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CASE REPORT

Classification of the spliceogenic *BRCA1* c.4096+3A>G variant as likely benign based on cosegregation data and identification of a healthy homozygous carrier

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Key Clinical Message

BRCA1, c.4096+3A>G was identified in a consanguineous Danish family with several cases of breast/ovarian cancer. In silico analysis and splicing assays indicated that the variant caused aberrant splicing. However, based on segregation data and the finding of a healthy homozygous carrier, we classify the BRCA1 c.4096+3A>G variant as likely benign.

Keywords

BRCA1, classification, genetics, oncology.

Germline mutations in the breast cancer gene 1 [*BRCA1*, MIM# 604370] is associated with a lifetime risk of 40–87% for developing breast cancer and a 22–65% lifetime risk of developing ovarian cancer in women [1]. Male *BRCA1* carriers do not seem to have a significantly increased risk of cancer, however, some studies show a trend toward an increased risk of prostate cancer [2, 3].

More than 5000 variants in the *BRCA1* gene have been identified (BRCA Exchange and ClinVar databases: http://brcaexchange.org/; https://www.ncbi.nlm.nih.gov/clinvar/?term=brca1%5Bgene%5D) through mutational screening as *BRCA1* was identified and cloned more than 20 years ago [4]. Variants can be classified into five classes, where Class 1 encompass variants that are "not pathogenic" or of "no clinical significance," Class 2 includes variants that are "likely not pathogenic" or of "little clinical significance," Class 3 are variants which are of "uncertain clinical significance" (VUS), the variants in Class 4 are "likely pathogenic," and the Class 5 variants are qualitatively described as "definitely pathogenic" [5, 6]. The ENIGMA consortium (Evidence-based Network for the Interpretation of Germline Mutant Alleles) has tailored the IARC 5-tier classification system specifically to the assessment of *BRCA1/2* variants by incorporating information from splicing assays, in silico data, cosegregation data as well as data regarding co-occurrence *in trans* with a known pathogenic mutation [7]. In April 2016, the ENIGMA consortium had received more than 3000 submissions of individual VUS in *BRCA1/2* [8], underlining the need for further research in this field.

In silico programs can to some extent predict the expected consequence of a VUS, however to classify variants as pathogenic or benign cosegregation analysis or functional analyses must be applied.

A large number of *BRCA1* variants were recently found to cause abnormal splicing [9]. Variants in the consensus acceptor and donor splice site are usually creating an abnormal splicing pattern resulting in an mRNA that is degraded by nonsense-mediated decay [10]. For intron variants located close to the splice site, in silico programs can help ascertain the effect of the variant, but in order to classify intron variants, functional analysis such as RT-PCR of RNA from blood samples or minigene assays must be performed [11, 12]. Even when an abnormal

mRNA transcript is produced, it can be a challenge to determine whether this transcript is of clinical relevance or an event that occurs in a normal cell [13].

Rosenthal et al. [14] reclassified three variants, one in each of the *BRCA1*, *BRCA2,* and *MSH2* genes, all initially suspected of being pathogenic. Based on additional family history, testing and/or renewed search in the literature, these variants were classified as benign or intermediate risk. Furthermore, they concluded that there is a need for a discussion of how to handle reevaluation of variants and where the responsibility for informing affected family members lie [14].

Here, we present a case in which a spliceogenic *BRCA1* variant is classified as likely benign after further genetic testing in the family.

We report a consanguineous Danish family with multiple cases of breast cancer and ovarian cancer (Fig. 1). The proband (V:2) was diagnosed with a borderline mucinous cystadenoma of the ovary at the age of 35 years and was referred to genetic counseling. At the age of 45, the patient additionally developed a squamous cell carcinoma on the left side of her back. The borderline mucinous cystadenoma does not fall into the *BRCA1* phenotype spectrum; however, being a first degree relative to a breast cancer patient in a family with a history of breast and ovarian cancer, further investigation was indicated. The *BRCA1* and *BRCA2* genes were sequenced, and a heterozygous variant was identified at the third nucleotide in the intron sequence after exon 11 in the *BRCA1* gene (c.4096+3A>G). The variant was localized in close proximity of exon 11's donor splice site, and in silico splicing prediction (MaxEntScan) indicated that the variant destroyed the donor splice site. Deletion/duplication analyses by multiplex ligation-dependent probe amplification (MLPA) of the *BRCA1* and *BRCA2* genes were also performed with a normal result.

Previous RT-PCR analysis on RNA from blood samples has shown that the *BRCA1* c.4096+3A>G variant increased skipping of *BRCA1* exon 11 (c.671_4096del) and increased expression of Δ11q, an isoform using an internal splice donor site at c.788 in exon 11 in contrast to control samples [15]. However, the allele-specific transcript expression was not assessed. Moreover, the presence of naturally occurring *BRCA1* isoforms lacking exon 11 has recently been described [13], adding to the complexity of assessing the effect of the variant.

