BRCA1 homozygous unclassified variant in a patient with non-Fanconi anemia: A case report

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Received March 13, 2017; Accepted November 20, 2017

DOI: 10.3892/ol.2017.7711

Abstract. The present case report discusses a woman affected by chronic lymphatic leukemia and breast cancer with a familial history of breast cancer; suspected to be hereditary breast and ovarian cancer (HBOC) syndrome. The patient underwent *BRCA1* and *BRCA2* genetic testing. Sequencing of *BRCA1* revealed the presence of the variant of unknown significance (VUS) c.3082C>T (p.Arg1028Cys) at homozygous state, whereas no mutations were detected in *BRCA2*. Multiplex ligation-dependent probe amplification confirmed the presence of two alleles. Although consanguineity between her parents was reported, which therefore supported the molecular data, her clinical phenotype was not suggestive of typical Fanconi anemia (FA), particularly of a *BRCA1*-linked FA. In the two cases reported in the literature, carriers of biallelic *BRCA1* mutation present a severe and quite typical phenotype. For this reason, the patient was offered a diepoxybutane test, where neither complex rearrangements nor multiradial formation were detected. We were therefore inclined to consider that *BRCA1* VUS as of little clinical significance.

Introduction

Even though the majority of cancers aren't linked to germline mutations, for some kind of tumours an inherited predisposition is known. In such situations, people carrying a mutation in specific genes are at increased risk of developing cancer. This is the case of the Hereditary Breast and Ovarian Cancer (HBOC) syndrome, where heterozygous carriers of a mutation in *BRCA1* or *BRCA2* genes face an increased risk of developing cancer in certain organs, especially breast (cumulative risk by age 70 years of 55 and 47% for *BRCA1* and *BRCA2*, respectively) and fallopian tubes/ovarian cancer (cumulative risk by age 70 years of 39 and 19% for *BRCA1* and *BRCA2*, respectively) (1). Even though heterozygous mutations in both *BRCA1* and *BRCA2* in the same individual have been seldom reported in HBOC syndrome, it is instead really rarer to observe in those genes homozygous or compound heterozygous carriers. To date the few described cases show a Fanconi anemia (FA) phenotype. FA is an autosomal recessive syndrome characterized by spontaneous chromosomal breaks and an increased sensitivity to radiation due to impaired DNA repair. FA patients usually present a wide spectrum of clinical features, mainly congenital anomalies, progressive pancitopenia and predisposition to malignancy. At present time, there are no less than 20 different genes linked to FA (where *FANCA* is the most frequently involved, and *BRCA1* is one of the rarest) and the list will probably grow in the future (2).

Because of the high genetic heterogenity of FA, in the case of a patient with a phenotype suggestive of FA, physicians usually prescribe laboratory test, like mitomycin C or diepoxybutane (DEB) test in order to confirm their clinical evaluation (3). If one of these tests detects the presence of a higher chromosomal breaks, a sequential study of each gene known to be causative of FA can be pursued until one mutation is detected in one of the FA genes.

Genetic testing has become quite common only in the last few years. Today we have a long way to go before learning about all genetic variations present in human being. For this reason, it is not so uncommon to detect a genetic variation never reported previously in PubMed or in gene-specific databases. In the 5-classes IARC (International Agency for Research on Cancer) classification, such variations belong to class 3, which means we are waiting for research studies aimed to demonstrate if they are pathogenic or likely pathogenic, a class 5 or class 4 variant, or not.

Such molecular results, called variant of unknown significance (VUS), represent a real challenge for clinicians. Because of the lack of knowledge of a possible link between cancer risk and the detected variation, clinical management usually should not be based on the molecular result of the test but on the patient's personal and family history of cancer (4).

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Key words: homozygous *BRCA1*, p.Arg1028Cys, Fanconi anemia, diepoxybutane test, hereditary breast and ovarian cancer syndrome

Here we present the case of a female patient referred to our cancer clinic because of her personal cancer history. The genetic test revealed the presence of a homozygous *BRCA1* VUS. The clinical phenotype of the patient is not linked to the typical FA features, and no complex rearrangements or multiradial formation were detected by DEB test, so we tend to consider that *BRCA1* VUS as of little clinical significance. Our data, together with further data that may be available in future studies, may therefore contribute to correctly define the clinical significance of this variant.

