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The silent mutation nucleotide 744 G → A, Lys172Lys, in exon 6 of *BRCA2* results in exon skipping

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Abstract Germ-line mutations in *BRCA2* predispose to breast and ovarian cancer. Mutations are widespread throughout the gene and include disease-causing mutations as frameshift, nonsense, splicing mutations and large genomic rearrangements. However a large number of mutations, including missense, silent and intron variants are of unknown significance. Here, we describe the functional characterization of a silent mutation (nucleotide 744 G → A/c.516 G → A, Lys172Lys) in exon 6 of *BRCA2* in a Danish family with breast and ovarian cancer. Exon trapping analysis showed that the mutation results in skipping of exon 6 and/or both exon 5 and 6, which was verified by RT-PCR analysis on RNA isolated from whole blood of the affected patient. We therefore conclude that the *BRCA2* silent mutation Lys172Lys is a disease-causing mutation.

Keywords Breast cancer · *BRCA2* · Silent mutation · Splicing · Exon trapping · RT-PCR

Introduction

Germ-line *BRCA2* (MIM# 600185) mutations confer a high risk for breast and ovarian cancer [1]. *BRCA2* is involved in several molecular pathways including homologous recombination and cell cycle control (reviewed in [2]). The *BRCA2* gene spans approximately 70 kb and is composed of 27 exons, which encodes a protein of 3,418 amino acids. In Denmark several different mutations in *BRCA2* have been identified, but only one case of a large genomic rearrangement has so far been reported [3, 4]. Most mutations in *BRCA2* have been listed in Breast Cancer Information Core (BIC) (<http://www.research.nhgri.nih.gov/bic/>), and include pathogenic mutations as frameshift and nonsense, but also a large number of missense, silent and intron variants of unknown significance. Genetic counseling to families containing variants of unknown significance are problematic and predictive testing can not be offered to healthy relatives. Therefore, studies are needed to clarify the function of such mutations.

Here we report the functional characterization of a *BRCA2* silent mutation located in exon 6 (nucleotide 744 G → A/c.516 G → A, Lys172Lys) in a Danish breast cancer patient. The mutation results in exon skipping and can therefore be regarded as a disease-causing mutation.

Materials and methods

Patients

A 64-year-old woman with breast cancer was referred to genetic counseling, since breast and ovarian cancer was observed in her family. Following verbal and written consent, blood samples were collected from the proband

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for mutation screening and after renewed consent a third blood sample was collected for RNA analysis. The family history was verified using the registry of the DBCG (Danish Breast Cancer Cooperative Group), hospital medical records and pathology reports, and genetic counseling was provided for family members.

BRCA1 and *BRCA2* screening

Genomic DNA was purified from whole blood using the QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. *BRCA1* and *BRCA2* screening was performed as recently described [5]. Sequence variations were verified in a new blood sample. The *BRCA2* mutation is numbered according to GenBank accession number NC_000059, in which A in the AUG start codon has number 229. Moreover, the *BRCA2* mutation is numbered according to GenBank accession number NC_000013.9 using the guidelines from the Human Genetic Variation Society (<http://www.hgvs.org/mutnomen>).

In silico analysis

The following three splice site prediction programs were used to predict the effect of the *BRCA2* nucleotide 744 G → A/c.516 G → A mutation on the efficiency of splicing: www.fruitfly.org/seq_tools/splice.html, www.cbs.dtu.dk/services/NetGene2 and www.umd.be/SSF. The genomic sequence spanning the *BRCA2* nucleotide 744 G → A/c.516 G → A mutation was submitted according to the guidelines of each program and default settings were used in all predictions.

Exon trapping analysis

pSPL3-*BRCA2*-wild-type and pSPL3-*BRCA2*-mutant plasmids, containing *BRCA2* exon 6 and flanking intron sequences, were constructed by PCR using purified DNA from human blood samples and the following oligonucleotides: *BRCA2*-F, 5'-ATCACGAATTCTCCAGCAGCTGAAATTTGTGAGTAC-3' and *BRCA2*-R 5'-GATCACGGATCCAAATATGTTTTGGTGTCTGACGACC-3'. The PCR products were treated with *Eco*RI and *Bam*HI and cloned into the pSPL3 vector (Gibco-BRL). All constructs were verified by sequencing. COS-7 cells were cultured and transfected as recently described [6]. After 48 h the cells were harvested and total RNA was isolated using Trizol reagent (Invitrogen). First strand cDNA was synthesized using 1 µg RNA, 20 µM oligo(dT) primer and M-MuLV reverse transcriptase (New England Biolabs). The cDNAs were amplified with pSPL3 vector-specific primers (5'-TCTGAGTCACCTGGACAACC-3' and 5'-ATCTCATGTGGTATTTGTGAGC-3') and the PCR products were

resolved on a 2% agarose gel. All experiments were repeated at least two times.

