How to measure homologous recombination deficiency in ovarian cancer

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Abstract
Defective DNA repair via homologous recombination (HR) is common in ovarian high grade serous carcinomas, and homologous recombination deficiency (HRD) represents an important therapeutic target in epithelial ovarian cancers (EOCs). The development of poly(ADP ribose) polymerase (PARP) inhibitors (PARPi) has been an important advance in the treatment of HR-deficient EOCs with the potential to change daily clinical practice. However, while germline and somatic mutations in *BRCA1* and *BRCA2* are still the most important mechanisms of HRD, alterations in other DNA repair pathways might also contribute to defective HR. In this review, we focus on current and emerging approaches for identifying and targeting HR-deficient EOCs, and discuss the challenges associated with these approaches.

Key words: HRD assays, ovarian cancer, molecular biology, PARP inhibitors

Introduction

Genomic and functional studies suggest that approximately 50% of ovarian high grade serous carcinoma (HGSCs) may exhibit defective DNA repair via homologous recombination (HR) at the time of diagnosis due to genetic and epigenetic alterations of HR pathway genes [1, 2]. Defective HR represents an important therapeutic target in HGSC as shown by the use of poly(ADP ribose) polymerase (PARP) inhibitors (PARPi), which display synthetic lethality when applied to HR-deficient cells [3, 4]. Here, we focus on current and emerging approaches for identifying and targeting HR-deficient epithelial ovarian cancers (EOCs), and discuss challenges associated with these approaches.

HGSC biology: molecular pathogenesis of epithelial ovarian cancer

Five distinct subtypes of ovarian cancer have been identified: high grade serous, low grade serous, clear cell, endometrioid and mucinous [5]; HGSC is by far the commonest subtype. HGSC is marked by universal mutation of the tumour suppressor gene *TP53* [6], as well as high levels of genomic instability [1]. Half of all HGSC carcinomas are estimated to have HR deficiency (HRD) at the time of diagnosis, with about 15% harbouring a germline mutation in *BRCA1*/2, 6% a somatic *BRCA1/2* mutation, and 20% a mutation in, or epigenetic silencing of, another HR gene [1, 7]. Epigenetic silencing of *BRCA1* via promoter hypermethylation occurs in approximately 10-20% of HGSCs and is mutually exclusive of *BRCA1* mutation; this implies that *BRCA* genes are frequently inactivated by either mutation or epigenetic silencing in EOC [1]. Other HR variations are infrequent (<2%), but might involve mutations in Fanconi anaemia (FA) genes (*PALB2*, *FANCA*, *FANCI*, *FANCL* and *FANCC*), in several genes of the RAD family (*RAD50*, *RAD51*, *RAD51C* and *RAD54L* etc.) and in other genes involved in HR pathway, including *ATM*, *ATR*, *CHEK1* and *CHEK2*.

Defective HR in EOC may also occur with alterations of other genes which are known to modulate the HR pathway and indirectly cause HR deficiency. For example, amplification and overexpression of EMSY, reported in 17% of HGSC [8], is another potential mechanism of HR deficiency. EMSY interacts with the transactivation domain of BRCA2 leading to inhibition of its transcriptional activity. It also co-localizes with BRCA2 at DNA damage sites and interacts with several chromatin remodelling proteins. The

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correlation between inactivating mutations of *CDK12* and HR deficiency has been elucidated [9]. *CDK12* is one of the most significantly mutated genes in EOC (3% of cases in the TCGA dataset) and is involved in the transcription of *BRCA1* and other HR genes; thus, when its activity is reduced, *BRCA1* expression is reduced as well as DNA repair by homologous recombination, and this finally leads to an increase of PARPi sensitivity [10].

Importantly, it should be highlighted that other underlying and less clear mechanisms might provoke defective HR in HGSC. Furthermore, variations in the nucleotide excision repair (NER) pathway have been found in up to 8% high grade serous EOCs as well as alterations of the mismatch repair (MMR) mechanism, which may be damaged in up to 3% of HGSCs [11].

