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Review Article

The ups and downs of DNA repair biomarkers for PARP inhibitor therapies

XiaoZhe Wang, David T. Weaver

On-Q-ity, Inc., 610 Lincoln St. Waltham, Massachusetts, 02451, USA.

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Abstract: PARP inhibitors are emerging as a valuable new drug class in the treatment of cancer. Recent discoveries make a compelling case for the complexity of DNA repair biomarker evaluation and underscore the need to examine at multiple biomarkers in a relational manner. This review updates the current trends in DNA repair biomarker strategies in use for the PARP inhibitors and describes the impact of many DNA repair biomarkers on PARP inhibitor benefit in the cancer clinic.

Keywords: PARP inhibitors, cancer, DNA repair, biomarker

Introduction

PARP inhibitors promise to be a valuable new drug class in the treatment of cancers, either as a single agent or in combination with other DNA-damaging agents including radiation therapy. It has been demonstrated that PARP inhibitors trigger significant anti-tumor reactivity and cause fewer side effects in treating aggressive, difficult-to-treat cancers, such as hereditary *BRCA1/2*-associated cancers, triple negative breast cancer (TNBC), and ovarian cancer. Two important considerations have propelled deeper investigation of PARP inhibitor biomarkers. First, PARP inhibitors may have utility beyond the relatively small proportion of cancer patients carrying BRCA mutations. How will we develop tests that broaden the recognition of additional patients who should be treated with this valuable drug class? Secondly, recent studies show that not all *BRCA1/2* carriers had a response to PARP inhibitors [1]. The challenge remains to develop an efficient and coordinated strategy to identify and measure effective biomarkers such that the patient population who are more likely to response to PARP inhibitor therapies may be identified. Traditional decision-making about cancer treatment is being redefined with the example of PARP inhibitor biomarkers and personalized medicine strategies.

DNA repair defects are often associated with cancer. DNA repair pathways are central to the responses to DNA damage caused by chemotherapy and radiotherapy. Therefore, the efficacy of cancer treatments is likely limited by the ability of cancer cells to repair such damage. One of the most important topics in translational research is the investigation of the DNA repair pathways that may influence responses to PARP inhibitor therapies and predict clinical outcome. The complexity of crosstalk between DNA repair pathways indicates that biomarker assays to detect the status of multiple DNA repair pathways could provide critical information regarding the sensitivity and resistance of cancer cells to PARP inhibitors. This review addresses recent updates to these approaches, describing the mechanisms of action of PARP inhibitors, and focusing on the DNA repair biomarkers that are potential candidates to stratify patient population likely to benefit from PARP inhibitor therapies.

DNA repair

DNA is constantly exposed to a variety of genotoxic stresses from cell metabolism and

the environment that cause damage. A vast number of DNA lesions may form that confer toxicities and mutagenesis if not repaired. To maintain genome integrity, six principal DNA repair pathways are used in all eukaryotes to repair single-strand breaks (SSBs) and double-strand breaks (DSBs): base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR), non-homologous endjoining (NHEJ), and translesion DNA synthesis (TLS). In addition, a network of DNA damage responses (DDR) orchestrates regulatory steps of DNA repair and forms a cross-functional purpose by coordinating backups or redundancies in the DNA repair network. In the simplest terms, BER, NER, or MMR pathways are involved in the repair of SSBs, while DSBs are repaired by NHEJ or HR pathways, either by ligating the broken DNA ends together or using templating recombination from the homologous DNA strand respectively. TLS enables the replication forks to bypass DNA lesions in order to avoid collapse, which would potentially cause mutagenesis. Fanconi anemia (FA)/BRCA pathway also coordinates the major pathways including HR, NER, TLS pathways following DNA interstrand crosslinks [2, 3].

DDR involves post-translational modification of protein complexes of DNA repair to regulate many steps of the DNA repair process. Cells activate a DNA damage response network coordinating chromatin-associated DNA repair with signaling to other cellular processes in response to different forms of DNA damage, including sensing, repairing, and feedback indicators of the completion of the DNA DSBs and damaged replication fork repair prior to cell division [4-6]. The DNA damage network contains complex and multifunctional pathways that involve complex post-translational modification enzymes, such as kinases, ubiquitin ligases, DUBs, methyl transferases, and some of these proteins may also serve specific purposes along the different DNA repair pathways [7].

DNA repair pathways play key roles in maintaining genome stability. These pathways do not operate at equivalent functional levels in cells because of considerably different DNA damage loads. For example, BER is the most active constitutive DNA repair pathway with frequent oxidative damage to DNA throughout the cell cycle and the genome. On the other hand, NHEJ that responds to as few as one DSB per cell, is of lower ongoing activity. Despite differing loads and roles, each of the DNA repair pathways is necessary for continuing a genome content and configuration.

DNA repair has frequently been implicated in tumorigenesis, deficiency in DNA repair genes is associated with high susceptibility to cancer, yet it is the tumor maintenance and therapy responsiveness features that may be most relevant to personalized medicine and diagnostics. Cancer cells exhibit genomic instability that is partially due to DNA repair pathway remodeling. Often, defects are demonstrated in one of these seven major DNA repair pathways. These features may be particularly meaningful towards identifying opportunities for patient therapies using agents that, by their mechanism of action, are interfering with DNA repair (**Figure 1**). It also should be noted that DNA damage by the classic means of DNA-toxic chemotherapies and radiotherapy causes a variety of DNA lesions. For example, chemotherapeutic agents such as cisplatin introduces intrastrand or interstrand crosslinks, and NER, HR, FA/BRCA, and TLS pathways are majorly involved in the repair of such damage. Since many cancer therapy strategies involve combination therapy, it is important to recognize the changed status of DNA repair in light of standard chemoradiotherapies and novel agents.

Role of PARP in DNA repair

Poly(ADP-ribose) polymerases (PARPs) are a family of enzymes that are involved in many cellular processes guided by an ability to modify various target proteins through the conversion of nicotinamide adenine dinucleotide (NAD+) into long poly(ADP-ribose) (PAR) chains coupled to the proteins. PARP1 is the best known member of an eighteen PARP domain protein family. PARP1 is a chromatin-associated enzyme that is involved in a number of distinct nuclear functions, such as DNA repair, regulation of chromatin structure and transcription, cell survival and cell death, maintenance of genome stability and pro-inflammatory signal transduction. PARP2, sharing homology with PARP1, also regulates different cellular processes, including DNA damage response. TNKS (Tankyrase 1) and its close homologue Tankyrase 2, are also PARP proteins in telomere maintenance, mitosis, and genomic stability, while the functions of many other PARP

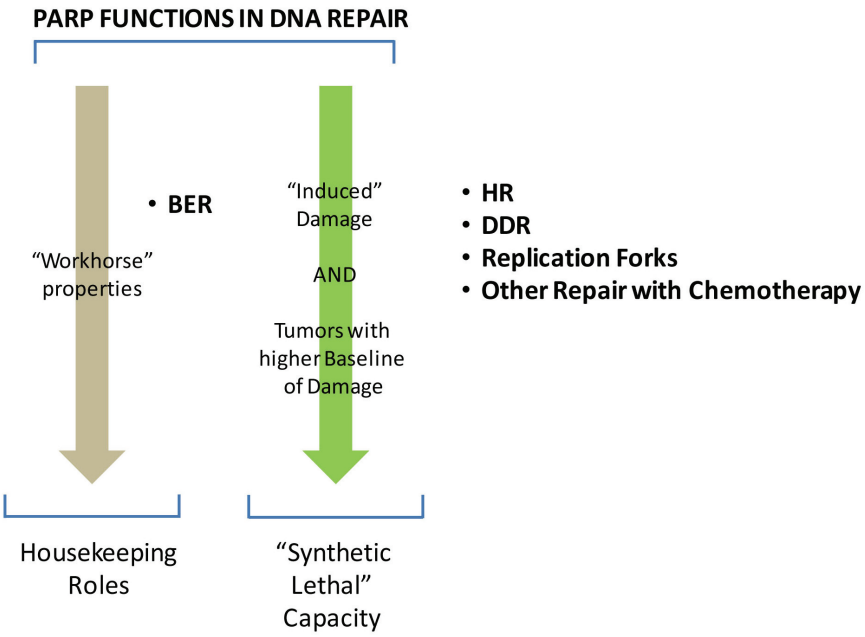

Figure 1. PARP in DNA Repair, Induced Damage, and Tumor Cells. PARP plays an important role in BER pathway. Tumors with HR or DDR defects are more dependent on PARP and BER to maintain genome integrity. Chemotherapy induces DNA damages, lack of repair results in cancer cell death, while PARP inhibition leads to an increased SSBs and will eventually lead to DSBs via replication forks collapse. Synthetic lethal association between PARP inhibition and HR, DDR deficiencies to selectively kill tumor cells provides rational of clinical development of PARP inhibitors.

enzymes is not understood [8-11].

PARP1 is by far the most abundant of the PARP family, responsible for >90% of the poly(ADP-ribosyl)ation activity in the cells of all higher eukaryotes. The most relevant function of PARP1 regarding cancer therapy is considered to be its role in several DNA repair processes (**Figure 1**). PARP1 is a key BER protein, but it also contributes to the two DSB repair pathways, NHEJ and HR repair, at replication forks [8, 10, 12]. PARP2 has been demonstrated to be also involved in BER, but is less active than PARP1, contributing only 5% to 10% of the total PARP activity in response to DNA damage [13]. Both PARP1 and PARP2 function as DNA damage sensors by binding rapidly to the site of damaged DNA to modulate a variety of proteins involved in DNA repair and other cellular processes [13, 14]. Double knockout PARP1 and PARP2 in mice results in an embryonic lethal phenotype, whereas the single gene knockouts are not lethal, suggesting essential physiological roles of PARP1 and PARP2 and some complementarity between the two proteins [15]. PARP1, containing a BRCT (BRCA1 C-terminal) repeat motif that overlaps with an automodification domain, and this motif is crucial for protein-protein associations during repair [16, 17]. PARP1 is activated by binding with high affinity to single-and double-stranded DNA breaks via its zinc fingers and catalyses poly(ADP-ribosyl)ation of various nuclear proteins. PARP1 was also found to protect DNA breaks and chromatin structure and recruit DNA repair proteins to sites of DNA damage [18, 19]. PARP1 heterodimerizes with PARP2 and forms DNA repair complexes with X-ray Cross Complementing factor 1 (XRCC1), histones, DNA ligase III, DNA polymerase β, ATM, p53, Mre11, and NBS1 to facilitate DNA repair [10, 17].

