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Additional molecular and clinical evidence open the way to definitive IARC classification of the BRCA1 c.5332G > A (p.Asp1778Asn) variant

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ABSTRACT

Objectives: In silico splicing analysis, a mini-gene assay and splicing data, obtained using RNA from blood samples, have shown that the *BRCA1 c.5332G > A* variant induces exon 21 skipping. However, despite these evidences, up to date, this variant is unclassified.

The aim of this study is to provide further molecular and clinical evidence for the *BRCA1 c.5332G > A* variant in a patient with high grade serous ovarian carcinoma (HGSOC) to allow a definitive classification of this variant.

Design and method: The effect of the *BRCA1 c.5332G > A* variant on RNA splicing was evaluated by amplifying regions of *BRCA1* from the cDNA of the patient. Loss of heterozygosity (LOH) in tumor tissue was also investigated.

Results: The *c.5332G > A* allele causes significantly aberrant splicing of the *BRCA1* exon 21. Evaluation of the *c.5332A* allele in tumor tissue highlights a possible loss of heterozygosity, supporting her pathogenic effect.

Conclusions: Our results regarding the *c.5332G > A* variant confirm that it contributed to predisposition and onset of ovarian carcinoma in the patient. We propose to classify this variant as *'likely-pathogenic'* (class IV).

1. Introduction

Most hereditary breast and ovarian cancer (HBOC) is due to germline pathogenic variants (PVs) within the *BRCA1/2* (*BRCA*) genes [1]. *BRCA* PV carriers face important and challenging decisions regarding cancer prevention, screening and early detection, menopausal hormonal management, risk-reduction surgical and pharmacological therapy based on Poly-ADP-ribose polymerase inhibitors (PARP-1) [2].

BRCA PVs are either nonsense, small insertion or deletion variants, larger gene rearrangements, splicing variants, or severely dysfunctional missense substitutions. Even silent substitutions could be pathogenic if they have a severe impact on the regulation of mRNA splicing.

However, extensive molecular screening of *BRCA* genes by next generation sequencing (NGS) often reveals novel or uncertain significance variants (VUS) [3,4]. The identification of a VUS is associated with a complicated cancer risk assessment, genetic counseling, and clinical management of the patients and their families. As most VUS occur at very low population frequencies, direct epidemiological measures, such as association studies, are often not adequately powerful to identify the variants associated with cancer predisposition [5].

Recently a quantitative "posterior probability model" for assessing the clinical relevance of VUS in *BRCA* genes that integrates multiple forms of genetic evidence has been developed [6]. Subsequently Lindor et al. [7] provided a detailed review of the components of this model and explain how these can be combined to calculate a posterior probability of pathogenicity for each VUS.

In addition, many VUS are located in highly conserved functional protein domains or affect transcript start or splice sites, resulting in possible either deletion of the exon or retention of the adjacent intron [8]. For these types of variants, bioinformatics predictions and

Abbreviations: BRCA, *BRCA1/2*; VUS, Uncertain clinical significance; HGSOC, High grade serous ovarian carcinoma; LOH, Loss of heterozygosity; HBOCs, Hereditary breast and/or ovarian cancers; PV, Pathogenic variant; PARP-1, Poly-(ADP-ribose) polymerase; NGS, Next generation sequencing; IARC, International agency for research on cancer; RT, Residual tumor; BEV, Bevacizumab; FFPE, Formalin-Fixed Paraffin-Embedded; MAF, Minor allele frequency

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functional assays using either mRNA derived from mutation carriers or by employing minigene constructs [9,10] are used to guarantee their correct classification.

Only the whole of these information allows to classify these *BRCA* VUS as "*likely pathogenic*", "*pathogenic*" or "*likely benign*" variants in according with the IARC (International Agency for Research on Cancer) classification.

In this context, in silico splicing analysis along with a validated mini-gene splicing assay have been investigated for the *BRCA1 c.5332G > A* (rs80357112, p.Asp1778Asn) variant by *Ahlborn* et al. [11] demonstrating that the *c.5332A* allele induces *BRCA1* exon 21 skipping. These data were in agreement with splicing data obtained using RNA from patient blood samples [12,13].

However, despite these evidences, the *c.5332G > A* variant is reported six times as a VUS and once as a pathogenic in *Clin Var* [14] and in BRCA Share™ [15] and to date it results not yet reviewed in the *BRCA Exchange* database [16]. In addition, the *c.5332G > A* variant has been reported by *Cherbal* et al. [17] for the first time in co-occurrence in trans with the deleterious *BRCA1* variant *c.798 799delTT(*p.Ser267-LysfsX19) in young breast cancer patient (30 years old) with a strong breast cancer history.

