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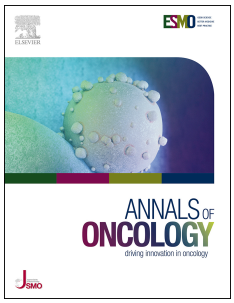

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The DNA Damage Response pathway as a land of therapeutic opportunities for colorectal cancer

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Abstract

Background:

Colorectal cancer (CRC) represents a major cause of cancer deaths worldwide. Although significant progress has been made by molecular and immune therapeutic approaches, prognosis of advanced stage disease is still dismal. Alterations in the DNA damage response (DDR) pathways are emerging as novel targets for treatment across different cancer types. However, even though preclinical studies have shown the potential exploitation of DDR alterations in CRC, systematic and comprehensive testing is lagging behind and clinical development is based on analogies with other solid tumors according to a tissue-agnostic paradigm. Recently, functional evidence from patient-derived xenografts and organoids have suggested that maintenance with PARP-inhibitors might represent a therapeutic opportunity in CRC patients previously responsive to platinum-based treatment.

Design:

In this review, we highlight the most promising preclinical data and systematically summarize published clinical trials in which DDR inhibitors have been used for CRC and provide evidence that disappointing results have been mainly due to a lack of clinical and molecular selection.

Conclusions:

Future preclinical and translational research will help in better understanding the role of DDR alterations in CRC and pave the way to novel strategies that might have a transformative impact on treatment by identifying new therapeutic options, including tailored use of standard chemotherapy.

Keywords

DNA Damage Response

PARP-inhibitors

PARPness

Organoids

BRCA

ATM

Colon cancer

Introduction

Colorectal cancer (CRC) is the third leading cause for cancer-related death in the Western world [1]. While 5-year survival rates are 85-90% for patients with localized CRC, they dramatically decrease to about 12% in patients with metastatic CRC (mCRC) [2]. Most effective treatment options for patients with metastatic CRC (mCRC) are chemotherapy regimens composed of 5-fluorouracil, oxaliplatin and/or irinotecan, in combination with targeted agents such as anti-angiogenic compounds (bevacizumab or aflibercept) or anti-EGFR drugs (cetuximab or panitumumab) according to *RAS/BRAF* status of the tumor [3, 4]. More recently, a triple combination of targeted agents against the MAPK pathway (panitumumab plus encorafenib plus binimetinib) proved its activity against *BRAF* mutant mCRC [5]. Moreover, *ERBB2* amplification has been identified as a druggable target with promising results from several phase II trials [6-8], and HER2-targeted combinations included in international guidelines for mCRC [9].

CRC carcinogenesis is due to a complex and well-characterized cascade of molecular events [10]. Among these, mismatch repair (MMR) alterations represent the carcinogenic event leading to 5-15% of all CRC cases [11]. According to MMR status, CRC can be classified into two major subtypes: microsatellite instable (MSI) or microsatellite stable (MSS). MSS and MSI CRCs are recognized as distinct diseases with different etiology and different treatment options [12]. Recent studies showed that CRC patients with microsatellite instability-high (MSI-H) tumors can benefit from checkpoint inhibitors, leading to FDA approval of pembrolizumab or nivolumab, alone or with the anti-CTLA4 ipilimumab [13-15]. However, this therapeutic innovation reaches few patients, since MSI-H mCRC accounts for 5% of patients and MSS tumors account for the remainder, with very poor prognosis and limited therapeutic options, especially when *RAS* or *BRAF* mutations are identified [3, 4]. Therefore, finding alternative and effective therapies for this group of CRC patients represents an urgent unmet clinical need. In this regard, refining molecular selection criteria to chemotherapy might lead to valuable options together with the discovery of new potential molecular targets that have been stagnant in recent years.

With the need for further advances, genomic alterations in the DNA damage response (DDR) pathway are emerging as a novel targets for treatment across different cancer types [16-18]. Platinum compounds and poly (ADP-ribose) polymerase-inhibitors (PARPi) are currently the two main classes of drugs active against cancer cells harboring DDR alterations, initially recognized in breast and ovarian cancers, and currently extended to prostate and pancreatic cancer [16-18]. In CRC, the role of DDR alterations is still widely unknown and only scarce and fragmented pieces

