BRCA2 Deep Intronic Mutation Causing Activation of a Cryptic Exon: Opening toward a New Preventive Therapeutic Strategy

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Abstract

Purpose: Diagnostic screening of the BRCA1/2 genes in breast cancer families is mostly done on genomic DNA. For families with a very strong family history and no mutation identified in the coding sequences or the exon–intron boundaries, BRCA1/2 transcripts’ analysis is an efficient approach to uncover gene inversion and pre-mRNA splicing defaults missed by conventional DNA-based protocols.

Experimental Design: We analyzed RNA from patients of negative BRCA families by reverse transcriptase PCR and identified an insertion in one family that we characterized by sequencing and by using a minigene splicing assay. More than 2,000 additional BRCA1/2 negative families were subsequently screened for this mutation using a dedicated PCR approach.

Results: Nine families were found to harbor a BRCA2 mutant transcript containing a 95-nucleotide cryptic exon between exons 12 and 13. This cryptic exon results from a new mutation located deep into intron 12, c.6937+594T > G, which reinforces the strength of a preexisting 5’ splice site, turning it into a perfect consensus sequence. It is systematically included in transcripts produced by the mutant allele in cells from mutation carriers or produced by a mutant splicing reporter minigene. The inclusion of the cryptic exon was prevented when we cotransfected the minigene with antisense oligonucleotides complementary to the 3’ or mutated 5’ splice sites.

Conclusion: This first deep intronic BRCA mutation emphasizes the importance of analyzing RNA to provide comprehensive BRCA1/2 diagnostic tests and opens the possibility of using antisense therapy in the future as an alternative strategy for cancer prevention. Clin Cancer Res; 18(18); 1–7. ©2012 AACR.

Introduction

Germline mutations in the BRCA1 and BRCA2 genes account for a significant fraction of familial breast cancer and ovarian cancer cases (1). The BRCA2 gene, similar to BRCA1, seems to function as a tumor suppressor or cellular "caretaker." It encodes a 3,418–amino acid protein that participates in the maintenance of genomic stability (2, 3) through its interaction with RAD51, a central enzyme in the DNA double-strand break repair pathway (4, 5). The BRCA2 transcript possesses a 10.2 kb coding sequence spread over 27 exons, including the exceptionally large (4.9 kb) exon 11. The vast majority of reported deleterious mutations in BRCA2 introduce a premature termination codon (PTC) into the open-reading frame (ORF; ref. 6) and thus target the transcript for degradation by the nonsense-mediated mRNA decay (NMD) pathway (7).

Diagnostic screening of the BRCA1/2 genes in breast cancer families is mostly done on genomic DNA. For families with a very strong family history and no mutation identified in the coding sequences or exon–intron boundaries, our laboratory pursues the investigations by analyzing BRCA1/2 transcripts. Doing so, we have identified a new mutation located deep into intron 12, c.6937+594T > G, which systematically activates splicing of a cryptic exon introducing PTC in the coding sequence. This mutation was subsequently identified in 8 additional French families. Specific antisense oligonucleotides (ASO) were used to block the cryptic exon inclusion generated by this deep intronic mutation in vitro. Our results suggest that ASO treatment could be an effective approach for...
restoring expression of BRCA alleles carrying deep intronic mutations.

**Patients and Methods**

**Patients**

All patients attended a visit with a genetic counselor in a family cancer clinic either in Lyon, Saint-Etienne, Grenoble, Chambéry, Dijon, or Besançon, France. Genetic testing for BRCA1/2 was proposed to women based on individual and/or family history, as published (8). Index cases (i.e., the first family member in whom complete BRCA1/2 gene screening was done) gave their informed consent for genetic testing. Patients were first screened using DNA extracted from a whole-blood sample collected on EDTA by a combination of high resolution melting curve analysis (9), direct sequencing (10), quantitative multiplex PCR of short fluorescent fragments (11), and multiplex ligation-dependent probe amplification (MRC Holland; ref. 12).