RNA analysis

A fresh blood sample was obtained from the patient. Total cellular RNA was isolated with Trizol (Invitrogen) according to the manufacturer's instructions. For reverse transcription-PCR (RT-PCR), cDNA was synthesized using random hexamer primers and M-MuLV reverse transcriptase (New England Biolabs) as described by the supplier. The cDNA were amplified with the *BRCA2* specific primers 5'-CACAGTGAAAATAAATGGAT-3' and 5'-CATGAGGAAATACAGTTTCAGATGC-3' resulting in a 317 bp wild-type fragment. The samples were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Finally, the bands were purified, cloned into pCR-Blunt II-TOPO (Invitrogen) and sequenced using an ABI3730 DNA analyzer (Applied Biosystems).

Results

The proband was referred to genetic counseling, since she was diagnosed with breast cancer at the age of 37 and again at the age of 65, and her father was diagnosed with breast cancer at the age of 79. Moreover her father's sister had ovarian cancer around the age of 60. Due to the family history we screened the entire coding region and the exon-intron boundaries of *BRCA1* and *BRCA2* from genomic DNA from the proband by dHPLC and sequencing and for large genomic rearrangements by MLPA analysis. The analysis identified a nucleotide 744 G → A/c.516 G → A mutation in *BRCA2*. The mutation is a silent mutation (Lys172Lys), but occurs at the last base in exon 6. To indicate whether the mutation could be disease-causing or not, three different splice site prediction programs was used as recently suggested [7] to predict the effect of this mutation on splicing of *BRCA2* exon 6. Since all three programs predicted the mutation to have an effect on splicing, functional characterization of the *BRCA2* nucleotide 744 G → A/c.516 G → A mutation on splicing was performed. A fragment including *BRCA2* exon 5 (50 bp) and exon 6 (41 bp) containing either the wild-type or the nucleotide 744 G → A/c.516 G → A mutation, respectively, 225 bp of intron 4 (IVS4), whole intron 5 (89 bp), and 241 bp of intron 6 (IVS6) was cloned into the mini-gene vector pSPL3 containing exons from *HIV-tat* under the control of the SV40 promoter (Fig. 1a) [8]. The wild-type and mutant constructs was then transfected into COS-7 cells. After 48 h mRNA was purified and examined by RT-PCR. The splicing products were separated on a 2% agarose gel. The wild-type construct should yield a product