PARP1 plays an essential role in cell survival in response to DNA damage (**Figure 2**). With low to moderate levels of DNA damage, PARP1 promotes cell cycle arrest and DNA repair. In the presence of extensive DNA damage, PARP1 meditates p53-regulated apoptosis and initiate cell death through necrosis [17, 20]. Activation of PARP1 is involved in very early DNA damage response, and its catalytic activity is rapidly increased by greater than 100-fold in response to DNA SSBs and DSBs [17, 21]. NAD+-dependant PARP1 activation results in the synthesis of long branched polymers of ADP-ribose (PAR) onto itself and other protein acceptors 15 to 30 seconds after DNA damage [22, 23]. PARP-mediated poly(ADP-ribosyl)ation is a very dynamic process as the polymer half-life is short, in the range of minutes. PAR is a heterogeneous, negatively charged linear or branched homopolymer of repeating ADP-ribose units linked

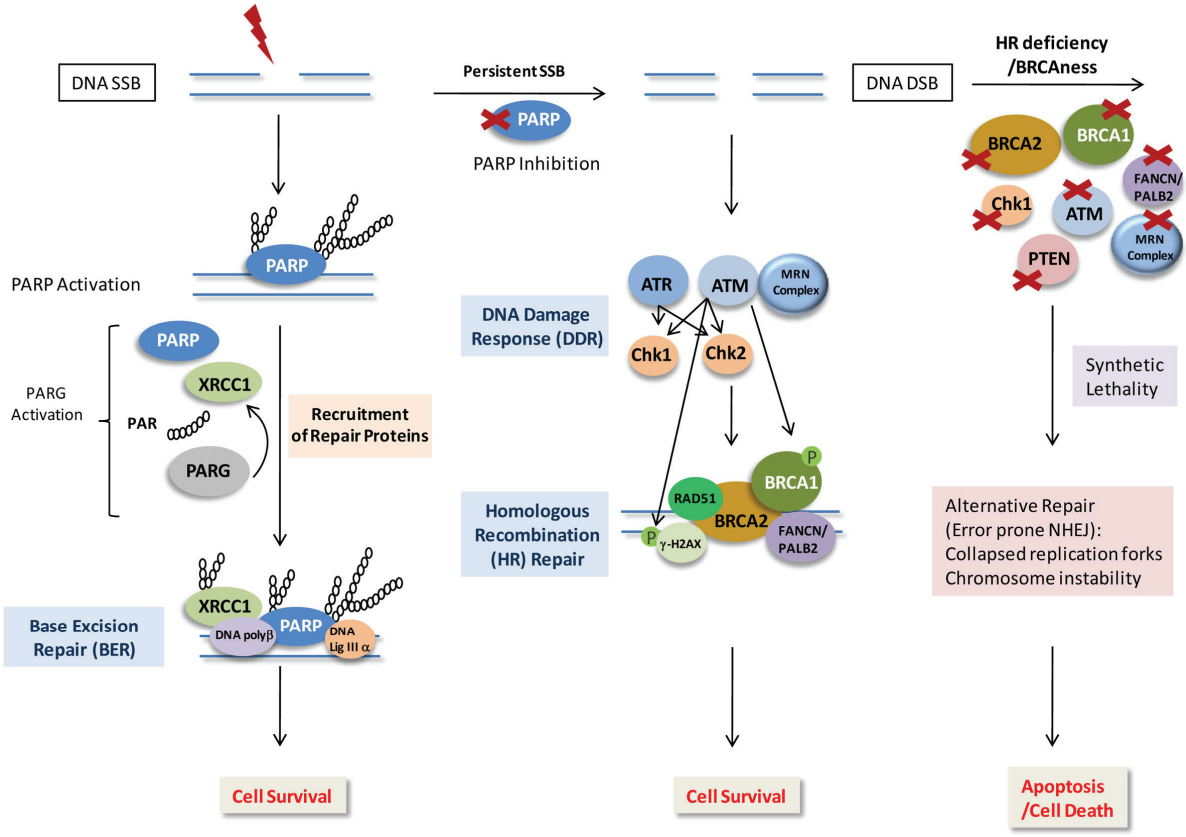

Figure 2. The role of PARP in DNA repair and connections to other proteins where there is evidence for synthetic lethality in cancer treatment. PARP is activated in response to DNA SSB by detecting and binding SSB to initiate the process of BER. Its catalytic activity results in poly(ADP-ribosyl)ation on itself and other key BER proteins such as XRCC1. Activated PARP then recruits other DNA repair proteins via poly(ADP-ribosyl)ation and direct interactions to facilitate DNA repair. The cellular levels of PAR are regulated by the opposing actions of PARP and PARG. Degradation of PAR polymer by PARG leads to release of modified proteins from damaged DNA. PARP inhibition causes an increase in persistent SSBs in DNA that are converted into DSBs. Both HR and DDR proteins are involved in the repair of DSBs. However, HR deficient cells or cells with BRCAness are unable to repair the accumulated DSBs caused by PARP inhibition, resulting in collapsed replication forks, chromosome instability and cell death.

by glycosidic ribose-ribose bonds [24]. Formation of PAR releases PARP1 from damaged DNA, and *in vitro* studies suggested that removal of PARP1 provides access for DNA repair proteins to damaged DNA and suppresses further PAR synthesis [25-27]. The levels of PAR are regulated by the opposing actions of PARPs and a poly(ADP-ribose) glycohydrolase (PARG), an enzyme that hydrolyzes the glycosidic linkages between the ADP-ribose units of PAR producing free ADP-ribose. PAR polymers are degraded immediately to ADP-ribose monomers upon the initiation of PAR synthesis. This rapid turnover strongly suggests that PAR synthesis and degradation is highly regulated [20, 23, 28] (**Figure 2**). PAR functions as a post-translational modification, a protein-binding matrix or a steric block. A variety of proteins involved in DNA repair or chromatin regulation including PARPs, topoisomerases, DNA-PK, XRCC1, p53, macroH2A1.1, ALC1, were found to bind PAR through PAR-binding motifs, indicating that dynamic and transient function of PAR may regulate activity of DNA repair proteins and other proteins or alter chromatin confirmation by PAR binding [10, 20, 29].

Mechanisms of action of PARP inhibitors

Synthetic lethality and BRCA1/2 deficiency: Proof-of-Concept studies

The foundation of the therapeutic utilities of PARP inhibitors is the mechanism of action of

the PARP proteins in DNA repair, and the biological principal of synthetic lethality (**Figure 1**). Synthetic lethality is a concept where the combination of mutations in two or more genes leads to cell death, and each mutation alone is not sufficient to cause cell death. Synthetic lethal attributes may specifically be targeted to a diseased state, such as cancer, broadening the ability to establish a therapeutic window for a drug. Several features of synthetic lethality are relevant to cancer drug action. First, a genetic deficiency (mutant) effect and a drug inhibitor effect may be viewed in a similar highly relevant context of the gene, its pathways and networks. Second, because genes often are described in their biological pathways, there is an opportunity to gain insights from the genetic connections in elaborating the likely process of an inhibitor's mechanism of action. Third, the understanding of the gene and inhibitor linkage via a pathway or network, unveils additional genetic changes that may also be valuable to other synthetic lethal associations. Each of these attributes is evident from the application of the synthetic lethal concept to PARP inhibitor therapies, as will be elaborated in this review.

BRCA1/2 mutant cells are extremely sensitive to PARP inhibitors, a finding that provided the preclinical rational of synthetic lethality between BRCA deficiency and PARP inhibition [30-32] (**Figures 1 and 2**). The DSB repair defects of *BRCA1/2* deficient cells are more dependent on PARP and BER to maintain genomic integrity, and loss of functions of both BRCA and PARP results in cell death [31-33]. Consequently, the BRCA DNA repair pathways have been validated in a number of studies with compelling antitumor activity of single-agent PARP inhibitors [31, 32].

BRCA1 and BRCA2 are tumor suppressor genes associated with genetically inherited breast cancer and have an important role in the repair of DNA DSBs and the maintenance of genomic stability [4]. Tumor cells lacking functional BRCA1 and BRCA2 are deficient in the repair of DNA DSBs by RAD51 mediated HR. BRCA1 is also involved in DNA damage signaling, cell cycle checkpoint regulation and functions as a scaffold to recruit DNA repair proteins, while BRCA2 interacts with RAD51 and translocates RAD51 to the site of DNA damage to initiate repair [4, 34-36]. PARP inhibition leads to persistent SSBs in DNA that are converted into DSBs at replication forks and can trigger sister chromatid exchange (SCE) [37-39]. A recent study shows that PARP1 and PARP2 play a role in detecting stalled or collapsed replication forks to recruit Mre11-Rad50-NBS1 (MRN) complex for resection and single stranded DNA (ssDNA) formation, which allows RAD51 loading on resected DNA to initiate HR. Thus, PARP is also involved in HR repair at replication forks, inhibition of PARP leads to increased DNA lesions that can cause stalling and collapse of the DNA replication machinery [40]. Loss of PARP function in BRCA1 or BRCA2 deficient cells results in the deficiency of repair of DSBs, which leads to cell cycle arrest and/or cell death. The accumulation of RAD51 nuclear foci after DNA damage is a hallmark reflecting an increased assembly of HR repair complexes to repair DNA lesions induced by PARP1 inhibition [24, 41]. PARP inhibition also activates ATM, and induces γ-H2AX foci in an ATM dependent manner [42]. The combined role of PARP1 in HR and SSB repair may explain the exceptionally strong synthetic lethal interaction between PARP and BRCA1/2 [6] (**Figure 2**).