**Translational Relevance**

Aberrant pseudoexon inclusion as a cause of human disease is more frequent than previously thought. These pseudoexon inclusions are ideal targets for RNA-based therapeutic technologies; splice-switching oligonucleotides targeted to the aberrant splicing elements have been shown to restore correct splicing and allow translation of the fully functional protein. Such oligonucleotides entered into clinical trials as a treatment for Duchenne muscular dystrophy. We report the identification of the first deep intronic mutation causing activation of a cryptic exon in one of the BRCA genes, more precisely in intron 12 of BRCA2. This mutation was subsequently identified in eight additional French families. Antisense oligonucleotides were used to suppress cryptic exon recognition in transcripts bearing a BRCA2 deep intronic mutation in transfected cells. Those findings open the possibility of using antisense therapy in the future as an alternative strategy for cancer prevention.

For mRNA analyses done as a second step, LCL were seeded at 400,000 cell/mL the day before puromycin or mock treatment. A total of 250 ng of RNAs isolated with the RNeasy midi kit (QIAGEN) were reverse transcribed using the Superscript III First-Strand Synthesis SuperMix (Life Technologies) with 65 ng of random hexamers and 210 ng of oligo(dT) primers. PCR was done with 2 μL of cDNA with Platinum Taq polymerase (Life Technologies) using primer pairs described in Supplementary Table S1; the 5 or 6 overlapping fragments generated, respectively, for BRCA1 or BRCA2 were separated on a 1.2% agarose gel and stained with ethidium bromide. Abnormal amplicons were purified and sequenced in both directions using the BigDye Terminator Cycle Sequencing V1.1 Ready Reaction kit on an ABI PRISM 3130XL Genetic Analyzer (Life Technologies). In some instances, the PCR fragments were cloned in bulk using the TOPO TA cloning kit (Life Technologies) before sequencing. Controls were always included in these experiments.

**Mutation characterization**

The BRCA2 exon 11/ex18 primer set gave rise to an extra PCR fragment with cDNA from the index case of Family A. Sequencing of this extra fragment showed that it contained a 95-nucleotide (nt) cryptic exon between exons 12 and 13 originating from intron 12 as determined by running a BLAST search. Genomic DNA from Family A index case was amplified with intron 12 primers: 5′-TGATGCGAT-TACCAGTCCAAGCTG-3′ (c.6937+348-c.6937+367) and 5′-GGGAATCCAAGCTGTAACAAA-3′ (c.6937+742-c.6937+761); the resulting 414-nt fragment containing the cryptic exon was sequenced. The full HGVS nomenclature for the identified mutation is the following: c.6937+594T > G (r.6937_6938ins6937+495_6937+589; p.Thr2314fs).

**Routine screening for the c.6937+594T > G mutation**

Genomic DNA was amplified with primers: 5′-GTGACAAAGCTTATTTCTATCATTGTTTTG-3′ (c.6937+495-c.6937+522) and 5′-TTCTTCCATTTGTGATTTTCTTTGG-3′ (c.6937+683-c.6937+710). The resulting 216-nt PCR fragment was analyzed by HRM and sequenced when showing an aberrant profile.

**Splice site score predictions**

The human BRCA2 exon 12 to exon 13 genomic sequence was analyzed for 5′ and 3′ splice sites using Splice Site Prediction by Neural Network (13, 14) and MaxEntScan (15, 16). Splicing enhancer and silencer elements were predicted using Human Splicing Finder (17, 18).

**BRCA2 minigene constructs**

The genomic sequence of human BRCA2 exon 12 to exon 13 was amplified by PCR from a Family A patient LCL using the following primers: 5′-CGGGATCCGGATGGGA-GAACCCTCAATCAAGAAGAACTAT-3′ (BRCA2 sequence underlined: c.7069-7095) and 5′-CGGGATCCGTTA-CGAAGGGTACACAGGTTACTCG-3′ (BRCA2 sequence underlined: c.7213-7234) and cloned into BamHI and...
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EcoRI sites in the pCDNA3.1 vector (Life Technologies). The wild-type minigene was obtained by site directed mutagenesis of the mutant minigene with the following primers: 5’-GTATGTTTCTAGGAAAATGTTTCTAGGAAAATAGCTAGATAG-3’ and 5’-GCTATCTACATTTTTCTATGAAAATCCTTTATCTGAAAGATAAC-3’. All constructs were verified by sequencing.

