A High Proportion of DNA Variants of BRCA1 and BRCA2 Is Associated with Aberrant Splicing in Breast/Ovarian Cancer Patients

David J. Sanz¹, Alberto Acedo¹, Mar Infante¹, Mercedes Durán¹, Lucia Pérez-Cabornero¹, Eva Esteban-Cardeñosa², Enrique Lastra³, Franco Pagani⁴, Cristina Miner¹, and Eladio A. Velasco¹

Abstract

Purpose: Most BRCA1/2 mutations are of unknown clinical relevance. An increasing amount of evidence indicates that there can be deleterious effects through the disruption of the splicing process. We have investigated the effect of aberrant splicing of BRCA1/2 on hereditary breast/ovarian cancer (HBOC).

Experimental Design: DNA variants were analyzed with splicing prediction programs to select putative splicing mutations. Splicing assays of 57 genetic variants were done by lymphocyte reverse transcription-PCR and/or hybrid minigenes in HeLa and nontumor breast epithelial cells.

Results: Twenty-four BRCA1/2 variants of Spanish HBOC patients were bioinformatically preselected. Functional assays showed that 12 variants induced anomalous splicing patterns, 6 of which accounted for 58.5% of BRCA1 families. To further evaluate the defective splicing of BRCA1/2, we analyzed 31 Breast Cancer Information Core Database (BIC) and two artificial variants that were generated by mutagenesis. Sixteen variants induced different degrees of aberrant splicing. Altogether, anomalous splicing was caused by 28 BRCA1/2 variants of all types, indicating that any DNA change can disrupt pre-mRNA processing. We show that a wide range of regulatory elements can be involved, including the canonical and cryptic splice sites, the polypyrimidine tract, and splicing enhancers/silencers. Twenty mutations were predicted to truncate the BRCA proteins and/or to delete essential domains, thus supporting a role in HBOC.

Conclusions: An important fraction of DNA variants of BRCA1/2 presents splicing aberrations that may represent a relevant disease-causing mechanism in HBOC. The identification of splicing disruptions by functional assays is a valuable tool to discriminate between benign polymorphisms and pathogenic mutations. Clin Cancer Res; 16(6); 1957–67. ©2010 AACR.

Inactivating mutations in BRCA1 (MIM 113705) and BRCA2 (MIM 600185) confer a high risk of developing breast and ovarian cancers (1, 2). Both genes are responsible for ~16% of the familial breast cancer risk (3). Genetic testing for BRCA1 and BRCA2 provides valuable information in determining the clinical management of breast/ovarian cancer patients. However, the data it provides are also difficult to interpret due to the identification of many DNA variants of unknown physiologic significance, or unclassified variants (UV) that hamper genetic counseling in hereditary breast/ovarian cancer (HBOC). The critical issue is to identify whether a given nucleotide change results in a benign polymorphism or a disease-causing mutation. In fact, approximately half of the 3,499 different sequence variations of the Breast Cancer Information Core Database (BIC) are UVs and determining their biological effect remains a challenge (4, 5).

The analysis of the deleterious effect of genetic variants in disease genes is usually focused on the predicted effect on protein structure and function. However, precise removal of introns from precursor mRNA or splicing is an essential step in eukaryotic gene expression. In spite of the low conservation of the basic splicing signals (donor/acceptor sites) in upper eukaryotes, exon recognition can be accurately achieved by additional cis-splicing regulatory elements (SRE) that promote (enhancers) or repress (silencers) exon inclusion (6, 7). The study of the connections between defective splicing and disease has become a

5 http://research.nhgri.nih.gov/projects/bic/
A powerful, combined strategy has been developed to study the potential effect of genetic variants on splicing efficiency: bioinformatic analysis followed by functional analyses by lymphocyte reverse transcription-PCR and/or ex vivo hybrid minigenes. We show that a large proportion of BRCA1/2 variants, mostly of unknown clinical significance, is associated with the anomalous splicing of their corresponding genes in hereditary breast/ovarian cancer. Anomalous splicing may therefore represent a relevant ethiopathogenic mechanism whose study may notably increase the proportion of hereditary breast/ovarian cancer families that may benefit from genetic counseling and tailored prevention protocols. Appropriate functional splicing assays should be incorporated to the screening of genetic diseases such as hereditary cancer to discriminate between benign polymorphisms and pathogenic mutations.

Central issue in biomedicine as a consequence of the growing list of point mutations linked to aberrant splicing (6–10). Any DNA variant that disrupts such elements can be a potential deleterious mutation (6). This feature is particularly interesting in synonymous mutations (no change in protein sequence) that have traditionally been considered innocuous polymorphisms. One of the first examples of mutations disrupting an SRE was the BRCA1 mutation c.5080G>T (11, 12), which was correlated with exon 18 skipping. Since then, numerous mutations affecting SREs have been reported in a wide range of inherited diseases, such as cystic fibrosis (MIM 219700), neurofibromatosis type I (MIM 162200), and hereditary cancer (7, 10, 13). An unexpectedly large percentage of mutations play a key role in human disease through the alteration of the pre-mRNA-processing step. In fact, it has recently been proposed that up to 60% of mutations that cause genetic diseases alter the splicing process (8, 9).

The numerous and diverse cis-acting splicing elements present in human genes, as well as the high density of mutations in BRCA1 and BRCA2 (approximately one mutation/seven nucleotides), make these genes excellent models to study the correlation between aberrant splicing and BOC. Our purpose was to reanalyze the BRCA genes from the splicing perspective. We thus examined the functional consequences on splicing of 24 BRCA variants carried by our patients and another 33 putative splicing variants that we created by direct mutagenesis. They were assayed by reverse transcription-PCR (RT-PCR) of lymphocyte mRNA and/or hybrid minigene experiments in human cell lines. We detected 28 variants that altered the splicing process by different mechanisms, suggesting that aberrant splicing of BRCA1/2 may represent an important ethiopathogenic mechanism in HBOC.