The first success in the clinic using synthetic lethal identifiers was achieved with the PARP inhibitor olaparib monotherapy to treat patients with *BRCA1/2* mutant tumors in a phase I exploratory study, which revealed that olaparib had antitumor effects only in *BRCA1/2* carriers with breast, ovarian, and prostate cancer [1]. Later results from phase II studies established proof of concept of selectively killing of HR-deficient cancer cells resulting in a substantial clinical benefit with minimal toxicity [43-45]. In addition to olaparib, clinical trials of other PARP inhibitors including PF-01367338, ABT-888, iniparib, MK4827, CEP-9722 as monotherapy in different types of cancer are ongoing or planned, which we will discuss later in detail in this review.

PARP inhibitor therapies targeting a BRCAness phenotype

In addition to hereditary *BRCA1/2* mutations, a synthetic lethal concept has been broadened to include sporadic cancers. In these diseases, patient tumors may acquire a BRCAness phenotype, defined as a general impairment of HR pathway or other HR related deficiencies in pathways such as DDR. BRCAness is the phenotypic characterization of these sporadic cancers that is shared with those that occur in carriers with mutations in *BRCA1* or *BRCA2* [46].

Possible mechanisms of inducing BRCAness that have already been reported include epigenetic hypermethylation of the *BRCA1* promoter [47-50], somatic mutation of *BRCA1/2* [49-51], methylation of the FA gene *FANCF* [52], and amplification of the gene *EMSY*, the protein product of which interacts with BRCA2 [46], or loss of function mutations in other genes in HR or DDR pathways, such as *PALB2*, *ATM* and *NBS1* [30, 53].

PALB2 (partner and localizer of BRCA2) was identified as both a breast cancer susceptibility gene and a Fanconi anemia gene (*FANCN*) [54, 55]. Mutations in *PALB2* have been associated with hereditary breast cancers. The *PALB2* promoter may be hypermethylated, and downregulation of PALB2 expression is found in both hereditary and sporadic breast cancers [56-59]. PALB2, a downstream player of FA/BRCA pathway, plays an important role in facilitating BRCA2 function. PALB2 directly functions in HR repair and is required for the assembly of BRCA2 and RAD51 nuclear foci [54, 55, 60]. PALB2 deficiency also results in hypersensitivity of cancer cells in response to PARP inhibitors treatment [53]. *Phosphatase and tensin homolog* (*PTEN*), one of most commonly mutated genes in human cancers, is a tumor suppressor gene and its protein product has recently been shown to be implicated in HR and the maintenance of genomic stability. PTEN loss of function mutations and loss of PTEN expression are more frequent in a range of hereditary and sporadic cancers [61]. Cancer cells lacking PTEN were found to have decreased levels of RAD51 foci formation and reduced capability in the repair of DSBs by HR. PTEN deficiency leads to HR deficiency and hypersensitivity to PARP inhibitors in tumor cells [62]. The sensitivity of cells to PARP inhibition could also be caused by the inability to sense DNA damage such as with other regulators in the same network, including ATM, Mre11/NBS1, ATR, Chk1 or Chk2 deficiency [30, 42, 63]. With these and other examples, loss of PARP activity leads to an increased number of DNA lesions repaired by HR and DNA damage response (DDR) pathways [30, 38, 42] (**Figure 2**). The observation that deficits in PALB2, PTEN, ATM, Mre11/NBS1, ATR, Chk1 or Chk2 resulted in sensitivity to PARP inhibition suggests that PARP inhibitors would be beneficial for a wider range of cancers with BRCAness phenotype such as dysfunction of genes involved in HR and DDR pathways.

The phenomena of BRCAness are recently being identified in an expanding list of cancers, and we advocate an increased attention to these genetic and epigenetic modifications in a more comprehensive way. Notably, BRCAness occurs not only in triple negative breast cancer but also in epithelial ovarian cancer and other types of cancer such as non-small cell lung cancer, head and neck cancer, prostate cancer and cervical carcinomas [46]. The BRCAness phenotypic characterization is emerging as a novel and attractive strategy for treating cancer patients with the targeted PARP inhibitors therapies.

Combination therapy with PARP inhibitors

PARP inhibitors are used as chemo/radiosensitizers in combination with radiation and/or chemotherapeutic agents such as the platinum compounds and the methylating agents. To date, PARP inhibitors such as olaparib, ABT-888, iniparib, PF-01367338, MK4827, CEP-9722, INO-1001 have been used in combination with chemotherapy or radiotherapy in phase I or phase II clinical trials to treat triple negative breast cancer, metastatic melanoma, malignant glioma, advanced colorectal cancer [64-67]. PARP inhibitors enhance the antitumor activity of ionizing radiation and DNA damaging chemotherapeutic agents. There are several potential mechanisms guiding the combination therapies: following exposure to chemotherapeutic agents, BER pathway of which PARP is a key component, can be activated, and may reverse the effects of chemotherapy, which leads to resistance to the therapy. The combination of PARP inhibitors and chemotherapy may exacerbate toxic effects, particularly if the effect is to induce DNA strand breaks. Certain agents, such as the platinum compounds and methylating compound (temozolomide) are in this category. For example, the majority of the DNA lesions caused by temozolomide are repaired by BER pathway [68]. Inhibition of PARP during temozolomide treatment prevents the repair by BER in cancer cells, and leads to tumor cell death. In a phase II study of metastatic melanoma, the combination of PF-01367338 with temozolomide was more myelosupressive than the expected profile with either agent alone, and preliminary results showed improved response rates and progression-free survival [69].

PARP inhibitors may also perform as therapeutic sensitizers to enhance chemo/radio sensitivity

and may delay resistance to treatment. This theory has been confirmed with a number of preclinical studies using various PARP inhibitors in tumor models [70-75]. A recent study showed that sensitization to ionizing radiation and the alkylating agent methylmethane sulfonate by olaparib was enhanced in DSB repair–deficient cells. Sensitization was DNA replication dependent and associated with defective repair of replication-associated damage in Artemis−/− and ATM−/− MEF cells [76]. Another study showed that the combination of PARP inhibitor and methylmethane sulfonate induced DSBs, led to activation of ATM/Chk2 and phosphorylation of histone 2AX (γ-H2AX), and formation of γ-H2AX foci correlated with PARP1 expression cells in S-phase [77].

Tumors contain a higher proportion of replicating cells than normal tissue. Sensitizing effect of PARP inhibition requires DNA replication, and therefore affects rapidly proliferating tumors more than normal tissues. Thus, PARP inhibitors have the potential to increase the therapeutic efficacy of chemotherapy and radiation therapy in a variety of tumor sites by increasing damage in highly replicating tumor cells, but sparing non-cycling normal tissue, which are often responsible for dose-limiting late damage after radiotherapy [74]. Therefore, the optimal dosage and scheduling of concurrent PARP inhibitor and therapeutic agent to treat cancer patients will require carefully designed clinical trials.

Current technologies to evaluate patient tumors

Current technologies such as high-throughput DNA microarrays, real-time quantitative reverse transcriptase (qRT)-PCR, protein microarrays followed by mass spectrometry, immunohistochemistry, immunofluorescence, are powerful tools to develop DNA repair protein expression profiling of patients' tumors that are sensitive to PARP inhibitors, and to identify and test DNA repair biomarkers of cancer patients associated with responsiveness to PARP inhibitor therapies at DNA, RNA and protein levels. Many of these technologies are accelerated by the availability of the complete human genome; however, due to the disparity created by tumor evolution, the DNA content of tumors is a moving target for PARP inhibitor therapies.

There are several aspects to consider in biomarker development strategy: 1) selection of the biological specimens to be used: for example, common clinical use of formalin fixed paraffin embedded (FFPE) tumor tissue samples are a valuable resource for discovery and validation of biomarkers because large numbers of samples with clinical outcome data can be rapidly acquired and analyzed [78, 79]. Circulating tumor cells (CTC) from the patient's bloodstream are emerging as a vital clinical tool in the diagnosis of malignancy, and in the monitoring of cancer progression and effect of cancer treatment [80] 2) determination of the biomarkers to be discovered; DNA, RNA, or protein can all be used as biomarkers, and the choice of biomarker has its relevant implications. 3) determination of predictive or prognostic biomarkers. Predictive biomarkers are measured at baseline to identify patients who are likely or unlikely to benefit from a specific treatment, while a prognostic biomarker provides information about the patients prognosis in the absence of treatment or in the presence of standard treatment [81]. 4) discovery, replication and validation of biomarkers.

High-throughput DNA microarray technology allows global analysis of gene transcript expression concurrently in one cancer tissue sample and sensitive measurement of biomarker gene panels. The number of DNA variations such as mutations in oncogenes, tumor-suppressor genes and DNA repair genes, single-nucleotide polymorphisms (SNPs), mitochondrial DNA aberrations, oncoviral markers can serve as DNA biomarkers [82]. However, both validity and the reproducibility of microarray-based clinical studies have been challenged based on enormous gene expression data generated from analysis and inadequate statistical analysis [83]. RNA based biomarkers expression patterns can be detected by qRT-PCR which represents a rapid and reliable method for the detection and quantification of mRNA transcription levels of a selected gene of interest. But technical irregularities such as RNA degradation and cross-linking, contamination with non-tumor cells and sample variability typical of FFPE tissues present challenges for gene expression diagnostic utilities.