Medical history of our patient. A 62-year-old woman was referred to our genetic clinic because of her past tumour history. Chronic lymphatic leukemia was diagnosed at age 48 and treated with chemotherapy and anti-CD20 monocolonal antibodies. At age 50 she was treated with hysterectomy and bilateral oophorectomy because of a huge uterine polyp, while at age 62 she underwent left mastectomy and hormonal therapy because of a diagnosis of multifocal lobular carcinoma associated with lobular carcinoma *in situ* LIN2.

Her mother and both maternal aunts were diagnosed with breast cancer (respectively, at 71, 74 and 60 years old) (Fig. 1). Her father had leukemia at age 84 and one paternal female cousin developed breast cancer aged 50+. Nothing else was relevant in the family, except referred kindred between parents (even though they were not first cousins).

BRCA mutation carrier probability was low (2.4% according to BOADICEA, 0.22% according to CaGene), but because of family history of breast cancers, we decided to offer her genetic testing for *BRCA1* and *BRCA2*. During counselling with the geneticist, the patient was informed about BRCA testing and all the possible personal and familial implications in case of the detection of a BRCA mutation; the patient decided to sign informed consent and underwent blood sample drawing.

Materials and methods

Sequencing and MLPA. Genomic DNA (gDNA) was extracted from blood sample by MagCore Super (Diatech LabLine SRL, Jesi, Italy) using MagCore Genomic DNA Whole Blood kit. Genomic DNA obtained from the patient was used for *BRCA1* and *BRCA2* sequencing in order to search for point mutations and small insertions/deletions. All coding exons and the intron/exon boundaries of *BRCA1* and *BRCA2* genes were amplified by PCR. All PCR fragments were simultaneously amplified at the annealing temperature of 60˚C, with the AmpliTaq Gold kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Sequencing was performed on purified PCR products by using BigDye® Terminator v.3.1 Cycle Sequencing kit (Thermo Fisher Scientific, Inc.) and run on 3730Xl DNA Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.), after purification with Agencourt CleanSeq®-Beckman Coulter. Sequences were analyzed by Mutation Surveyor® Software (v5.0.1; SoftGenetics, LLC., State College, PA, USA).

Because heterozygous large deletions or duplications can go undetected by conventional PCR based sequencing of gDNA, we searched for possible rearrangements by using the MLPA assay. MLPA was performed by using SALSA P002(D1)-*BRCA1* MLPA kit and SALSA P045(B3)-*BRCA2* MLPA kit (MRC-Holland, Amsterdam, The Netherlands), following manufacturer's instructions. MLPA products were run on the 3730Xl DNA Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the Gene Mapper Module (Applied Biosystems; Thermo Fisher Scientific, Inc.). Results were then analysed by the Gene Marker Software (v2.6.3; SoftGenetics, LLC.).

In silico analysis of the missense mutation. Pathogenicity of the variant was predicted with bioinformatic tools based on the impact of aminoacid substitutions on the structure and function of proteins and on the degree of conservation of aminoacid residues along the same protein in different species. The effect of the variant c.3082C>T (p.Arg1028Cys) on *BRCA1* protein was predicted using: Mutation Taster (http://www.mutationtaster.org), PolyPhen (http://genetics.bwh.harvard.edu/pph), SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html), Align GVGD (http://agvgd.iarc.fr/agvgd_input.php) and HCI Breast Cancer Genes Prior Probabilities (http://priors.hci.utah.edu/PRIORS). Possible effect on mRNA splicing was predicted by using: MaxExScan (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html), Human Splicing Finder (http://www.umd.be/HSF), Gene Splicer (http://www.cbcb.umd.edu/software/GeneSplicer/gene_spl.shtml) and NNSPLICE v.0.9 (http://www.fruitfly.org/seq_tools/splice.html).

DEB test. In order to highlight the possible increased rate of chromosome breakage and radial forms, a DEB test was performed. The analysis was carried out following the European and Italian Guidelines (Specific Constitutional Cytogenetic Guidelines ECA July 2012-www.e-c-a.eu-, Linee guida diagnosi citogenetica 2013-www.sigu.net-). Peripheral blood lymphocyte cultures in the absence or presence of a nontoxic concentration (0.01 and 0.1 μg/ml) of DEB, chromosome preparation and QFQ staining were made according to standard procedures (5). A total of 100 metaphases were analyzed for each harvested cultures scoring: the rate of cells with chromosome breakage, the mean of chromosome/chromatid breaks/gaps, multiradial formations, acentric and dicentric fragments, rings, endoreduplicated chromosomes and premature chromosome condensation were evaluated for each metaphase. Case results were compared with those derived from the normal control.

