A commentary on the discrepancy between blood and tumor *BRCA* testing: an open question

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A commentary on the discrepancy between blood and tumor *BRCA* testing: an open question

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Running title: Understanding discordant *BRCA* test cases.

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Molecular evaluation of *BRCA1/2* (*BRCA*) genes represents a well-known example of precision oncology. The availability of Poly ADP Ribose Polymerase inhibitors (PARPi) as target therapy option for several *BRCA* mutated cancers types (e.g. ovarian, breast, prostate, and pancreatic)[1] changed the course of*BRCA* testing over the last years. In this context, an emerging path of molecular evaluation is represented by the *BRCA* testing performed directly on tumor tissue (t*BRCA*): this increased the chance to identify more patients with higher likelihood of benefiting from PARPi treatment. This approach leads to the simultaneous identification of both constitutional and somatically acquired variants, with a lower turnaround time: the identification of *BRCA*pathogenic variants (PVs) could lead to a secondary "reflex" germline*BRCA* (g*BRCA)* testing in order to assess personal and familiar risks. In contrast, performing g*BRCA* as first molecular test causes the loss of a relevant proportion of patients with tissue acquired *BRCA* PVs, needing of a following tumor test[2;3].

However, challenges exist in t*BRCA* that may lead to inefficient germline variant call. A recently published paper by Kordes *et al.* reported a pancreatic adenocarcinoma patient with a germline*novel BRCA2 c.516+4A>G* variant classified as deleterious by the authors based on *in silico* and functional data[4]. Also the tumor tissue was sequenced in order to achieve the enrolment criteria for a clinical trial of Olaparib in

combination with pembrolizumab (KEYLYNK-007). Unexpectedly, the germline variant was not stated in the final report. The authors took into account all the relevant basis of the experienced discrepancy, without identifying a confident reason.

In our opinion it is crucial to investigate about the reliability of t*BRCA* in the identification of both somatic and germline variants. Inspired by the recently published commentary of Gourley[5] and taking into account that several troubling cases of discrepancy between blood and t*BRCA* testing have been reported in literature, we collected the recent relevant studies covering the comparison between g*BRCA* and t*BRCA* to give a critical opinion about some shared key points of the somatic testing that could affect the final genotyping and reporting (Table 1).

Major reasons of discrepancies are related to: (1) differences in input DNA quality, (2) type of *BRCA* gene alteration, (3) inherent limitations of the Next Generation Sequencing (NGS), (4) bioinformatics pipeline features (e.g. the ability to predict the occurrence of Copy Number Alterations (CNAs) and the evaluation of the intron/exon boundaries), and finally (5) the issues related to the *BRCA*variants interpretation and classification.

To date, t*BRCA* testing is mainly performed on two sample types: Fresh Frozen Tissue (FFT) and Formalin-Fixed Paraffin-Embedded (FFPE). Here, we focused on t*BRCA* performed on FFPE being the most common tissue type used for clinical diagnostic purpose. Pre-analytical procedures regarding fixation step, tissue section size, and neoplastic cell content assessment, are well-known crucial aspects of the t*BRCA* testing reliability. In fact, sub-optimal DNA quality represents a relevant reason of inaccuracy of t*BRCA* and also it is the cause of around 5% of FFPE t*BRCA* NGS testing fails, with the consequent need of additional new samples[9]. In Bekos *et al.* only the retesting of a newly extracted tumor DNA solved two cases of discrepancies with g*BRCA* : the *BRCA1c.1881_1884del* variant was not recognized due to poor NGS quality data related to the input materials, as well as for the*BRCA2 c.8537_8538del* variant[2]. Also in Care*et al.* the test failure rate was related to fixation methods or storage of FFPE material[8]. *Ad hoc*recommendations for the "ideal" starting tissue material are available[9;14].

Furthermore, different approaches should be used in the analytical step for the *BRCA* genes amplification and sequencing, with several types of sequencing chemistries (e.g. amplicon-based, capture-based), platforms (e.g. Illumina, IonTorrent) and data analysis pipelines (e.g. full-coding regions or *hot spot* analysis, different size of splice site region analysed, CNAs detection). Each one of these could be characterized by specific pitfalls that affect the downstream bioinformatics variants filtering and calling. For example, in amplicon-based approaches, a reason leading to the missing of a variant detection may be related to the experimental design of the primers distribution along the genomic region of interest. Variants located at the 3' or 5' ends of overlapping amplicons could be covered by only one read and could be consequently identified with a "strand bias" flag and filtered out at the bioinformatics quality check[3].

Also the use of different bioinformatics pipeline for the NGS data analysis derived from the germline and the somatic tests of the same patient could be the cause of apparent inconsistent results. For example, in a large cohort of patients affected by several types of malignancies and analysed for the evaluation of the utility of germline test following tumor test, Lincoln *et al.* identified several cases of discrepancies between the two tests (n=4)[15]. Among these, the germline *BRCA2 c.8967_8973del* variant was not detected in tumor sequencing due to the characteristic of somatic panel (*hot spot* type), not comparable to the germline one. Moreover, in case of discrepancy involving splice site variants could be useful to check the concordance of the splice site region size included in the germline and somatic bioinformatics pipelines[3]. Regarding data analysis, it should be acknowledged that some tumor testing platforms filter out germline variants in the final reports in order to improve the accuracy of somatic variant calling.