The proteome contains more independent variables than the genome and transcriptome as proteins are considerably more diverse than DNA or RNA. There are estimated to be between 20,000 and 25,000 human protein-coding genes [84]. Proteins carry more information

than nucleic acids due to alternative splicing and post-translational modifications of species of protein from each gene. Moreover, many physiologic changes are mediated post-transcriptionally and will not be revealed at the nucleic acid level. Therefore, protein biomarkers have a significant impact in cancer diagnostics and therapies. Proteomics technology coupled with high-resolution liquid chromatography (LC) and high-performance mass spectrometry (MS) has enable thousands of proteins to be identified in biofluids. Proteomic strategies are attracting increasing interest to be used for the identification of tissue and serum markers to be used for early disease detection and to follow treatment effects and disease progression; however, highly abundant protein albumin in serum and plasma is always a problem of false positive. It has been very challenging to do quantitative analysis of FFPE tissue using this LC-MS method in clinics due to the limited amount of protein that can be extracted from FFPE samples and other factors such as throughput, accuracy and precision [85]. Immunohistochemistry (IHC) is widely used to detect protein expression levels in FFPE tissues to identify therapeutic biomarkers for prediction and prognosis. There have been many improvements of IHC that include effective antigen retrieval methods, increasingly sensitive detection systems and several pretreatments before antibody immunostaining so that the antigens that are modified by formalin fixation can be recovered. In addition, antibody specificity is one of the key components to ensure the success of IHC staining. Tumor tissue contains a mixture of tumor cells, inflammatory cells, stroma, blood vessels, and other non-malignant elements. Because the specific location of the target within tissue can be determined by IHC, IHC along with high throughput automation image analysis offer a great advantage for assessment of morphology and biomarker expression in a tumor-specific manner on a given patient specimen. Tissue microarrays (TMAs) allow assessment of protein expression in multiple tissue specimens on a single slide that minimizes the variability and increases the high throughput. The advantage of TMAs is its higher degree of precision and throughput feature that provide for the clinical analysis. IHC on TMAs analysis can be measured either manually or by automation using digital pathology platforms and correlation of these data to other available clinical data would allow better prediction of patient outcome, which have become an established and powerful tool for cancer biomarker discovery [85]. Quantitative immunofluorescence (IF) labeling on FFPE tissue has the capability for multiple labeling and is of higher resolution due to the fluorophores being directly conjugated to the antibody, this method has been applied in various studies, particularly in TMAs achieved by the development of computer assisted fluorescence imaging systems [86, 87].

RNA interference (RNAi) screen allows systematic gene and/or pathway analysis in tumor cells and have the potential to identify novel determinants of drug response. Several RNAi studies have unveiled novel pathways and molecules for therapeutic targets in various tumor types [88-92]. With the development of RNAi libraries composed of reagents that allow targeting a wide range of transcripts, it is now possible to conduct high-throughput screens (HTS) that simultaneously interrogate phenotypes associated with the loss of function of many genes.

Biomarkers of DNA repair

To understand the role of DNA repair biomarkers in cancer progression, their implication in cancer treatment such as the prediction of response to therapies and its correlation to clinical outcome has become one of the main areas in personalized medicine. Assessment of the activity of DNA repair pathways that may influence treatment response and predict clinical outcome in tumor cells may identify new therapeutic targets and influence clinical decision making. It has been shown that DNA repair proteins are frequently changed in human cancers, indicated by measurements of DNA, RNA, protein determinations of biopsies. An increasing number of studies on DNA repair pathways including DNA repair gene expression profiling, mutation status of DNA repair genes, expression levels of DNA repair proteins, nuclear foci status of DNA repair proteins, and DNA repair capacity have been demonstrated to have a predictive value for treatment outcome or the response to therapies in different types of cancer [93-97].

DNA repair is a complex multistep process requiring many DNA repair proteins to act in concert to maintain genome integrity. The impact of DNA repair biomarkers from multiple DNA repair

Table 1. Potential informative DNA repair biomarkers relevant to PARP inhibitor therapies. Biomarkers in bold indicate they are potentially more relevant to PARP inhibitor therapies

DNA Repair Pathways	HR/FA	BER	NHEJ	MMR	NER	TLS	DDR
Key Biomarkers Relevant to PARP Inhibitor Therapies	**BRCA1** **BRCA2** **PTEN** **USP11** **PALB2** **53BP1** **RAD51 foci** **FANCD2/foci** **DSS1** **RAD54** **RPA** XRCC2 XRCC3 FANCF EMSY	**PARP1** **PARP2** **PAR** DNA Ligase III XRCC1	**DNA-PK** **Ku70/80** DNA Ligase IV	**MLH1** MSH2 MSH6 PMS2	**XPF** **ERCC1** **XPA**	Rev1 pol η pol β pol κ	**ATM** **ATR** **Chk1** **Chk2** **Mre11/NBS1** **γ-H2AX foci** **p53** **MK2/pMK2** PCNA

pathways on treatment response and cancer survival offers opportunity to evaluate patient tumor samples and determine their status of DNA repair pathways prior to and during therapy for individual patients. Most PARP inhibitors target both PARP1 and PARP2, PARP1/2 are critical DNA repair enzymes responsible for the sensing and repair of single-strand DNA breaks via short-patch BER pathway. Changes to other DNA repair pathways in cancer increase the dependence on the PARP enzymes in BER pathway. To kill tumor cells selectively by PARP inhibitors, DNA repair modulation will have to be targeted against tumors with suboptimal DNA repair. Therefore, knowledge of the status of multiple DNA repair pathways is important to determine DNA repair profiling of patients and may discriminate patients with likelihood to respond to PARP inhibitors. Currently, a number of DNA repair biomarkers are the potential informative biomarkers relevant to PARP1 inhibitor therapies (**Table 1**).

Biomarkers involved in HR pathway

Human tumors use homologous recombination more than normal cells. HR repair proteins are often dysregulated in cancer. For example, a high proportion (up to 50%) of sporadic epithelia ovarian cancers could be deficient in HR due to genetic or epigenetic inactivation of HR genes [98, 99]. Tumor cells with HR deficiency (such as *BRCA1/2*, *PTEN*, *PALB2* mutated cancer cells) are hypersensitive to PARP inhibitors, resulting in killing of tumor cells based on the synthetic lethality principle [30-32, 53, 62]. Importantly, tumor cells from sporadic cancers with BRCAness phenotype are also sensitive to PARP inhibitors [46]. A recent study identified a 60-gene signature profile for BRCAness in familial and sporadic ovarian cancers that correlated with platinum and PARP inhibitor responsiveness [100]. *FANCF* promoter methylation has been detected in several types of sporadic cancer as a BRCAness phenotype, including ovarian, breast, head and neck, non-small cell lung and cervical carcinomas [52]. Fanconi anemia (FA) *FANC* genes knockout mouse fibroblasts were shown to have sensitivity to PARP inhibitors [30]. Since FA deficient cells derived from FA patients were found to have a mild defect in HR [101], further validation of the sensitivity to PARP inhibitors using human FA derived cell lines is warranted. BRCA1 and BRCA2 have been demonstrated to collaborate in FA/BRCA pathway [102, 103], therefore, targeting FA deficiency for treatment with PARP inhibitors has its potential clinical implication [104]. Ubiquitin modification and deubiquitination at the sites of DSBs has emerged as an essential regulator of cell signaling and DNA repair [105, 106]. Using synthetic lethal siRNA screening approaches, the deubiquitylating enzyme USP11 was recently identified to be involved in HR repair of DSBs. Silencing USP11 led to HR defects, spontaneous DNA damage and hypersensitivity to PARP inhibition [107]. Deficiency in other known HR pathway proteins such as DSS1, RAD54, RPA, XRCC2, XRCC3 may also show similar synthetic lethal relationship with PARP inhibition

[30, 108].

The BRCT protein 53BP1 (p53 binding protein 1), which associates with Mre11, BRCA1 and γ-H2AX, is important in HR and NHEJ to repair DSBs, and also required for DDR, it plays an integral role in maintaining genomic stability [109-112]. Two recent studies show a new role for 53BP1 as an inhibitor for HR. Also, 53BP1 controls the sensitivity of BRCA1-deficient cells to PARP inhibitors, providing a mechanism of resistance to therapies involving PARP inhibition and DNA damaging agents [111, 112]. Moreover, absence of 53BP1 was found to correlate with triple negative breast tumors [111].

DNA repair capacity varies among individual cancer patients, and is strongly associated with chemosensitivity. For example, acquired resistance to PARP inhibitor or cisplatin in *BRCA1*- or *BRCA2*-mutated tumors was associated with secondary mutations in these genes that restore the wild-type reading frame [113-115]. The HR pathway is the central to the repair of DNA damage generated by PARP inhibitors. Defects in HR pathway are associated with hypersensitivity to PARP inhibitors and other chemotherapeutic agents [6, 31, 32, 41, 116], indicating that HR competence could be a potential indicator for chemosensitivity. Therefore, identification of HR status in patient samples is important for the use of PARP inhibitors. RAD51 mediated HR plays an important role in the repair of DNA lesions caused by PARP1 inhibition. RAD51 is a key enzyme for HR and absolutely critical for cellular survival, mice defective in RAD51 or other major components of HR repair are embryonic lethal [117-119]. RAD51 forms a nucleoprotein filament with the 3' overhanging ssDNA of the resected DSB, which invades a homologous sequence of the sister chromatid to facilitate DNA sequencing and restoration of DNA to its original form [34, 36]. DNA damage induced RAD51 nuclear foci formation is the hallmark for HR-mediated DSB repair, and the levels of RAD51 nuclear foci reflect HR efficiency. HR deficient cells fail to form DNA damage- induced RAD51 nuclear foci [24, 120-123]. In contrast, inhibition or loss of PARP in HR intact cells results in increased RAD51 foci formation, confirming a hyper recombination phenotype in these cells [38]. Upregulation of RAD51 was found in a wider range of tumors [124], which most likely correlates with drug resistance of these tumors. Increased RAD51 expression majorly detected as increased RAD51 foci formation appears to be increased transcription of the RAD51 gene and possibly its post-translational modifications [122, 124, 125]. A functional RAD51 IF assay based on the levels of RAD51 foci formation in primary cultures of epithelial ovarian tumor was developed. This assay demonstrated correlations between RAD51 foci and *in vitro* responsiveness to the treatment with PARP inhibitor [98]. In another study, RAD51 nuclear foci detected by IF assay were scored as the proportion of proliferative cells to predict the response to neoadjuvant chemotherapy in breast cancer biopsies, the results showed that defective HR, as indicated by low RAD51 foci, may be one of the factors that underlie sensitivity to anthracycline based chemotherapy [126]. DNA repair proteins often form nuclear foci in response to DNA damage, during S phase or after DNA damage, RAD51 localizes in nuclear foci with other DNA repair proteins including BRCA1, BRCA2, PALB2, FANCD2 [54, 123, 127-130]. Furthermore, inactivation of the FA/BRCA pathway, which is frequently found in cancer, can be detected by the inability of the affected cells to form FANCD2 foci in response to DNA damage [102, 131]. There is currently great interest in using FANCD2 foci formation as a functional biomarker to predict the sensitivity of cancer cells to cross-linking drugs such as cisplatin [2]. In addition to the repair of DNA crosslinks, FANCD2 can also be activated by forming nuclear foci in response to DSBs such as with chemotherapy, radiotherapy or PARP inhibitors [102, 104, 131]. A biomarker assay was developed by Powell's group for detection and measurement of DNA damage induced BRCA1, RAD51, FANCD2 foci in sporadic breast cancer biopsies using IF assay. Interestingly, they found three of the foci-defective tumors were triple-negative breast cancer, the absence of such foci was closely correlated with likely defects in the BRCA1 pathway [132]. **Figure 3** shows an example of FANCD2 as a biomarker that can be detected by IHC and IF.