For these reasons, this study aims to provide further molecular and clinical evidence for this variant in a patient suffering from high grade serous ovarian cancer syndrome. In fact, we provided evidence that *c.5332A* allele causes significantly aberrant splicing of the *BRCA1* exon 21. In addition, we studied the *c.5332G > A* variant at tissue level, highlighting a possible loss of heterozygosity, supporting her pathogenic effect.

In conclusion, this molecular approach could be help for a possible classification of the *c.5332G > A* as "*likely pathogenic*" variant, in order to allow patient to be administered with the PARP-1 inhibitors treatment and, finally, to identify healthy family members potentially at-risk, who could therefore start with a follow-up and personalized program of cancer prevention.

2. Case presentation

A 41-years-old patient with a medical history of stage IIIC ovarian cancer presented in our Hospital for suspicious of peritoneal recurrence. Her mother died at the age of 72 from cancer of unknown origin, suspecting an ovarian disease. The patient had been submitted to cytoreductive surgery 18 months before: a bloc pelvic resection was performed by total abdominal hysterectomy, bilateral salpingo-oophorectomy, pelvic peritonectomy, and rectosigmoid colectomy with colorectal anastomosis using a stapling device, diaphragmatic bilateral peritonectomy, omentectomy. At that moment, a RT (residual tumor) = 0 was achieved, while the histopathological examination revealed the presence of a poorly differentiated epithelial serous ovarian adenocarcinoma. The patient was tested for germline mutations in *BRCA* genes after written informed and signed consent. Postoperatively, the patient was submitted to six cycles of adjuvant platinum- and taxane-based chemotherapy, followed by 8 cycles of Bevacizumab (BEV) maintenance.

After 8 cycle of BEV, the imagistic studies performed for the increasing value of CA-125, revealed suspicious of peritoneal recurrent disease. At that time the CA-125 was 159 U/ml, where the normal range is < 35 U/ml. She underwent a laparoscopy where diffuse carcinomatosis with innumerable small bowel serosal and mesenteric nodules were noted. The tumor biopsy was evaluated for somatic *BRCA* status. The patient has been candidate to a second line of chemotherapy platinum based.

3. Materials and methods

3.1. Germline DNA/mRNA and somatic DNA extraction

Genomic and somatic DNA were isolated from peripheral blood and Formalin-Fixed Paraffin-Embedded (FFPE) HGSOC sections, respectively, using an automated device (MagCore HF16 Plus, Diatech Lab Line, Jesi, Italy).

Total RNA was isolated from peripheral blood using the TRIzol® protocol (Thermo Fisher Scientific, Waltham, MA, USA). DNA and RNA concentrations and quality were determined using Qubit™ dsDNA HS Assay and RNA HS Assay Kit, respectively (Life Technologies, Thermo Fisher Scientific Inc.).

3.2. BRCA testing

BRCA analysis was performed using the Devyser BRCA kit (Devyser, Hägersten, Sweden). Sequencing reactions were carried out on the MiSeq instrument (Illumina, CA, USA) and NGS data were processed using the Amplicon Suite software (SmartSeq s.r.l, Novara, Italy). In addition, *BRCA* large genomic rearrangements were also investigated, as reported [18,19].

3.3. Reverse transcription PCR (RT-PCR), cDNA detection and Sanger sequencing

We analyzed the patient's RNA to determine whether the *BRCA1 c.5332A* allele impaired the normal splicing process of exon 21.

Total RNA was retro-transcribed by employing the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Basel, Switzerland) using 500 ng of total RNA and oligo (dT) primers.

CDNA amplification was performed using the PCR Master Mix (Promega Corporation, Madison, WI USA) and a primer pair manually designed to amplify *BRCA1* exons 19–24. In particular, the forward (F-1: 5′-AGGCAAGATCTAGAGGGAACC-3′) and the reverse (R-1: 5′-GGG TCACCCAGAAATAGCTAAC-3′) primers annealed on the 19/20 exon junction and the exon 24, respectively. The amplified *BRCA1* WT and mutated alleles were distinguished by capillary electrophoresis on Experion™ Automated Electrophoresis System (BioRad, Hercules, CA, USA) following the manufacturer's instructions [20].

In addition, another primer which anneals on the 20/22 exon/exon junction (F-2: 5'-GAAAACTGGTATACCAAGAAC-3′) was used to demonstrate *BRCA1* exon 21 skipping. Primer's specificity was verified using Primer Blast site [21].