Materials and Methods

Patients, nucleic acid isolation, and mutation detection. Breast/ovarian cancer patients of 688 unrelated families were selected in the Genetic Counselling Unit according to the criteria previously described (14). Written informed consent was obtained from all patients before blood extraction.

DNA and RNA were purified from peripheral blood lymphocytes by using the QiAamp DNA and RNA blood mini kits (Qiagen), respectively. Mutation analysis of BRCA1/2 was carried out by heteroduplex analysis on an ABI3130XL capillary DNA sequencer (Applied Biosystems; ref. 15). Mutation nomenclature follows the guidelines of the Human Genome Variation Society.6

Splicing prediction programs. Mutant and normal sequences were analyzed with several bioinformatic tools to identify potential splicing mutations. Disruption/creation of splice sites was evaluated with NNSPLICE7 (16). Analysis of putative SREs was done with two Web-based resources: ESEFinder,8 which detects exonic splicing enhancers (ESE) for the SR proteins SF2/ASF, SC35, SRp40, and SRp55 (17), and the ESRsearch tool,9 which identifies enhancers/silencers (18). Besides its own algorithms, ESRsearch integrates those of RESCUE-ESE10 (19), PESX11 (20), and detection of known regulatory motifs. To ascertain the evolutionary conservation of ESE motifs, human BRCA1 and BRCA2 sequences were aligned with those of other organisms using CLUSTALW12 (21).

Reverse transcription-PCR. Lymphocyte RNA was retrotranscribed with the SuperScriptII kit (Invitrogen). Amplification was conducted with Platinum Taq (Invitrogen) and flanking exonic primers (Supplementary Table S1). Twelve unrelated lymphocyte RNAs and normal breast RNA were used as controls.

Construction of minigenes. Mutant and wild-type (wt) exons of BRCA1/2 and flanking intronic sequences were amplified with PfuUltra High fidelity polymerase (Stratagene) and primers containing a 5′-tail with a restriction site for Xhol, BamHI, or BglII (Supplementary Fig. S1 and Table S2). After restriction enzyme digestion, fragments were cloned into the exon-trapping vector pSPL3 (Invitrogen) to transform Escherichia coli DH5α cells. To confirm the fidelity of the cloned sequences, plasmids were sequenced with the Big Dye 3.1 Sequencing kit (Applied Biosystems) and primers PSPL3-SEQ-FW (5′-CTTGGGATGTTGATGAT-3′) and PSPL3-SEQ-RV (5′-TTGCTTCCCTTCCCCA-3′). Minigenes were transfected into HeLa and nontumorigenic breast epithelial cells (MCF10A; ATCC, LGC Standards; ref. 22) with Lipofectamine (Invitrogen). RNA was purified

6 http://www.hgvs.org/mutnomen/
7 http://www.fruitfly.org/seq_tools/splice.html
8 http://nulab.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home
9 http://esrsearch.tau.ac.il/
10 http://genes.mit.edu/burgelab/rescue-ese/
11 http://cubweb.biology.columbia.edu/pess/
12 http://www.ebi.ac.uk/Tools/clustalw2/index.html
with Nucleospin-RNA-II (Macherey-Nagel) and RT-PCR was done as indicated above. Normal and anomalous bands in agarose gels were quantified with the Quantity One software v4.5.2 (Bio-Rad) and sequenced. 

Site-directed mutagenesis. Direct mutagenesis was carried out according to the PCR mutagenesis protocol\(^{13}\) with PfUUltra polymerase. Previously constructed minigenes of exons 5-6-7, 13, and 14 of BRCA1 and 3, 5-6-7, and 18 of BRCA2 were used as templates to generate 31 mutations from the BIC database (Supplementary Data). Experiments were done by triplicate and densitometric results of exon inclusion/exclusion bands between wt and mutant minigenes and between HeLa and MCF10A cells were compared with a t test.

Results

A total of 167 DNA variants were detected in 688 breast and ovarian cancer unrelated patients, 49 of which (103 unrelated families) were classified as deleterious. All the variants were analyzed with the NNSPLICE (splice sites) and ESEfinder and ESRsearch (enhancers/silencers). Twenty-four mutations were selected because they affected presumed SRE motifs or created/disrupted splice sites (Supplementary Table S3; Table 1).

Analysis of putative splicing variants of our breast/ovarian cancer families

Splice site mutations. Six variants affected the natural splice sites (Table 1), BRCA1 c.211A>g, c.212+1G>A, c.5153-1G>A, c.5277+1G>A, and c.7007G>A, and one created a cryptic donor site, BRCA2 c.1763A>G. These variants were tested by lymphocyte RT-PCR and/or hybrid minigenes, except for c.5277+1G>A, because patient RNA or DNA were not available (23). All of them induced aberrant splicing of their exons (Table 1; Fig. 1A; refs. 24–26). Apart from the classic mutations of the donor and acceptor splice sites, two variants should be highlighted. First, the novel BRCA2 missense variant c.1763A>G (p.N588S, exon 10) affected a nonconserved residue of the BRCA2 protein\(^{14}\) and could be considered neutral a priori. However, this DNA change was predicted to create a strong cryptic donor site, which led to an alternative transcript with an in-frame deletion of 147 nucleotides (predicted effect: p.N588S and loss of 49 amino acids; Fig. 1). Second, c.68-7T>A affected the polypyrimidine tract of the acceptor site of BRCA2 intron 2. Minigene analysis revealed partial exon 3 skipping (Supplementary Fig. S2) that had also been reported in fibroblasts (27).

