

Integrated RNA-based functional characterization facilitates reclassification of BRCA1/2 variants of uncertain significance in a clinical cancer cohort

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Abstract

Background:

Variants of uncertain significance in *BRCA1* and *BRCA2* remain a major challenge in hereditary breast and ovarian cancer genetic testing, particularly when their effects on pre-mRNA splicing are unclear. Computational prediction can facilitate the identification of potentially splice-altering variants but may yield discordant results and cannot fully resolve their clinical significance. This study aimed to characterize the splicing consequences of selected *BRCA1/2* variants and evaluate the contribution of functional RNA evidence to variant interpretation.

Results:

Sixty unique *BRCA1/2* variants of uncertain significance identified among 1,762 patients with cancer were evaluated for splice-altering potential, and five candidate variants were selected for functional analysis. Minigene assays identified two splice-altering *BRCA2* variants, c.476-3C > A and c.8332-13T > G, whereas *BRCA2* c.632 – 18_632-17del and c.7435 + 20G > A and *BRCA1* c.4676-6T > C predominantly retained normal splicing. The c.476-3C > A variant predominantly induced exon skipping. In contrast, c.8332-13T > G generated a more complex splice pattern comprising exon skipping and activation of two cryptic 3' splice acceptor sites. Exon skipping caused by c.8332-13T > G resulted in the in-frame deletion p.(Ile2778_Gln2829del). RNA sequencing further characterized these aberrant splice-junction patterns and confirmed the complex transcript profiles observed in the minigene assays. Structural analysis and molecular dynamics simulations were subsequently used to characterize the native *BRCA2*–DSS1 structural environment surrounding the region deleted in p.(Ile2778_Gln2829del). Familial segregation provided additional supportive evidence for the c.8332-13T > G variant. Integration of functional RNA findings with predicted protein consequences and familial evidence supported classification of c.476-3C > A and c.8332-13T > G as likely pathogenic and pathogenic, respectively, while the three splice-neutral variants were classified as likely benign.

Conclusions:

Experimental RNA analysis can resolve *BRCA1/2* variants that remain difficult to interpret from sequence information alone. In this study, combined minigene analysis, transcript quantification, and RNA sequencing revealed complex variant-specific splice patterns and provided functional evidence supporting reclassification of two *BRCA2* variants as pathogenic and three splice-neutral variants as likely benign. These findings also demonstrate that disruption of normal splice-site recognition can redistribute usage among competing splice sites and generate multiple transcripts with distinct molecular consequences. Integrating RNA-based functional evidence with predicted protein consequences and familial data may therefore improve the clinical interpretation of selected *BRCA1/2* variants of uncertain significance.

Introduction

Germline pathogenic variants in *BRCA1* and *BRCA2* are among the most well-established genetic determinants of hereditary cancer predisposition, particularly hereditary breast and ovarian cancer (HBOC) syndrome^[1, 2]. The *BRCA1* and *BRCA2* proteins play essential roles in maintaining genomic stability through homologous recombination-mediated repair of DNA double-strand breaks, and loss-of-function alterations in these genes compromise DNA damage repair and increase susceptibility to tumor development^[3, 4]. Individuals carrying pathogenic *BRCA1/2* variants have substantially elevated lifetime risks of breast, ovarian, prostate, and other cancers^[1]. With the widespread implementation of next-generation sequencing (NGS) in hereditary cancer testing, germline *BRCA1/2* analysis has become an important component of cancer risk assessment, genetic counseling, surveillance strategies, and therapeutic decision-making^[5], including the selection of targeted treatments such as poly(ADP-ribose) polymerase (PARP) inhibitors^[6]. Therefore, accurate interpretation of *BRCA1/2* genetic variants is critical for translating genomic information into clinically actionable outcomes.

Despite the clinical importance of *BRCA1/2* genetic testing, the interpretation of many identified variants remains challenging. The increasing application of NGS has resulted in the identification of a large number of rare *BRCA1/2* variants, among which a

considerable proportion are classified as variants of uncertain significance (VUS)^[7] according to the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) guidelines^[8]. VUS represent genetic alterations for which available evidence is insufficient to determine whether they impair gene function or contribute to disease risk, thereby limiting their clinical utility. Unlike pathogenic or benign variants, VUS cannot be directly used to guide cancer risk management, surveillance strategies, or therapeutic decisions^[9]. Therefore, generating reliable functional evidence to clarify the clinical significance of VUS remains a critical unmet need in hereditary cancer genomics.

Although computational prediction tools and population databases have greatly facilitated variant interpretation, the clinical significance of many *BRCA1/2* VUS remains unresolved^[10, 11]. This challenge is particularly evident for variants whose functional consequences cannot be directly inferred from DNA sequence information, including non-canonical splice-region variants and variants that may influence RNA processing^[12]. Functional characterization of such variants is therefore essential to bridge the gap between genomic identification and clinical interpretation^[13].

Aberrant pre-mRNA splicing represents an important mechanism through which genetic variants can disrupt gene function and contribute to disease development^[14]. Although variants affecting canonical splice sites are generally recognized as splice-disrupting alterations, variants located outside classical splice regions, including deep intronic, synonymous, and some exonic variants, may also alter RNA processing through activation of cryptic splice sites^[15], exon skipping^[16], intron retention^[17], or generation of abnormal transcript isoforms. These splicing abnormalities may result in altered protein sequences, loss of functional domains, or impaired protein activity^[18]. In *BRCA1* and *BRCA2*, accumulating evidence indicates that splice defects contribute to the pathogenicity of a subset of variants^[19]; however, the RNA-level consequences of many VUS remain difficult to determine because splicing outcomes cannot always be accurately predicted from genomic sequence alone^[20]. In particular, disruption of canonical splice-site recognition may alter the use of nearby cryptic sites and generate multiple transcript isoforms, making the molecular consequences of a variant more complex than a simple prediction of splice-site loss or gain. Experimental characterization is therefore required not only to establish whether a variant alters splicing, but also to determine which splice sites are used and what transcripts are consequently produced.

Recent advances in computational approaches, including splice prediction algorithms such as SpliceAI, have improved the prioritization of variants with potential effects on RNA processing^[21]. However, these *in silico* predictions provide probability-based assessments and cannot fully capture the complexity of transcript alterations. Variants predicted to affect splicing may generate diverse molecular consequences, including cryptic splice-site activation, multiple abnormal transcript isoforms, or subtle changes in transcript usage, which require experimental validation for accurate interpretation^[22, 23]. Minigene reporter assays enable direct assessment of variant-induced splicing alterations, whereas RNA sequencing allows more comprehensive characterization of aberrant splice junctions and transcript isoforms. In addition, structural characterization of splice-derived protein alterations may extend RNA-level findings by providing insight into their potential consequences for protein architecture and molecular interactions. Thus, an integrated strategy combining computational prediction, RNA-based functional assays, and structural analysis may facilitate a more comprehensive interpretation of *BRCA1/2* VUS.

In this study, we systematically investigated the functional consequences of *BRCA1/2* VUS identified in a clinical cancer cohort by integrating computational predictions, RNA-based functional assays, and structural analyses. We first characterized the spectrum of clinically identified *BRCA1/2* VUS and evaluated their potential effects on pre-mRNA splicing using multiple computational prediction tools. Selected candidate variants were subsequently examined using minigene assays, and splice-altering variants were further characterized by RNA sequencing to define their aberrant transcript patterns. Structural modeling and molecular dynamics simulations were further used to investigate the structural context and potential functional consequences of a splice-derived *BRCA2* protein alteration. Finally, functional findings were integrated with familial evidence to support clinical variant interpretation and reclassification. By resolving both splice-site usage and the resulting transcript profiles, we sought to determine how RNA-based functional evidence could improve the interpretation of *BRCA1/2* VUS that remain inconclusive from sequence information alone.