Sanger sequencing was performed with an Applied Biosystems 3500 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA). Sequences analysis was carried out with the SeqScape Software v2.5.

3.4. Tumor loss of heterozygosity (LOH) associated with the c.5332G > A variant

Since LOH is the footprint of the mechanism of tumorigenesis for patients inheriting a deleterious *BRCA* variant [22], we analyzed by NGS the FFPE sample of the disease relapse of the patient, to evaluate a possible tumor *BRCA* LOH and obtain further evidence of pathogenicity for the *c.5332A* allele in this patient.

4. Results

4.1. BRCA testing on germline and somatic DNA

The nomenclature of the *c.5332G > A* variant is based on the *BRCA1* cDNA sequence (NCBI Reference Sequence: NM_007294.3; *GRCh37*) (Fig. 1A), according to the recommendations of the Human Genome Variation Society. Sanger sequencing was used to confirm the presence of the *c.5332G > A* variant on a second independent sample

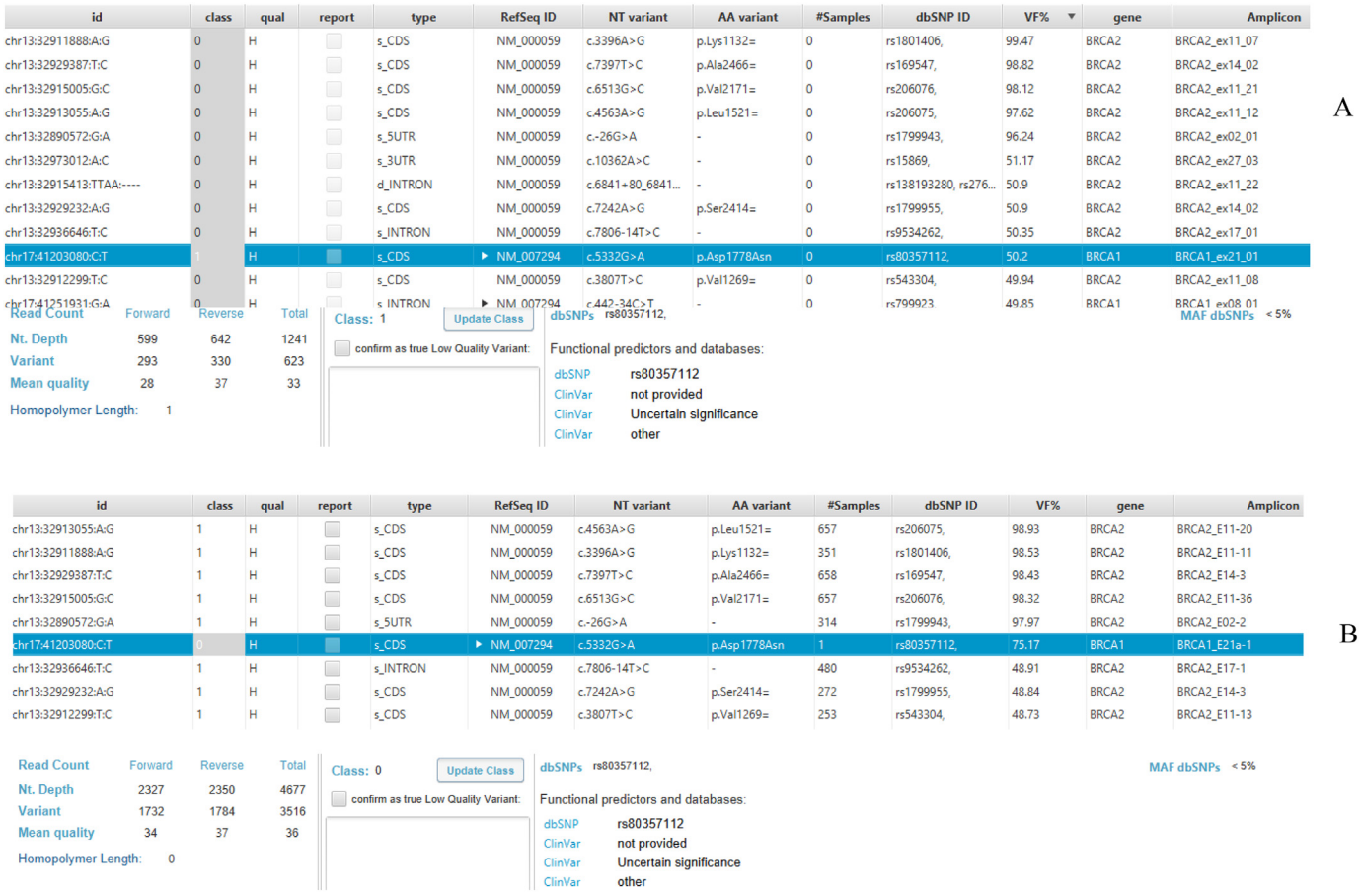

Fig. 1. NGS *BRCA* results: germline (A) and somatic genome (B) from blood and FFPE samples, respectively.

of the patient.