DNA double strand break repair

The recognition and subsequent repair of DNA damage is necessary for normal cellular function and genomic stability. It has been demonstrated that acquired or inherited defects in DNA repair pathways result in an increased lifetime risk of cancer [12]. Several pathways have been identified for DNA repair, and they are engaged variably to repair single- (SSB) and double-strand (DSB) DNA breaks. In particular, three mechanisms are involved in DSB repair: nonhomologous end joining (NHEJ) repair, HR repair (HRR) and microhomology-mediated NHEJ (MMEJ), also termed alternative NHEJ (alt-NHEJ). MMEJ is different from the other repair pathways as it requires a 5-25 base pair microhomologous sequences to align the broken strands before joining [13].

These mechanisms are complex and require the accurate temporal and spatial synchronization of a numerous proteins which are involved in both DNA repair processes and in cell cycle control, until the repair is complete. HRR is an error-free pathway that uses a homologous DNA template to repair DSB and is initiated by end resection of the DNA ends to generate a long stretch of single-strand DNA for strand invasion. HRR can only be utilised when cells enter S and G2 because cyclin-dependent kinases are needed for promoting end resection and to activate HRR, and a homologous donor strand is available. The majority of DSB arise during DNA replication when a replication fork encounters an unrepaired SSB; the HRR pathway, together with the nuclear enzyme, PARP-1, is particularly important for repairing these collapsed replication forks [3, 14]. Double-strand DNA breaks are recognized by ATM kinase, which phosphorylates downstream targets including CHEK2, p53, BRCA1 and H2AX. BRCA1, assisted by BARD1 and BRIP1, acts as a scaffold that organizes the remaining proteins to the site of repair. The MRN complex, which consists of MRE11, RAD50 and NBS1, then resects the DNA to form 3' overhangs that are bound by RPA. BRCA2 is recruited with the assistance of PALB2 and loads RAD51 onto RPA-coated DNA with the assistance of RAD51B, RAD51C and RAD51D. The RAD51 nucleoprotein filament then invades the homologous DNA strand in a process called strand invasion, allowing the remaining DNA repair to occur with the use of the sister chromatid as a template for error-free repair [15]. Thus, BRCA1 and BRCA2 are crucial to the accurate execution of HR repair.

Assays of HR function

Multiple studies have demonstrated that tumours with loss of BRCA1/2 function have a higher sensitivity to platinum compounds and PARPi [16-18]. However, many HGSC that do not have a mutation in *BRCA1/2* or another HR gene show *BRCA* mutant-like behaviour [10, 19]. Therefore, defining those tumours with defective HR might help in the clinical management of these patients. Indeed, in order to offer PARPi to all HGSC patients with defective HR, considerable efforts have been made to identify somatic signatures of abnormal HR. Although there are gold-standard laboratory assays of HR function (e.g. DR-GFP [20] and RAD51 [2] focus formation assays), these are not suitable for routine use in clinical samples. In particular these functional HR deficiency assays demand a live tumour sample that can be grown for several days, which precludes the use of fixed or paraffin embedded specimens. There are three other approaches that may be of clinical use: response to platinum chemotherapy, genomic scarring and next generation sequencing (NGS) of HR genes.

Loss of BRCA1/2 function is associated with response to platinum chemotherapy. In single agent olaparib studies, response to PARP inhibition was correlated with platinum sensitivity, with more frequent and durable responses in tumours that had relapsed in the platinum-sensitive timeframe (>6 months following last platinum exposure) [21]. Similarly, Ledermann et al. showed clearly that maintenance olaparib produced a highly significant progression-free survival (PFS) advantage compared to placebo when administered to women with relapsed HGSC who had responded to platinum chemotherapy in the relapse setting, independently of *BRCA* mutation status [22]. Thus, at a first approximation, true platinum sensitivity is able to identify patients who may benefit from PARP inhibition. However, this is only applicable for maintenance treatment where prior platinum response has been demonstrated, and will not be applicable in the first-line set-

ting where patients have undergone complete debulking and have no visible disease to allow platinum response to be assessed. Moreover, in the treatment setting, Gelmon et al. demonstrated robust responses in some patients with relapsed HGSC who were *BRCA* wild-type and who had relapsed in the platinum-resistant timeframe [23], indicating that the simple clinical criteria of 'platinum-sensitive' and 'platinum-resistant' relapse are inadequate. Thus, there is a need to develop robust assays that can assess HR status.