Materials and Methods

Study cohort and identification of *BRCA1/2* VUS

A total of 1,762 individuals undergoing germline *BRCA1/2* testing were enrolled in this study. Peripheral blood samples were collected after genetic counseling and written informed consent. Germline *BRCA1/2* sequencing was performed using the AmoyDx *BRCA1/2* Kit on an Illumina MiSeq platform, covering all coding exons and flanking intronic regions. Single-nucleotide variants and small insertions/deletions were identified using the corresponding analysis pipeline, and suspected large genomic rearrangements were further evaluated by multiplex ligation-dependent probe amplification (MLPA). Variants classified as variants of uncertain significance (VUS) according to the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) framework were retrieved for subsequent analysis. Clinical information was obtained from electronic medical records, and additional family history was collected by telephone follow-up. The study was approved by the Ethics Committee of Zhongshan Hospital, Fudan University (B2023-042), and was conducted in accordance with the Declaration of Helsinki.

Variant Nomenclature

All *BRCA1* and *BRCA2* variants and their corresponding transcript- and protein-level consequences were described according to the Human Genome Variation Society (HGVS) nomenclature recommendations. Reference sequences NM_007294.4 and NP_009225.1 for *BRCA1*, and NM_000059.4 and NP_000050.3 for *BRCA2* were used throughout the study. Variant descriptions were verified using Mutalyzer 3^[24].

In silico splice prediction

All identified *BRCA1* and *BRCA2* VUS were subjected to *in silico* analysis to evaluate their potential effects on pre-mRNA splicing. Variants were annotated according to their genomic location and predicted molecular consequence. Spliceogenic effects were assessed using four complementary prediction algorithms: Human Splicing Finder (HSF)^[25], MaxEntScan (MES)^[26], NNSplice^[27], and SpliceAI^[21]. For HSF, MES, and NNSplice, predicted splice-site strengths were compared between wild-type and variant sequences to evaluate disruption of native splice sites or activation of nearby cryptic splice sites. SpliceAI delta scores for donor gain, donor loss, acceptor gain, and acceptor loss were used to estimate the probability of variant-induced splicing alterations.

Variants showing evidence of a potential splice-altering effect were prioritized for experimental validation using minigene assays. Candidate selection was based on the overall evidence from computational predictions, with particular consideration of variants showing consistent splice-altering predictions across multiple algorithms.

Minigene Construction

Wild-type minigene constructs containing the corresponding *BRCA1* or *BRCA2* genomic regions were generated by cloning the target exons together with flanking intronic sequences into the pTB splicing reporter vector. For each candidate variant, genomic fragments encompassing the affected exon(s) and approximately 300–500 bp of upstream and downstream intronic sequences were amplified and cloned into the pTB vector via homologous recombination (BGI, Shenzhen, China).

Variant-containing constructs were generated from the corresponding wild-type plasmids using the same homologous recombination strategy. All wild-type and mutant constructs were verified by Sanger sequencing before functional analysis.

In vitro Minigene Splicing Assays

HEK293T cells were seeded in 6-well plates at a density of 2×10^5 cells per well 24 h before transfection. Wild-type and mutant minigene constructs were transiently transfected using linear polyethylenimine (PEI; Yeasen, China) following the manufacturer's instructions. At 6 h post-transfection, the medium was replaced with fresh DMEM supplemented with 10% fetal bovine serum (FBS; Gibco). Total RNA was extracted 24 h post-transfection using TRIzol reagent followed by phenol–chloroform purification. cDNA was synthesized using Hifair® III 1st Strand cDNA Synthesis SuperMix for qPCR (Yeasten, China). Splicing products were amplified using vector-specific primers, separated by agarose gel electrophoresis, and further quantified by fluorescent fragment analysis using denaturing capillary electrophoresis.

RNA Sequencing Analyses

Total RNA extracted from transfected HEK293T cells was assessed for RNA integrity using an Agilent 2100 Bioanalyzer, and samples with an RNA integrity number (RIN) > 6.8 and an OD260/280 ratio > 2.0 were used for library preparation. Sequencing libraries were prepared according to the manufacturer's standard protocol and subjected to paired-end 150-bp sequencing on DNBSEQ-T7 platform (Novogene, Beijing, China). Raw sequencing reads were subjected to quality filtering and adapter trimming and subsequently aligned using STAR in two-pass mode to a custom reference comprising the human reference genome GRCh37/hg19 and the corresponding pTB minigene sequences. This strategy allowed minigene-derived transcripts to be distinguished from endogenous *BRCA2* transcripts. Alignment files were processed using SAMtools and Picard to generate indexed BAM files for downstream splice-junction analysis. Reads mapping to the transfected minigene constructs and spanning the targeted *BRCA2* regions or their associated splice junctions were extracted for transcript-level characterization. Aberrant splice junctions and alternative transcript structures were identified based on split-read evidence and visualized using Integrative Genomics Viewer (IGV, v2.8.0) and sashimi plots. RNA sequencing was used to characterize variant-associated cryptic splice-site usage, exon skipping, and alternative transcript isoforms identified by the minigene assays.

Molecular Modeling Analysis

The AlphaFold3-predicted wild-type *BRCA2*–*DSS1* complex served as the starting structure for molecular dynamics (MD) simulations^[28]. Because the splice-derived *BRCA2* p.(Ile2778_Gln2829del) alteration resulted in extensive disruption of the predicted local structure and did not yield a sufficiently reliable mutant complex for comparative simulation, MD analysis was therefore restricted to the wild-type *BRCA2*–*DSS1* complex to characterize the native structural environment associated with the deleted region. All-atom MD simulations were performed using GROMACS v2022.03^[29] with the AMBER99SB-ILDN force field^[30]. The complex was solvated in a periodic box using the TIP3P water model^[31], maintaining a minimum distance of 1.2 nm between the protein and the box boundary, and Na⁺ ions were added to neutralize the system. Energy minimization was performed using the steepest-descent algorithm. Short-range electrostatic and van der Waals interactions were calculated with a cutoff of 1.0 nm, long-range electrostatic interactions were treated using the particle mesh Ewald method^[32], and bonds involving hydrogen atoms were constrained using the LINCS algorithm^[33]. The system was sequentially equilibrated for 100 ps under the NVT ensemble and 100 ps under the NPT ensemble at 300 K and 1 bar, followed by a 200-ns production simulation with a 2-fs integration time step. Trajectory frames were recorded every 10 ps.

Trajectory analyses included backbone root mean square deviation (RMSD), residue-level root mean square fluctuation (RMSF), radius of gyration (Rg), solvent-accessible surface area (SASA), and intermolecular hydrogen bonds between *BRCA2* and *DSS1*. Hydrogen bonds were defined using a donor–acceptor distance of < 3.5 Å and a donor–hydrogen–acceptor angle of > 120°. Gibbs free-energy landscapes were constructed using RMSD and Rg as reaction coordinates to characterize the predominant conformational states sampled during the simulation. Binding energetics were further estimated using the molecular mechanics/Poisson–Boltzmann surface area (MM/PBSA) method implemented in gmx_MMPBSA^[34, 35], using representative frames extracted from the equilibrated portion of the trajectory. The calculated binding free energy (ΔG_{bind}) was decomposed into van der Waals, electrostatic, polar solvation, and nonpolar solvation contributions.

Reclassification of *BRCA1/2* Variants

BRCA1/2 variants were reclassified according to the recommendations of the ClinGen Sequence Variant Interpretation (SVI) Splicing Subgroup^[36]. Experimentally observed abnormal splicing was evaluated using PVS1_Strength (RNA), with the applicable evidence strength determined by the observed transcript profile, the relative abundance of normally and aberrantly spliced transcripts, and the predicted protein consequence of the resulting transcript(s). For appropriate intronic or synonymous variants, BP7_Strong (RNA) was applied when experimental analysis demonstrated normal splicing. Familial segregation evidence was incorporated using PP1 at the corresponding evidence strength, where applicable. ACMG/AMP classifications were established both before and after incorporation of functional RNA evidence together with available segregation evidence, to evaluate their contribution to variant reclassification.