Taken together, gene mutation status or functional loss of BRCA1, BRCA2, 53BP1 and BRCAness (such as PTEN, EMSY, USP11, PALB2, FANCF, DSS, RAD54, XRCC2, XRCC3) in a group of patient specimens would serve as predictive markers which allow tailoring of PARP inhibitors treatment to the DNA repair profile of individual tumors. Measurement of expression

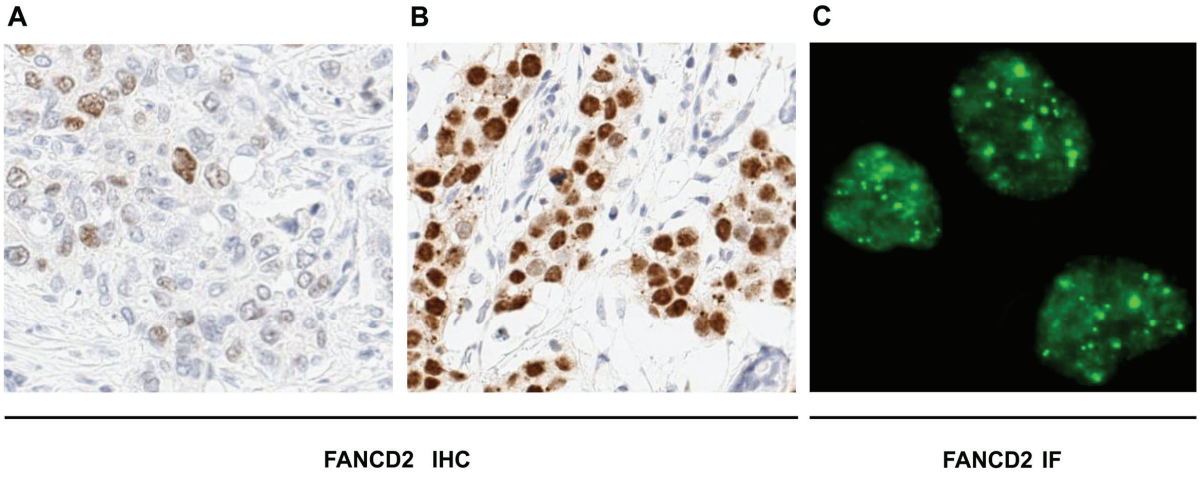

Figure 3. The FANCD2 DNA repair protein as a biomarker. **A**. IHC of FFPE from a breast cancer patient biopsy with low levels of FANCD2 observed; **B**. Similar IHC from another breast cancer patient where high levels of FANCD2 are found; **C**. Detection of FANCD2 nuclear foci by IF in cancer cells.

levels of HR repair proteins listed in **Table 1** and the levels of nuclear foci formation of HR proteins such as RAD51, FANCD2 for HR competence in patient tumors prior to, during and after PARP inhibitor therapies may identify effective and informative biomarkers that predict response and clinical outcome to PARP inhibitor therapies.

Biomarkers involved in BER pathway

PARP1 and PARP2 are the only two enzymes in PARP superfamily that have been implicated in the repair of DNA damage by BER pathway. Formation of PAR by PARPs mediated-poly(ADP-ribosyl)ation results in releasing of PARPs from damaged DNA. PAR is a potentially powerful biomarker to indicate PARPs activity. Levels of PAR are associated with PARPs activity, low levels of PAR may have low DNA repair capacity [12, 25, 28, 29]. A pharmacodynamic assay was developed to detect cellular levels of PAR in both tumor specimens and peripheral blood mononuclear cells (PBMCs) [133]. This robust, quantitative and sensitive enzyme-linked immunosorbent assay (ELISA) has been applied to assess the efficacy of various dose levels of the PARP inhibitors ABT-888, olaparib during clinical trials including ongoing trials with topotecan and cyclophosphamide, each of which includes measurement of PAR as a pharmacodynamic endpoint [1, 133, 134]. These measurements showed a significant correlation between the effects of the PARP inhibitor in PBMCs and the tumor samples, raising the possibility that blood samples could be used as tumor surrogates following PARP inhibition. In the future, similar tests could be a potential biomarker to monitor CTC from patient's blood prior to, during and after PARP inhibitor therapies [135]. In addition, it has been reported that PARPs expression and activity are up-regulated in a variety of human tumors, including glioblastoma [136], malignant lymphoma [137], hepatocellular carcinomas [138], breast [139], ovarian[140], and cervical cancers[141]. Strong PARP expression detected by IHC was determined in 76% of cases expression in a cohort of ovarian serous carcinomas and this group correlated with a poorer outcome compared to patients with low expression [140]. PAR levels can also be detected by IHC [139, 142]. In a phase 0 clinical trial study, expression levels of PAR and PARP1 were evaluated by IHC in patient FFPE specimens with refractory solid tumors and lymphomas treated with PARP inhibitor ABT-888. Reduced PAR levels and upregulated expression of PARP1 in tumor were significantly associated with ABT-888 treatment. Given the effect of ABT -888 on both PAR and PARP1, it was suggested that an absolute or relative change in the ratio of PAR to PARP1 may be an appropriate measurement for evaluating the pharmacodynamic effect of PARP inhibition in human tumor cells

[142]. A recent small clinical study investigated PARP activity and expression, it draws attention to the results obtained in clinical trials where PARP activity used as a pharmacodynamic marker of PARP inhibition could reflect the effect of a chemotherapeutic on PBMCs rather than the effectiveness of a tested PARP inhibitor [143]. In addition, XRCC1 which forms heterodimers with PARP1, interacts with many BER proteins. XRCC1-/- cells were found to be sensitized by PARP inhibition [144]. Therefore, measurement of expression levels and mutation status of BER proteins (**Table 1**) such as PARP1, PARP2, PAR, XRCC1 is of importance and should be proceeded with caution, which could facilitate the cancer diagnosis in order to stratify patient population.

Biomarkers involved in DDR pathway

Both ATM and ATR kinases are key regulators to sense DNA damage and initiate the subsequent protein kinase cascade. There are two major parallel pathways: ATM-Chk2 pathway is activated primarily to DSBs induced by ionizing irradiation, while ATR-Chk1 pathway responds to agents that could cause SSBs or stalled DNA replication forks, such as ultraviolet light and hydroxyurea. It has been demonstrated that there is an active cross talk between ATM and ATR pathways, and some agents have been shown to be able to activate both pathways [145]. The emerging evidence indicates that the concept of synthetic lethality is also applied to the effect of PARP inhibitors on selectively killing tumor cells with DDR deficiency, tumor cells with deficiency of DDR such as ATM, Chk2, Mre11/NBS1, ATR, Chk1, are hypersensitive to PARP inhibitors [30, 42, 63]. ATM is activated by PARP inhibitor-induced collapsed replication forks and may function upstream of HR in the repair of certain types of DSBs [63]. It was reported that ATR signaling mediates an S phase checkpoint after methylated DNA damage in combination with inhibition of PARP [146]. The histone H2AX, a key protein of the cellular response to DNA damage, recruits DNA repair proteins to the sites of DNA damage in a phosphorylation dependent manner. Phosphorylated H2AX at serine 139 termed γ-H2AX, forms nuclear foci after exposure to exogenous DNA damage agents that induce DSBs [147]. γ-H2AX has been considered as a DNA DSBs marker to evaluate the efficacy of various DSB-inducing compounds and radiation, and its foci are known to be involved in the repair of DSBs by HR and NHEJ pathways [148]. Monitoring DSBs formation in a cell by detecting the levels of γ-H2AX foci formation has become a sensitive means to monitor cancer progression and treatment since many therapeutic agents either induce DSBs directly (such as radiation, bleomycin, doxorubicin) or create different types of DNA damage that can lead to DSBs formation (such as PARP inhibitors, gemcitabine, cisplatin) [149]. Inhibition of PARP leads to γ-H2AX foci accumulation in an ATM dependent manner [42]. γ-H2AX is an active pharmacodynamic biomarker currently being developed by NCI. Assays to measure levels of γ-H2AX foci have been developed: one ELISA-based method using an electrochemoluminescent detection system (an assay derived from the Meso Scale Discovery Technology or MSD assay) to measure γ-H2AX in tumors biopsies after irradiation was recently reported [150]. A high-throughput screening system, called the RABIT (Rapid Automated Biodosimetry Tool), using a γ-H2AX IF assay to directly measure DSBs level, was developed, which would allow the screening of 6,500 samples a day [151]. With these assays, the levels of γ-H2AX foci can be measured in tumors after the treatment with PARP inhibitors.