To date, it is reported in dbsnp [23] and neither reported in ExAC [24], gnomAD [25] and nor 1000G browser [26]. Recently, this variant is been reported once in a worldwide study containing data from approximately 29,700 unrelated individuals with different population origin [27].

BRCA testing on FFPE DNA (containing > 80% tumor cells, after histological revision performed by an expert pathologist) of the disease relapse highlighted the *c.5332G > A* with a variant allele frequency (VAF) of 75% (Fig. 1B), leading to support a possible loss of heterozygosity of the WT allele.

4.2. Sanger sequencing was used to confirm the skipping of the BRCA1 exon 21

All cDNA samples (patient and controls) showed an amplification product of 395 bp (Fig. 2A-B), corresponding to the expected amplification of the *c.5332G* allele. Moreover, an additional band shorter than full-length transcript appeared in the mutated cDNA of the patient (Fig. 2B). This event could be explained by an alternative splicing process of the exon 21, hypothesizing the skipping of this exon. To demonstrate this event, a second amplification was performed and Sanger sequencing was used to highlight that the additional band was the resulting of the amplification of the exons 20, 22, 23 and 24 (Fig. 2C).

5. Discussion

Screening for *BRCA* PVs is a common practice for high-risk breast and/or ovarian cancer families. However, test results may be ambiguous due to the presence of VUS in the concurrent absence of clearly cancer-predisposing variants. This scenario considerably hampers cancer risk estimation and clinical management. Therefore, it is necessary to functionally characterize and to develop appropriate VUS classification tools.

Many VUS represent putative splicing alterations and are assessed by in silico prediction and analyzed using mRNA derived from variant carriers. In many cases, RNA is not available from the patient and the sequence variant can be examined by mini-gene splicing analysis, which has been shown to be a valid method for investigating the impact of an alteration on the splicing pattern [9,10].

The *BRCA1 c.5332G > A* variant involves the AG dinucleotides at the 3′- ends of the exon 21 and in silico splicing analysis along with a validated mini-gene assay have demonstrated that the *c.5332A* allele induces *BRCA1* exon 21 skipping [11]. These data are in agreement with splicing data obtained using RNA from patient blood samples [12,13]: however, despite these evidences the *c.5332G > A* variant is to date unclassified. A possible cause of the non-definitive classification of this variant is the finding that natural occurring BRCA1 isoforms lacking exon 21 exist [28]. In addition, another possible cause is the absence of clinical data of patients carrying this variant. In fact, the *c.5332A* allele presents a global minor allele frequency (MAF) of 0.0006% as reported by Kaviar (Known VARiants) tool [29].

Furthermore, the *c.5332G > A* variant has been reported by *Cherbal* et al… [17] in co-occurrence in trans with the deleterious *BRCA1* variant *c.798_799delTT* (p.Ser267LysfsX19) in a young breast cancer patient (30 years old) showing a strong breast cancer history. *Cherbal* et al speculated on this finding to hypothesize that *c.5332G > A* is a benign variant. In light of all molecular evidence regarding the variant *c.5332G > A*, we believe that this statement is