ASO transfection

HeLa cells were plated at 150,000 cells per well in 6-well plates. 0.4 μg of wild-type or mutant minigenes was transfected together with 100 nmol/L of ASO (TriLink Biotechnologies; 5’ splice site ASO: 5’-TCATGACATACATCCTTTAC-3’; 3’ splice site ASO: 5’-TTGTCCACCTACAGAAAAATA-3’; control ASO: 5’-TGAAGTCTTTCG-TATAAGTAG-3’) using Jetprime (Polyplus). Cells treated or not with puromycin were washed with PBS1X and collected 48 hours after transfection. All transfections were done in triplicates.

BRCA2 intron 12 splicing pattern analysis

One microgram of total RNA isolated from human LCL or transfected HeLa cells using the Nucleospin RNA II kit (Macherey-Nagel) was reverse transcribed using Expand Reverse Transcriptase (Roche Diagnostics) with 1 μg of oligo(dT) primers (Promega) according to the manufacturer’s instructions. The splicing pattern of BRCA2 intron 12 was analyzed by a 30-cycle PCR using Platinum Taq polymerase (Life Technologies) and the following primers: (i) for endogenous BRCA2 transcripts, a forward primer in exon 11 (5’-TTTTATCATGTTCCGAAAAT-3’) and a reverse primer either at exons 13 to 14 junction (5’-GACTTGGGTATTCGGCAGAC-3’); 3’ splice site ASO: 5’-TTGTCCACCTACAGAAAAATA-3’; control ASO: 5’-TGAAGTCTTTCG-TATAAGTAG-3’) using Jetprime (Polyplus). Cells treated or not with puromycin were washed with PBS1X and collected 48 hours after transfection. All transfections were done in triplicates.

Results

Mutation identification and characterization

Our laboratory provides a diagnostic test for identifying mutations in the BRCA1 and BRCA2 genes in breast and/or ovarian cancer families recruited by genetic counsellors mainly in the Rhône-Alpes region. For high-risk families, when the screening of the coding sequence and exon–intron boundaries turns out to be negative, this test includes a complete analysis of the BRCA1 and BRCA2 transcripts to identify, in particular, pre-mRNA splicing defaults. While analyzing a series of patients from high-risk breast cancer families by reverse transcriptase PCR (RT-PCR), we identified in one of them a BRCA2 mutant transcript whose sequencing showed the aberrant inclusion of a cryptic exon between exons 12 and 13. The 95-nt sequence of the cryptic exon corresponded to a sequence from intron 12 (r.6937+495_6937+589). Sequencing a 414-nt region of intron 12 encompassing the cryptic exon using genomic DNA from the index case revealed a deep mutation, c.6937+594T>G, also present in the affected sister of the index case (Fig. 1A).

We then screened patients belonging to more than 2,000 BRCA1/BRCA2 negative breast and/or ovarian cancer families for this mutation by using specific PCR primers and identified 8 additional families bearing the c.6937+594T>G mutation. These 9 positive families, among which 7 were breast only and 2 were breast/ovarian cancer families, contained 23 female breast and 2 ovarian cancer cases in total. Predictive tests were done in 6 families; in total, the mutation was identified in all of the 13 affected women tested, diagnosed with unilateral breast cancer at age 30, 31, 35, 37, 40, 45, 50, 57, 57, with bilateral breast cancer at age 41 and 44, or with ovarian cancer at age 40 and 52. This variant (rs191253965, genomic coordinates Chr13 32919384T>G in the GRCh37/hg19 assembly) was identified in 5 individuals in the course of the 1000 Genomes Project (http://www.1000genomes.org; ref. 19) in a dataset of 1,092 genomes.

The inclusion of the cryptic exon introduces PTC in the BRCA2 coding sequence (Supplementary Fig. S1A) and the mutant transcript therefore fulfils the requirements to be degraded by NMD (20). It could nevertheless be detected by PCR in the LCL from 2 patients belonging to distinct families carrying the c.6937+594T>G BRCA2 mutation and...
using primers located in surrounding exons (Fig. 1B, lanes 5 and 7), although in low abundance, and using primers specific to the cryptic exon (Fig. 1B, lanes 13 and 15). Upon puromycin treatment of the cells to nonspecifically inhibit NMD, the amount of mutant transcript considerably increased in the 2 patient cell lines (Fig. 1B, lanes 5–8 and lanes 13–16), suggesting NMD-mediated degradation of the mutant transcript. Transcripts containing the cryptic exon could not be detected by PCR in 2 control LCL with primers located in surrounding exons (Fig. 1B, lanes 1 and 2) but could be detected with specific primers (Fig. 1B, lanes 9–12), thus suggesting that this exon can be included in normal cells although at almost undetectable levels. In the control LCL treated with puromycin, the level of cryptic exon–containing transcripts increased (Fig. 1B, lanes 9–12), but never reached the levels observed in the patient LCL bearing the c.6937+594T>G mutation (Fig. 1B, lanes 13–16).