PARP inhibition sensitizes p53-deficient breast cancer cells treated with doxorubicin [152]. Loss of p53 renders cells dependent on MAPKAP kinase 2 (MK2) signaling for survival after DNA damage, MK2 is activated and phosphorylated at Thr-334 site by p38 MAPK in response to DNA damage induced by chemotherapeutic agents [153]. A recent study from Yaffe's group shows that nuclear Chk1 activity is essential to establish a G2/M checkpoint, while cytoplasmic MK2 activity is critical for prolonged checkpoint maintenance through a process of posttranscriptional mRNA stabilization. MK2 is found to be activated in human tumor samples [154]. The importance of p53, MK2/pMK2 in DDR pathway, their roles in apoptosis and the fact that p53 was mutated in a large proportion of human cancers make them strong candidate biomarkers relevant to PARP inhibitor therapies.

Collectively, DDR proteins (**Table 1**) are potential powerful biomarkers relevant to PARP inhibitor therapies. Assays to identify the DDR genes mutation status or expression levels of the DDR proteins could serve a guide to determine cancer patients' likelihood of response to PARP

inhibitor therapies.

Biomarkers involved in other DNA repair pathways

Detection of the status of other DNA repair pathways using DNA repair proteins in NHEJ, MMR, NER and TLS pathways as potential biomarkers may also provide useful information to enrich DNA repair profiling of cancer patients, and contribute to the effort to discriminate a subset of patients who would benefit from PARP inhibitor therapies (Table 1).

For example, PARP has also been implicated in the alternative NHEJ pathway of DSBs repair [155]. PARP inhibitors inhibit NHEJ pathway, and greatly decrease DNA-dependent protein kinase (DNA-PK) activity. Poly(ADP-ribosyl)ation of DNA-PK by PARP1, and phosphorylation of PARP1 by DNA-PK also occur, suggesting a reciprocal regulation [156]. PARP inhibition also sensitized DNA Ligase IV knockout MEF cells to methylmethane sulfonate treatment and promoted replication-independent accumulation of DSBs, repair of which required DNA Ligase IV. Additionally, Ku80 deficient cells were sensitized to ionizing radiation by PARP inhibition [76].

PARP1 was also reported to affect two of the other DNA repair pathways: NER and MMR [157, 158]. NER pathway is involved in efficient repair of SSBs and repairs lesions such as interstrand and intrastrand breaks induced by many chemotherapeutic agents, such as cisplatin. Cells with defective NER are hypersensitive to platinum agents and enhanced NER pathway is one of the mechanisms of platinum resistance [159, 160]. PARP inhibitor enhanced lethality in XPA deficient cells after UV irradiation [161]. MMR gene deficiency results in increased resistance to many anticancer therapies [13I. MLH1 in MMR plays a role in signaling PARP-dependent cell death [162]. PARP inhibitors have a greater impact on the temozolomide sensitivity of MMR-deficient than MMR-proficient tumor cells, where it overcame their resistance to temozolomide [163]. Cells proficient in MMR were found to be more sensitive to single agent olaparib than are microsatelite instability (MSI) cells [164].

Taken together, evaluation of DNA repair biomarkers from each DNA repair and damage signaling pathway in cancer patient biopsies prior to, during and after treatment with PARP inhibitors may be critical. Therefore, integrating the multiple pathways information that associated with clinical outcome will assist in discriminating a subset of patients who would benefit from PARP inhibitors therapies.

Clinical trials race ahead

Most PARP inhibitors are competitive inhibitors of NAD+ at the enzyme active site. The early generation of PARP inhibitors, such as the nicotinamide analogue 3- aminobenzamide (3-AB), lacked selectivity and potency, and their use in the clinic was limited. More specific and potent PARP inhibitors have been developed using structure activity relationships and crystal structure analysis to modify 3-AB with variable biochemical, pharmacokinetic and PARP selectivity properties [25, 165]. Also, new chemotypes have been discovered and optimized by the classical drug development paradigms. A number of clinical trials are now underway to test the efficacy of PARP inhibitors, such as PF-1367338, ABT-888, olaparib, iniparib, INO-1001, MK-4827 and CEP-9722.

The first inhibitor of PARP used in human trials is PF-1367338 (previously known as AG014699) that was developed by Pfizer and was shown to potentiate the cytotoxicity of temozolomide and irinotecan in preclinical models. A phase I clinical trial of PF-1367338 in combination with temozolomide in patients with advanced solid tumors demonstrated antitumor activity of PF-1367338. This study also established PARP inhibition levels to a biologically effective dose by quantitative immunologic detection of the cellular levels PAR in surrogate tissue (peripheral blood mononuclear cells) and further validating corresponding PARP inhibition in melanoma. Comet assays was applied to assess DNA strand breaks for DNA damage levels [166]. Additional clinical trials using PF-1367338 either as a single agent in *BRCA1* and *BRCA2* carriers with locally advanced or metastatic breast cancer, advanced ovarian cancer or in combination with several chemotherapeutic regimens (such as carboplatin, paclitaxel, cisplatin, pemetrexed, epirubicin, cyclophosphamide) in advanced solid tumors, are ongoing.

A phase I study of treating *BRCA1/2* associated

breast, ovarian or prostate cancers using oral olaparib was the first to show antitumor activity of PARP inhibitor as a single agent in the absence of chemotherapy. Olaparib (previously known as KU-0059436 and AZD2281) developed by KuDOS Pharmaceuticals and later by AstraZeneca, is orally active inhibitor of PARP1 and PARP2 with up to 1000-fold selective potency in isogenic preclinical models [31, 32]. In the phase I study, PARP inhibition was evaluated in pharmacodynamic studies by means of a functional assay involving the analysis of PAR levels in PBMCs and tumor cell lysates after treatment. Values were all normalized to the amount of PARP1 protein present. In addition, the formation of γ-H2AX foci was evaluated in patients receiving doses of 100 mg or more of olaparib twice daily prior to, and at multiple time points after treatment on plucked eyebrow-hair follicles. Induction of γ-H2AX foci was found after 6 hours of olaparib treatment, indicating that PARP inhibition was rapidly associated with downstream induction of collapsed DNA replication forks and DNA DSBs, consisting with preclinical models [1]. In a phase I study for the treatment of *BRCA* mutation carrier patients with advanced ovarian cancer by the same group, olaparib resulted in high antitumor response and disease stabilization rates, suggesting that resistance to platinum decreases sensitivity to olaparib and the platinum-free interval in patients with *BRCA*-mutated ovarian cancer may be associated with response to olaparib [43]. In addition to undergoing clinical trials for the treatment of *BRCA1* and *BRCA2* mutation carriers with advanced tumors, Olaparib is being entered in clinical trials of treating patients with ovarian, pancreatic, prostate and colorectal tumors and melanoma. Olaparib has the potential for use as a single agent or in combination with platinum-based DNA-damaging agents and cytotoxic drugs, as well as radiotherapy. Two parallel multicentre phase II studies of olaparib in *BRCA1* and *BRCA2* mutation carriers with advanced or metastatic breast and recurrent epithelial ovarian cancer recently confirmed significant therapeutic efficacy and established proof-of-concept for targeting cancers in *BRCA* mutation carriers with PARP inhibitors [44, 45]. A number of clinical trials involving combination of olaparib with carboplatin and paclitaxel, topoisomerase inhibitors, gemcitabine and bevacizumab in advanced solid tumors are ongoing. Several efficacy studies using olaparib with paclitaxel, irinotecan, liposomal doxorubicin and cediranib to treat patients with recurrent ovarian or triple negative breast, gastric, and colorectal cancers are planned. A phase I study to compare the bioavailability of two oral formulations of olaparib in advanced solid tumor cancer patients is also underway.

ABT-888 (Veliparib, Abbott), an oral potent inhibitor of both PARP1 and PARP2, was the first anticancer compound to be evaluated in a phase 0 clinical trial in patients with advanced malignances. ABT-888 demonstrated good oral bioavailability with a half-life of several hours and crosses the blood-brain barrier. PARP activity was measured based on PAR levels using a validated ELISA pharmacodynamic assay and IHC to determine pharmacokinetic profile of ABT -888. Treatment with ABT-888 resulted in significant decrease of PAR levels and increased expression level of PARP1 [134, 142]. One of current clinical trials aims to identify suitable patients by measuring foci formation of FANCD2 and γ-H2AX in the FFPE tumors treated with ABT -888 either alone or in combination with chemotherapy [104]. A number of phase I/II clinical trials are ongoing that use ABT-888 as a single agent or in combination with chemotherapeutic agents including carboplatin, paclitaxel, cisplatin, temozolomide, topotecan, cyclophosphamide, for recurrent and/or metastatic breast, ovarian epithelial, colorectal cancers and glioblastoma.

Iniparib (previously BSI-201) developed by BiPar, and now Sanofi-Aventis, was the first PARP inhibitor to enter phase III clinical trials for breast and non-small lung cancers. Iniparib is a potent inhibitor of PARP1 and possible other enzymes through an irreversible, covalent modification. This inhibitor has a different mechanism of action from other PARP inhibitors, because it forms a covalent bond. Iniparib, either alone or in combination with chemotherapy, had significant antitumor activity in preclinical studies *in vitro* and *in vivo*. Iniparib is being evaluated in multiple phase II and phase III clinical trials in breast, ovarian, uterine, and brain tumors [167]. The phase III trial, initiated in July, 2009, is a multi-center, randomized trial designed to evaluate the safety and efficacy of iniparib when combined with gemcitabine and carboplatin as first-, second-, and third-line therapy in women with metastatic TNBC. Another randomized phase III trial of gemcitabine/carboplatin with or without iniparib in patients

with previously untreated advanced squamous cell lung cancer is ongoing. Preliminary data on TNBC are promising, phase I clinical trials in patients with solid tumors demonstrated that treatment with iniparib was associated with minimal toxicity. A randomized phase II clinical trial reported by Sanofi-Aventis demonstrated 71.7% of patients in 120 women metastatic TNBC receiving iniparib in combination with gemcitabine and carboplatin showed clinical benefit. Combination of iniparib to gemcitabine and carboplatin also improved tumor response, progression-free survival and overall survival in this cohort of patients [168]. Phase I/II study of iniparib in combination with temozolomide to treat patients with newly diagnosed malignant glioma is ongoing. Several phase II clinical trials of iniparib as a single agent or in combination with gemcitabine and carboplatin/cisplatin chemotherapy are ongoing in other tumor types, such as ovarian and uterine cancer, non-small cell lung cancer and glioblastoma.