Results

Using Sanger sequencing and MLPA we were able to find only the presence of the variant c.3082C>T (p.Arg1028Cys) in *BRCA1*. Unexpectedly, this VUS was detected at homozygous state. MLPA confirmed the presence of two alleles in the corresponding exon and the variant was detectable also with a different couple of primers in a further PCR reaction.

In our case, even though the presence of a homozygous *BRCA1* mutation was likely to be due to consanguinity in her parents, the result was not compatible with the typical clinical presentation of a FA, especially of *BRCA1* related-FA.

As we usually do, we invited the patient for a second visit in order to discuss the result of her test. Because clinical features of our patient suggested that the reported *BRCA1* variant was

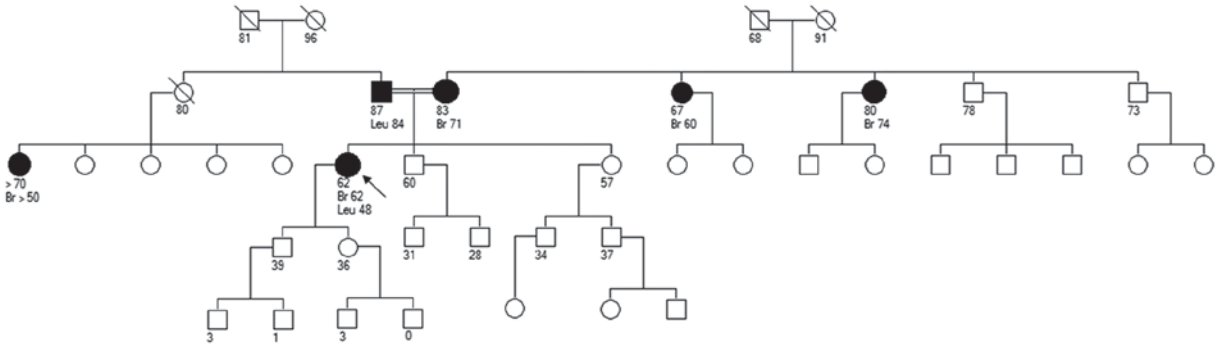

Figure 1. Pedigree of our patient, drawn with software CancerGene by University of Texas Southwestern Medical Center (Dallas, TX, USA) and The BayesMendel Group. The arrow indicates the proband. Asymptomatic subjects are represented with open symbols, while affected subjects with filled symbols; males are shown with squares, females with circles. Numbers below symbol indicate current age or age at death; where number is missing, age was unknown. Br, breast cancer; Leu, leukemia.

more likely a variant of little clinical significance instead of a pathogenic one, we invited the woman to undergo DEB test in order to definitively rule out FA.

The DEB testing was performed and a normal female karyotype was detected in all analyzed metaphases. The chromosomal instability has been evaluated in 100 metaphases without DEB, in 100 metaphases after incubation with 0.01 μg/ml DEB and in 100 metaphases after incubation with 0.1 μg/ml DEB. The frequency of chromosomal breakage detected with and without DEB was below 1%, and no complex rearrangement or multiradial formations were detected. The standard of our laboratory of chromosomal breakage is as high as 3%.

Discussion

The variant we detected is very rare and is now reported in ExAC database (http://exac.broadinstitute.org) with a frequency of 0.0025%.

This VUS shows substantial disparity of classification among open accessible database (6): in fact, it has been described as Likely benign in ClinVar, as a VUS in BIC and UMD. In LOVD 3.0 the variant has been reported as affecting function (+/?), according to evolutionary conservation analysis (7).

Bioinformatics tools, used to predict the impact of the VUS detected, gave conflicting results: Polyphen software predicted that the variant is possibly damaging (with a score of 0.587). The Polyphen scoring system considers a variant as 'benign' below the score of 0.5, as 'possibly damaging' between 0.5 and 0.85, as 'probably damaging' over 0.85. Mutation Taster and SIFT programs predicted that the variant is tolerated, based on sequence homology and the physical properties of aminoacids. HCI Breast Cancer Prior Probabilities predicted a weak or null probability of pathogenicity from damage to the protein sequence. Align GVGD class for this substitution is C15 (likely neutral). The tool combines the biophysical characteristics of aminoacids and protein multiple sequence alignments to predict where missense substitutions in genes of interest fall in a spectrum from enriched deleterious to enriched neutral. Possible effect on mRNA splicing was instead excluded by all the bioinformatics tools.

For all unclassified variants, it is important to collect useful data to correctly define the classification. The presence of this variant at homozygous state may probably help this difficult task.