Ovarian HGSC exhibits a high burden of genomic aberration. Mainly, there are two important types of aberrations: (1) regional copy number aberrations (CNAs) and (2) structural rearrangements. CNAs are described as loss or gain in the number of delineated, subchromosomal section of DNA copies. Whereas structural rearrangements are defined as changes into precise location or orientation of known DNA sequence. Structural rearrangements include DNA recombinations (material exchange between homologous regions), DNA translocations (exchange of material between non-homologous regions) and DNA inversions (change into defined sequence orientation) [24].

The potential outcome of DNA recombinations is the regional loss of heterozygosity (LOH), in which one parental copy of heterozygous DNA region is lost and the other is preserved. This leads to an imbalance in the ratio of parental alleles from the normal 1:1 and a cell exhibits a 'structural chromosomal instability' [25].

Three independent DNA-based measures of genomic instability have been developed, based on LOH [26], telomeric allelic imbalance (TAI) [27], and large-scale state transitions (LST, a measure of the number of chromosomal breaks between adjacent regions of ≥10 Mb) [28]. Importantly, the sum of the three scores was better in discriminating HR deficient from non-deficient tumours. There was also a linkage between genome signature to platinum sensitivity [27] and/or *BRCA1* inactivation [22-24], and a significant correlation between the three scores was identified [29].

On this basis, the Myriad myChoice HRD has been developed [6], which uses an unweighted sum of the three independent DNA-based measures of genomic instability above mentioned in order to indentify an HRD score [30]. The relationship between response to therapy and the presence of germline *BRCA* mutation (*gBRCAm)* or HRD score (defined as HRD score ≥42) has been confirmed in three studies focused on neoadjuvant platinum containing therapy in triple-negative breast cancer patients [30].

A second assay has been developed by Foundation Medicine, using formalin-fixed paraffin-embedded (FFPE) tissue. The NGS assay combines somatic *BRCA* status as well as the percentage of genome-wide LOH, quantified in approximately 3500 single-nucleotide polymorphisms (SNPs) throughout the genome, to define these subgroups: *BRCA* mutant (deleterious germline or somatic), *BRCA* WT/LOH high or *BRCA* WT/LOH low. Foundation has partnered with Clovis Oncology to develop a companion diagnostics (CDx) in parallel with the clinical development of rucaparib in the ARIEL2 and 3 studies (NCT01968213) (see below).

However, it has been suggested [10] that these assays might have shortcomings as they are not able to detect the reversal of HR deficiency that may appear when cell become resistant to platinum and PARPi, for example through acquisition of secondary revertant mutations that restore BRCA1/2 function, which have been detected in both cell lines and tumour samples [31, 32].

Beyond genome scars and functional tests, other strategies are currently under investigation, including gene-expression profile (GEP) signatures and protein signatures of "HRness" within tumours. Interestingly, Konstantinopoulos developed a 60-gene expression signature of *BRCA*-ness in sporadic or germline *BRCA*-associated EOC [33]. This signature was able to predict platinum responsiveness in 8 out of 10 patient-derived tumour specimens and was then validated in 70 patients with sporadic disease: it was found that patients with the *BRCA*-like profile had an increase in both disease-free survival and overall survival (OS) compared with patients with a non-*BRCA*-like profile. Further studies are needed to confirm these findings and also to evaluate whether these gene expression signatures are truly predictive of response to therapy rather than being only prognostic.

