High-resolution melting analysis coupled with next-generation sequencing as a simple tool for the identification of a novel somatic BRCA2 variant: a case report

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Abstract
In a 72-year-old woman with no associated personal or family history of breast and/or ovarian cancers, we identified a novel somatic pathogenic BRCA2 variant (c.18_28delAGAGAGGCCAA, p.Lys6Asnfs*4) using next-generation sequencing (NGS). The variant allele frequency (VAF) was 16%, and Sanger sequencing was unable to identify this variant. Adopting a high-resolution melting analysis strategy coupled with NGS, we successfully highlighted the presence of the c.18_28delAGAGAGGCCAA allele.

Keywords: High-resolution melting analysis; next-generation sequencing; novel somatic BRCA2 variant

Testing BRCA1/2 (BRCA) genes on formalin-fixed paraffin-embedded (FFPE) or fresh tissue (FT) samples permits the simultaneous assessment of both somatic and germline variants using an easily-accessible material that is routinely available in any pathology laboratory worldwide. FFPE and FT samples are histologically heterogeneous1, while tumor-specific DNA contains varying proportions of contaminating DNA from normal cells.

Next-generation sequencing (NGS) methods have the potential to detect variants at low admixture levels, offering a potential solution to this challenging type of analysis2. Because of the poor quality of extracted DNA and to a low sequencing signal, variants in DNA from FFPE and FT sources are difficult to confirm using Sanger sequencing. Furthermore, these types of sources cannot be re-analyzed by NGS because of the small amount of DNA. To avoid considering these variants to be PCR artefacts, it is highly recommended to use alternative methodologies.

In this context, we used high-resolution melting analysis (HRMA) as a simple, cost-effective, rapid and sensitive method to confirm a novel somatic BRCA2 variant (c.18_28delAGAGAGGCCAA, p.Lys6Asnfs*4) that was previously identified by NGS in a patient with high-grade serous ovarian cancer (HGSOC).

The present study involved a 72-year-old woman who presented to an oncologist with a complex right ovarian mass and elevated CA-125 level. Her gynecological history was negative. A transvaginal and transabdominal ultrasound examination revealed a multilocular solid cyst with >10 locules, papillary projections, and irregular surface with a Color Score of 4. Computed tomography of her abdomen and pelvis showed a 10 × 5 cm right ovarian mass and diffuse peritoneal enhancement, consistent with...
peritoneal carcinomatosis. Ovarian cancer was suspected, and the patient consented to complete surgical staging. She underwent a total abdominal hysterectomy, bilateral salpingo-oophorectomy, partial pelvic peritonectomy, and radical omentectomy 2 months after her initial presentation. The surgery was largely uncomplicated, with no significant hemostasis or coagulation issues, and optimal cytoreduction was achieved. Surgery was followed by six cycles of chemotherapy with paclitaxel and carboplatin. Written informed consent was obtained to allow BRCA testing to be performed after the pathological diagnosis of HGSOC was made.

DNA was extracted from FT HGSOC sections from areas with a minimum neoplastic cellularity of 70% using the MagCore Genomic DNA Tissue Kit by MagCore HF16 Plus (Diatech Lab Line, Jesi, Italy). The DNA concentration and quality were determined using a Qubit dsDNA HS assay (Thermo Fisher Scientific, Waltham, MA, USA). BRCA analysis was performed using the Devyser BRCA kit (Devyser, Hägersten, Sweden). Sequencing reactions were carried out on the MiSeq instrument (Illumina, CA, USA). NGS data were processed using the Amplicon Suite software (SmartSeq s.r.l., Novara, Italy) with the parameters of aligning reads to the HG19 reference genome and to generate run metrics, including the depth of sequencing, total read count, and quality. In addition, BRCA large genomic rearrangements were also investigated as previously reported.

Sanger sequencing and PCR-HRMA were performed on an ABI 3500 Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific) and the LightCycler® 480 Real-Time PCR System (Roche Diagnostics, Basel, Switzerland), respectively.

The WT allele is longer by 11 nucleotides compared to the c.18_28delAGAGAGGCCAA allele; this DNA size difference allows the allele separation by capillary electrophoresis.
For this reason, we analyzed the PCR products on an Experion™ Automated Electrophoresis System (BioRad, Hercules, CA, USA) following the manufacturer’s instructions. BRCA testing obtained by NGS and multiplex ligation-dependent probe amplification did not reveal any known pathogenic variants (PVs). However, the patient carried a c.18_28delAGAGAGGCCAA variant in exon 2 of the BRCA2 gene. The nomenclature of the variant is based on the BRCA2 cDNA sequence (NCBI Reference Sequence: NM_000059.3; GRCh37) according to the recommendations of the Human Genome Variation Society (HGVS, http://www.hgvs.org/). The average NGS read depth for the sample was ~10,000×, with a minimum and maximum depth of 2600× and 32,400×, respectively. The c.18_28delAGAGAGGCCAA allele showed a read depth of ~1400× on a total read count of 8700, resulting in a VAF of 16%.