SRE mutations. We also evaluated 17 variants that disrupted putative SREs according to the ESEfinder and ESRsearch (Supplementary Table S3; Table 1). Five of them (29.4%) showed different types of splicing alterations in RT-PCR analysis. The new BRCA1 variants c.4379G>A (silencer creation) and c.4392T>A (enhancer disruption) appeared together in two unrelated patients. Lymphocyte RT-PCR revealed two aberrant products that corresponded to the skipping of exon 15 and exons 14+15 (Fig. 1B). BRCA1 c.5123C>A showed partial in-frame skipping of exon 18 (Fig. 1B and C) in lymphocytes. This mutation eliminated one SC35 and one conserved SRp55 enhancers (ESEfinder) and generated one exonic splicing silencer (ESS; ESRsearch). RT-PCR analysis of the new BRCA2 mutation c.439C>T (p.Q147X) showed significant exon 5 removal (Fig. 1B). It was suggested that this mutation disrupted one Srp40 motif and created two silencers. The synonymous mutation c.9234C>T disrupted one Srp40 motif, although it also created ESSs. Lymphocyte RT-PCR showed partial exon 24 skipping (data not shown). Finally, the nonsense mutation c.145G>T, which disrupted one conserved SF2/ASF motif, induced exon 3 skipping in minigene assays (Fig. 2A).

The remaining 12 variants did not show any remarkable splicing consequence (Supplementary Table S3). The feature common to most negative SRE variants was the absence of evolutionary conservation of the disrupted elements. In fact, several of these variants had previously been suggested as nondeleterious DNA changes, such as BRCA1 c.4535G>T, and BRCA2 c.125A>C, c.223G>C, c.1114C>A, c.7397C>T, and c.8182G>T (4, 5, 28). Despite that BRCA2 c.7994A>G and c.9375C>G disrupted conserved ESEs, they did not affect splicing probably because they are present in exons with strong splice sites, which are supposed to be less dependent on SREs (29).

To summarize, 31 unrelated patients carried 12 different splicing variants, 7 of which affected splice sites (BRCA1- c.211A>G, c.212+1G>A, and c.5153-1G>A and c.5277+1G>A, and BRCA2-c.68-7T>A and c.7007G>A, and one created a cryptic donor site, BRCA2 c.1763A>G). These variants were tested by lymphocyte RT-PCR and/or hybrid minigenes, except for c.5277+1G>A, because patient RNA or DNA were not available (23). All of them induced aberrant splicing of their exons (Table 1; Fig. 1A; refs. 24–26). Apart from the classic mutations of the donor and acceptor splice sites, two variants should be highlighted. First, the novel BRCA2 missense variant c.1763A>G (p.N588S, exon 10) affected a nonconserved residue of the BRCA2 protein\(^{14}\) and could be considered neutral a priori. However, this DNA change was predicted to create a strong cryptic donor site, which led to an alternative transcript with an in-frame deletion of 147 nucleotides (predicted effect: p.N588S and loss of 49 amino acids; Fig. 1). Second, c.68-7T>A affected the polypyrimidine tract of the acceptor site of BRCA2 intron 2. Minigene analysis revealed partial exon 3 skipping (Supplementary Fig. S2) that had also been reported in fibroblasts (27).

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To summarize, 31 unrelated patients carried 12 different splicing variants, 7 of which affected splice sites (BRCA1- c.211A>G, c.212+1G>A, and c.5153-1G>A and c.5277+1G>A, and BRCA2-c.68-7T>A, c.1763A>G and c.7007G>A, and one created a cryptic donor site, BRCA2 c.1763A>G). These variants were tested by lymphocyte RT-PCR and/or hybrid minigenes, except for c.5277+1G>A, because patient RNA or DNA were not available (23). All of them induced aberrant splicing of their exons (Table 1; Fig. 1A; refs. 24–26). Apart from the classic mutations of the donor and acceptor splice sites, two variants should be highlighted. First, the novel BRCA2 missense variant c.1763A>G (p.N588S, exon 10) affected a nonconserved residue of the BRCA2 protein\(^{14}\) and could be considered neutral a priori. However, this DNA change was predicted to create a strong cryptic donor site, which led to an alternative transcript with an in-frame deletion of 147 nucleotides (predicted effect: p.N588S and loss of 49 amino acids; Fig. 1). Second, c.68-7T>A affected the polypyrimidine tract of the acceptor site of BRCA2 intron 2. Minigene analysis revealed partial exon 3 skipping (Supplementary Fig. S2) that had also been reported in fibroblasts (27).

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\(^{13}\) http://www.methodbook.net/PCR/PCRmut.html

\(^{14}\) http://agvgd.iarc.fr/BRCA2_Align.htm

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www.aacjrournals.org

Clin Cancer Res; 16(6) March 15, 2010

Published OnlineFirst March 9, 2010; DOI: 10.1158/1078-0432.CCR-09-2564

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cause an in-frame deletion of 49 amino acids of unknown importance because it does not affect any recognized functional domain. Finally, three variants were correlated with partial splicing outcomes with different interpretations. Only BRCA1 c.5123C>A (p.A1708E) was previously catalogued as deleterious by protein functional assays and statistical evaluations (4, 31). Thus, it is plausible that the pathogenicity of this variant may rely on both protein and splicing anomalies.

Splicing analysis of BIC and artificial mutations

To further evaluate the incidence of defective splicing in BRCA1/2, we analyzed mutations from the BIC database. For this purpose, we included BIC mutations from exons 5–6–7, 13, and 14 of BRCA1 and 3, 5–6–7, and 18 of BRCA2, which were evaluated for splice site disruption or creation (NNSPLICE), disruption of conserved ESEs, and ESS creation (ESEfinder and ESRsearch). Thirty-one putative splicing mutations were selected that comprised 28 nucleotide substitutions (22 missense, 3 nonsense, 1 synonymous, 2 intronic variants), one intronic deletion, and, as examples, two frameshift deletions. Another two artificial variants (BRCA1 c.165G>A and BRCA2 c.439C>A) were designed with the ESEfinder and ESRsearch to target specific SREs. All of them were generated by site-directed mutagenesis taking advantage of previous wt minigene plasmids.

