.	BC (70y)	
	P9		CF (46y)	Mother	n.d.	BC (35y)	
				Maternal aunt	n.d.	BC (31y)	
				Maternal grandmother	n.d.	BC (55y)	
				Mother	n.d.	BC (44y)	
	P10		BC (47y) (SBR II, RE+, RP+)	Maternal grandmother	n.d.		
				Mother	n.d.		

table continues

Effect on splicing	Variant	Proband	Patient clinical and pathology data	Family data	Co-segregation data ¹	
					Carrier (Y) or non-carrier (N)	Breast cancer (BC), or other cancer (CC, PC) or cancer-free (CF)
				Sister Brother Brother Maternal cousin	N	BC (53y)
					Y	CF (62y)
					N	CF (59y)
					n.d.	BC (55y)
					Carrier (Y) with breast cancer (BC)	
					Carrier (Y) and Cancer-free (CF)	
					Non-carrier (N) with breast cancer (BC)	

CF, cancer-free; BC, Breast Cancer; Bilat., Bilateral; OC, Ovary Cancer; CC, Colon Cancer; PC, Prostate Cancer; Y, carrier of the variant; Y^o, obligate carrier of the variant; N, non-carrier of the variant; N^o, negative for both variants;
¹Co-segregation data relative to women over 45 of age are indicated in bold and in colors as specified on the right.