Sanger sequencing, which was used to confirm the presence of the c.18_28delAGAGAGGCCAA allele, did not reveal this allele (Fig. 1). By contrast, high-resolution melting profiles for the patient showed a specific melting behavior compared to the FT samples (n = 10) that did not carry this variant. Each experiment is reported in duplicate. The same forward primer used for sequencing was used for PCR-HRMA, while the PCR-HRMA-reverse primer was 3’-TCAATTGAGAGATACATAGA-3’. The PCR-HRMA primers were designed using Primer3 software (http://bioinfo.ut.ee/primer3) and certified as high molecular-quality products via HPLC purification (Eurofin MWG Operon, Ebersberg, Germany).

Fig. 2 We used capillary electrophoresis to verify the presence of the c.18_28delAGAGAGGCCAA allele. Although it was difficult to distinguish the results because of this method’s lower resolution, amplification of the somatic mutated DNA gives two PCR products (b); the 218-bp peak of the WT allele and 207-bp peak corresponding to the BRCA2 c.18_28delAGAGAGGCCAA allele compared to the size marker (a). Normalized and shifted melting curves (c) and normalized and temperature-shifted difference plots (d) of the c.18_28delAGAGAGGCCAA allele are shown. Melting profile evaluation of the patient shows a specific melting behavior, as observed in both the normalized and the temperature-shifted and difference plots compared to the FT samples (n = 10) that do not carry this variant. Each experiment is reported in duplicate. The same forward primer used for sequencing was used for PCR-HRMA, while the PCR-HRMA-reverse primer was 3’-TCAATTGAGAGATACATAGA-3’. The PCR-HRMA primers were designed using Primer3 software (http://bioinfo.ut.ee/primer3) and certified as high molecular-quality products via HPLC purification (Eurofin MWG Operon, Ebersberg, Germany).
molecular diagnosis of this somatic pathogenic BRCA2 variant made our patient eligible for therapeutic treatments based on poly ADP ribose polymerase, which is a valuable option with promising activity in recurrent ovarian cancer patients and at the different stages of this disease.

**HGV Database**

The relevant data from this Data Report are hosted at the Human Genome Variation Database at https://doi.org/10.6084/m9.figshare.hgv.1946

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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**References**

1. Nik-Zainal, S. et al. Breast Cancer Working Group of the International Cancer Genome Consortium: the life history of 21 breast cancers. *Cell* 149, 994–1007 (2012).

2. Sims, D., Sudbery, I., Ilott, N. E., Hegar, A. & Ponting, C. P. Sequencing depth and coverage: key considerations in genome analyses. *Nat. Rev. Genet.* 15, 121–132 (2014).

3. Minucci, A. et al. Clinical impact on ovarian cancer patients of massive parallel sequencing for BRCA mutation detection: the experience at Gemelli hospital and a literature review. *Expert Rev. Mol. Diagn.* 15, 1383–1403 (2015).

4. Concolino, P. et al. Advanced tools for BRCA1/2 mutational screening: comparison between two methods for large genomic rearrangements (LGRs) detection. *Clin. Chem. Lab. Med.* 52, 1119–1127 (2014).

5. Minucci, A. et al. Identification of RFLP G6PD mutations by using micro-capillary electrophoretic chips (Experton). *J. Sep. Sci.* 31, 2694–2700 (2008).

6. Kaufman, B. et al. Olaparib mono therapy in patients with advanced cancer and a germline BRCA1/2 mutation. *J. Clin. Oncol.* 33, 244–245 (2015).

7. Minucci, A., Concolino, P., Giardina, B., Zuppi, C. & Capoluongo, E. Rapid UGT1A1 (TA)(n) genotyping by high resolution melting curve analysis for Gilbert’s syndrome diagnosis. *Clin. Chim. Acta* 411, 246–249 (2010).

8. Minucci, A. et al. High resolution melting analysis is very useful to identify BRCA1 c.4964_4982del19 (rs80359876) founder calabrian pathogenic variant on peripheral blood and buccal swab DNA. *Mut. Diagn. Ther.* 21, 217–223 (2017).

9. Kabalaike Nyia, M. H. et al. Sensitive high-resolution melting analysis for screening of KRAS and BRAF mutations in iranian human metastatic colorectal cancers. *Asian Pac. J. Cancer Prev.* 17, 5147–5152 (2016).

10. Cree, I. A. et al. Guidance for laboratories performing molecular pathology for cancer patients. *J. Clin. Pathol.* 67, 923–931 (2014).