of data about their clinical impact are available [19, 20]. Germline pathogenic variants of *BRCA1* are rising as a risk factor for CRC, as *BRCA1/2* alterations have been associated with early-onset CRC [21, 22]. Recent studies suggest that a subset of CRCs is characterized by germline and/or somatic genetic defects in DDR genes [23-26]. The prevalence of somatic DDR defects in CRC has been reported, ranging between 10 to 30% [20, 25, 27, 28] . Retrospective studies have also reported a significant correlation between an *ATM* polymorphism and survival with advanced lines of chemotherapy [29] and between some *ATM* mutations and better prognosis [30]. However, different from other cancer types, none of DDR alterations is associated with approved therapy in CRC treatment. When considering analysis of the transcriptome and the four consensus molecular subtypes (CMS) of CRC, the prevalence of alterations in MMR genes is highest in MSI-hypermutated tumors classified as CMS1 [31-33]. Further studies have shown a significant prevalence of ATM mutations in 7% of non-hypermutated tumors in CMS3 [34] and a consistent DDR pathway downregulation in CMS4 [35]. Considering the poor prognosis and the limited treatment options for CMS3 and CMS4, targeting DDR in this large subset of CRC might represent a valuable therapeutic opportunity.

In this article, we aim to draw a map exploring DDR pathway in CRC by showing preclinical data and systematically review of early clinical evidences available to highlight the most promising avenues for clinical leveraging of DDR alterations in this tumor.

The DNA Damage Response pathway

The DDR pathway represents a complex network of effectors involved in DNA repair and cell cycle checkpoint control, governing the correct execution of DNA replication and cell proliferation. The recognition and repair of damaged DNA involves the cascade activation of a tightly controlled sequence of actions including DNA damage sensing, DNA repair and cell cycle checkpoint delay or arrest [36, 37]. An intact DDR system is required to maintain genomic integrity and vulnerabilities in the DDR genes might constitute a potential actionable target for cancer treatment. Depending on the insulting agent, different types of damage can be recognized in DNA, often resulting in single-strand (SSB-DNA) or double-strand breaks (DSBs).

Various groups of effectors are activated to re-establish the correct sequence of DNA. Mismatch Repair (MMR), base excision repair (BER), and nucleotide excision repair (NER) represent the key pathways involved in SSB-DNA repair, while homologous recombination (HR) and non-homologous

end joining (NHEJ) pathways operate for the repair of DSBs [38]. A third pathway responsible for rescuing damaged DSB-DNA is called translation DNA synthesis [39] .

Given the complexity of the DDR pathways and the availability of many reviews focused on this topic [20, 38, 40, 41], an exhaustive description is beyond the scope of this manuscript. Instead, we will focus on the key players that appear most relevant to the clinical applicability for CRC treatment, as shown in **Figure 1**.

DSBs represent the most deleterious type of DNA damage [41]; after the recognition of the damaged DNA site, a complex network of proteins is recruited to slow down DNA fork progression before the cell enters into the G2 phase [42]. The MRN complex (MRE11-NBS1-*RAD50*) is one of the first complexes to be engaged to the lesion and afterwards the DNA damage mediator ataxia telangiectasia mutated (*ATM*) is recruited to trigger cell cycle blockade and DNA repair by HR system [43, 44]. DSBs can alternatively be repaired by NHEJ system by recruiting the Ku70/80 complex, followed by DNA-PK activation [45]. ssDNA derived either from SSBs forming the DSBs or from stalled replication forks will be coated by Replication Protein A (RPA), protecting the DNA against degradation and recalling the ATR-ATRIP complex that will unleash cell cycle checkpoint control by interacting with downstream kinase proteins such as CHK1 and WEE1 [43] (**Figure 1**).

Pharmacological modulation of DDR pathways - Inactivation of proteins belonging to the HR system can make tumor cells dependent on effectors belonging to the NHEJ pathway, such as PARP proteins, leading to a synthetic lethal response when PARPi is applied. This type of interaction was described for the first time in two seminal papers in 2005 [46, 47] and the application of this experimental evidence has been observed in clinical trials between 2009 and 2010 [48-51]. Only recently, the use of PARPi has entered into the clinical practice following FDA approval for the treatment of a subgroup on tumors, such as ovarian, breast and recently pancreatic cancers, carrying defects in the HR genes, while in CRC the use of PARPi-based treatment is still lagging behind [16, 52, 53].

The PARP family includes 17 isoforms of nuclear proteins that are classified on the base of their structure and specific function [54]. PARP1 is the most characterized protein of the family; however, other members of the family such as PARP2 might play similar or partly overlapping activities [55, 56]. PARP1 is generally activated during the early phase of DNA damage recognition where it is recruited to repair SSB. Once bound to the altered DNA, PARP1 increases its catalytic activity and exploits NAD+ substrate to synthesize polymers of poly(ADP-ribose) (PAR) that are

transferred (PARylation) to acceptor proteins, such as PARP itself, histone H1 or transcriptional factors[57] .

