Tumor BRCA testing in ovarian cancer and EQA scheme: our experience of a critical evaluation

Elisa De Paolis1 · Paola Concolino1 · Maria Elisabetta Onori1 · Concetta Santonocito1 · Claudia Marchetti2,3 · Anna Fagotti2,3 · Giovanni Scambia2,3 · Andrea Urban1,3 · Angelo Minucci1,4

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Abstract
Next generation sequencing (NGS) is a widespread molecular biology method integrated into clinical practice to detect genetic variants, for diagnostic and prognostic purposes. The scheduled external quality assessments (EQA) is integral part of clinical molecular laboratory quality assurance. The EQA provides an efficient system to compare analytic test performances among different laboratories, which is essential to evaluate consistency of molecular test. EQA failures demands targeted corrective action plans. In this context, the complexity of the NGS techniques requires careful and continuous quality control procedures. We report a tumor BRCA1/2 (tBRCA) testing benchmark discrepancy provided by the European Molecular Genetics Quality Network in our laboratory during a round of EQA for somatic mutation testing of BRCA genes in relation to ovarian cancer. The critical analysis emerging from the tBRCA EQA is presented. We underline that harmonization processes are still required for the EQA in the molecular biology field, especially if applied to the evaluation of methods characterized by high complexity.

Keywords Next generation sequencing · Tumor BRCA testing · Ovarian cancer · EMQN · EQA · Allelic dropout

Introduction
Next generation sequencing (NGS) are beginning to replace traditional molecular techniques [1]. Giving the implementation of NGS in clinical settings, best practice guidelines are now available to introduce quality management processes. Quality management includes policies established with the aim to provide accurate laboratory test, as internal quality control and external quality assessment (EQA) programs. In particular, EQA involves the examination of the laboratory procedures by a third-party accreditation process, and are available for the majority of well-established technologies [2]. It is an inter-laboratory comparison that may extend throughout all phases of a testing cycle including interpretation of results [3]. EQA provides the examination of different types of external samples depending on the scheme version. For the germline scheme, laboratories generally receive genomic DNA extracted from cell lines. In somatic schemes, genetically engineered samples are generally provided. These artificial controls can be obtained by homogeneously mixing mutant versus wild-type (WT) cell lines at defined allelic ratios, which closely mimics the formalin-fixed paraffin-embedded (FFPE) tissue block [4].
Tumor BRCA testing in ovarian cancer and EQA scheme: experience in our Molecular and Genomic Diagnostics Unit

In order to evaluate tumor \( BRCA1/2 \) (tBRCA) testing performed in our Molecular and Genomic Diagnostics Unit (MGDU) as a predictive biomarker of response to platinum-based-chemotherapy and PARP-inhibitors in ovarian cancer patients [5], we recently joined the disease-based Somatic Ovarian 2020 scheme of the European Molecular Genetics Quality Network (EMQN) [6]. The EMQN offers disease-specific and method-based EQA schemes when participating once a year to three different samples per EQA exercise. Participants are assessed on their ability to correctly genotype, interpret, and report the molecular results using their usual laboratory report format [2].

Our MGDU routinely assesses the \( BRCA \) molecular status using the amplicon-based library preparation kit Devyser \( BRCA \) (Devyser, Stockholm, Sweden) on Illumina MiSeq NGS platform [7, 8].

After uploading the EMQN scheme results, a discrepancy emerged between our \( BRCA \) genotyping and those expected in one of the samples provided by the scheme.

The validated sample genotype declared the presence of the \( BRCA1 \) NG_005905.2 (NM_007294.4):c.68_69del (rs80357914, p.(Glu23ValfsTer17)), variant with an expected variant allele frequency of 20%. Our NGS analysis failed to identify the presence of this variant in the sample.