More recently, based on the fact that abrogation of BRCA1 and BRCA2 function leads to a characteristic set of mutational signatures, a new HRD assay (HRDetect) has been developed which was able to identify six distinguishing mutational signatures predictive of *BRCA1*/*BRCA2* deficiency [34]. In the paper from Davies et al., it was found that integrating all of the classes of mutational signatures reveals a larger proportion of individuals harbouring *BRCA1*/*BRCA2* deficiency. In fact, although only 22 patients were originally recruited with known germline *BRCA1*- or *BRCA2*-null cancers, HRDetect revealed an additional 33 tumours with a germline mutation, 22 tumours with a somatic mutation bringing the total number of *BRCA1*/*BRCA2*-deficient tumours to 124 (22%). Large-scale population-based studies as well as application in clinical trials are required to gather proper population estimates.

Clinical trial data

Several trials have been conducted with the aim of identifying HR deficient patients who might benefit from PARP inhibition. In addition to the results obtained with olaparib mentioned above, data from two other PARPi are now available and have the potential to change our daily practice.

Niraparib (MK-4827) is a selective PARP-1 and -2 inhibitor (PARP1/2 Ki 3.2/4.0 nM) [35] which has been investigated in both germline *BRCA* mutated (*gBRCAm*) and *BRCA* wild-type EOC [36, 37]. In October 2016, the results of the phase III ENGOT (European Network for Gynecological Oncological Trial groups)-OV16/NOVA trial were published [17]. NOVA examined the role of niraparib as maintenance treatment following response to platinum-based chemotherapy in patients with relapsed HGSC. Patients were stratified based on germline *BRCA* mutation status, and randomised 2:1 to receive niraparib or placebo. In order to identify *BRCA* wild-type patients who might benefit from niraparib, the myChoice HRD assay [6] was applied to archival tumour tissue samples, and used to classify the population of patients in the *gBRCAm* wild-type cohort as being either HRD positive or negative. The trial enrolled 553 patients, with 203 in the *gBRCAm* group (138 allocated to oral niraparib 300 mg and 65 to placebo) and 350 patients in the non-*gBRCAm* cohort (234 allocated to oral niraparib 300 mg and 116 to placebo). The study met its primary endpoint with a significant improvement of PFS compared with placebo. Median PFS with niraparib compared to placebo was 21.0 *versus* 5.5 months (hazard ratio [HR] 0.27; $p<0.0001$) in the *gBRCAm* group (n=203); 9.3 *versus* 3.9 months (HR 0.45; $p<0.001$) in the non-germline *BRCA* mutated group (n=350). Median PFS in the non-*gBRCA* cohort was 12.9 *versus* 3.8 months (HR 0.38; $p<0.001$) in the subgroup who were classified as HRD positive, and 6.9 *versus* 3.8 months (HR 0.58; p=0.0226) in HRD-negative (n=134) patients.

Compared to placebo, niraparib also significantly prolonged the second PFS, time to first subsequent treatment, and chemotherapy-free interval in the mutation and mutation-free groups, as well as in the HRD subgroup.

In March 2017, the U.S. Food and Drug Administration approved niraparib for the maintenance treatment of recurrent epithelial ovarian, fallopian tube or primary peritoneal cancer, who have had complete or partial response to the last platinum-based chemotherapy, regardless of *BRCA* or HRD status [38].

The first PARPi to be administered in humans was rucaparib. Two important trials are currently ongoing for rucaparib in ovarian cancer: the phase II and phase III ARIEL2 and ARIEL3 (NCT01891344 and NCT01968213, respectively). ARIEL2 is a two-part study; part 1 has been completed and part 2 is currently enrolling. Part 1 investigated oral rucaparib as single agent treatment in recurrent platinum-sensitive high-grade epithelial ovarian, primary peritoneal, or fallopian tube cancer [18]. As previously mentioned, it also assessed the capability of an integrated Foundation Medicine HRD assay to predict response to rucaparib by the prospectively defining three subgroups, defined above.