Results

Landscape of *BRCA1/2* variants of uncertain significance and their predicted spliceogenic potential

A total of 60 unique *BRCA1/2* variants of uncertain significance (VUS) identified from 1,762 cancer patients were included in this study (Fig. 1A). Among these variants, 35 were located in *BRCA2* and 25 in *BRCA1* (Fig. 1B). Missense variants represented the predominant category (43/60, 71.7%), followed by intronic variants (6/60, 10.0%) and UTR/flanking region variants (4/60, 6.7%) (Fig. 1C). SpliceAI analysis showed that most variants had low predicted splice-altering potential, with 56 variants exhibiting Δ scores < 0.20 , whereas only four variants showed moderate-to-high splice prediction scores (Fig. 1D). When stratified by variant category, higher SpliceAI scores were mainly observed among intronic variants, while most coding variants showed limited predicted effects on splicing (Fig. 1E). Among the six intronic VUS, five variants located in splice-relevant intronic regions were selected for functional evaluation; the remaining *BRCA1* c.-19-7G > A variant, located in the non-coding 5' region, was not included in the minigene analysis. The five variants examined were *BRCA2* c.632 – 18_632-17del, c.7435 + 20G > A, c.8332-13T > G, and c.476-3C > A, and *BRCA1* c.4676-6T > C. Their potential effects on pre-mRNA splicing were further evaluated using SpliceAI, HSF, MaxEntScan, and NNSPLICE (Table 1). The predicted effects varied considerably among algorithms. *BRCA2* c.8332-13T > G showed the most prominent predicted splice-altering effect, with SpliceAI scores of 0.89 for acceptor loss and 0.49 for donor loss, together with reduced native splice-site scores predicted by HSF, MaxEntScan, and NNSPLICE. *BRCA2* c.476-3C > A was predicted to weaken the native splice site, most prominently by MaxEntScan. Notably, *BRCA2* c.7435 + 20G > A had negligible SpliceAI scores but was predicted by HSF to create a de novo splice site, illustrating discordance between prediction algorithms. In contrast, *BRCA2* c.632 – 18_632-17del and *BRCA1* c.4676-6T > C showed relatively limited predicted effects on native splice-site strength across most algorithms. The variability and discordance among computational predictions underscored the need for direct experimental assessment of the splicing consequences of these variants.

Table 1 <i>In silico</i> prediction of splice-altering effects of selected <i>BRCA1/2</i> variants														
Variant	Splice AI				Splice Site	HSF			MaxEnt			NNSPLICE		
	DL	DG	AL	AG		N	V	%	N	V	%	N	V	%
<i>BRCA2</i> : c.632 – 18_632-17del	0	0.01	0.07	0	c.632	90.71	90.71	0	7.91	7.91	0	0.99	0.98	1.01
<i>BRCA2</i> : c.7435 + 20G > A	0	0	0	0	c.7435	79.41	79.41	0	5.64	5.64	0	0.91	0.91	0
					c.7435 + 17	0	80.56	De novo	0	0	0	0	0	0
<i>BRCA2</i> : c.8332-13T > G	0.49	0	0.89	0.17	c.8332	85.17	73.58	13.61	4.26	0.54	87.32	0.89	0.68	23.60
<i>BRCA2</i> : c.476-3C > A	0.31	0	0.26	0.05	c.476	87.38	87.38	0	10.49	6.02	42.6	0.91	0.84	7.69
<i>BRCA1</i> : c.4676-6T > C	0.01	0	0.06	0.50	c.4676	87.59	87.59	0	10.2	10.2	0	0.72	0.72	0
Abbreviations:														

Functional validation identifies splice-altering *BRCA1/2* variants by minigene assays

To experimentally evaluate the splice-altering effects of the selected *BRCA1/2* variants, genomic fragments containing the target exons and flanking intronic sequences were cloned into the pTB minigene reporter vector and analyzed in HEK293T cells (Fig. 2A). Five candidate variants, including *BRCA1* c.4676-6T > C and *BRCA2* c.476-3C > A, c.632 – 18_632-17del, c.8332-13T > G, and c.7435 + 20G > A, were examined by RT-PCR (Fig. 2B). Among these variants, *BRCA2* c.476-3C > A and c.8332-13T > G generated distinct

aberrant transcript patterns compared with their corresponding wild-type constructs, whereas *BRCA1* c.4676-6T > C, *BRCA2* c.632 – 18_632-17del, and c.7435 + 20G > A showed no apparent splice alterations.

Capillary electrophoresis further resolved and quantified the transcript isoforms generated by the two splice-altering variants (Fig. 2C–F), and the identities of the aberrant transcripts were confirmed by Sanger sequencing. *BRCA2* c.476-3C > A predominantly induced exon skipping, along with an additional transcript containing a 50-bp intronic insertion spanning c.476 – 141 to c.476 – 92 (Fig. 2C and 2E). *BRCA2* c.8332-13T > G produced a more complex splice pattern, comprising exon skipping and two additional transcripts generated through activation of cryptic 3' splice acceptor sites at c.8397 and c.8430 (Fig. 2D and 2E). The exon-skipping transcript generated by c.8332-13T > G resulted in an in-frame deletion of 52 amino acids, p.(Ile2778_Gln2829del). Quantification by capillary electrophoresis demonstrated substantial shifts from the normal transcript toward aberrant isoforms for both variants, whereas the other three variants predominantly retained normal splicing (Fig. 2F). These results identified c.476-3C > A and c.8332-13T > G as splice-altering *BRCA2* variants, with each variant producing a distinct pattern of aberrant splice-site usage.

RNA sequencing resolves complex aberrant transcript patterns induced by *BRCA2* splice-altering variants

To further characterize the transcript patterns generated by the two splice-altering *BRCA2* variants, RNA sequencing was performed on wild-type and variant minigene constructs. Sashimi plot analysis revealed distinct alterations in splice-junction usage for both variants, consistent with the transcript patterns identified by RT-PCR and capillary electrophoresis (Fig. 3A–D). For *BRCA2* c.476-3C > A, RNA sequencing detected aberrant splice junctions predominantly corresponding to exon skipping, together with additional junctions associated with intronic sequence inclusion, whereas the wild-type construct predominantly generated the canonical transcript (Fig. 3A and C). These findings demonstrated that c.476-3C > A alters splice-site selection and generates multiple aberrant transcript isoforms.

Similarly, *BRCA2* c.8332-13T > G produced a complex pattern of alternative splice-junction usage, comprising exon skipping and activation of two cryptic 3' splice acceptor sites at c.8397 and c.8430 (Fig. 3B and D). These junctions corresponded to the aberrant transcript isoforms identified by minigene and capillary electrophoresis analyses. RNA sequencing therefore provided junction-level characterization consistent with the splice patterns identified by RT-PCR and capillary electrophoresis, while further resolving the heterogeneous transcript architectures generated by alternative splice-site usage.

Structural and dynamic characterization of the *BRCA2*–*DSS1* complex

Following identification of the splice-derived *BRCA2* p.(Ile2778_Gln2829del) alteration, we examined the structural context of the affected region within the *BRCA2*–*DSS1* complex. Because the deletion substantially altered local folding and precluded reliable modeling of the mutant complex, molecular dynamics simulations were performed on the wild-type *BRCA2*–*DSS1* complex to characterize the native interaction environment. During the 200-ns simulation, the complex underwent an initial conformational adjustment followed by stabilization, with the backbone RMSD reaching a plateau during the later phase of the trajectory (Fig. 4A). The radius of gyration decreased from its initial value and subsequently remained relatively stable (Fig. 4B), while SASA showed an overall decline during the latter part of the simulation (Fig. 4C), together indicating a more compact equilibrated conformation.