Despite this evidence, we received an EMQN communication that explains the observed controversial result. The artificial FFPE tumor sample provided contained a cell line engineered to generate the \( BRCA1 \) c.68_69del variant. The engineering process used to generate the variant (undertaken by Horizon Discovery, Cambridge, UK), left a small piece of artificial DNA (the “engineering scar”) of approximately 90 bp in the intron upstream of the variant. The insertion site was positioned at the \( BRCA1 \) c.80+48_c.80+49 site and caused the mis-priming of a PCR primer used by the Devyser \( BRCA \) kit (Devyser AB, Stockholm, Sweden). Consequently, this engineering “scar” meant that this kit was unable to detect the \( BRCA1 \) c.68_69del variant for any laboratory using. This could potentially lead a laboratory to fail the EQA scheme; however, this did not occur because EMQN had detected the error during their assessment process and no laboratory using this kit was penalized. The technology used to make this artificial material has subsequently been superseded by the use of CRISPR system, which does not leave an engineering “scar”.

**Question:** Why did the Devyser kit not identify the c.68_69del variant in the EQA sample?

Taking into account that poor performance in the EQA represents a relevant alert, we decided to explore the discrepancy experienced in the EMQN exercise. In particular, we reported the experimental workflow adopted to evaluate the EMQN \( BRCA1 \) genotype via alternative methods considering the technical information about the artificially development of the external sample. We undertook this effort to exclude that the failure in the identification of the EMQN \( BRCA1 \) variant, attributable to the analytical incompatibility of the EQA scheme and the Devyser kit, cannot occur in human tissue samples in an appropriate clinical setting.

The c.68_69del variant falls into the exon 2 of the \( BRCA1 \) gene. The Devyser \( BRCA \) kit amplifies this region via two different amplicons, which are only partially overlapping. The \( BRCA1_E2-1 \) amplicon has the sense primer that matches the region including the insertion site c.80+48_c.80+49, precluding its effective amplification (Fig. 1). Because the NGS results were obtained only by the WT allele, as a consequence of the allelic dropout (ADO), we

![c.68_69del](image)

**Fig. 1** Integrative genomics viewer (IGV) visualizations of the \( BRCA1 \) sequence surrounding the region of interest (Human hg19) and containing the two partially overlapping Devyser amplicons \( BRCA1_E2-1 \) and \( BRCA1_E2-2 \)
missed the sequencing information regarding the c.68_69del allele. We decided to better understand the type of molecular lesion affecting the specific BRCA1 region. We firstly designed ad hoc primer pair including the c.80+48_c.80+49 site (Fig. 2). Sanger sequencing of this PCR product highlighted again a WT sequence demonstrating that the engineering extends beyond the insertion site (at least up to the antisense primer). Therefore, this molecular alteration cannot occur in extracted human genomic DNA samples. However, since some BRCA1 polymorphisms are reported in the c.80+48_c.80+49 position (e.g. c.80+48 C>T, rs200513210, and c.80+49 C>G variants), the possibility of a failure in the amplification of this region using the Devyser BRCA kit remains open. Certainly, the minor allele frequency of these polymorphisms seems to be very low and they are never reported in linkage with the c.68_69del variant.

Results
Identification of the c.68_69del variant by alternative PCR/NGS approach

Since the Devyser kit was unable to identify the c.68_69del variant, we investigated and confirmed the presence of the declared BRCA1 variant using an alternative PCR/NGS molecular approach. In particular, we adopted the Hereditary Cancer Solution CE-IVD kit (SOPHiA Genet- ics, Saint-Sulpice, Switzerland), a NGS capture-based target enrichment of 26 cancer related genes (ATM, APC, BARD1, BRCA1, BRCA2, BRIP1, CDH1, CHEK2, EPCAM, FAM175A, MLH1, MRE11A, MSH2, MSH6, MUTYH, NBN, PALB2, PIK3CA, PMS2, PMS2C, PTEN, RAD50, RAD51C, RAD51D, STK11, TP53, XRCC2). As shown in Fig. 3a, this kit correctly detected the c.68_69del variant (showing a variant allele frequency of about 8%). In addition, NGS data provides also information regarding part of the BRCA1 intron 2, including the c.80+48_c.80+49 site. Analyzing NGS results for this region (Fig. 3), a WT sequence was observed highlighting that the WT allele had been preferentially sequenced and confirming that the vector insertion region is wide extending beyond the c.80+48_c.80+49 site, as already assumed after performing Sanger sequencing.