Cryptic exon characteristics

The BRCA2 mutation c.6937+594T>G is located within a preexisting 5’ splice site that defines, together with a strong 3’ splice site, the boundary of a cryptic exon (Fig. 2). The T>G mutation occurs at position +5 of the 5’ splice site and strongly enhances the splice site strength according to the splice site prediction algorithms (Neural Network and Max Entropy), rendering it identical to the consensus 5’ splice site sequence. Intron 12 contains many potential 5’ splice sites, together with wild-type (Fig. 3B, lanes 1–3) or mutant minigenes (Fig. 3B, lanes 4–6) and the resulting transcripts were analyzed by RT-PCR. Both 5’ and 3’ ASO were effective in promoting skipping of the cryptic exon in the mutant minigene (Fig. 3B, lane 4–6), indicating that the mutation is sufficient to promote inclusion of the cryptic exon in nearly all the transcripts produced by the mutant BRCA2 gene.

**In vitro characterization of splicing elements and oligo therapy assays**

To confirm that the BRCA2 c.6937+594T>G mutation alone is sufficient to promote the inclusion of the cryptic exon, we have generated 2 minigenes containing exons 12 to 13 including intron 12 (Fig. 3A) by amplifying the corresponding genomic region of a patient carrying the c.6937+594T>G mutation (mutant minigene) and then reverting the mutation by site-directed mutagenesis (wild-type minigene). Following transient transfection of the wild-type minigene, intron 12 was properly spliced (Fig. 3B, lane 3). In the presence of the c.6937+594T>G mutation, the most abundant transcript form contained the cryptic exon and the wild-type transcript was hardly detectable (Fig. 3B, lane 6), indicating that the mutation is sufficient to promote inclusion of the cryptic exon in normal cells although at almost undetectable levels. In the control LCL treated with puromycin, the level of cryptic exon–containing transcripts increased (Fig. 1B, lanes 9–12), but never reached the levels observed in the patient LCL bearing the c.6937+594T>G mutation (Fig. 1B, lanes 13–16).

Discussion

From the mid-1990s onward, screening of the BRCA1 and BRCA2 genes has been routinely done in genetic diagnostic laboratories to identify mutations predisposing to breast and ovarian cancer. All laboratories analyze the coding sequence and intron–exon junctions by direct sequencing or prescreening methods followed by sequencing. Most of them, at least in Europe, also search for large rearrangements using semiquantitative PCR-
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Figure 3. Correction of the cryptic exon inclusion by antisense oligonucleotides (ASO) in a BRCA2 minigene reporter system. A, schematic representation of the BRCA2 minigene containing exons 12 to 13 and the intervening sequence. PCR primers positions are indicated by arrows, ASO positions by combs. B, RT-PCR analysis of BRCA2 transcripts expressed after transient cotransfection of 5’, 3’, or control (C) ASO and of WT or mutant minigenes (i.e., carrying the c.6937+594T > G mutation) with primers indicated in A. Cells were treated with puromycin before lysis. The corresponding transcript structure is represented on the right. GAPDH was used as a loading control. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; WT, wild type.

Based methods such as MLPA (12) or QMPSF (11). In some laboratories, mRNA analyses are conducted for high-risk families as a second step when no mutation is identified on genomic DNA. Indeed, mRNA analysis represents an effective tool for the identification of yet undiscovered molecular defects of the BRCA1/2 genes such as gene inversion, or splicing alterations. With regard to the detection of this latter class of mutations, much progress has been accomplished in the recent years because of the development of in silico tools that can more or less efficiently predict the impact on splicing of variants of unknown significance (VUS) detected through the analysis of genomic DNA (25). However, splicing alterations resulting from deep intronic mutations are missed at the present time given that only approximately 100 nt of the intronic regions surrounding exons are investigated.