Residue-level RMSF analysis showed relatively limited fluctuations across the modeled *BRCA2* region, whereas *DSS1* displayed greater flexibility, particularly at its terminal regions (Fig. 4D–E). Intermolecular hydrogen bonds between *BRCA2* and *DSS1* were maintained throughout the simulation, although their number fluctuated dynamically over time (Fig. 4F). Gibbs free-energy landscape analysis identified a predominant low-energy conformational basin corresponding to the equilibrated region of the trajectory (Fig. 4G–H). MM/PBSA analysis yielded a favorable binding free energy ($\Delta G_{\text{bind}} = -56.17$ kcal/mol), with substantial favorable electrostatic and van der Waals contributions partially offset by polar solvation energy (Fig. 4I). Collectively, these results support a dynamically stable native *BRCA2*–*DSS1* interaction and provide structural context for the potential functional relevance of the splice-derived deletion.

Integrated RNA and familial evidence refines the classification of selected *BRCA1/2* variants

Cascade genetic testing was performed in available family members of the two splice-altering *BRCA2* variant carriers. *BRCA2* c.8332-13T > G was detected in affected maternal relatives of the proband, although an unaffected carrier was also identified,

indicating supportive but incomplete co-segregation (Fig. 5A). *BRCA2* c.476-3C > A was identified in the proband with ovarian cancer, as well as in two affected and one unaffected relative, also indicating incomplete co-segregation (Fig. 5B).

Integration of the experimental and familial findings further refined the interpretation of these variants (Fig. 5C and Table 2). *BRCA2* c.8332-13T > G generated a complex aberrant splice pattern comprising exon skipping and activation of two cryptic 3' splice acceptor sites. The exon-skipping transcript resulted in the in-frame deletion p.(Ile2778_Gln2829del), and structural analysis characterized the native *BRCA2*–*DSS1* environment surrounding the deleted region. Familial segregation provided additional supportive evidence. *BRCA2* c.476-3C > A predominantly induced exon skipping, together with an additional transcript containing a 50-bp intronic insertion, with the aberrant transcripts predicted to disrupt the normal reading frame. Incorporation of the RNA functional evidence together with available familial evidence supported reclassification of *BRCA2* c.8332-13T > G as pathogenic and *BRCA2* c.476-3C > A as likely pathogenic. In contrast, *BRCA2* c.632 – 18_632-17del, c.7435 + 20G > A, and *BRCA1* c.4676-6T > C predominantly retained normal splicing, providing functional RNA evidence against a detectable splice-disrupting effect in the minigene assay and supporting their classification as likely benign (Table 2).

Table 2
ACMG/AMP classification of selected *BRCA1/2* variants before and after incorporation of functional RNA evidence

HGVS	Before functional RNA analysis		Splicing assay	Predicted protein consequence	After functional RNA analysis	
	ACMG/AMP classification	ACMG/AMP criteria			ACMG/AMP classification	ACMG/AMP criteria
<i>BRCA2</i> : c.632 – 18_632-17del	LB	PM2, BP6, BP7	Normal	No predicted protein consequence	LB	PM2, BP6, BP7, BP7_Strong(RNA)
<i>BRCA2</i> : c.7435 + 20G > A	LB	PM2, BP6, BP7	Normal	No predicted protein consequence	LB	PM2, BP6, BP7, BP7_Strong(RNA)
<i>BRCA2</i> : c.8332-13T > G	LP	PS4, PM2	Abnormal	p.(Ile2778_Gln2829del)	P	PS4, PM2, PVS1_Moderate(RNA), PP1_Moderate
<i>BRCA2</i> : c.476-3C > A	VUS	PM2, PP3	Abnormal	p.(Val159GlyfsTer10)	LP	PM2, PVS1_Strong(RNA), BS4
<i>BRCA1</i> : c.4676-6T > C	VUS	PM2, BP4	Normal	No predicted protein consequence	LB	PM2, BP4, BP7_Strong(RNA)
Variants were initially classified according to ACMG/AMP criteria without consideration of the functional RNA assay results. Splicing outcomes were subsequently incorporated into variant interpretation according to ClinGen Sequence Variant Interpretation Splicing Subgroup and <i>BRCA1/2</i> -specific recommendations. Experimentally demonstrated aberrant splicing was evaluated using PVS1(RNA) at an evidence strength determined by the predicted consequence of the resulting transcript, whereas experimentally demonstrated normal splicing was incorporated as BP7_Strong(RNA), where applicable. Familial segregation evidence was incorporated using PP1 at the corresponding evidence strength. Predicted protein consequences were derived from the experimentally observed transcript alterations. P, pathogenic; LP, likely pathogenic; VUS, variant of uncertain significance; LB, likely benign.						

Discussion

Germline *BRCA1/2* testing is increasingly used in cancer risk assessment and treatment decision-making, but variants of uncertain significance (VUS) remain a major obstacle to the clinical interpretation of genetic testing results^[37]. In this study, 60 unique *BRCA1/2* VUS were identified among 1,762 patients undergoing germline testing, and five variants with potential splice-altering effects were selected for functional analysis. Two *BRCA2* variants, c.476-3C > A and c.8332-13T > G, produced complex aberrant

transcripts involving alternative splice-site usage, while the other three variants showed splicing patterns comparable to their wild-type constructs. Capillary electrophoresis and RNA sequencing further resolved the composition and relative abundance of these transcripts. Integration of the RNA findings with other available evidence supported reclassification of all five variants. Our findings further indicate that interpretation of intronic variants requires consideration not only of whether normal splicing is disrupted, but also of which alternative splice sites are used and the relative abundance of each resulting transcripts.

The comparison between computational predictions and experimental results also showed that predicting whether a variant affects splicing is not equivalent to predicting the transcript that will ultimately be produced^[38, 39]. Current algorithms are useful for identifying changes in canonical splice-site strength or the emergence of potential cryptic sites, but several competing sites may coexist within the same local sequence^[36, 40]. This was evident for both splice-altering *BRCA2* variants in our study. *BRCA2* c.476-3C > A predominantly induced exon skipping, while also promoting the use of a cryptic intronic splice site that resulted in inclusion of a 50-bp intronic sequence. An even more complex pattern was observed for c.8332-13T > G, which induced exon skipping together with activation of two nearby cryptic 3' splice acceptor sites, generating multiple aberrant transcripts. These observations suggest that the consequence of a splice-region variant is determined not simply by weakening or strengthening a single site, but by changes in competition among the native site and surrounding potential splice sites^[41]. Thus, evaluation of an intronic variant should consider not only its effect on the canonical splice site, but also the strength and position of nearby potential donor or acceptor sites^[42]. Computational prediction therefore remains useful for selecting variants for functional testing and formulating hypotheses about possible splice-site usage, but the actual pattern and relative use of these sites need to be established experimentally. This distinction is also consistent with current ClinGen recommendations, which separate computational evidence of spliceogenicity from experimentally observed RNA consequences^[36].

Minigene assays were used to determine whether the predicted splice effects could be reproduced experimentally^[15, 43]. This approach is particularly useful when patient-derived RNA is unavailable or unsuitable for analysis and has been applied extensively to *BRCA1/2* splice variants^[20, 44]. In our study, HEK293T cells provided a reproducible system in which the wild-type and variant constructs could be examined under the same experimental conditions. The clear differences between wild-type and mutant constructs for c.476-3C > A and c.8332-13T > G support a direct effect of these variants on splice-site selection. However, minigene assays do not fully reproduce endogenous *BRCA1/2* splicing. Pre-mRNA splicing is influenced by the cellular repertoire of RNA-binding proteins and splicing factors, and HEK293T cells may not reproduce the regulatory environment of breast or ovarian tissues^[45]. In addition, the pTB constructs contain only the target exon and limited flanking intronic sequences and therefore lack long-range regulatory elements and the native chromatin context that may influence exon recognition. These limitations are particularly relevant when several splice sites compete for recognition, because the relative abundance of individual isoforms may be more sensitive to cellular and sequence context than the mere presence or absence of the splice defect itself^[46]. We therefore considered the minigene assay primarily as evidence that a variant alters splicing, rather than as a precise quantitative recapitulation of endogenous *BRCA1/2* transcripts proportions.