A well-known cause of g*BRCA* /t*BRCA* non-concordance resulted from the challenge in the bioinformatics calling of CNAs in tissue samples[2;3;15]. NGS sensitivity in CNAs detection mostly depends on DNA quality, tumor heterogeneity, library preparation, type of algorithms, and size of rearrangement. As a consequence, the somatic bioinformatics pipeline must require computational algorithms developed *ad hoc* and specific

characteristics of sequencing raw data (e.g. maximum amount, coverage uniformity and sufficient reads depth)[1]. Even if the majority of methods are optimized for somatic CNAs identification[6;8], attention should be given in the comparison of blood and tissue tests results[13]. As an example, Bekos *et al.* failed to identify in the tumor sample a verified pathogenic germline deletion of *BRCA1* exon 20. Only a careful re-evaluation of the bioinformatics variant calls finally revealed the deletion and leaded to the correction of the report[2].

Relevant role in the evaluation of non-concordant results is played by the post-analytical step involving the *BRCA* variants interpretation. Complex issues underlying the classification of*BRCA* variants exist. The American Collage of Medical Genetics (ACMG) and the Association for Molecular Pathology (AMP) have established the best practice for germline variant interpretation providing the well-known classification using a five-tier system[16]. Conversely, the interpretation of somatic variants should be focused on their impact on clinical care. Specifically, evidence-based categorization of somatic variants released by the AMP, the American Society of Clinical Oncology (ASCO), and the College of American Pathologists (CAP) includes a four-tier system: (1) variants of strong clinical significance (level A and B of evidence); (2) variants of potential clinical significance (level C and D of evidence); (3) variants of unknown clinical significance; (4) benign of likely benign variants[17]. To date, with the publication of an increasing number of large-scale tumor sequencing projects, a plenty of information is being collected into several public databases useful for the querying about the significance of a*BRCA* variant. Cancer-specific variant databases are available as: BRCAexchange, OncoKB, Catalog of Somatic Mutations in Cancer, My Cancer Genome, cBioPortal, Memorial Sloan Kettering Cancer Center, International Cancer Genome Consortium, and VARSOME. Likewise, constitutional variant databases available are mainly: ClinVar, Human Gene Mutation Database, ENIGMA, Leiden Open Variation Database, and VARSOME. Differences in the germline- and somatic-based annotation may exist between the abovementioned tools. Consequently, the risk of non-concordant annotations of a *BRCA* variant could occur. This is crucial in the comparison between the same molecular test performed by different labs and it is exacerbated in the case of t*BRCA* and g*BRCA* concordance evaluation: variants that met germline guidelines[16] to be considered pathogenic may not meet the criteria[17] to be considered oncogenic in the somatic test. This situation could more likely affect the missense Variants of Unknown Significance (VUSs)[15]. As reported by Bekos *et al.* , after the inclusion of *BRCA* VUSs in the secondary data analyses, the concordance rate of tumor testing compared to germline one decreased, mainly due to VUSs classification[2]. Moreover, in a large study investigating the differences in variant interpretation between germline and somatic variants accounted in several cancer-related genes, Moody*et al.* highlighted a relevant percentage of discrepancies in variants classification. Among these, the authors reported four*BRCA2* variants with discordant somatic/germline annotations[15].

In a retrospective cohort of 57 subjects tested for both germline and somatic *BRCA* status, Kim *et al.* highlighted one case of a germline variant not identified in the tissue evaluation[10]. This discrepancy derived from a true reversion of the germline *BRCA1* variant accounted via restoration of the wild-type allele in the tissue cells. Finally, t*BRCA*should follow specific criteria that maximize molecular information, improving the clinical relevance of the test and giving a more comprehensive interpretation of each variant. With these purposes, peculiar role is played by the "naturally occurring" *BRCA*splicing isoforms. As we recently described for the $BRCA1c.788G>T$ variant, complex considerations should be done for rare variants that not only are different germline and somatic annotations, but also are characterized by variability in final effect and annotation in the context of all gene relevant transcripts[19].

In conclusion, we underline as the systematic and careful checking of tumor tissue suitability could prevent and solve non-concordance cases. Moreover, the robust identification of *BRCA* variants in FFPE sample correlates with the confidence of the bioinformatics pipeline adopted for the variant filtering and calling, especially for the CNAs detection. In addition, translation of variant calls into clinical decisions relies on proper annotations and discrepancies in classifications of specific variants between tumor and germline contexts could represent a relevant pitfall.

We argue that only harmonized guidelines encompassing the abovementioned methodological and post-analytical steps could solve the *BRCA*germline and somatic testing bias. In our laboratory, *BRCA*genetic

testing is routinely performed on blood, FFT and FFPE samples[1]. In many cases, we routinely analyze matched blood and tissue samples belonging from the same patient, in order to perform an efficient *BRCA* test comprehensive of both germline and somatic evaluation. This approach pointed out also the relevance of multi-disciplinary and skilled resources for a solid molecular characterization of the tumor. Together with the need of standardization, we suggest as performing *BRCA* molecular test at both germline and somatic levels in the same laboratory could improve the reliability of the entire molecular path taken by the patient and his clinicians.

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Table caption list:

Table 1. Comparative studies between germline and tumor *BRCA* testing results.

The table shows the recent relevant studies investigating the concordance in the identification of *BRCA* germline variants between germline and tissue tests. For each reference study, the number of subjects with paired tumor and germline *BRCA* tests is reported, together with the cancer and specimen types. According to the study, we reported the methodological pipelines adopted, if available. The table also shows details about the germline findings not reported by tumor test.

Contribution to authorship

Initial draft of manuscript, RM; manuscript writing and

approval, RM and FG

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