We report in the manuscript the identification of a new mutation located deep into BRCA2 intron 12, c.6937+594T > G, through the analysis of transcripts of an index case belonging to high-risk breast cancer families. This mutation activates a cryptic 5’ splice site and leads to the inclusion of a 95-nt exon that disrupts the BRCA2 ORF and introduces several PTC in the coding sequence. As expected according to the present accepted rules, most of the mutant transcripts are degraded by the NMD pathway, c.6937+594T > G was subsequently identified in 8 other French families using a dedicated PCR approach. Therefore, it seems imperative to evaluate the occurrence of this mutation in other regions of France and in other countries as well, as many recurrent mutations are shared among European populations.

Some splicing defects might not affect all the produced transcripts but merely enhance the occurrence of an alternative or a cryptic splicing event. The causality of variants leading to such partial effects is difficult to assess in molecular diagnostic as it is usually not known how detrimental they are. Here, 3 arguments are in favor of a deleterious effect of the c.6937+594T > G mutation: when NMD is inhibited, (i) in the absence of the mutation, the transcript containing the cryptic exon is present in negligible amount compared with the canonical isoform; (ii) in carriers, mutant and canonical transcripts seem to be expressed at approximately the same level; (iii) a minigene containing exon 12, mutated intron 12, and exon 13 expresses mainly a transcript containing the cryptic exon. We can deduce from these observations that the cryptic exon is nearly systematically included in transcripts deriving from the mutant BRCA2 allele in carriers of the c.6937+594T > G mutation, which can therefore be considered as a true deleterious mutation.

The therapeutic potential of ASO for correcting splicing defects that cause genetic diseases has been investigated in great depth in recent years. As all proteins are encoded by mRNA, ASO have the potential to target many diseases for which no drug is currently available. In this study, we were interested in splice-switching ASO blocking the access of the spliceosome machinery to RNA to module the splicing outcome. ASO have the potential to redirect a specific splicing event to prevent the production of a truncated or mutated protein, or to generate a specific protein isoform. Systemically delivered splice-switching ASO have been proven to be effective in vivo in animal models of β-thalassemia (26, 27), spinal muscular atrophy (SMA; ref. 28, 29), and Duchenne muscular dystrophy (30), and are currently tested in human clinical trials. Deep intronic mutations, like the one in BRCA2 described in this study, are the perfect candidates for targets for therapeutic correction as they are not located in the coding sequence or near the constitutive splice sites and can thus be corrected to restore the production of the normal protein. Improvements in the ASO chemistries that increased the stability, efficacy, and cellular uptake over the past few years (23) have made it possible to envision this type of therapeutic approach for a variety of genetic disorders. A typical ASO drug candidate is a modified approximately 20-nt long RNA molecule bearing chemical modifications to render it neutral and resistant to degradation by cellular enzymes, thus increasing the in vivo stability (23). Recent studies suggest that the newest ASO are stable for up to several months in animal models of SMA after single or double dose injections, which can be done subcutaneously to be distributed systemically or can be injected into a specific organ or compartment (29). ASO therapy as a preventive approach for carriers of BRCA1/2 mutations will not be achieved in the near future, but it is an attractive less invasive alternative to prophylactic mastectomy and ovariectomy that are the main preventive treatments today. Of course, additional studies will be required for each specific mutation to test, in appropriate animal models, the ASO efficiency and stability in vivo in the organ of interest, and to predict the potential clinical outcome and benefits for breast and/or ovarian cancer patients.
Implementation of technological innovations has had great repercussions on BRCA1/2 diagnostic testing over the years. Recently, sequencing capacities have grown massively with next generation approaches, and the systematic investigation of BRCA1 and BRCA2 intronic sequences is becoming feasible. However, this will increase the number of screened nucleotides by a factor of 14 and 8, respectively, and will result in the identification of growing numbers of VUS, which already represent a major challenge with regard to interpretation. For the time being, RNA analysis remains the most suitable approach to identify deep intronic mutations leading to splice defects that are more and more commonly identified in human diseases (31). Identification of the first BRCA mutation of this type opens the door to future-targeted therapeutic solutions for prevention of BRCA-related cancers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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