Confirmation of the c.68_69del variant via by an orthogonal method: a targeted Sanger sequencing

In order to confirm the presence of the BRCA1 c.68_69del variant via Sanger sequencing, we designed an ad hoc PCR primer pair that ensured the amplification of the mutated c.68_69del allele. The antisense primer was designed to pair in the exon/intron junction of the BRCA1 exon 2, before the c.80+48_c.80+49 site generating a small fragment, compatible with DNA quality from FFPE material (Fig. 2). As shown in Fig. 4, this strategy allowed us to correctly identify the c.68_69del variant in the EQA sample.

Concluding remarks

During an EQA program from EMQN for tBRCA testing, our MGDUnit failed to identify the somatic BRCA1 c.68_69del variant. All laboratories that used the Devyser BRCA kit obtained the same result. The EMQN did not assign the category of poor performance to any laboratory that failed to detect the c.68_69del variant using this kit. They acknowledged that the material used contained an artificial engineering scar that caused mis-priming of a PCR primer. We decided to explore this result using alternative methods.

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**Fig. 2** The BRCA1 sequence surrounding the region of interest (LRG.292t1) is showed, with the exon 2 boundaries reported in bracket. The declared BRCA1 variant c.68_69del and the insertion site c.80+48_c.80+49 are also depicted (boxes). The primer pair designed for the first Sanger sequencing reaction are reported in italic as: forward: 5’-TGGATTTATCTG CTCTTCGC-3’; reverse: 5’-AGGAGATAATCATAGGAATCCC-3’. The primer pair used in the second Sanger sequencing reaction are reported in bold as: forward: 5’-GTGTTAAAGTTCATTGGAACAG-3’; reverse: 5’-TCT TGT GCT GAC TTA CCAG-3’. The latter allow the correct identification of the BRCA1 variant. The primers were designed by Primers 3 software (http://primer3.ut.ee/)
encompassing different PCR/NGS approaches in addition to the traditional Sanger sequencing technique. Firstly, an attempt using Sanger sequencing was made to identify the vector insertion region without success. Secondly, a different PCR/NGS method was used allowing to correctly identify the EMQN variant declared. Third, we confirmed the presence of the EMQN c.68_69del variant using a targeted Sanger sequencing designing ad hoc an PCR/sequencing primer pair.

Sequencing of BRCA genes is the prominent approach for routine screening of genomic variations in ovary, breast, pancreas and prostate cancer patients [9, 10]. Participation to EQA allows monitoring of laboratory performance in an inter-laboratory level. In addition, EQA participation is an integral part of the quality framework of diagnostic laboratories, required by the International Organization for Standardization (ISO 15,189) [11].

A crucial aspect of EQA is the nature of the external source of quality control material. The ideal EQA sample should be obtained from clinical specimens, most closely representing real testing in a clinical laboratory [2]. However, the use of FFPE clinical tissue samples for large-scale formal EQA studies is nearly impossible due to the limited amount of tissue samples available from a given patient. In addition, the fixation process usually yields degraded DNA and sequence artifacts are frequently detected. Consequently, appropriate quantities of high-quality and stable FFPE clinical EQA samples are difficult to obtain [2]. In light of these critical issues, artificial samples, engineered to contain variants of interest, are often used in EQA schemes [4]. Here, we underlined that the DNA sequences flanking the mutations of interest in the artificial fragment must be identical to the in vivo genomic DNA sequences. In fact, this feature should allow to confirm the NGS results using a Sanger sequencing approach. In this study, only ad hoc Sanger sequencing allowed confirming the EMQN NGS results.

