BIOMARKERS OF HOMOLOGOUS RECOMBINATION DEFICIENCY IN THE ERA OF PARP INHIBITORS

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ABSTRACT

Homologous Recombination Deficiency (HRD) was initially described in cancers with germline mutations of BRCA1 and BRCA2 and thereafter in both sporadic and hereditary cancers carrying mutations or epigenetic inactivation of other genes involved in HR. Since cancers harbouring HRD are particularly susceptible to PARP inhibitors (PARPi), identifying methods to detect HRD that can accurately predict clinical sensitivity to PARPi beyond BRCA1/2 mutations has been challenging. In this review, we describe the HRD biomarkers identified up to now, pointing out strengths and weaknesses of each associated assay. Multigene panel testing, genomic scar assays and the most recent functional assays developed in the last ten years are associated with several drawbacks, mainly due to the possible restoration of HR proficiency and tumor heterogeneity. The use of functional assays on samples obtained from liquid biopsy could overcome these issues, providing a dynamic readout of HRD status and helping in clinical decision-making especially in the recurrent setting. Composite HRD scores involving two or more biomarkers would be probably required to define “HRDness” and to predict response to PARPi alone or in combination regimens.
INTRODUCTION

Homologous recombination (HR) is a fundamental pathway that allows error-free repair of double-stranded DNA breaks (DSBs). HR operates during S and G2 phase of the cell cycle when a homologous sister chromatid is available as template and relies on many proteins including BRCA1 and BRCA2, MNR complex (MRE11/RAD50/NBS), RAD51, ATM, ATR, PALB2, BRI1, and BARD1 (1). HR deficiency (HRD) induces activation of the more error-prone template-independent non-homologous end-joining (NEJH) pathway, which results in the accumulation of additional mutations and chromosomal instability (2).

HRD was initially described in cancers with germline mutations of BRCA1 and BRCA2 (BRCA1/2) (3). However, germline or somatic mutations or epigenetic inactivation of other genes involved in HR can lead to HRD in both sporadic and hereditary cancers, broadly termed BRCAAness (4, 5). Cells with mutant BRCA1/2 are exquisitely sensitive to poly-(ADP-ribose) polymerase (PARP) enzyme PARP inhibitors (PARPi) (8, 9). The PARP1 subunit binds single-stranded DNA breaks (SSBs) and then organizes their repair by synthesising PAR chains on target proteins (the so-called PARylation) (10). Inhibition of PARP1 promotes SSBs, which, if unrepaired, consequently lead to DSBs by collapsing of the stalled replication fork during DNA replication (11). PARPi act mainly in a double way: by inhibition of the catalytic activity of PARP1, which results in synthetic lethality in cells with impaired HR, and by trapping PARP1 at sites of DNA damage (12, 13).

Other mechanisms of HR impairment beyond BRCA1/2 mutations can similarly confer PARPi sensitivity; however, identifying methods to detect HRD that can accurately predict clinical sensitivity to PARPi has been challenging (14-16). BRCA1/2 mutations and/or HRD status have been evaluated in clinical trials with PARPi (16-18). Multiple genomic biomarkers have been evaluated to presume the presence of HRD; although promising, these biomarkers are inadequate predictors of response to PARPi, with clinical benefits observed both with and without HRD as measured by current clinical assays (19). In this review, we aim to describe the HRD biomarkers identified up to know, pointing out strengths and weaknesses of each associated assay, and key challenges in the clinical use of HRD testing. An overview of HRD assays and their biological principles are summarized in figure 1.

GERMLINE AND SOMATIC MUTATIONS IN HR-RELATED GENES

Testing for germline and somatic mutations in BRCA1/2 and other HR-related genes may be used to infer the presence of HRD. Germline BRCA1/2 (gBRCA) mutations are present in 13-15% of epithelial non-mucinous ovarian cancer (OC) patients and an additional 5-7% of OC harbour somatic BRCA1/2 (sBRCA) mutation that have arisen during cancer development or progression (20, 21). BRCA1/2 mutant cells show clear evidence of HRD in vitro (22, 23). The main randomised clinical trials indicate that gBRCA mutations remain the best clinical biomarker for response to PARPi while limited data are available on sBRCA mutations alone, although the clinical outcomes for patients with sBRCA mutations were similar to those with gBRCA mutations (24-33). Retrospective analysis from Study 19 identified bi-allelic inactivation in > 80% of cases of sBRCA mutation and mutations were predominantly clonal, suggesting that sBRCA mutations arise early in tumorigenesis (34).