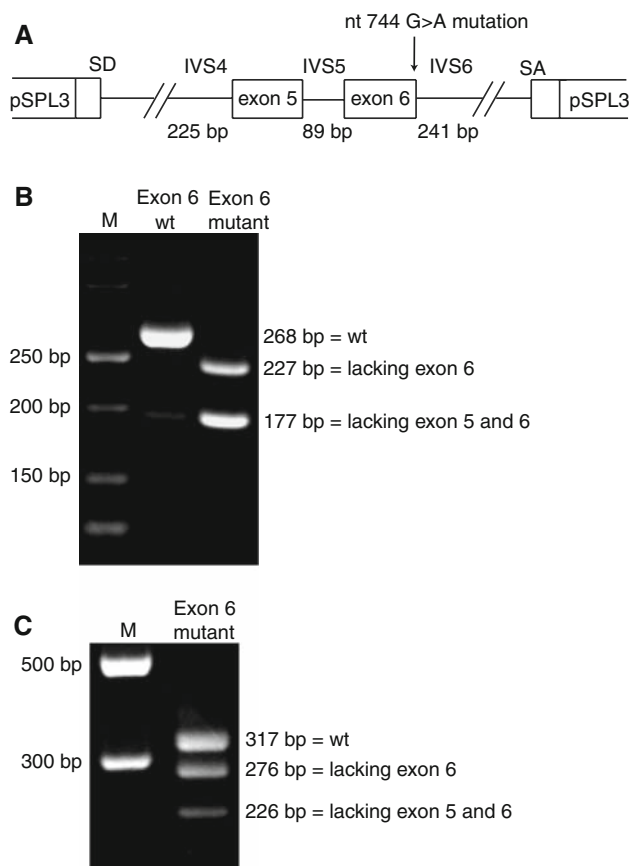


Fig. 1 Exon trapping and RT-PCR analysis. **a** Structure of the exon trapping vector pSPL3 including the *BRCA2* exon 5 and 6, 225 bp of intron 4, whole intron 5, and 241 bp of intron 6, respectively, containing the wild-type or the nucleotide 744 G → A/c.516 G → A mutation. **b** COS-7 cells were transfected with pSPL3-*BRCA2*-exon 6 wild-type or pSPL3-*BRCA2*-exon 6 mutant plasmids. Total RNA was isolated, RT-PCR analysis was performed and the PCR products were resolved on a 2% agarose gel. The 268 bp product corresponds to wild-type exon 6 (unaltered splicing), while the 227 bp product corresponds to the exclusion of exon 6, and the 177 bp product correspond to the exclusion of exon 5 and 6. The sizes of the DNA marker are indicated to the *right*. **c** RT-PCR was performed on RNA purified from whole blood from the proband. The cDNA was amplified with specific *BRCA2* primers. The sample was separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Three RT-PCR products (317, 276, and 226 bp) were obtained from the patient. The sizes of the DNA marker are indicated to the *left*. The PCR products were cloned and sequence analysis revealed that the 317 bp band corresponded to the wild-type allele, while the 276 and 226 bp bands corresponded to a transcript lacking exon 6 or exon 5 and 6, respectively

of 268 bp, while a product of 177 bp is expected if exon 5 and 6 is excluded from the transcript (exon skipping). The normal wild-type *BRCA2* exon 6 generated one transcript comprising the expected 268 bp and a very faint band of 177 bp, while the *BRCA2* exon 6 nucleotide 744 G → A/c.516 G → A mutant yielded two bands of 227 and 177 bp, respectively (Fig. 1b). Sequencing revealed that the 227 bp

band lacked exon 6, while the 177 bp band lacked both exon 5 and 6. To provide direct evidence for the aberrant splicing, RNA was isolated from whole blood of the affected patient and RT-PCR was performed before the products were analyzed on a 1% agarose gel (Fig. 1c). Three PCR products were amplified from the patient. Cloning and sequence analysis showed that the 317 bp band contained exon 5 and 6 and therefore corresponded to the wild-type allele, while the 276 and 226 bp bands lacked exon 6 or exon 5 and 6, respectively (data not shown).

Discussion

The splicing process is an essential step in the expression of most genes. Several intronic *cis*-acting elements are involved in the splicing of a typical intron. These include the splice acceptor site, the splice donor site, the branch point, the polypyrimidine tract as well as intronic splicing enhancers and silencers (ISE/ISS) (reviewed in [9]). Moreover exonic *cis*-acting elements known as exonic splicing enhancers and silencers (ESE/ESS) have been identified (reviewed in [10]).

In this study we examined a silent mutation (Lys172-Lys) present in *BRCA2* at the last base in exon 6 next to the splice donor site. The mutation is reported once in the BIC database as a variant of unknown significance. A few other mutations have been reported in this area, including nucleotide 744 + 7 A → G, nucleotide 744 + 10 C → T, nucleotide 744 + 18 T → C, nucleotide 744 + 18 T → G, and nucleotide 744 + 21 A > T (<http://www.research.nhgri.nih.gov/bic/>) [11], but the function of these variants are currently unknown. In contrast, the nucleotide 744 + 1 G → A mutation in the splice donor site has been shown to be disease-causing [12]. Splice site prediction programs predicted the *BRCA2* nucleotide 744 G → A mutation to disrupt the splicing of exon 6 and therefore functional studies of this mutation was carried out. Using exon trapping and RT-PCR analysis we show that the mutation results in skipping of exon 6 and of exon 5 and 6. The skipping results in a frameshift and a premature termination at codon 154 (skipping of exon 5 and 6) or codon 168 (skipping of exon 6), which most likely will result in nonsense-mediated mRNA decay (NMD) as previously shown for *BRCA1* and *BRCA2* transcripts with premature stop codons [13, 14]. We therefore conclude that the *BRCA2* nucleotide 744 G → A/c.516 G → A, Lys172Lys silent mutation can be classified as a disease-causing mutation. Predictive testing for the mutation is now being offered to family members at risk.

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