In the ClinVar database, reports classify the variant as either pathogenic, likely pathogenic or as a VUS, however using the ENIGMA classification system, we initially classified the variant as a VUS (Class 3). Therefore to further clarify the role of the *BRCA1* c.4096+3A>G variant, we decided to test other family members to see if the variant segregated with breast/ovarian cancer. However, the family members were informed that the variant was of uncertain significance beforehand. Family members who had inherited the variant were offered surveillance programs according to national guidelines for mutation carriers, whereas family members who had not inherited the variant were risk evaluated based on the family pedigree. The index patient's cousin (V:6), who had developed breast cancer at the age of 47, was tested and she did not carry

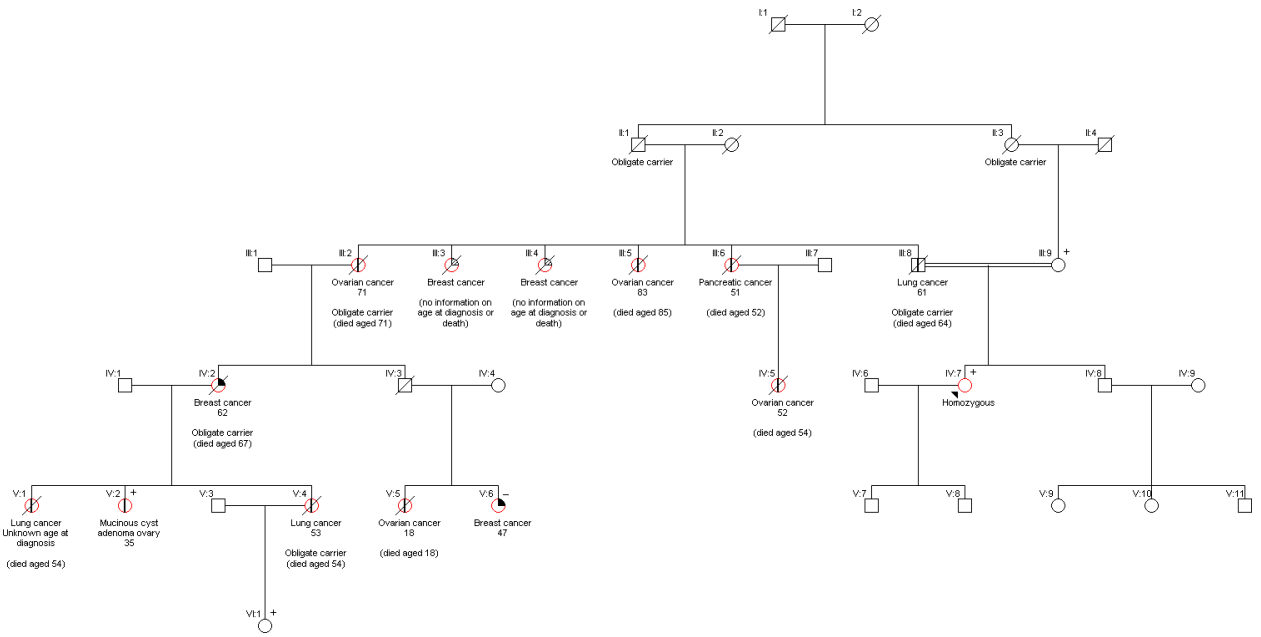

Figure 1. The pedigree of the family is shown and the diagnosis is noted under each individual with the age of diagnosis. □ : Male. ○ : Female. + : Carrier of the *BRCA1* c.4096+3A>G variant. – Not a carrier of the *BRCA1* c.4096+3A>G variant.

the variant. Complete screening of *BRCA1*, *BRCA2*, *PTEN*, *RAD51C*, *TP53*, and *CDH1* was performed, and no pathogenic mutations were identified.

A 58-year-old healthy cousin of the index patients mother (IV:7) was tested and found to be homozygous for the *BRCA1* c.4096+3A>G variant (Fig. 2). The finding was verified by the use of two sets of PCR primers as well as target sequence capture followed by NGS analysis.

Clinical examination of the cousin revealed a single café-au-lait spot on her right crus (present from early childhood), and no congenital malformations, no mental retardation or dysmorphic features, normal extremities, and especially normal thumbs with no additionally fingers or toes. Her height was 164 cm, corresponding to the height of her parents, and she had normal educational background and worked in the same research office for 40 years. A standard chromosome analysis was performed, showing a normal female karyotype and no visible chromosomal breakage.