In vitro studies showed that beyond BRCA1/2, mutations in other HR-related genes can also confer an HRD phenotype and increased sensitivity.
to platinum and/or PARPi (35). Cancer-associated mutations in *PALB2*, *BARD1*, *BRIP1*, *RAD51B*, *RAD51C*, *RAD51D*, *ATM*, *FAAP20*, *CHEK2*, *FAN1*, *FANCE*, *FANCM*, and *POLQ* (20,36,37), are potential biomarkers of HRD in cancer but how much these genes impact on PARPi response in vivo is still being defined due to the relative rarity of non-BRCA HR-related genes mutations (19). For example, mutations or methylation of *RAD51C* were identified in OC patients with clinical PARPi responses (38), and patients harbouring *RAD51C/D* mutations had long-term responses with rucaparib (39). *ATM* pathogenic variants are associated with olaparib response in OC and prostate cancer (40, 41). Germline genetic testing is recommended for all women with OC, ideally with genetic counselling (18, 42). The blood-based assay Myriad Genetics BRACAnalysis CDx platform (Myriad Genetics; Salt Lake City, UT) has been FDA approved to identify OC patients with suspected pathogenic gBRCA variants eligible for treatment with olaparib (43). The phase III studies of PARPi in OC (Study 19 (33) and the NOVA trial (24)) and breast cancer (BC) (OlympiAD (25)) used BRACAnalysis to establish gBRCA mutation status (table I). Multigene germline panels, which extend the analysis to other genes as-

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<td>ARIEL3 (28)</td>
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**Table I.** HRD biomarkers and relative assays used in clinical trials of PARPi in ovarian cancer.
GENOMIC SCAR ASSAYS

The loss of HR function and consequent defective DNA repair induces chromosomal aberrations, irrespectively of which component of the pathway was lost. “Genomic scars” of HRD consist of specific patterns of mutations and structural chromosomal aberrations, including rearrangements, insertions, and deletions in the genome (51). Current genomic scar assays are based on a combination of different genomic profiling techniques including array-based comparative genomic hybridization (aCGH), single nucleotide polymorphism (SNP) genotyping, and next generation sequencing (NGS).

aCGH of structural chromosomal rearrangements

The aCGH assay detects genomic copy number variation (CNV) in tumors (52). An aCGH genomic profiles analysis of primary BC identified four subgroups, two of which were enriched for BRCA1/2 deficiency (53). However, only two-thirds of BRCA1-like tumors harbour either BRCA1 mutation or promoter methylation. A BRCA1-like aCGH signature predicted favourable response to platinum, suggesting that this signature identifies a wider spectrum of HRD tumors (54). The BRCA1-like and BRCA2-like profiles were later combined to create a BRCA-like aCGH score that was evaluated ret-

PLATINUM SENSITIVITY

Platinum sensitivity in vitro is a feature of HRD, and BRCA1/2 mutant OC and BC have increased platinum sensitivity (7, 47). As platinum is a key component of first line chemotherapy in OC, prior platinum sensitivity has been considered a surrogate clinical marker for prediction of PARPi efficacy (48). For example, in the phase III NOVA trial, Niraparib conferred a benefit in all subsets of platinum-sensitive OC, also in non-BRCA mutated patients (24). However, PARPi sensitivity does not completely overlap with platinum sensitivity in all cases (figure 2) (26). Considering cancers with defects in nucleotide excision repair, the response to platinum therapy does not confer a concurrent PARPi sensitivity (49). On the other hand, there is also a fraction of platinum-resistant patients who maintain PARPi sensitivity (50).
respective in a BC clinical trial, where BRCA-like aCGH patients showed a statistically significant benefit from high-dose platinum-based therapy (55). Up to now, aCGH assays have not been evaluated in the context of PARPi.