Patients were subdivided into three groups *BRCA* mutant (deleterious germline or somatic), *BRCA* WT/LOH high (LOH ≥14%), or *BRCA* WT/LOH low (LOH <14%). The primary endpoint was PFS, which was longer in rucaparib-treated patients with *BRCA* mutations (12.8 months; 95% confidence interval [CI] 9.0-14.7) or *BRCA* WT/LOH high platinum-sensitive disease (5.7 months; 95% CI 5.3-7.6) than in *BRCA* WT/LOH low carcinomas (5.2 months; 95% CI 3.6-5.5; $p<0.0001$ for *BRCA* mutant *vs* *BRCA* WT/LOH high; p=0.011 for *BRCA* mutant *vs* *BRCA* WT/LOH low).

It was also found that the proportion of patients who achieved a response (objective response by combined Response Evaluation Criteria in Solid Tumors [RECIST] and cancer antigen [CA]125) was similar irrespective of whether the *BRCA* mutation was germline (85%) or somatic (84%) or whether a patient had a *BRCA1* (86%) or *BRCA2* (82%) mutation. Moreover, all 4 patients with a mutation in *RAD51C* were classified as LOH high, and 3 of these 4 showed a RECIST response, suggesting that mutation in this gene has an effect similar to that of *BRCA1* and *2* mutations.

It should also be underlined that there were also tumours with mutations in other HR genes classified as LOH low and which did not respond. So, it could be hypothesized that not all genes within the HR pathway are equivalent and therefore just relying on panel sequencing might not be sufficient.

Confirmed RECIST responses were more frequent in the LOH high subgroup (24 of 82 patients [29%; 95% CI 20-40]) than in the LOH low subgroup (7 of 70 patients [10%; 95% CI 4-20; p=0.0033]), and the duration of response was longer in the LOH high subgroup (10.8 months; 95% CI 5.7–not reached) than in the LOH low subgroup (5.6 months; 95% CI 4.6-8.5; p=0.022).

A recent presentation compared efficacy results between the pre-specified genomic LOH (14%) and a 'refined' LOH cut-off point (16%) and demonstrated an improvement in the selection of patients most likely to benefit from rucaparib (ORR of 80% in patients with *BRCA* mutations for both LOH cut-offs but an improvement in ORR

from 35 to 39% in patients with a *BRCA*-like LOH-high signature and from 13 to 14% in patients without a *BRCA* mutation or a *BRCA*-like low signature) [39].

The HRD assay is also being prospectively tested in an extension (Part 2) of ARIEL2, in which rucaparib is administered in patients with platinum-sensitive, platinum-resistant, or platinum refractory cancers, who have received at least three but not more than four prior lines of therapy and have had a treatment-free interval of more than 6 months following first-line chemotherapy. The primary endpoint of ARIEL2 Part 2 is response rate. More importantly, the HRD test is also being applied prospectively in ARIEL3 (NCT01968213), a study of maintenance rucaparib in women with relapsed platinum-sensitive HGSC. In addition, a phase III study, ARIEL4 (NCT02855944), is ongoing, investigating rucaparib *versus* chemotherapy in patients with relapsed platinum-sensitive *gBRCAm*-only ovarian cancers who have received at least two lines of chemotherapy.

In December 2016, based on the positive results of the ARIEL clinical trials and other rucaparib studies, the FDA approved rucaparib as treatment for women with advanced ovarian cancer who have already been treated with at least two chemotherapies and have a *BRCA1* or *BRCA2* mutation identified by an approved companion diagnostic test [40].

Conclusions

Overall, response to PARPi seems to be strongly related to defective HR: broadly, those patients who present with a defect of function of the genes linked with HR pathways should achieve the greater benefit from PARP inhibition. This approximation holds for germline and somatic mutation in *BRCA1* or *BRCA2* and possibly *RAD51C*, but is not perfectly confirmed when HR is assessed using panel sequencing alone. HRD assays, based upon genomic LOH and other measures of genomic instability, show great promise, especially when added to *BRCA1/2* mutation state, but further refinement and validation is required for routine clinical use. In the maintenance setting, response to platinum chemotherapy, as demonstrated for both olaparib and niraparib, remains a powerful and useful phenotypic predictor of HRD and, therefore, of potential PARP sensitivity.

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Conflicts of Interest

The Authors declare there are no conflicts of interest in relation to this article.

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