Splicing functional assays showed that 16 variants (48.5%) caused different degrees and types of aberrant splicing (P<0.05, 5 variants; P<0.005, 10 variants; and P=0.13 for BRCA2-c.518G>T; Supplementary Figs. S2 and S3; Table 2; Figs. 2B and 3). Three variants affected the natural splice sites (BRCA1-c.212+3A>G, c.302-3C>G, and BRCA2-c.8331G>A), five created cryptic sites (BRCA2-c.467A>G, c.518G>T, c.7988A>T, c.8035G>T, and c.8168A>G), one disrupted the polypyrimidine tract (BRCA2-c.426-12del5), and seven affected ESE/ESS

Table 1. Bioinformatic analysis and RT-PCR results of mutations affecting pre-mRNA processing of BRCA1 and BRCA2

<table>
<thead>
<tr>
<th>DNA variant (BIC)/type</th>
<th>No. fam.</th>
<th>Carriers</th>
<th>Untested relatives</th>
<th>Clinical characteristics†</th>
<th>Bioinformatic study SREs‡</th>
<th>mRNA effect§</th>
<th>Protein effect∥</th>
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<tr>
<td>BRCA1</td>
<td></td>
<td></td>
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<tr>
<td>c.211A&gt;G (330A&gt;G)/Mis</td>
<td>5</td>
<td>5 BC +3</td>
<td>8 BC, 1 Cx, 1 CRC</td>
<td>Splice site disruption/</td>
<td>Ex 5del22</td>
<td></td>
<td>PT (p.Cys64X) +</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>cryptic donor site 22</td>
<td>Ex 5 skipping</td>
<td></td>
<td>IFD (p.Phe46_Arg71del) -Ring Finger</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>nt upstream-</td>
<td>(Ly-RT/MG)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>(NNSPLICE)</td>
<td></td>
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</tr>
<tr>
<td>c.212+1G&gt;A (331+1G&gt;A)/IVS</td>
<td>3</td>
<td>2 BC+3 OC</td>
<td>3 BC, 2 OC, 1 Bla, 1 end, 2 BC</td>
<td>Splice site disruption/</td>
<td>Ex 5del22</td>
<td></td>
<td>PT (p.Cys64X) +</td>
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<td>cryptic donor site-</td>
<td>Ex 5 skipping</td>
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<td>(NNSPLICE)</td>
<td>(Ly-RT/MG)</td>
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<tr>
<td>c.4379G&gt;A/Mis + c.4392T&gt;A/Syn</td>
<td>2</td>
<td>2 BC</td>
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<td>ESE creation-(PESX)</td>
<td>Ex 15 skipping</td>
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<td>PT (p.Ser1496GlyfsX13) + IFD (p.Ala1453_Leu1588del)-SCD</td>
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<td>ESE disruption/ SRp40,SC35-(ESEfinder)</td>
<td>Ex 14-15 skipping</td>
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<td>ESS creation-(PESX/ESRsearch)</td>
<td>Partial exon</td>
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<td>c.5123C&gt;A (5242C&gt;A)/Mis</td>
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<td>IFD (p.Val1719X)</td>
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<td></td>
<td></td>
<td>downstream-</td>
<td>(NNSPLICE)</td>
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<tr>
<td>c.5153-1G&gt;A (5272-1G&gt;A)/IVS</td>
<td>7</td>
<td>10 BC, 2 bBC, 2 OC + 1 CRC</td>
<td>10 BC, 4 OC, 4 GC, 10 others</td>
<td>Splice site disruption-</td>
<td>1 nt deletion</td>
<td></td>
<td>IFD (p.His1732_Lys1759del)-BRCT + PT (p.Lys1759_ile1760ins29)</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>(NNSPLICE)</td>
<td>(Ly-RT)</td>
<td></td>
<td></td>
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<tr>
<td>c.5277+1G&gt;A (5396+1G&gt;A)/IVS</td>
<td>1</td>
<td>1 BC (5 OC; ref. 23)</td>
<td>1 BC</td>
<td></td>
<td>Retention 87 nt intron 20,</td>
<td>Ex 20 skipping</td>
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<td>(NNSPLICE)</td>
<td>(Ly-RT)</td>
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<tr>
<td>BRCA2</td>
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<tr>
<td>c.68-77&gt;A (296-77T&gt;A)/IVS</td>
<td>2</td>
<td>2 BC</td>
<td>5 BC, 1 GC, 1 leukemia</td>
<td>Polypyrddmide tract-(NNSPLICE)</td>
<td>Partial ex 3 skipping</td>
<td></td>
<td>IFD (p.Asp23_Leu105del)-PBD + wt</td>
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<td></td>
<td></td>
<td>tract-(NNSPLICE)</td>
<td>(MG)</td>
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BRCA1-c.165G>A, c.178C>T, c.439C>A, c.455C>A, c.470del5, and c.473C>T). These mutations had been reported 54 times in the BIC database. Most induced important or total aberrant splicing of their exons (12 of 16), but four (BRCA2-c.455C>A, c.518G>T, c.7988A>T, and c.8168A>G) caused a partial effect (Supplementary Figs. S2 and S3; Fig. 2B).

BRCA1-c.211A>G, c.212+1G>A, and c.212+3A>G caused a similar splicing defect in minigene experiments (Tables 1 and 2; Fig. 3): exon 5 skipping and loss of 22 nucleotides due to the use of a cryptic donor site. In contrast, other exon 5 variants, such as c.165G>A and c.178C>T, just induced exon skipping.