**SNP-based “genomic-scar” assays**

In 2012, three studies reported SNP-based CNV assays to assess up to three types of genomic scarring patterns (56-58). Loss of heterozygosity (LOH) is the absence of one of two gene alleles at a heterozygous site or uniparental disomy due to inaccurate repair of sister chromatids during the S/G2 phase of cell cycle. A study in OC detected that a “HRD-LOH” score defined by the number of LOH regions of more than 15 Mb and shorter than the whole chromosome was associated with BRCA1/2 deficiency (56). Large-scale transitions (LST) are chromosomal breaks between adjacent genomic regions longer than 10 Mb (after exclusion of region shorter than 3 Mb). Break points may be caused by chromosomal inversions, deletions, duplications, translocations, or other rearrangements. BRCA1/2 and RAD51C deficient BC show higher LST than sporadic cancers (57, 59). Telomeric allelic imbalance (TAI) considers subchromosomal regions displaying allelic imbalance extended to one of the telomeres but not crossing the centromere longer than 11 Mb. TAI is consequence of aberrant chromosomal end fusion due to inappropriate end-joining during mitosis. TCGA data show elevated TAI in gBRCA mutated OC and higher levels of TAI correlates significantly with response to neoadjuvant platinum-based chemotherapy in triple-negative BC (58). HRD-LOH, LST, and TAI are correlated each other (60) and are associated with BRCA1/2 deficiency independently and when combined into a single score (56-61).

Several combination HRD score have been described (62), with most data for the three-factor combination scar assay by Myriad Genetics (60). The Myriad myChoice test joins a combined HRD score called “Genomic Instability Score” (GIS) with mutation and rearrangement analysis of BRCA1/2. GIS consists of the unweighted sum of LOH, LST, and TAI which produces a continuous score between 0 and 100. A threshold for GIS was decided on a pooled set of BC and OC in which HRD was defined as biallelic BRCA1/2 loss of function. A score of 42 corresponded to the 5th percentile of the set of known BRCA-mutant tumors, therefore a score of ≥ 42 was established to denote HRD and a score of < 42 was considered HR-proficient (63). Several PARPi clinical trials have incorporated the myChoice test (**Table I**), with a score of ≥ 42 considered HR deficient in most trials (24, 29, 32). This assay was FDA-approved as a companion diagnostic for niraparib in relapsed OC and for olaparib with bevacizumab in newly diagnosed patients following front-line therapy (19).

The FoundationFocus™ CDx BRCA LOH assay was applied in clinical trials of rucaparib (28, 38) (**Table I**) and it has been approved as a complementary diagnostic to determine tumor HRD status. In this assay, a percent genomic LOH is calculated based on the fraction of genome regions with LOH. The optimal cut-off from analysis of OC (56) was 14% genomic LOH, which was prospectively validated in the ARIEL2 study, where progression-free survival was longer in the LOH-high subgroup compared with LOH-low (38). In a subsequent phase III study of rucaparib ARIEL3, the cut-off was adjusted to 16% genomic LOH as the threshold to identify HRD tumors (28).

The combined HRD score and the percent genomic LOH only partial correlated in predicting HRD status (64). Both HRD tests have several drawbacks, since they estimate the likelihood of HRD in the tumor based on evidence of genomic scarring. However, genomic alterations induced by HRD are permanent, even if functional capacity of HR is restored, for example in case of reversions in BRCA1/2, hence HRD testing via one of these assays on archival tumor may not represent the current HRD status of the cancer cells. Furthermore, HRD test results may not perfectly predict PARPi response due to PARPi resistance mechanisms which overcome HRD. Finally, HRD tests can have false positives or negatives due to technical factors, empiric threshold to classify HRD patients not accurate for all and heterogeneity in HRD between biopsy site and other disease sites (19).

**NGS-based mutational signatures**

Cancer types carry distinct mutational signatures which reveal the impact of different mutational processes including aging, UV light, and DNA damage repair and replication defects. A set of mutational signatures were detected from whole-exome sequencing of human tumors using NGS and computational technologies (65, 66). One of these, “signature 3” is enriched in cancers with BRCA1/2 mutations and other mechanisms of HRD and has
been shown to exist in several cancers, including BC, OC, pancreatic, prostate, and gastric. It has been proposed as a biomarker for HRD (67, 68). A computational tool called Signature Multivariate Analysis (SigMA) can identify the presence of signature 3 on targeted gene panel data and does not require whole-exome sequence data. However, the sensitivity for identification of signature 3 is only 74% (67).

HRDetect (69) was developed using whole genome sequence data from BRCA mutant and wild-type (control) BC samples. The algorithm uses information from all four aCGH genomic profiles and incorporates a weighted score of microhomology mediated deletions, base substitutions/rearrangements signatures and the HRD score (as used in Myriad myChoice HRD). Using a probabilistic cut-off of 70%, HRDetect predicted BRCA deficiency with a sensitivity of 98.7% in BC and reaching 100% in OC and pancreatic cancer validation cohorts. There is some evidence that the HRDetect score can predict clinical outcome and response to platinum therapy in BC but its ability to predict PARPi benefit has not yet been established (70, 71).