The presence of multiple abnormal products also raises an important issue in the interpretation of splice-altering variants. A single variant does not necessarily generate a single aberrant transcript, particularly when several alternative splice sites become available after normal splice-site recognition is disturbed^[47]. In our study, capillary electrophoresis quantified the relative abundance of individual products, whereas RNA sequencing resolved the corresponding splice junctions and transcript structures. This was particularly informative for c.8332-13T > G, for which several transcript isoforms resulted from alternative use of two cryptic 3' splice sites. The combination of these approaches was therefore useful for distinguishing transcript heterogeneity that could not be adequately resolved by gel electrophoresis alone. Products of similar size may represent different splice events, while minor isoforms may be difficult to resolve on conventional gels. Previous *BRCA1/2* minigene studies have likewise identified multiple transcripts arising from the same variant through exon skipping, intron retention, and alternative splice-site usage^[12, 44, 48]. Importantly, defining transcript composition is relevant not only to describing the molecular consequence of a variant but also to interpreting RNA evidence, because partial splice defects retaining normally spliced transcript cannot necessarily be treated in the same manner as complete loss of normal splicing^[49].

The protein consequences of aberrant splicing also need to be considered when interpreting variants that generate in-frame transcripts^[17]. Unlike splice events that introduce a frameshift and premature termination codon, an in-frame deletion may preserve

protein translation but still remove residues required for the structure or function of an important domain^[50]. This is relevant to *BRCA2* c.8332-13T > G, which generated an aberrant transcript predicted to result in a 52-amino-acid deletion, p.(Ile2778_Gln2829del), within the *BRCA2* DNA-binding domain. The C-terminal DNA-binding domain of *BRCA2* contains a helical domain and three oligonucleotide-binding folds, with the Tower domain extending from *OB2*, and forms a structurally organized region involved in DNA binding and interaction with *DSS1* during homologous recombination^[51]. Structural mapping placed the deleted segment within the *OB2*–Tower region and close to the *DSS1*-associated structural environment. The wild-type *BRCA2*–*DSS1* complex remained stable during the 200-ns simulation, supporting the structural integrity of this region within the modeled native complex. Although these analyses do not directly demonstrate that p.(Ile2778_Gln2829del) disrupts *BRCA2*–*DSS1* binding, the location and extent of the deletion argue against interpreting the transcript as functionally preserved based solely on the maintenance of the reading frame. Collectively, these structural analyses do not directly demonstrate that p.(Ile2778_Gln2829del) abrogates *BRCA2* function, but they firmly establish that the deleted segment resides within a structurally ordered and dynamically stable region of the *BRCA2*–*DSS1* interface. Nonetheless, functional studies of the mutant protein will be required to determine whether this deletion affects *DSS1* interaction, DNA binding, or homologous recombination activity.

The identification of defined cryptic splice sites also raises the possibility that some intronic *BRCA1/2* variants may be amenable to RNA-level splice correction^[52]. Because the primary defect lies in aberrant splice-site selection rather than in alteration of the genomic coding sequence itself, these variants provide sequence-defined targets for splice modulation^[53]. Antisense oligonucleotides (ASOs) can mask aberrantly activated splice sites or nearby regulatory elements and redirect splice-site usage toward the normal transcript^[54]. This strategy has also been explored for *BRCA2*: a deep-intronic *BRCA2* variant, c.6937 + 594T > G, was previously shown to activate a cryptic exon, and ASOs targeting the aberrant splice sites prevented cryptic exon inclusion in a minigene system^[52]. The cryptic sites identified for c.476-3C > A and c.8332-13T > G therefore represent potential targets for a similar strategy. Such correction remains to be tested experimentally, and restoration of normal splicing would need to be accompanied by recovery of protein function before any therapeutic implication could be established. Nevertheless, precise definition of variant-induced splice junctions may have value beyond classification by revealing molecular targets for future splice-correction studies^[55].

The RNA results had direct consequences for variant classification, but their interpretation depended on both the type and extent of the observed splice defect. For c.476-3C > A and c.8332-13T > G, the abnormal transcripts provided experimental evidence of altered *BRCA2* pre-mRNA processing, while predicted protein consequences and available familial data provided additional information for classification. Interpretation becomes more difficult when several transcripts coexist or when a proportion of normally spliced transcript is retained. Current ClinGen SVI recommendations allow RNA evidence to be incorporated through PVS1_Strength(RNA) when experimentally observed transcripts are expected to result in loss of function, but the appropriate evidence strength depends on the resulting transcript profile and its predicted protein consequences^[36]. Multiple transcripts, partial splice defects, and retention of functional transcript therefore require more cautious interpretation than complete loss of normal splicing. Conversely, experimental demonstration of normal splicing can provide benign RNA evidence for appropriate variants through BP7_Strong(RNA). In our study, incorporation of the RNA findings together with other available evidence supported classification of c.476-3C > A and c.8332-13T > G as likely pathogenic and pathogenic, respectively, whereas the three splice-neutral variants as likely benign. Thus, RNA analysis contributed in both directions: abnormal splicing provided evidence toward pathogenicity, whereas a well-controlled negative result helped exclude the predicted splice-disrupting mechanism for the remaining variants.

Several limitations should be considered when interpreting our findings. First, the splicing assays were performed using minigene constructs in HEK293T cells rather than patient-derived RNA. Although this system allows the effect of an individual variant to be examined under controlled conditions, it does not reproduce the complete genomic context or tissue-specific regulation of endogenous *BRCA1/2* transcripts. Accordingly, the relative abundance of individual isoforms observed here should not be assumed to represent their proportions *in vivo*. Second, only five candidate variants were functionally examined, and the present data are not sufficient to directly compare the diagnostic performance of the different splice prediction algorithms. Third, structural analysis of p.(Ile2778_Gln2829del) was based primarily on mapping the deleted region onto the wild-type *BRCA2*–*DSS1* complex. Because a sufficiently reliable mutant complex could not be obtained for comparative simulation, the structural findings provide biological context for the deletion rather than direct evidence of impaired *BRCA2* function. Finally, familial data were available for only a

subset of carriers, limiting the contribution of segregation analysis to variant classification. Patient-derived RNA and direct assays of *BRCA2* function, particularly homologous recombination activity, would provide important additional evidence for the two splice-altering variants.

In summary, our study shows that experimental analysis of RNA splicing can resolve a subset of *BRCA1/2* VUS that remain difficult to interpret from sequence information alone. Our findings further illustrate that spliceogenicity is not simply a binary property of a variant: disruption of normal splice-site recognition can redistribute usage among nearby competing sites and generate several transcripts with different molecular consequences. Combining minigene analysis with transcript quantification and RNA sequencing allowed these complex splice patterns to be resolved and provided evidence for variant reclassification. For variants generating in-frame products, interpretation should additionally consider the structural and functional importance of the affected protein region rather than relying on preservation of the reading frame alone. Together, these findings support the incorporation of RNA-based functional analysis into the interpretation of selected *BRCA1/2* VUS and underscore the importance of considering local splice-site competition and transcript heterogeneity when evaluating intronic variants.

Declarations

Ethical procedures

The study was approved by the Ethics Committee of Zhongshan Hospital, Fudan University (B2023-042), and was conducted in accordance with the Declaration of Helsinki.

Consent for publication

Not Applicable.

Competing interests

The authors declare no conflicts of interest.

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Author Contribution

H.J., J.T., and Y.Z. performed experiments, analyzed data, and drafted the manuscript. B.W., W.G., and X.S. contributed to experimental procedures and data interpretation. H.Z. conceived and supervised the study, provided critical feedback, and contributed to manuscript revisions. All authors reviewed and approved the final version of the manuscript. H.J. and J.T. contributed equally as first authors. X.S. and H.Z. served as corresponding authors.

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Data Availability

The RNA sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) repository under BioProject PRJNA1528889 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1528889>).