MK4827, developed by Merck, inhibits both PARP 1 and PARP2. In a xenograft model of *BRCA1* deficient cancer, MK4827 was well-tolerated *in vivo* and demonstrated efficacy as a single agent [169]. A Phase I study of MK-4827 is currently ongoing in patients with advanced solid tumors. A Phase Ib dose escalation study of MK4827 in combination with carboplatin, carboplatin/paclitaxel and carboplatin/liposomal doxorubicin in patients with advanced solid tumors is recruiting participants.

CEP-9722 from Cephalon, is a prodrug of CEP-8983 that is a novel 4-methoxy-carbazole inhibitor of the PARP1 and PARP2 with antineoplastic activity. CEP-9722 enhances the accumulation of DNA strand breaks and promotes genomic instability and apoptosis. CEP-9722, when combined with temozolomide or irinotecan, inhibited the growth of glioblastoma or colon carcinoma tumor cells. CEP-9722 attenuated PAR accumulation in glioma xenografts in a dose- and time-related manner, indicating CEP-9722 is an effective chemosensitizing agent [170]. A phase I study of CEP-9722 either as a single agent or in combination with temozolomide is currently being tested in patients with advanced solid tumors.

INO-1001 developed by Inotek, functions as the orphan drug for cardiovascular postoperative complications of aortic aneurysm repair. Based on company's news release, extensive preclinical *in vivo* studies have shown that the PARP-blocking activity of INO-1001 protects tissues from ischemia, reperfusion injury, and inflammatory damage. Several Phase I and Phase II trials showed that INO-1001 was safe and well tolerated, with no incidence of serious adverse event. A small phase I trial of the combination of INO-1001 with temozolomide in 12 patients with advanced melanoma was recently reported that the combination were hepatic toxicity and myelosuppression. This combination is being evaluated in patients with malignant glioma.

Ups and downs: personalized PARP inhibitor therapies with companion biomarkers

Disruption of DNA repair increases chromosome breaks and mutagenesis, and leads to genome instability. Tumors that are deficient in one or more DNA repair pathways appear to rely more than normal cells on the remaining functional DNA repair pathways to repair DNA damage induced either endogenously or exogenously to survive. For example, tumors tend to use homologous recombination relatively more than normal cells [171]. On the other hands, tumors in patients with *BRCA1* or *BRCA2* mutations are defective in HR. Tumors with HR deficiency or BRCAness are hypersensitive to PARP inhibitors, providing a "synthetic lethality" rationale for cancer therapy [30-32] (**Figure 4**).

Resistance to PARP inhibitors

It has been demonstrated that elevated DNA repair capacity in tumor cells is associated with resistance to drug or radiation, which significantly limits the efficacy of these agents in most diseases [6, 172, 173]. Not all of the cancer patients would respond to PARP1 inhibitors treatment. In phase I study, a group of 19 patients with a documented BRCA mutation, including breast, ovarian, and prostate malignancies were found to have a 47% response rate and a 63% clinical benefit rate [1]. There may be several alternative mechanisms for resistance to PARP inhibitors in cancer patients, revealed by patient tumor DNA repair profiling (**Figure 4).** Overall, most of these mechanisms are likely to apply to all of the PARP inhibitors, as a class of drug effect.

The studies from the Ashworth and Taniguchi groups provided insight into the resistance

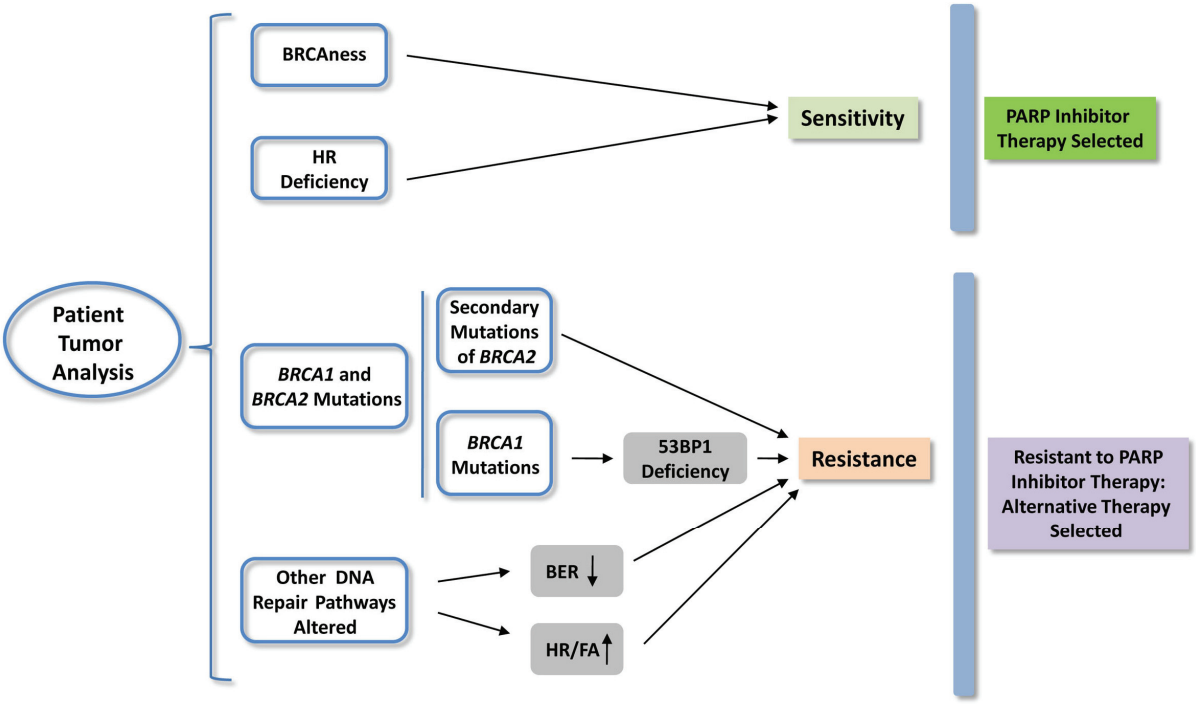

Figure 4. Patient predisposition to PARP inhibitor therapy benefit and development of resistance. Biomarker analysis of patient tumor samples could reveal DNA repair profile of individual patients, and may discriminate patients with likelihood to respond to PARP inhibitors. Tumors in patients with HR deficiency and BRCAness are hypersensitive to PARP inhibitors, it would be optimal to select this subset of patients for PARP inhibitor therapy. On the other hand, patient tumors that have secondary mutations of *BRCA2*, or *BRCA1* mutations containing 53BP1 deficiency or other DNA repair pathways altered such as downregulation of BER or upregulation of HR/FA, would be resistant to PARP inhibitors, such group of patients needs alternative therapy other than PARP inhibitor.

mechanism of PARP inhibitors or cisplatin in *BRCA2* deficient tumor cells with potential clinical implications. PARP inhibitor-resistant clones derived from *BRCA2* deficient pancreatic cancer cell line, and carboplatin-resistant ovarian tumors from *BRCA2* mutation carriers, were found to be acquired by deletion of a mutation in *BRCA2* that restored the open reading frame of *BRCA2* and expressed new BRCA2 isoforms. Reconstitution of *BRCA2* deficient cells with these revertant *BRCA2* alleles rescued PARP inhibitor sensitivity and HR deficiency, supported by a capability to form RAD51 foci after treatments with PARP inhibitor and IR [114]. Secondary mutations in *BRCA2* that restore wild-type *BRCA2* reading frame were also found in cisplatin-resistant *BRCA2* mutated breast cancer cell lines and pancreatic cancer cell line which were also cross-resistant to PARP inhibitor. Both drug resistant clones were able to form RAD51 foci after exposure to IR. Furthermore, recurrent ovarian tumors from *BRCA2* mutation carriers acquired cisplatin resistance were found to have undergone reversion of its *BRCA2* mutation [113]. Therefore, patients who can acquire additional mutations of *BRCA2* would restore HR functionality, which may result in resistance to PARP inhibitor treatment, whereas platinum-resistant *BRCA2*-mutated tumors without secondary *BRCA2* mutations may remain sensitive to PARP inhibitors [113, 114]. These elements of resistance are a rationale for DNA repair profiling to better direct patient treatment in the course of PARP inhibitor therapy.

Recently, two studies shed light on another resistance mechanism of PARP inhibitors in patients with *BRCA1* mutations that also implications for cancer therapy [111, 112]. 53BP1 was found to inhibit HR repair in *BRCA1* deficient cells, loss of *53BP1* increased HR capacity in *BRCA1* mutant cells, rescued RAD51 foci formation after IR treatment, and promoted RPA phosphorylation in a manner dependent on ATM

and CtIP. When *53bp1* was deleted in mice, the sensitivity of *BRCA1*-deficient cells to a PARP inhibitor was reversed. Loss of *53BP1* in *BRCA1* deficient cells resulted in significant tumor formation in *BRCA1* deficient mice [112]. The effect of 53BP1 is specific to BRCA1 function, as 53BP1 depletion did not alleviate proliferation arrest or checkpoint responses in *BRCA2*-deleted cells [111]. Many *BRCA1* deficient tumors overexpress RAD51 [124], which might indicate partial restoration of DSBs. Reduced 53BP1 expression was found in subsets of sporadic triple-negative and BRCA-associated breast cancers. Loss of *53BP1* is another secondary mutation that renders *BRCA1* mutant cells HR competent and resistant to PARP inhibitors [111]. Therefore, resistance to PARP inhibitors can be acquired from secondary gain-of-function mutations in the synthetic lethal partner or other genes involved in the complex HR pathway rather than the direct drug target (PARP inhibitors). The studies also suggest that additional DNA repair inhibitors, such as ATM inhibitors, could serve as a second line of chemotherapy for PARP inhibitor-resistant tumors [112].