There is strong pre-clinical evidence that mutation-based assays that use information from multiple mutation types could outperform existing scar assays (for example the GIS had a sensitivity of 60% (63)). A major limitation, however, is the need of fresh frozen material while most trial samples are formalin fixed paraffin embedded (FFPE). A second limitation is that mutation-based assays remain genomic scar assays, so they by definition reflect the historical presence of HRD and do not provide information about current HR status that can be restored through different mechanisms as above mentioned.

**FUNCTIONAL ASSAYS**

Functional assays have the potential to provide a dynamic indicator of the actual HR status, giving the challenge of measuring a single downstream event that would reflect proficiency of multiple upstream components of HR (16). The most used experimental system in this setting has been quantification of RAD51 nuclear foci. RAD51 is a DNA recombinase which act as a downstream HR protein facilitating DNA strand invasion into the sister chromatid and consequent faithful DSBs repair. Reduced DNA damaged-induced nuclear RAD51 foci has been associated with BRCA1/2 deficiency as well as PARPi responses, both in OC and BC laboratory models and in small cohorts of patient samples (72-74).

One of the most frequently used RAD51-based functional HRD tests that has been validated on different tumor and specimen types is the RE-combination CAPacity (originally termed Repair CAPacity) or RECAP test (75-78). However, this test relies on the use of fresh tumor tissue and requires ex vivo induction of DNA damage; so, a RAD51 score on FFPE tumor tissue has been developed (79-81). The RAD51 score is dependent on the combination of two parameters: the percentage of geminin-positive GMN+ cells (an S/G2 phase cell proliferation marker (82)) with RAD51 foci and the number of RAD51 foci per nucleus. In BC samples, an RAD51 score threshold of 10% GMN+ cells with RAD51 foci and a cut-off of five foci/nucleus showed the best correlation with PARPi response in gBRCA1 patient-derived xenografts and gBRCA1/2 patient samples (79). This outcome was confirmed by a second study that identified all BRCA1/2-deficient BC tumors as HRD (80). An RAD51 score threshold of 15% GMN+ cells with RAD51 foci and a cut-off of five foci/nucleus showed the best correlation with PARPi response in gBRCA1 patient-derived xenografts and gBRCA1/2 patient samples (79).

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**FUTURE PERSPECTIVES**

Although promising, the current available biomarkers (multigene panel testing, genomic scar and functional assays) are inadequate predictors of response to PARPi, with clinical benefits observed both with and without HRD (84). Moreover, the current HRD assays do not provide a dynamic readout and are only valid for the time point at which the cancer tissue sample is obtained, usually at diagnosis, and do not consider tumor heterogeneity. Considering cancer’s capacity to continuously evolve and to develop therapy resistance, functional assays are expected to be able to detect acquired resistance to PARPi due to HR restoration in HRD tumors. The so-called liquid biopsy that sample circulating tumor cells or circulating tumor DNA may overcome these issues. Hence, analysing feasibility of functional assays on multiple and serial samples obtained from liquid biopsy could majorly impact in clinical decision-making in the recurrent setting.

Several mechanisms of resistance to PARPi have been suggested, with only reversions in BRCA1/2 clinically proved; however, other mechanism different from HR restoration have been described in preclinical models (85). Moreover, several ongoing clinical trials are investigating the combination of PARPi (especially Olaparib) with inhibitors of the replication stress, particularly ATR inhibitors (86), in order to elicit additive or synergistic effects and possibly overcome PARPi resistance. Given the complexity of the HR pathway and its interaction with cell cycle regulation, response to stress replication, and other DNA damage repair pathways it is unlike that one single biomarker will suffice: in all likelihood, composite HRD scores involving two or more biomarkers would be required to define “HRDness” and to predict response to PARPi alone or in combination regimens.

**CONCLUSIONS**

The need of predictive markers of response to PARPi is raising alongside with the increasing use of PARPi in clinical practice and the emerging of resistance to these agents. However, the current available biomarkers to infer the presence of HRD, including multigene panel testing, genomic scar and functional assays, are not able to accurately predict clinical sensitivity to PARPi. In the next future, the implementation of composite HRD scores involving multiple biomarkers identified on tumor samples from liquid biopsy will be challenging.

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**Availability of data and materials**

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**Authors’ contribution**

All the authors contributed equally to conception, data collection, analysis and writing of this paper.

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