Hypersensitivity to the clastogenic effect of DNA cross-linking agents can be considered as a unique marker for the diagnosis of FA. This cellular characteristic is still utilized as a diagnostic test to identify the preanemic patient as well as the patient with aplastic anemia or leukemia who may or may not have the common physical stigmata associated with FA; in this situation, we thought it could be useful also to provide additional data regarding the possible effect of the detected mutation and, therefore, its pathogenicity. A variety of chemical agents can be used to test for DNA cross-link sensitivity but DEB analysis is the preferred test for FA diagnosis, since it has the highest sensitivity and specificity compared to other agents (8,9).

Data obtained by DEB-test performed in our patient does not support the hypothesis that she is affected by FA. As ever with a laboratory test, also DEB-test has its own limits. For example, results interpretation of the chromosome breakage test may be complicated by the presence of a cellular mosaicism, a condition in which there are two cell populations, one with increased sensitivity to DEB and the other with normal levels of chromosome breakage.

Clinical features of our patient supported this negative DEB result. Therefore, we didn't advise the patient to undergo more intensive breast screening but to continue standard five years follow-up. Direct gene testing was not offered to other family members because the detection of that *BRCA1* VUS would not change their clinical management.

We decided to perform a literature review on FA, in order to see what are the typical presentations of a *BRCA1*-linked FA. Despite that the presence of a homozygous/compound heterozygous pathogenic mutation in *BRCA1* is commonly considered embryonic lethal, we were able to find just few case reports of carriers of such genetic defect.

The first ever reported patient (10) with a homozygous biallelic *BRCA1* mutation (c.2681_2682delAA; p.Lys894Thrfs*8) is a Scottish woman who developed breast cancer at age 32 and subsequently contralateral breast cancer. Nonetheless, Greenberg's equipe recently re-sequenced her DNA and the mutation was found only at heterozygous state. Therefore this Scottish woman actually does not have a homozygous biallelic *BRCA1* mutation.

The second report (11) is a woman who developed papillary serous ovarian carcinoma at 28 years old; the cancer was found to be extremely sensitive to the interstrand crosslinking agents like carboplatin. Additional features were adult height of 150 cm, developmental delay with limited speech at 4 years of age and dysmorphic features.

This woman had multiple relatives who developed cancer. The sister of the paternal grandfather and one paternal aunt were affected by breast cancer, while the sister of the maternal grandfather had bilateral breast cancer as well as ovarian cancer.

From the molecular point of view, this woman had a known deleterious mutation in *BRCA1* (c.2457delC; p.Asp821Ilefs*25), a VUS *in trans* on *BRCA1* (c.5209T>C; p.Val1736Ala) as well as a VUS in *BRCA2* (c.971G>C; p.Arg324Thr). Even though the co-occurrence in *BRCA1* of a VUS *in trans* with a pathogenic variant suggests that the VUS should not be considered clinically relevant, residue conservation across vertebrate species, loss of heterozygosity, DNA double strand break data lead Greenberg's equipe to consider the c.5209T>C variant as hypomorphic and therefore supported their hypothesys of a biallelic heterozygous mutations in *BRCA1*.

The last report (12) is still from Greenberg, who described the case of a woman with multiple dysmorphic features and anomalies, developmental delay, intellectual disability and a personal history of breast cancer at age 23.

The cancer familiy history was significant in the maternal side, since both the mother and one maternal aunt had ovarian cancer at the age of 50.

This woman was found to carry a frameshift mutation (c.594_597del; p.Ser198Argfs*35) on one *BRCA1* allele and a missense mutation (c.5095C>T; p.Arg1699Trp), previously described as pathogenic, on the other *BRCA1* allele. DEB test supported the diagnosis of FA and, therefore, the pathogenicity of the frameshift mutation detected.

For our team, DEB test's result and literature review supported the hypothesis that our patient was not affected by FA and therefore we were pretty confident to exclude a possible relationship between the *BRCA1* mutation detected in the woman and her personal history of cancer.

We offered to our patient a tailored surveillance program according to her personal and family history of cancer (rather than based to her molecular profile) and we did not suggest testing to her relatives.

If our clinical case observation will be confirmed by further evidence, this might contribute to better understanding and more appropriate clinical management of similar families.

Acknowledgements

Authors wish to thank the patient, as she accepted to trust in our lateral thinking plan (and undergo DEB testing) and for allowing us to publish her data so as to help her family and other people who may in future take advantage of the work that has been done. The authors would like to to thank Edda Opack for English revision of the study and Ms. Alessandra Rossi for technical support.

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