Interestingly, the amino acid change p.W31C (BRCA2-c.93G>T) was previously established to disrupt the DNA repair function of BRCA2 (32). However, we have found that c.93G>T induced exon 3 skipping and thus the Cys31 codon will not be present in the mature mRNA (Supplementary Fig. S2).

Four of six variants of BRCA2 exon 5 displayed aberrant splicing. BIC mutations at codon 147 (c.440A>G and c.441A>T) did not affect exon 5 splicing as c.439C>T, despite the fact that they disrupted the same SRp40 motif (Fig. 2B). However, neither variant created a silencer as with c.439C>T (ESRsearch). We then designed mutation c.439C>A (SRp40 disruption and creation of three ESSs; Table 2) that also induced exon 5 skipping (Fig. 2B).

Four of nine variants caused abnormal splicing of BRCA2 exon 18 (Supplementary Fig. S3). Three of them created cryptic splice sites, causing a deletion of 298 nucleotides (c.8035G>T) and a partial loss of 164 (c.8168A>G), whereas c.7988A>T induced partial exon skipping (reported in

<table>
<thead>
<tr>
<th>DNA variant* (BIC)/type</th>
<th>No. fam.</th>
<th>Clinical characteristics †</th>
<th>Bioinformatic study SREs‡</th>
<th>mRNA effect§</th>
<th>Protein effect∥</th>
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<tbody>
<tr>
<td>c.145G&gt;T (373G&gt;T)/non</td>
<td>1</td>
<td>2 BC, 3 BC, 1 OC</td>
<td>ESE disruption/SF2/ASF-(ESEfinder/PESR)</td>
<td>Ex 3 skipping (MG)</td>
<td>IFD (p.Asp23_Leu105del)-PBD</td>
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<tr>
<td>c.439C&gt;T (667C&gt;T)/non</td>
<td>1</td>
<td>1 BC, 1 BC, 1 bBC, 2 OC</td>
<td>ESE disruption/SRP40-(ESEfinder)</td>
<td>Ex 5 skipping (Ly-RT/MG)</td>
<td>PT (p.Pro143GlyfsX22)</td>
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<tr>
<td>c.1763A&gt;G (1991A&gt;G)/mis</td>
<td>1</td>
<td>1 bBC</td>
<td>Cryptic donor site 147 nt upstream (NNSPLICE)</td>
<td>Del 147 nt-ex10 (Ly-RT)</td>
<td>IFD (p.Asn588 Gly637del, p.N588S)</td>
</tr>
<tr>
<td>c.7007G&gt;A (7235G&gt;A)/mis</td>
<td>1</td>
<td>3 BC</td>
<td>Splice site disruption (NNSPLICE)</td>
<td>Ex13 skipping (Ly-RT)</td>
<td>PT (p.Gly2313AlafsX40)</td>
</tr>
<tr>
<td>c.9234C&gt;T (9462C&gt;T)/Syn</td>
<td>1</td>
<td>1 BC</td>
<td>ESE disruption/SRP40-(ESEfinder/PESR)</td>
<td>Partial ex24 skipping (Ly-RT)</td>
<td>PT (p.Val3040AspfsX18)+ wt</td>
</tr>
</tbody>
</table>

*Mutations described at the BIC database are shown in bold. The rest of the mutations were described by our group (Infante et al., 2006). Descriptions of mutations follow the nomenclature guidelines of the Human Genome Variation Society. BIC nomenclature is shown between parentheses. Types of mutations are indicated as follows: syn, synonymous; non, nonsense; mis, missense.
†BC, breast cancer; bBC: bilateral breast cancer; Bla, bladder cancer; Cx, cervix cancer; CRC, colorectal cancer; GC, gastric cancer; OC, ovarian cancer. Mutation c.7007G>A has been found in another family with bilateral breast cancer and male breast cancer (Thomassen et al., 2006).
‡Nt, nucleotide. Splicing prediction programs are shown between brackets: NNSPLICE, PESR, Rescue-ESE, PESX, PESR, and ESRsearch. Full data of bioinformatic study are available upon request.
§Method between brackets: Ly-RT, Lymphocyte RT-PCR; MG, Minigene assay; Ex; Exon; Del, deletion. Effects of mutations c.211A>G, c.5153-1G>A, c.5277+1G>A, and c.7007G>A on lymphocyte RNA have also been reported (Tesoriero et al., 2005; Vega et al., 2001; Thomassen et al., 2006; and Beristain, et al., 2007).
∥Mutation designations (protein level) according to the Human Genome Variation Society (www.hgvs.org) are between parentheses. Protein Effect: PT, Protein Truncation; IFD, In-frame deletion; BRCT, BRCA1 C-Terminal Domains (amino acids 1,646-1,859); PBD, PALB2 Binding Domain; SCD, SQ-cluster domains (amino acids 1,280-1,524) that are preferred sites of ATM phosphorylation.
ref. 33), but also a deletion of 345 nucleotides. Finally, c.8331G>A affected the donor site and provoked total exon 18 skipping. None of the five missense mutations directed to conserved ESE significantly altered splicing, although variant c.8165C>G (34) showed a weak exon 18 skipping in minigene context, also detected by other authors (33, 35).

Six positive splicing mutations showed simultaneous ESE disruption/ESS creation by bioinformatic analysis (BRCA1-c.178C>T, BRCA2-c.93G>T, c.439C>A, c.455C>A, c.470del5, and c.473C>T). Only the synonymous mutation BRCA1-c.165G>A was an apparent strict ESE mutation (three motifs) that caused exon 5 skipping (Fig. 3).