All PARP inhibitors currently in clinical development structurally mimic the nicotinamide moiety of NAD substrate, resulting in inactivation of PARP1 catalytic activity, pADPr synthesis, and DNA trapping[58], with consequent stalling of the DNA replication fork and DNA breaks formation. The efficacy of PARPi has also emerged in BRCA wild-type cancers carrying defects in other genes belonging to the HR family. These alterations have been associated with the so-called *BRCAness* phenotype, later described in this review.

More recently, pharmaceutical companies have increased their attention to other DDR players that act as initial DNA damage sensors and mediators such as ATM, ATR and DNA-dependent protein kinase (DNA-PK), all belonging to the phosphatidylinositol 3-kinase-related kinases (PIKKs) family (**Figure 1**). Together with them, also the major DDR interactor downstream ATR, CHK1, has acquired relevance in the drug market, while CHK2, an effector of ATM signaling controlling the G1/S checkpoint, still lacks a specific inhibitor [59]. In parallel, the WEE1 kinase inhibitor has gained a significant application through its ability in impairing WEE1 in delaying progression between cell cycle phases as the gatekeeper of G2 arrest, with the aim of unleashing mitosis and genomic instability, ultimately leading to tumor cell apoptosis.[60]. Interestingly, and with immediate translational impact, few studies have shown as co-occurrence of pharmacological inhibition and defective function of two DDR effectors, such as ATM plus PARP1 or ATR plus XRCC1/ATM/CHK1/ERCC1/WEE1, might be synthetic lethal in preclinical models [61-65] (**Table 1**). Important to future applications in CRC treatment, tumors characterized by biallelic loss-of-function alterations in genes involved in DDR might be sensitive to DNA damage-inducing agents such as platinum-based compounds, similar to that observed in gynecological cancers [49].

Biomarkers of "PARP-ness" - The identification of patients with clinically meaningful DDR deficiency is an unmet clinical need in oncology. In solid tumors, *BRCA1/2* mutations are the best predictive biomarkers to identify patients achieving benefit from treatment with PARPi [40]. However, while some patients lacking *BRCA1/2* mutations can benefit from PARPi, others carrying mutant BRCA1/2 do not benefit from them [66, 67]. The term "*BRCAness*" indeed defines a homologous recombination-deficient (HRD) phenotype beyond the *BRCA* pathway and, therefore, the "*BRCAness*" term should be considered equivalent to "*HRDness*" [40, 68]. Further broadening the spectrum of patients likely gaining benefit from PARPi, the term "*PARPness*" defines

responsiveness to PARPi beyond HRD, potentially owing to PARP trapping or PARP activity abrogation in processes different from base-excision repair (BER), for example alterative-NHEJ or replication-fork protection [69-71]. Besides this terminology, proper identification of patients likely to respond to PARPi is still lacking.

The main clinical criteria adopted in all trials to identify PARPness is tumor sensitivity to platinum agents [25, 72].Beyond this, at least two different molecular approaches have been exploited to identify PARPness in tumors: 1) detection of genetic alterations and "genomic scars" by means of DNA sequencing panels and NGS technologies, 2) analysis of mutational signatures [73-78] (**Figure 2**).

The "*MyChoice HRD*" by Myriad Genetics and the "*FoundationFocus CDx* $_{BRCA\ LOH}$" by Foundation Medicine are two companion diagnostic next generation sequencing (NGS)-based HRD tests able to detect and measure both single nucleotide variants and "genomic scars" in DNA samples obtained from patients[73]. In particular, the first one assesses the presence of somatic mutations in BRCA1 and BRCA2 and of genomic scars such as loss of heterozygosity (LOH), telomeric allelic imbalance (TAI), and large-scale state transitions (LST). The unweighted sum of these three independent DNA-based measures of genomic instability, historically determined by SNP-based array data [79], identifies a HRD score, also named Genomic Instability Score (GIS), that has been set at ≥42 to likely identify those tumors carrying mutant BRCA or showing high genomic instability as confirmed in three studies focused on neoadjuvant platinum containing therapy in triple-negative breast cancer patients [76] and in recurrent ovarian cancers treated with niraparib [80]. The second assay can detect mutations in *BRCA1/2* and the percentage of genomic LOH in DNA extracted from patients 'samples [67, 74]. In the ARIEL 3 study, in ovarian carcinoma carrying mutant BRCA or LOH score ≥16, rucaparib significantly improved progression-free survival (PFS) vs placebo [67, 81]. No data regarding prevalence of genomic scars in CRC are available. Interestingly, Foundation Medicine has recently developed the Lynparza HRR-HRD assay [82, 83], which assesses a panel of 15 HR genes (*BRCA1, BRCA2, ATM, RAD51B, RAD51C, RAD54L, RAD51D, FANCJ/BRIP1, FANCL, PALB2, BARD1, CHEK1, CHEK2, CDK12, PPP2*) and that might be considered for future studies exploiting HRD in CRC.