PARP inhibitors increase antitumor efficacy when used in combination with chemotherapeutic agents. However, the addition of the PARP inhibitors does not alleviate development of patient resistance to the combination therapy. A recent study investigated the potential resistance mechanism of the treatment with the combination of temozolomide and the PARP inhibitor ABT-888. Colorectal carcinoma HCT116 cells resistant to the combination treatment (HCT116R) were found to have increased ability to repair DSBs and depend on RAD51 for proliferation and survival, HCT116R cells were defective in BER, and failed to generate PAR in response to the treatment with ABT-888. Decreased levels of PARP1 mRNA and increased levels of mRNA coding various HR proteins including RAD51, FANCA, FANCG, BLM, BRCA1, and BRCA2 in the resistant clone were found, in addition, HCT116R cells were more resistant to radiation than the parental HCT116 cells [174].

Patient stratification and pharmacodynamic benefit of tracking biomarkers

Patient stratification involves the use of biomarkers to discriminate subsets of the patient population most likely to respond to a given therapy. In the clinic, Biomarker assays for responder/nonresponder patient stratification are useful to determine the appropriate treatment. Relatively little biomarker information is currently available for candidate cancer patient stratification for PARP inhibitors. One of the major challenges in PARP inhibitor therapies is how to identify biomarkers for the subset of the responder population with non-*BRCA* mutant, BRCAness and HR deficient cancers. Despite the early stage of the diagnostics capabilities for PARP inhibitor therapies, it is valuable and important to develop properly validated and robust biomarker assays to assist oncologists in making treatment choices for individual patients.

Assays to measure HR proficiency and PARP activity *in vivo* will be vital to the primary or acquired resistance to PARP inhibitors in the clinical studies. Pharmacodynamic biomarker assays to measure levels of PAR, γ-H2AX foci, RAD51 foci *in vivo* were recently developed [98, 133, 135, 150] and applied in several clinical studies [1, 134, 166, 175]. For example, the drug effect of PARP inhibitors can be determined via a robust validated immunoassay ELISA or IHC to quantify PAR levels in patient tumor biopsies and blood cells, and the consequences of PARP inhibition can be detected in tumor and blood cells by IF to quantify the levels of γ-H2AX foci in order to assess the extent of stalled and collapsed replication forks and DSBs, or the levels of RAD51 foci in order to assess HR competence. Further clinical studies are needed to evaluate if failure to form nuclear foci of RAD51, γ-H2AX or other DNA repair proteins is a predictor of sensitivity to PARP inhibitors and if tumor cells with constitute high levels of nuclear foci of DNA repair proteins would indicate resistance to PARP inhibitors. The systematic use of PAR, γ-H2AX, RAD51 and other DNA repair biomarkers in tumor biopsies or patient blood prior to, during and post treatment may discriminate patient populations responding or resistant to PARP inhibitors.

There is considerable interaction, crosstalk and overlap between DNA repair pathways in response to different types of DNA damage. For example, crosstalk between HR, NHEJ, DDR pathways in the repair of DSBs or crosstalk between BER, alkyltransferases and DNA dioxygenases in the repair of alkylation damage, are also likely to contribute to resistance mecha-

nisms in tumors, which is a limitation for combating more advanced tumors [176]. DNA lesions induced by chemotherapeutic agents and radiation can be repaired by a variety of DNA repair pathways. Tumor cells utilize DNA repair pathways to survive in response to chemotherapy or radiation, elevated activity of DNA repair pathways in tumor cells often leads to resistance to treatments [6, 172, 173]. It is important to realize that the efficacy of PARP inhibitor therapies can be modulated by interrelationship of DNA repair pathways. Compensation of repair in the absence of one DNA repair pathway by another DNA repair pathway in tumors often leads to selective toxicity in a subgroup of cancers in response to specific cancer therapy. The use of potent, orally active PARP inhibitor olaparib as monotherapy in phase I to treat the *BRCA1* and *BRCA2* mutant carriers demonstrated synthetic lethality of HR repair defective cells when BER was blockade by PARP inhibition [1]. Resistance to platinum-based chemotherapy in the clinic is a major challenge for cancer therapy. Platinum sensitive tumors may indicate defects in HR and NER pathways, while resistance to platinum agents may be caused by enhanced NER and MMR deficiency [160]. Tumors that are sensitive to platinum agents may depend more on functional PARP activity, resistance to platinum decreases sensitivity to PARP inhibition and high doses of cisplatin may overcome the ability of PARP to repair the cisplatin induced DNA breaks, leading to cell death with dysfunctional HR. There was a significant association between the clinical benefit rate and platinum-free interval across the platinum-sensitive, resistant, and refractory subgroups when treated with olaparib in combination with platinum [43]. Iniparib, when combined with gemcitabine /carboplatin in patients with metastatic TNBC significantly improved clinical benefit rate, progression-free survival and overall survival, compared with gemcitabine / carboplatin treatment alone [168]. Although complex, monitoring the status of DNA repair pathways by systematically evaluating multiple DNA repair biomarkers in patient tumors would reveal important information about treatment and personalized therapies.

Proceed with caution

In this review, we have discussed current trends in DNA repair biomarker strategies for patient selection and prediction in PARP inhibitor therapies. Systematic evaluation of multiple DNA repair biomarker panels in patient specimens will lead to improved prediction and monitoring of patient response to PARP inhibitor therapies and guide clinical decision-making. Thus, targeted therapy using PARP inhibitors will prove beneficial only in specific patient subsets as defined by their DNA repair biomarker signatures.

This endeavor must proceed with caution. Further understanding of these DNA repair pathways will improve the development of therapeutic strategies that kill tumors with increased specificity and efficacy. The effective stratification biomarkers from different DNA repair pathways measured specifically in tumor would be necessary to determine patients' response to PARP inhibitors. It is also essential to identify informative biomarkers with loss of specific post -translational modifications present in the DNA repair pathways, or those that indicate increased or decreased activity of the targeted DNA repair pathway. Moreover, it is important to develop robust, tumor specific assays such as pharmacodynamic assays to measure DNA repair biomarkers in patient samples prior to, during and after treatment with PARP inhibitors, which would allow the accurate assessments of DNA repair biomarkers in a tumor-specific manner to predict and monitor response to PARP inhibitor therapies. One of the challenges to biomarker discovery is tumor heterogeneity that would affect tissue-based biomarker assessment and analysis, which may influence the association between a biomarker and an outcome. It is thought that tumor cell heterogeneity arises in cancer cell populations as a result of genetic instability. Therefore, levels of biomarkers may differ among multiple biopsies of the same tumor. It is likely that tumor heterogeneity is highly dependent on biomarker analyzed and caution should be used when making determinants of biomarker expression in a tumor using patient FFPE tumor samples.

From a therapeutic prospective, appropriate planning and design of biomarker studies must consider how biomarkers may be used as targets and how to develop multivariate assays that can aid in a better understanding of how multiple DNA repair pathways can affect the progression or therapeutic response of an individual's unique cancer. Careful selection of the most informative and reproducible biomarkers

and validation of the assays used to assess those biomarker profiles in subsequent clinical studies are all critical components that can maximize the chances of predicting drug efficacy in cancer patients. If a predictive biomarker is to be co-developed with the drug, then the phase 1 and phase 2 studies should be designed to evaluate the candidate biomarkers and assays, to select one, and then to perform analytical validation of the assay prior to launching the phase 3 trial. Accomplishing all of this prior to initiation of a phase 3 trial can be very challenging [81].

Because of the complexity and crosstalk between DNA repair pathways, single biomarker models may not be sufficient to predict the benefit of PARP inhibitor therapies. A combination of DNA repair biomarkers would provide crucial information of the status of different DNA repair pathways in PARP inhibitor treatments, and is expected to be more robust than a single biomarker. Biomarker discovery, replication and validation studies to develop effective, integrated multiple pathways algorithms that associated with clinical outcome in cancer patients treatment are essential to stratify subsets of patients who would benefit from PARP inhibitor therapies and guide clinical diagnosis. Furthermore, a careful, proper implementation of the biomarker–stratified design and reliable, effective statistical analysis are crucial for assessment of biomarker clinical utility.

The frustration and the potential

PARP inhibitors are a new class of drug showing considerable promise with results in a number of clinical trials for treating several types of cancer. Yet, there are still many challenges facing the success of using PARP inhibitors in cancer therapies. As we discussed in this review, resistance to PARP inhibitors is a major hurdle, as is the prospect that not all patients benefit from the PARP inhibitor therapies. A major important solution to these barriers is to build biomarker testing into patient tumor identification, and to use the biomarker panels during treatment. The incorporation of biomarker strategies involving serial biopsies in clinical studies may be critical in further elucidation of resistance mechanisms. That, in turn, would aid in the identification of biomarkers to guide clinical design and management and help overcome treatment failure. Identification of subsets of patients that will benefit from these new PARP inhibitors and development of tests that broaden the recognition of additional patients who should be treated with PARP inhibitors using larger cohorts are of importance and challenging. Moreover, PARP inhibitors may have utility outside the relatively small proportion of cancer patients carrying *BRCA* mutations. Another major challenge in the coming years is to identify tumors with non-*BRCA* mutant, BRCAness and HR deficiency. Utilization of the knowledge of DNA damage and repair networks and determination of which DNA repair pathways are abrogated in sporadic tumor subtypes may allow identification of biomarkers early enough to predict response to PARP inhibitors and sensitization of PARP inhibition. Moreover, development of robust biomarker assays that provide a quantitative assessment of the likelihood of PARP inhibitor therapies benefit is critical to bridge biomarkers to clinical outcome, which will be very useful for identification of adequate predictive algorithms. Discovery, replication and validation of candidate biomarkers to predict responders will lead to identifying the additional subset of patient population most likely to respond to PARP inhibitors and ultimately will guide clinical diagnosis. Future studies focusing on defining and incorporating of biomarker strategies in clinical planning and design of PARP inhibitor therapies would have a great impact on distinguishing optimal patient populations.

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Please address correspondence to: XiaoZhe Wang, PhD, On-Q-ity, Inc., 610 Lincoln St., Waltham, Massachusetts, 02451, USA. E-mail: xiaozhe.wang@on-q-ity.com

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