Seven variants were predicted to truncate the BRCA proteins (Supplementary Table S4; Table 2); three variants were suggested to cause in-frame deletions of essential domains (Ring Finger of BRCA1 and PALB2 binding domain of BRCA2), two variants were predicted to produce both effects; and one missense change (p.D2723G) with a partial splicing outcome was previously shown to inactivate BRCA2 (33). Moreover, four variants (BRCA1-c.212+3A>G, c.302-3C>G, BRCA2-c.7988A>T, and c.8168A>G) were previously estimated with odds in favor of causality of at least 120:1. Altogether, these observations support the pathogenicity of 13 of these splicing variants. The disease causality of the remaining three variants was uncertain because one of them (c.467A>G) deleted three nonconserved residues of BRCA2 and two were nonconserved missense variants (BRCA2-c.455C>A and c.518G>T) that caused partial splicing outcomes.

Regulation of alternative splicing is tissue specific and it is well known that tumor cells show global aberrant splicing patterns (36). We proceeded to confirm all the results in nontumor cells from the target tissue of the disease. The positive mutant minigenes were transfected into nontumor breast cells (MCF10A). All anomalous events were corroborated, albeit some quantitative differences between both types of cells were observed (Fig. 3). For example, mutations c.165G>A, c.178C>T, and c.212+1G>A of BRCA1 exon 5 led to almost total exon 5 skipping in MCF10A, whereas this effect was apparently less pronounced in HeLa. However, the differences in band intensities were not statistically significant (Fig. 3).

Discussion

A high proportion of BRCA1/2 variants reported in the BIC database has not been classified as either deleterious or neutral. In this work, we have developed a powerful combined strategy to study the potential effect of genetic variants on splicing efficiency: bioinformatic analysis followed by functional analysis (average successful rate of 49.1%). Predictions of the splicing software can be considered useful, but only complementary, tools for identifying candidate splicing mutations because they must
necessarily be confirmed by a functional assay. ESEfinder and ESRsearch were not so precise because they systematically recognize all the motifs that can act as putative regulators. Their sensitivity can be increased by filtering the output data with other parameters such as strict evolutionary conservation of the ESE motif, strength, and proximity of the splice sites (7, 37, 38).

**Splicing functional assays.** We found that 28 of 57 variants exhibited different degrees and types of aberrant splicing (Tables 1 and 2). The minigene assay is a straightforward and reliable method for studying potential splicing mutations without the need of patient RNA (39). Results of lymphocyte RT-PCR were reproduced in minigene experiments in variants tested by both methods (24–26, 33, 35, 40–43), except for quantitative differences of aberrant isoforms.

All anomalous events of HeLa-minigenes were confirmed in breast epithelial cells—MCF10A (Fig. 3). These results allowed us to discard possible artifacts derived from the global alteration of splicing patterns in tumor cells that could have masked the real splicing consequence (36). Given that nontumor breast tissue samples from BRCA carriers are practically unavailable, the use of minigenes in MCF10A cells is an excellent approach for reproducing the splicing outcome in this tissue. Finally, the combination of minigene and PCR mutagenesis techniques allows the splicing analysis of any DNA variant reported worldwide, as well as mapping of key SREs.

**Disruption of SREs.** A wide variety of splicing elements were involved: the natural splice sites (7 mutations), the polypyrimidine tract (2 mutations), and cryptic splice sites (7 mutations); and different exonic SRE motifs (12 mutations), illustrating the high complexity of exon recognition that depends on multiple parameters (29). Moreover, an important fraction of variants affected exonic motifs (12 SREs and 7 cryptic splice sites), a fact that supports the idea that any DNA change should be investigated in depth. According to ESEfinder and ESRsearch, many different SRE motifs were involved in aberrant splicing: SC35, SRp40, SRp55, SF2/ASF, hnRNPB, and other SREs without a known binding protein. Most functional SREs were placed in close proximity to the splice sites, in keeping with the previously reported position effect of these elements (38). Computational SRE search produced conflicting results (enhancer disruption/silencer creation).

![Fig. 2. Splicing functional assays of hybrid minigenes of BRCA2](image-url)
Table 2. BIC and artificial mutations engineered by site-directed mutagenesis that showed aberrant splicing in minigene assays