The use of massive parallel sequencing has led to the identification of molecular signatures revealing peculiar patterns left on the cancer genome by different molecular processes associated with PARPness [84]. Alexandrov and coworkers described the "*signature 3*" as a genomic marker of HRD [85]. This signature is capable of classifying missense *BRCA1/2* mutations and is associated

with *BRCA1* and *RAD51C* silencing by promoter methylation, while ATM or CHK2 inactivation do not contribute to signature 3 [86-88]. More recently, a new HRD assay (HRDetect) based on whole genome sequencing has been developed, showing its ability to identify six different mutational signatures characterized by structural variants ("*rearrangement signatures*") predictive for BRCA1/ BRCA2 deficiency and potentially improving the selection of patients sensitive to PARP inhibition [89]. In another seminal work from the same group, 254 triple-negative breast cancers (TNBCs) were classified by HRDetect mutational-signature-based algorithm and HRDetect-high tumors were found to be associated to germline/somatic mutations of BRCA1/BRCA2, BRCA1 promoter hypermethylation, RAD51C hypermethylation or biallelic loss of PALB2 [90].

At least in breast cancer, the advent of HRDetect with analysis of whole genome defects has significantly refined the sensitivity in predicting BRCAness, thus improving the positive predictive value (PPV) of HRD testing [91, 92]. Genomic scars per se provide in fact either a stable or a transient snapshot of the HRD status of the tumor that could be bypassed by later acquisition of molecular alterations conferring restoration of the HR phenotype and likely not captured by scar analysis. For this reason, while testing based on genomic scar analysis can offer a good negative predictive value (NPV), indicating as negative those patients that would not likely respond to the treatment, its PPV could be hampered by the presence of other, sometimes hard to identify genomic variations [93].

In summary, evolution of sequencing technologies and the use of extended gene sequencing panels has improved the ability to detect variations not only in cancer susceptibility genes, but also in DDR genes, beyond BRCA1 and BRCA2 [94] . However, a major limitation to this method is given by variants of unknown significance (VUS) in DDR genes that are hard to interpret and functionally assess, thus hampering actionability of NGS results. Moreover, NGS is not able to identify epigenetic silencing of DDR genes [26]. It should be mentioned that both whole exome sequencing (WES) approach and analysis of WES-based molecular signatures have been tested in MSS CRC cell lines, but the outcome has resulted inconclusive, not being fully and clearly predictive for sensitivity to oxalipatin and olaparib [19]. Analysis of whole genome features might open up new avenues to the definition of DNA damage response inhibitors (DDRi) sensitivity in CRC [95].

As an alternative to these approaches, functional biomarker analysis based on dynamic assays might be useful to assess DDR deficit. At preclinical level, two main types of functional analysis have been evaluated: the assessment of γH2AX and RAD51 foci following irradiation-induced DNA

damage [96, 97] and the detection of DSB repair by mean of an engineered plasmid-based system (pDR-GFP) [19, 98]. Although some limitations occur with the former method, such as the incapability of identification of ATM-mutant tumors likely benefitting from PARPi [18, 44], it has been recently reported as a potential surrogate marker for detecting HR deficiency both in breast and CRC [19, 96]. The detection of DSB repair by pDR-GFP, although nicely discriminating between HR proficient and deficient cells, presents several technical limitations such as amenability for cell transfection, drug selection and long-term propagation[19].

A further limitation for clinical feasibility and routine use of both tests is the requirement of generation of DNA damage, such as exposure to ionizing irradiation in case of the RAD51 foci assay, and the use of a second plasmid expressing a DNA-cutting enzyme (SceI) to trigger DSBs in the GFP sequence inserted in the first plasmid. Altogether, these functional tests appear to be the most promising to identify PARPness in CRC as well as in other histologies. However, the lack of prospective validation and concerns regarding their large-scale feasibility constitute major issues for their translation into the clinic [73].

Direct testing on preclinical models (i.e. organoids or patient-derived xenografts) directly derived from CRC patients might represent a more reliable way to understand sensitivity or resistance to DDR inhibitors in CRC [19]. These models could offer the valuable advantage of dynamically monitoring tumor growth and evolution over time or under drugs selective pressure (**Figure 2**).