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Figures

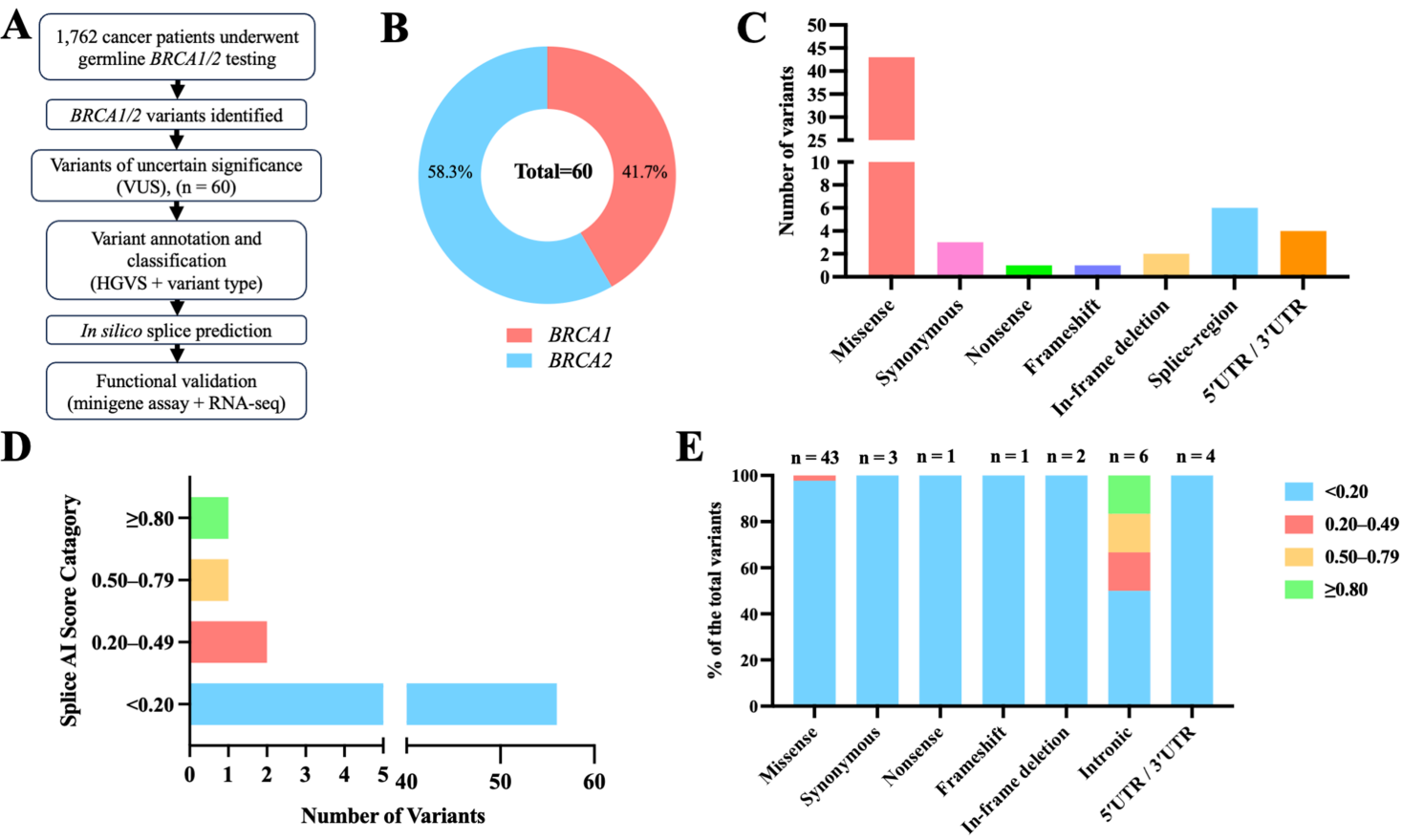


Figure 1

Landscape of *BRCA1/2* variants of uncertain significance and predicted spliceogenic potential in a hereditary cancer cohort

(A) Overview of the study workflow. Germline *BRCA1/2* variants identified in cancer patients were annotated and analyzed through computational splice prediction, followed by functional validation of candidate splice-altering variants.

(B) Distribution of unique *BRCA1/2* variants according to the affected gene.

(C) Classification of *BRCA1/2* variants according to molecular consequence, including missense, synonymous, nonsense, frameshift, in-frame deletion, intronic, and UTR/flanking region variants.

(D) Distribution of SpliceAI Δ scores among all identified *BRCA1/2* variants. Variants were categorized into four groups according to predicted splice-altering probability (<0.20 , $0.20-0.49$, $0.50-0.79$, and ≥ 0.80).

(E) Distribution of SpliceAI prediction categories across different variant classes. The proportion of variants within each category is shown according to molecular consequence.

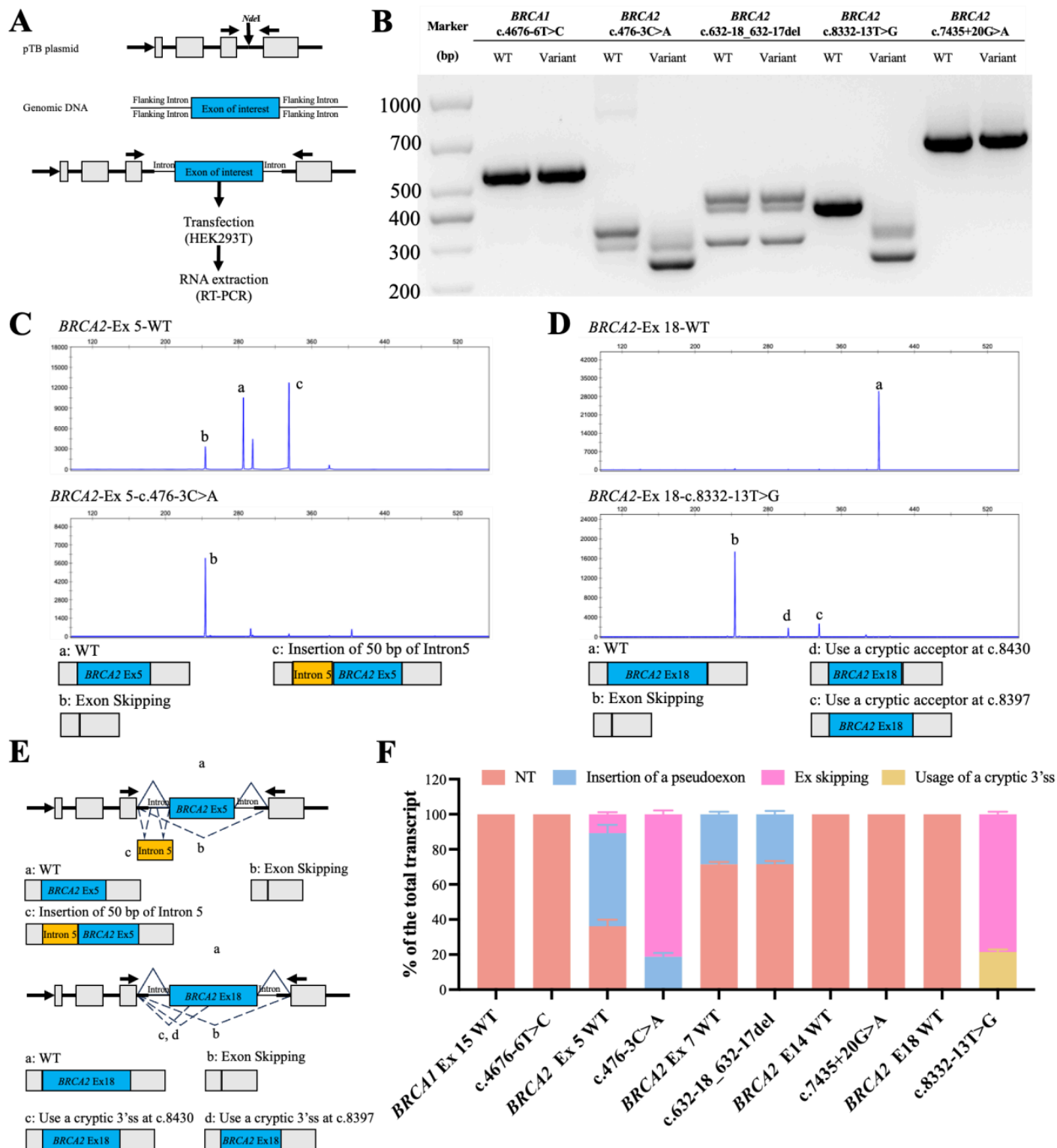


Figure 2

Functional characterization of candidate *BRCA1/2* splice-altering variants using minigene assays.