She was related to the proband both on paternal side (III:8) but also on her maternal side (III:9) as her parents were first cousins. Her mother (III:9) was a carrier of the *BRCA1* c.4096+3A>G variant, and she was 87 years old and without cancer. It was not possible to test the father, but he was an obligate carrier based on the pedigree. This case is an example of a *BRCA1* variant which is assessed possibly pathogenic due to data from in silico predictions and splicing assays, but in which family testing revealed that the variant did not segregate with the disease and even more important a healthy 58-year-old female relative was shown to be homozygous for the *BRCA1* c.4096+3A>G variant (IV:7). Biallelic mutations in the *BRCA1* gene are very rare, and they cause severe disease, for example Fanconi Anemia, which is characterized by growth retardation, skeletal and organ malformation, aplastic anemia (caused by bone marrow failure), and increased risk of cancer development (particularly leukemias) [16, 17]. Besides a single "cafe-au-lait" spot, the clinical examination of IV:7 revealed none characteristics seen in Fanconi Anemia. In this case, IV:7 was homozygous for the *BRCA1* variant, and homozygosity for a pathogenic *BRCA1* mutation has traditionally been considered incompatible with life. Therefore, the likelihood that the *BRCA1* c.4096+3A>G variant is pathogenic is considered to be low. We therefore reclassified the variant as a class 2 variant according to the IARC 5-tier classification system, even though reduced penetrance of the variant cannot be excluded. In this regard, it should be noted that any *BRCA1* allele that permits 20–30% of tumor suppressor function recently has been suggested not to increase the risk of breast or ovarian cancer [18]. Correct evaluation of novel variants in genes associated with cancer is crucial in order to give patients and family members the correct treatment/surveillance. This is particularly important in a time where the use of exome/genome sequencing is increasing and as a consequence of this so are the number of reported variants in high-risk cancer genes. Ideally, a more elaborate setup should be rolled out, for example including functional analyses if possible (RNA sequencing or at protein level e.g., evaluating protein–protein interactions) when assessing a novel variant [19]. If functional analyses were performed, the nature of a novel variant may be assessed correctly initially, but this setup is not always possible at least in a diagnostic laboratory. If the nature of the variant cannot be assessed through further analyses Rosenthal et al. [14] suggests a compromise in which to opt for surveillance programs but not preventive surgeries. Family studies with cosegregation analysis are not always possible, but this reported case illustrates the importance of including this aspect in the assessment of pathogenicity of variants.

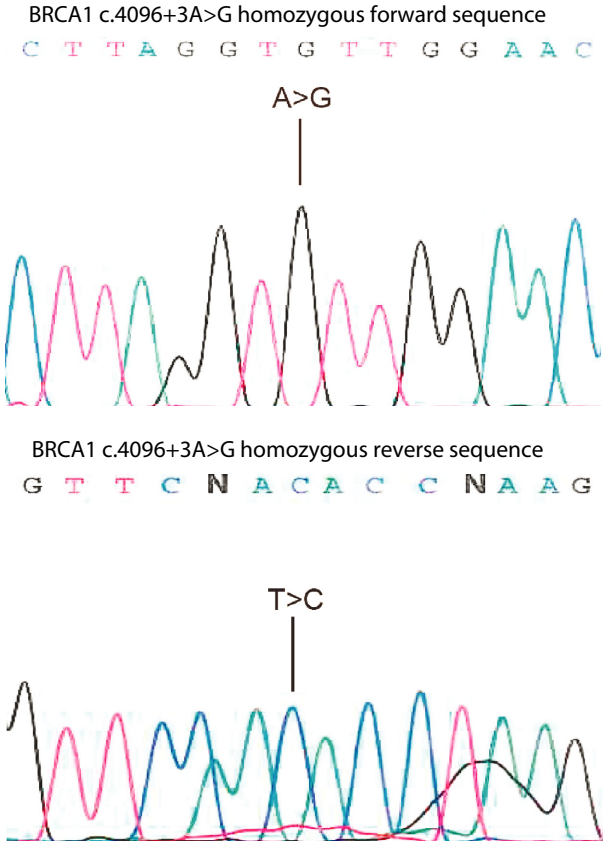

Figure 2. Identification of the *BRCA1* c.4096+3A>G variant in a homozygous carrier. DNA was purified from individual IV7, and Sanger sequence analysis was carried out on an ABI 3730 DNA Analyzer. Both the forward and reverse electropherograms are shown. The variant is indicated with an arrow.

Authorship

AMG: conceived the case. AB: drafted the initial manuscript. AY and TvOH: performed the genetic screening

and the in silico analysis of the variant. All authors contributed to the writing, rewriting, and revision process.

Conflict of Interest

The corresponding author has participated in a board meeting arranged by AstraZeneca. The other authors declare no conflict of interest. All authors had access to the data and played a significant role in writing the manuscript.

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