Moreover, as here described, some anecdotal evidences reported in literature indicate that NGS could not always optimally perform with DNA isolated using some external source [2] and errors, commonly associated to the assay design, could occur. ADO is a common phenomenon that reduces the efficiency of PCR-based targeted sequencing. It was first described as a “partial amplification failure” causing a potential source of misdiagnosis for both dominant and recessive diseases [12]. The ADO phenomenon involves selective allele amplification during the PCR process. The presence of single nucleotide variants in primer binding sites may lead to the complete or partial lack of amplification of the single allele, while the second may “drop” out during the PCR. Generally, the amplicon-based NGS strategy consists in multiplex PCR producing overlapping amplicons and providing a full and uniform coverage of all exons and exon/intron junctions of genes. This method assures a higher coverage of insertions/deletions and also an accurate analysis of copy number variants. However, the ADO remains one of the most relevant technical limitation of different PCR-based methods, as depicted elsewhere [13]. Moreover, several cases of unrecognized polymorphic variants located in the binding sites of the amplification primer that preclude the detection of specific mutations, are reported in literature. For example, one EQA scheme for Cystic Fibrosis evidenced that a laboratory-developed test could not accurately detect the c.621+1G>T mutation declared in the quality assessment [14]. Similar evidence was reported for BRCA testing [15] and for hereditary hemochromatosis [16]. In all these cases, the involved laboratories used the observed discrepancies as an opportunity to review their analytical process. The experienced ADO caused by the mutagenesis method using the Devyser BRCA kit reinforce the need of a careful optimization of the NGS workflow. In our Devyser BRCA NGS diagnostic workflow, the monitoring of ADO events is obtained via the identification of a strong and deep deletion signal obtained in the NGS copy number variant evaluation involving an amplicon among all the overlapping amplicons covering one exon. In this type of suggestive situation, we perform an alternative NGS test using a capture-based method, as described for the purpose of this paper.

Considering that the library preparation kit used in our molecular workflow is an amplicon-based type, at first we suspected the occurring of an ADO event. However, in the light of what has been reported, the ADO turns out to be exclusively dependent on the artificial nature of the external EMQN sample. Our efforts to understand the unexpected EQA scheme result, underlined the educational aspect of the EQA process, which generally helps laboratories to detect errors in their protocols and adopt corrective strategies improving the molecular assay outcome.

**Summary**

In this paper, we focused on an EMQN scheme used for the EQA of somatic variant detection in BRCA genes. In this context, our MGDUnit experienced a failure in EQA performance. However, a careful and critical evaluation of
this error help us to better understand if corrective actions, as redesigning tests or adjustment of methods, are needed.

We underlined that the adoption of complex testing methods, as NGS, should require implementation or optimization of the actual EQA design. Ideally, major attention should be paid to the plethora of amplification and NGS approaches available, ensuring that the success of an EQA is independent from the type of NGS or library preparation approach used.

New EQA approaches could be useful to ensure the continued quality in case of advanced and complex technologies. For example, a new prospective emerges from EQA schemes that use FFPE samples derived from untransformed cells modified using the CRISPR/Cas9 system without dramatically altering the original DNA sequence [2].

In conclusion, an appropriate EQA material could be useful to assure high-quality NGS testing in clinical laboratory settings. With this regard, each laboratory should take part to EQA schemes in order to critically analyze and improve the quality of own work. We would like to point out that a critical approach is always recommended to test the laboratory skills and abilities.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study complied with the Ethical Principles for Medical Research Involving Human Subjects according to the World Medical Association Declaration of Helsinki and was certified by the Committee of the Applicable Institution of the Fondazione Policlinico Universitario Agostino Gemelli IRCCS, Rome.

Consent to publish All authors have given their consent to participate in this report and submit it to Molecular Biology Reports.

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