(A) Schematic workflow of the pTB minigene assay for evaluating variant-induced splicing alterations.

(B) RT-PCR analysis of wild-type (WT) and variant constructs for the five selected *BRCA1/2* variants. Aberrant transcripts were observed for *BRCA2* c.476-3C>A and c.8332-13T>G.

(C) Capillary electrophoresis of *BRCA2* c.476-3C>A, showing exon skipping and a 50-bp intronic insertion (c.476-141_c.476-92).

(D) Capillary electrophoresis of *BRCA2* c.8332-13T>G, showing exon skipping and activation of two cryptic 3' splice acceptor sites.

(E) Schematic representation of the aberrant transcript structures generated by *BRCA2*c.476-3C>A and c.8332-13T>G.

(F) Relative abundance of normal and aberrant transcript isoforms determined by capillary electrophoresis.

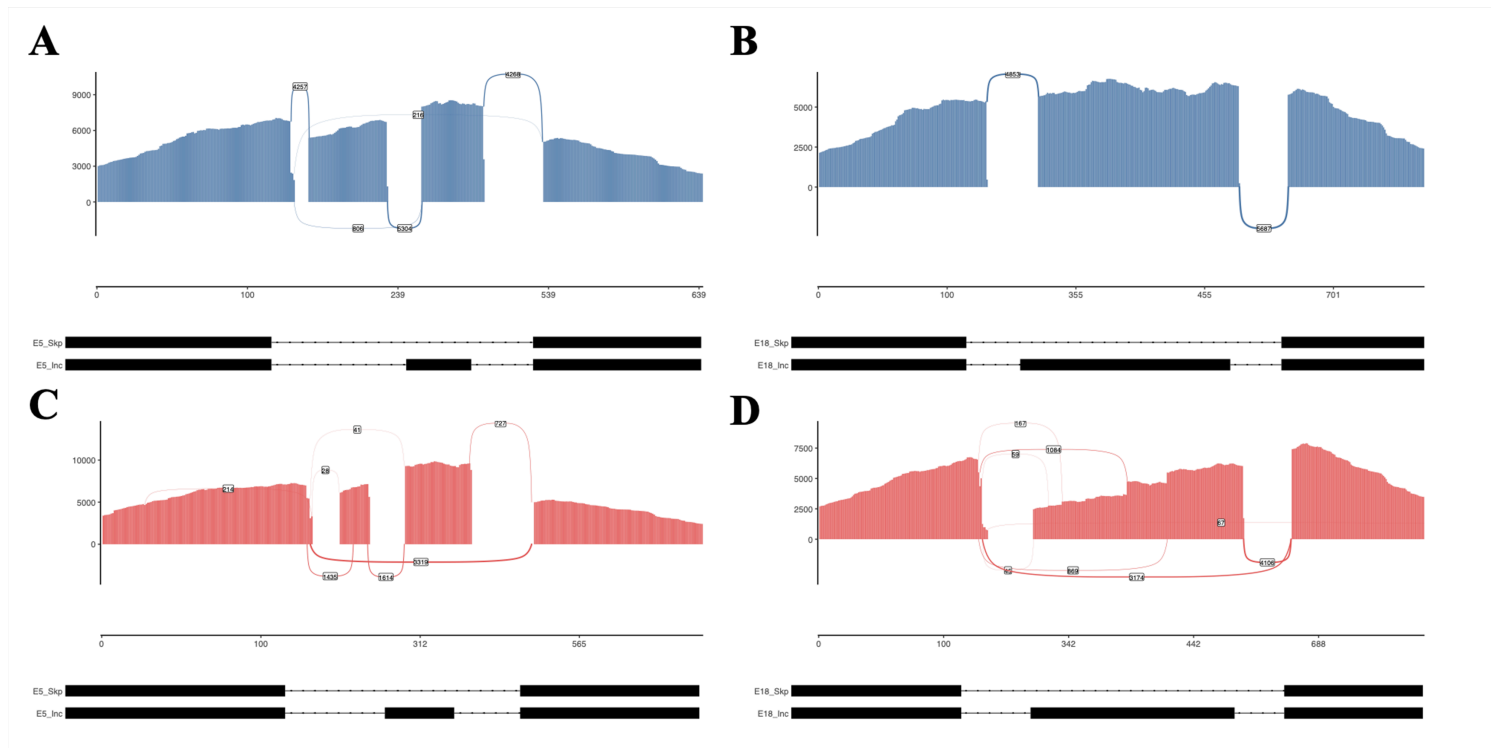


Figure 3

RNA sequencing reveals complex aberrant splice-junction patterns induced by *BRCA2* splice-altering variants.

(A) Sashimi plot of the wild-type *BRCA2* exon 5 minigene construct, showing predominant canonical splice-junction usage.

(B) Sashimi plot of the wild-type *BRCA2* exon 18 minigene construct, showing predominant canonical splice-junction usage.

(C) Sashimi plot of the *BRCA2* c.476-3C>A construct, revealing multiple aberrant splice junctions corresponding to exon skipping and intronic sequence inclusion.

(D) Sashimi plot of the *BRCA2* c.8332-13T>G construct, showing exon skipping and alternative usage of two cryptic 3' splice acceptor sites. Arc labels indicate the numbers of junction-spanning reads supporting individual splice junctions.