<table>
<thead>
<tr>
<th>DNA variant (BIC)-type of mutation*</th>
<th>Exon No. of BIC records</th>
<th>Bioinformatic study SREs†</th>
<th>mRNA effect</th>
<th>Protein effect‡</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BRCA1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.165G&gt;A (284G&gt;A)/Syn</td>
<td>5 —</td>
<td>ESE disruptions/2 SRp40, SF2-(ESEfinder)</td>
<td>Ex5 skipping</td>
<td>IFD (p.Phe46_Arg71del)-Ring Finger</td>
</tr>
<tr>
<td>c.178C&gt;T (297C&gt;T)/non</td>
<td>5 4</td>
<td>ESE disruptions/2 SF2, SRp55-(ESEfinder) ESS creation-(Rescue-ESE)</td>
<td>Ex5 skipping</td>
<td>IFD (p.Phe46_Arg71del)-Ring Finger</td>
</tr>
<tr>
<td>c.212+3A&gt;G (331+3A&gt;G)/IVS</td>
<td>I-5 13</td>
<td>Donor site disruption-(NNSPLICE)</td>
<td>Ex5del22 +</td>
<td>PT (p.Cys64X) +</td>
</tr>
<tr>
<td>c.302-3C&gt;G (421-3C&gt;G)/IVS</td>
<td>I-6 3</td>
<td>Cryptic acceptor site-(NNSPLICE)</td>
<td>2 nt insertion</td>
<td>PT (p.Ser72GlyfsX17)</td>
</tr>
<tr>
<td><strong>BRCA2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.93G&gt;T (321G&gt;T)/mis</td>
<td>3 1</td>
<td>ESE disruption/SC35-(ESEfinder/PESR) ESS creation-(ESRsearch)</td>
<td>Ex3 skipping</td>
<td>IFD (p.As23_Leu105del)-PBD</td>
</tr>
<tr>
<td>c.426-12del5 (654-12del5)/IVS</td>
<td>I-4 1</td>
<td>Polypyrimidine tract-(NNSPLICE)</td>
<td>Ex5 skipping</td>
<td>PT (p.Pro143GlyfsX22)</td>
</tr>
<tr>
<td>c.439C&gt;A (667C&gt;A)/mis</td>
<td>5 —</td>
<td>ESE disruption/SRp40-(ESEfinder) 3 ESS creation-(PESX)</td>
<td>Ex5 skipping</td>
<td>PT (p.Pro143GlyfsX22)</td>
</tr>
<tr>
<td>c.455C&gt;A (683C&gt;A)/mis</td>
<td>5 1</td>
<td>ESE disruption/SRp40-(ESEfinder) ESS creation-(PESX)</td>
<td>Partial ex5</td>
<td>PT (p.Pro143GlyfsX22) +</td>
</tr>
<tr>
<td>c.467A&gt;G (695A&gt;G)/mis</td>
<td>5 8</td>
<td>Cryptic donor 9 nt upstream-(NNSPLICE) 9 nt del ex5</td>
<td>9 nt del ex5</td>
<td>IFD (p.As1p6_Ser158del; N.C.)</td>
</tr>
<tr>
<td>c.470_474del5 (698del5)/fr</td>
<td>5 1</td>
<td>ESE disruptions/SF2,SC35, SRp55-(ESEfinder) ESS creation-(Rescue-ESE)</td>
<td>Ex5 skipping</td>
<td>PT (p.Pro143GlyfsX22)</td>
</tr>
<tr>
<td>c.473C&gt;T (701C&gt;T)/mis</td>
<td>5 4</td>
<td>ESE disruptions/SF2,SRp55-(ESEfinder) 5 ESS creation-(Rescue-ESE)</td>
<td>Ex5 skipping</td>
<td>PT (p.Pro143GlyfsX22)</td>
</tr>
<tr>
<td>c.518G&gt;T (746G&gt;T)/mis</td>
<td>7 1</td>
<td>Cryptic donor site-(NNSPLICE)</td>
<td>Partial ex7</td>
<td>PT (p.Gly174SerfsX19)+</td>
</tr>
<tr>
<td>c.7988A&gt;T (8216A&gt;T)/Mis</td>
<td>18 9</td>
<td>Cryptic donor site-(NNSPLICE)</td>
<td>Partial loss of 345 nt +Partial ex18</td>
<td>IFD (p.Glu2663_Lys2777del) +</td>
</tr>
<tr>
<td>c.8035G&gt;T (8263G&gt;T)/mis</td>
<td>18 1</td>
<td>Cryptic donor site-(NNSPLICE)</td>
<td>Loss of 298 nt</td>
<td>PT (p.Tyr2664PhefsX43)+</td>
</tr>
<tr>
<td>c.8168A&gt;G (8396A&gt;G)/mis</td>
<td>18 6</td>
<td>Cryptic donor site-(NNSPLICE)</td>
<td>Partial loss of 164 nt</td>
<td>PT (p.Gly2724PhefsX3)+</td>
</tr>
<tr>
<td>c.8331G&gt;A (8559G&gt;A)/Syn</td>
<td>18 —</td>
<td>Splice site disruption-(NNSPLICE)</td>
<td>Exon 18 skipping</td>
<td>PT (p.Tyr2664PhefsX43)</td>
</tr>
</tbody>
</table>

Abbreviations: Ex, exon; del, deletion; nt, nucleotide.
*Artificial mutations 284G>A and 667C>A are italicized, whereas BIC mutations are shown in bold. BIC nomenclature of mutations is shown between parentheses. Types of mutations are indicated as follows: syn, synonymous; non, nonsense; mis, missense; fr, frameshift. DNA change c.8331G>A (8559G>A) of the BIC database is incorrectly assigned to codon 2776 (p.K2777K).
†Splicing prediction programs are shown between brackets: PESR, Rescue-ESE, PESX, and ESEfinder. Full data of bioinformatic study are available upon request. Evolutionarily conserved binding motifs of SR proteins are underlined.
‡Mutation designations (protein level) according to the Human Genome Variation Society are between parentheses. Protein effect: PT, protein truncation; IFD, in-frame deletion; PBD, PALB2 binding domain; DBD, DNA Binding domain; N.C., not conserved; P.F., missense changes that affect the protein function.
in 10 positive mutations, but the significance of these data can only be solved experimentally. For example, in silico analysis of BRCA1 c.5080G>T predicted the disruption of one SF2/ASF enhancer, but it has recently been shown that the mutant sequence specifically binds to the repressor factors hnRNP A1/A2 and DAZAP1 (11, 12). We found that all the mutations at codon 147 of BRCA2 disrupted the same SRp40 motif, but only two (c.439C>T and c.439C>A), which created silencers, caused exon 5 skipping, suggesting that the binding of repressors is the underlying molecular mechanism. These results also illustrate the fine balance between positive and negative determinants necessary for exon identity (44). Alternatively, strict ESE mutations seemed to be BRCA1 c.165G>A and BRCA2 c.145G>T, which only disrupted conserved ESEs and were correlated with skipping of their respective exons (Figs. 2 and 3).

The influence of the genomic context is another factor that regulates the splicing process (7). A representative example is the “splicing interdependence” of the exon cluster 34 to 38 of the Neurofibromatosis type 1 gene, where a mutation of exon 37 (6792C>G) causes skipping of exons 36+37 and exon 37. Our results suggest that variants c.439G>A, c.439T>A of BRCA1 exon 14 triggered skipping of exon 15 and exons 14+15. The exact, underlying mechanism is unknown and should be confirmed with a minigene containing both exons.