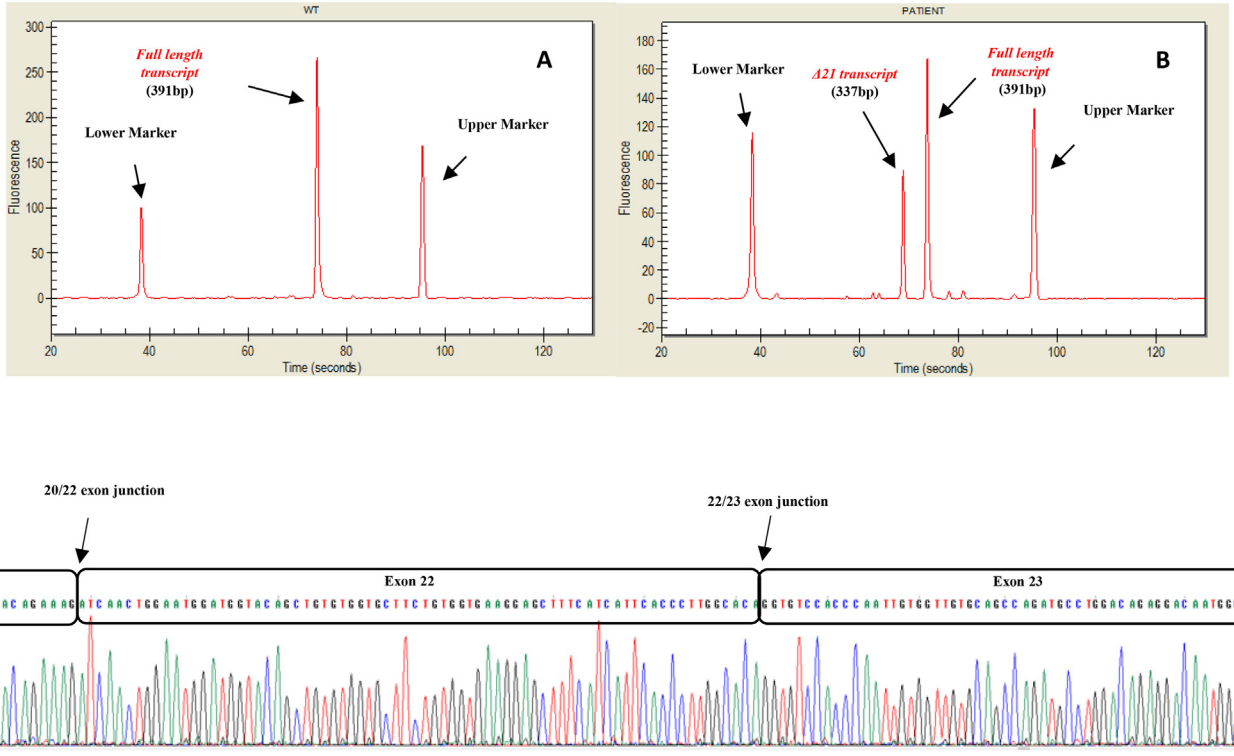

Fig. 2. Capillary electrophoresis of the WT (A) and proband (B) cDNAs and Sanger sequencing of the *c.5332A* allele (cDNA) (C).

misleading. In fact, it would be easy thinking that when a VUS segregates *in trans* with a deleterious variant, it can be reclassified as a benign variant. Nevertheless, in this case we cannot completely accept this interpretation: in fact, *Cherbal et* did not conduct the family segregation study of the co-segregating variants. We underline as young age (30y) of the *Cherbal's* proband and the strong family history can make us suspect that both variants have contributed to the onset of BCs in all family members. In this case the contribution of both variants to increased cancer risk could have been evaluated only through a detailed family segregation analysis. In addition, patients with *BRCA1*-related Fanconi anemia and very young onset breast or ovarian cancer were shown to be compound heterozygotes for a *BRCA1* truncating mutation and a *BRCA1* missense allele retaining partial DNA repair activity [30,31]. The lack of additional information on the family reported by *Cherbal* et al. does not completely exclude the presence of the Fanconi-like phenotype.

In this study, we report an Italian patient with personal history of ovarian cancer and carrying the *BRCA1 c.5332G > A* variant. We confirmed the presence of an aberrant transcript due to exon 21 skipping by using mRNA from blood of the patient.

In order to reinforce these data, we performed NGS by FFPE DNA to evaluate a possible LOH as footprint of the mechanism of tumorigenesis due to presence of the *c.5332A* allele.

6. Conclusions

To date the *BRCA1 c.5332G > A* variant is unclassified, although the unfavorable impact on splicing has already been demonstrated previously. Our data obtained on the patient's RNA confirmed that the variant strongly affects the splicing reactions of the exon 21. In addition, the LOH data obtained by FFPE seems to associate a pathogenic role for this variant.

Moreover, since the B*RCA1* variant c.*5332 + 1G > A,* that induces exon 21 skipping, is definitively classified as pathogenic in the *BRCA exchange* database although minor amount of naturally occurring transcript lacking this exon exists, we can try to classify *BRCA1 c.5332G > A* as *'likely pathogenic'* variant (class IV). Moreover, we are aware as the need of further molecular evidence, (as the possibility that the aberrant transcript produces both products (Δ21 and full length transcripts), could help to definitively demonstrate the deleterious effect of this variant.

Declaration of competing interests

The authors declare they have no competing interests.

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