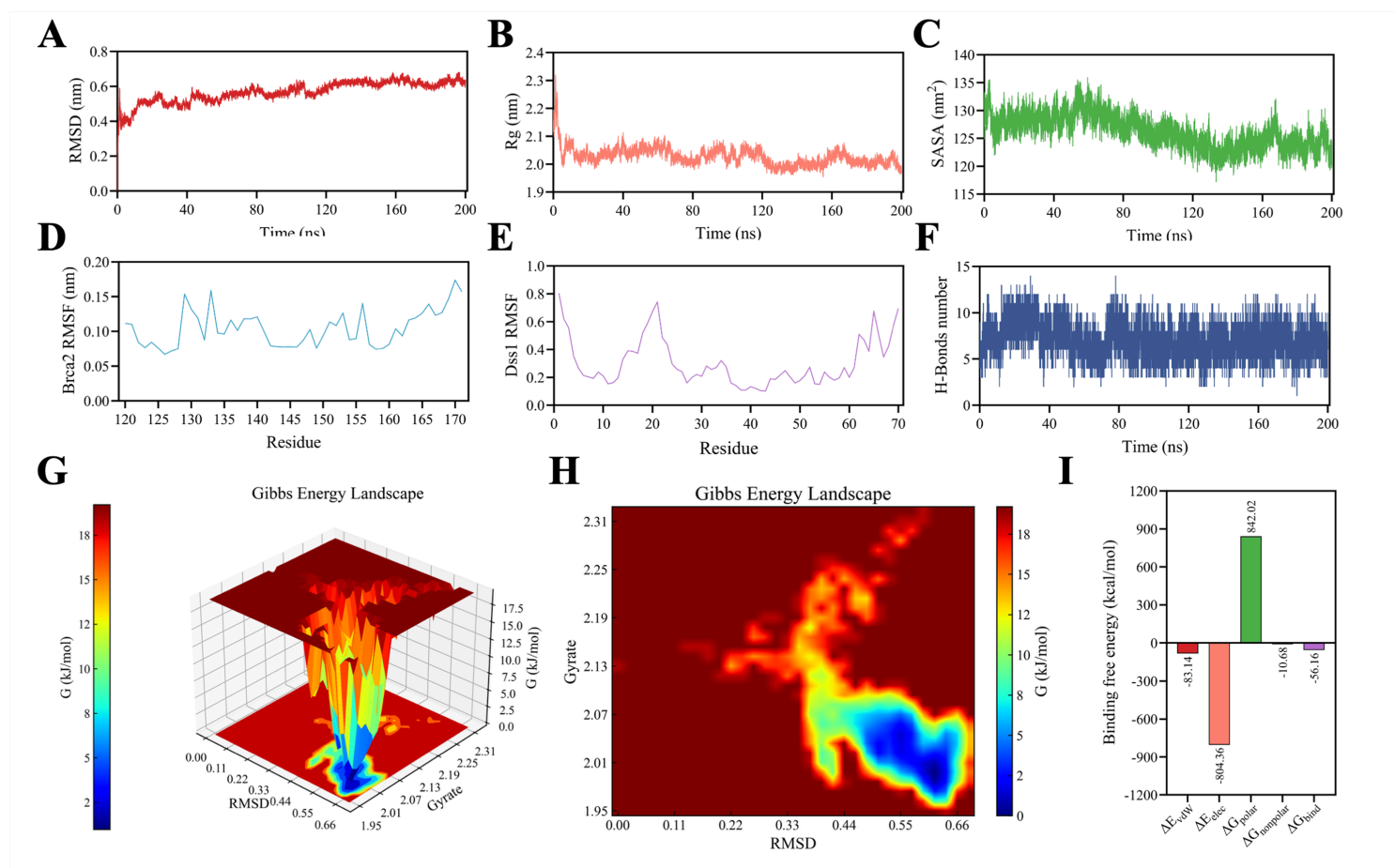


Figure 4

Molecular dynamics and energetic characterization of the BRCA2-DSS1 complex associated with the splice-derived deletion region.

(A) Root mean square deviation (RMSD) of the BRCA2-DSS1 complex during the 200-ns molecular dynamics simulation, reflecting the overall conformational deviation of the complex from its initial structure.

(B) Radius of gyration (Rg) of the BRCA2-DSS1 complex throughout the simulation, representing changes in the overall structural compactness of the complex.

(C) Solvent-accessible surface area (SASA) of the complex over the 200-ns trajectory, reflecting changes in solvent exposure during conformational equilibration.

(D) Residue-level root mean square fluctuation (RMSF) profile of BRCA2, showing local conformational flexibility across the modeled BRCA2 region.

(E) Residue-level RMSF profile of DSS1, illustrating residue-specific fluctuations of DSS1 during its interaction with BRCA2.

(F) Dynamic changes in the number of intermolecular hydrogen bonds between BRCA2 and DSS1 throughout the simulation, reflecting the persistence of interfacial hydrogen-bond interactions.

(G) Three-dimensional Gibbs free-energy landscape constructed using RMSD and Rg as reaction coordinates to characterize the conformational states sampled by the BRCA2-DSS1 complex.

(H) Corresponding two-dimensional Gibbs free-energy landscape, with low-energy regions representing energetically favorable conformational states sampled during the simulation.

(I) MM/PBSA binding free-energy analysis of the *BRCA2*–*DSS1* complex, showing the contributions of van der Waals interactions (ΔE_{vdW}), electrostatic interactions (ΔE_{elec}), polar solvation (ΔG_{polar}), and nonpolar solvation ($\Delta G_{nonpolar}$) to the total binding free energy (ΔG_{bind}).

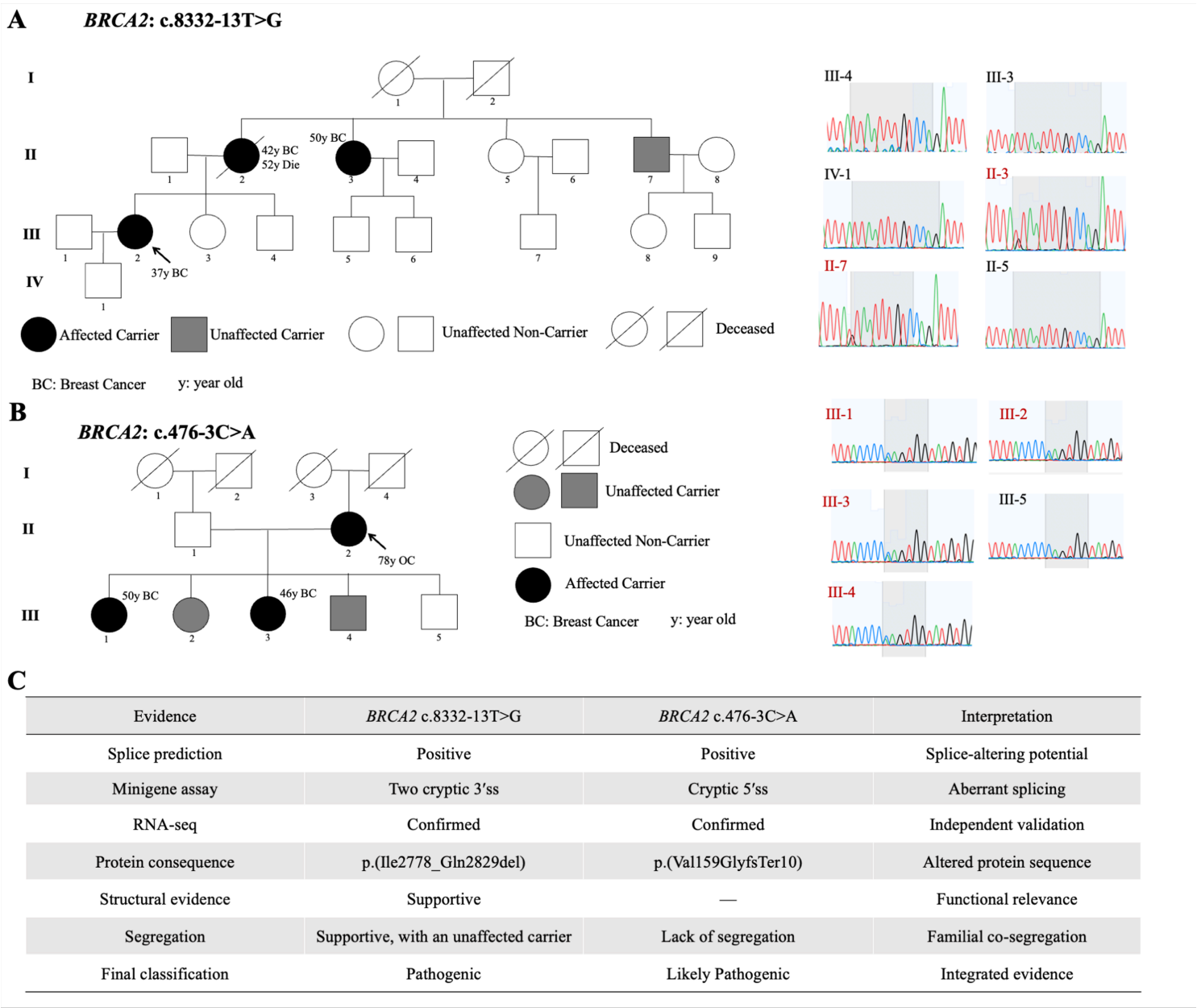


Figure 5

Pedigree analysis and integrated interpretation of two splice-altering *BRCA2* variants.

- (A) Pedigree and cascade Sanger sequencing of *BRCA2* c.8332-13T>G. The variant showed supportive but incomplete familial segregation, with an unaffected carrier identified among the available family members.
- (B) Pedigree and cascade Sanger sequencing of *BRCA2* c.476-3C>A. The variant was detected in the proband with ovarian cancer and in two affected family members with breast cancer, providing supportive evidence of familial co-segregation.
- (C) Integrated interpretation of the two variants based on splice prediction, minigene assays, RNA sequencing, predicted protein consequences, structural evidence, and familial segregation. The two variants were ultimately classified as pathogenic and likely pathogenic.

Supplementary Files

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