Reclassification of mutations. Reclassification of UVs as deleterious under the splicing viewpoint may notably increase the proportion of HBOC families who may benefit from tailored prevention protocols. Thirteen missense, 3 nonsense, 4 synonymous, 1 frameshift, and 7 intronic variants produced abnormal splicing patterns, indicating that any DNA change should be regarded as a potential splicing mutation. Of these, 20 (12 missense, 4 synonymous, and 4 IVS) were previously considered UVs or mere polymorphisms. Consequently, splicing should be considered a primary mechanism of pathogenicity to be investigated in UVs. Nevertheless, effects on protein function must not be disregarded because several negative missense changes affected strongly conserved amino acids, supporting their functional importance. Actually, several missense changes were formerly shown to inactivate BRCA1/2 at the protein level (31, 33). Another interesting example is missense c.5123G>A (p.A1708E), which inactivates BRCA1 through a double mechanism: alteration of the protein function (disruption of the BRCT domain; ref. 31) and a decrease of the exon 18 splicing efficiency (Fig. 1) by the binding of specific repressors (41). In addition, BRCA2 c.8168A>G (p.D2723G) of exon 18 (DNA binding domain) was shown to inactivate BRCA2 at the protein level (33), but we also found a splicing isoform with a deletion of 164 nucleotides. Intriguingly, the missense mutation p.W313C (c.93G>T) was reported to disrupt the interaction BRCA2-PALB2, which is essential for recombinational repair and checkpoint functions (32). Conversely, our minigene experiment shows that this mutation induces the almost complete in-frame skip of BRCA2 exon 3 by disruption of SREs (Table 2); thereby, this amino acid change will not be present in the majority of mutant proteins and BRCA2-PALB2 interaction would be impeded as well. It would also be interesting to test p.W31R (c.91T>C), which also abolished PALB2-BRCA2 interaction (32), but our bioinformatic analysis indicated no disruption of the ultraconserved SC35 motif of c.93G>T. Reclassification of missense and protein truncation mutations (e.g., BRCA2 c.439C>T or c.470del5) as splicing alterations might also have an effect in the penetrance and expressivity of such mutations. The reduced penetrance of BRCA1 c.211A>G lends further support to this hypothesis (45).

Genetic susceptibility to breast/ovarian cancer. A high proportion (28 of 57, 49.1%) of assayed mutations disrupted pre-mRNA processing. This suggests that the prevalence of splicing mutations may have been vastly underestimated. If we make a short review of the BIC mutations of exons 5–6, 13, 14 of BRCA1 and 3, 5–6, 7, and 18 of BRCA2, we find 26 variants of the AG/GT canonical splice sites that, together with our data of such exons, 4 BIC mutations of our patients (c.211A>G, c.5153C>A, c.68–7T>A and c.145G>T) and 14 positive engineered BIC mutations, account for 44 splicing variants. They therefore constitute 22.1% of variants of those exons reported in the BIC database, which may be even higher, given that only two frameshift mutations
(c.470_474delACTCA and c.594_595delAT) were examined in our study. In comparison, the low-penetrance genes ATM, CHK2, PALB2, and BRI1 scarcely make any contribution, with ~50 mutations to breast cancer susceptibility after the analysis of a large number of families (Human Gene Mutation Database),\(^5\) accounting for <2.3% of the familial risk of breast cancer (3). Moreover, splicing is the major pathogenic mechanism of BRCA1 in our HBOC families (58.5% of BRCA1 families), although the mutations c.211A>G, c.212+1G>A, c.5153-1G>A, and c.5123C>A are very frequent in our population (21 families; refs. 24, 30). Furthermore, typical screening protocols only scan ~25 Kb of the 160 Kb of the genomic sequences of BRCA1/2, so deep intronic mutations may be missed. For example, the GTAA deletion of intron 20 of the ATM gene promotes the exonization of a cryptic exon (46). However, other authors have suggested a minor role for such mutations in the molecular spectrum of BRCA1/2 (47).

One important question is to elucidate the carcinogenic power of mutations with incomplete splicing effects. Variants with strong effects (20 variants; Supplementary Table S4) are probably deleterious because they inactivate BRCA1/2 functions through protein truncation and/or deletion of essential protein domains. Moreover, 12 variants have been predicted to be deleterious by an integrated evaluation approach (Supplementary Table S4; ref. 4), which provides additional support to our findings. Nevertheless, the involvement in breast cancer susceptibility of weaker splicing variants, such as c.68-7T>A or c.518G>T of BRCA2 or missense changes with incomplete effects on protein function, is more uncertain (27). This question should be addressed by a more comprehensive study, including supplementary functional assays and epidemiologic and statistical analyses (4, 35). Partial splicing mutations might contribute to the breast and ovarian cancer genetic spectrum, providing low/moderate cancer susceptibility alleles that might act synergistically with other low-penetrance alleles to increase the general risk of breast cancer. All these parameters should be integrated in a unique model of breast and ovarian cancer risk, which would include susceptibility and protector alleles (48), as well as environmental and life-style factors, with a view to providing individual risk assessment, which seems to be one of the most laborious undertakings.

In conclusion, we have shown that defective splicing is an important inactivating mechanism of the BRCA genes. Our results also suggest that careful interpretation is needed for the pathologic nature of DNA variants, particularly when changes in protein function cannot be evaluated. The implantation of simple, cost-effective splicing assays in the genetic diagnostic laboratory may contribute to the classification of variants of unknown clinical significance. Finally, splicing analysis of human disease genes also contributes to the basic knowledge of the regulatory mechanisms of this process.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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\(^{5}\) [http://www.hgmd.cf.ac.uk/ac/index.php](http://www.hgmd.cf.ac.uk/ac/index.php)