In summary, there is still no consensus on which is the best assay to be used to identify cancers more likely bearing meaningful DDR deficits leading to PARPness. Different tests have been evaluated but no one has yet emerged as a clear winner among the others. More efforts are currently ongoing to increase the efficiency and practicability of these tests, but major issues of clinical feasibility still need to be addressed.

Landing in terra incognita: DDR in colorectal cancer

Preclinical evidences – The efficacy of PARPi has been so far been proven in different tumor types harboring alterations in genes belonging to the HR pathway[52]. DDR genetic defects in CRC have been historically and more frequently associated to MMR alterations often causing MSI as reported from the analysis of 526 CRC cases in The Cancer Genome Atlas Colon Adenocarcinoma (COAD) and Rectal Adenocarcinoma (READ) PanCancer Atlas datasets [27]. Defective MMR/MSI-H CRCs, which account for around 15% of CRCs and 5% of metastatic CRCs, are considered to have favorable prognosis and better survival [99], and recently they have been approved for

immunotherapeutic treatment [100]. The remaining 85% MSS CRC still represents an unmet medical need and harnessing defects in DDR genes might represent a new hope to treat this subgroup with poor prognosis and limited therapeutic options. So far, toxicity of drug combinations or lack of patient selection has hindered the clinical development of PARPi in CRC [101-104]. Only a few works have assessed, in a limited number of preclinical CRC models, the effects of PARP or other DDR inhibitors. This emphasizes the critical need for the identification and validation of predictive biomarkers of response to DDRi in a larger number of clinically relevant models for better patient selection and stratification.

McAndrew and colleagues have shown a direct correlation between deficiency of RAD54B, an effector of the HR pathway and a direct interactor of RAD51, and sensitivity to olaparib, but only one cell model was used [105]. Direct correlation between lack of the DDR sensor ATM and olaparib sensitivity has been shown by Wang and colleagues in three CRC cell lines (SKCO-1, LoVo and HCT116), where further shRNA-mediated mechanistic experiments show that p53 depletion could enhance PARPi sensitivity in *ATM*$^{-/-}$ cells [106].

PARP sensitivity might also be related to HR deficiency due to increased DDR protein heterodimers instability. This is the case described by Ozden and colleagues [97], showing that CRC cells expressing higher level of a splice variant of BARD1 and likely a more unstable BARD1/BRCA1 complex are more susceptible to PARP inhibition.

Other groups have investigated the potential synergistic effects exerted by the concomitant use of olaparib and chemotherapeutic agents such as oxaliplatin and SN-38 (the active metabolite of irinotecan). Xu and colleagues have functionally analyzed the effects of olaparib, alone or in combination with oxaliplatin, in one CRC cell line (SW480), but no molecular DDR biomarker analysis was provided [107]. By analyzing subsets of MSI and MSS CRC cell lines, different groups have demonstrated that PARP inhibitors can potentiate SN38 cytotoxicity irrespective of the MMR status [108-110], and Tahara and colleagues show that this effect could be even amplified in those cells carrying defects in HR proteins such as RAD51 [109]. On the other hand, Augustine and colleagues, while agreeing on the synergistic effect of PARPi (rucaparib instead of olaparib) with SN38, do observe a more prominent effect in MSI compared to MSS cells [111]. This study moreover assesses whether concomitant versus sequential administration is more effective in treating CRC and further experiments in mice show the efficacy of concurrent use of rucaparib and SN-38 in controlling tumor growth. Interestingly, and in contrast with previous findings, they claim that no synergy is observed when PARPi and oxaliplatin are administered in combination. Other

DDRi such as ATM inhibitors have been shown to be effective in combination with SN38 [112], in particular in those patient-derived xenografts (PDX) models showing primary resistance to irinotecan. In addition, they noted an interesting association between efficacy of combinatorial treatment and presence of *PIK3CA* activating mutations, which may warrant a more extended analysis in clinically relevant models.

The seminal work performed by Dietlein and colleagues [113] has shown how addiction in KRAS and BRAF mutant cells of different tissue origin to checkpoints controlled by CHK1 and MK2 can be exploited as an Achille's heel to inhibit proliferation of tumors characterized by poor prognosis and limited therapeutic options. These promising results have been presented in a limited number of CRC models and further validation in a larger cohort of CRC cell lines and patient-derived models might confirm the effective synergism of these checkpoint inhibitors.

All these studies are informative and provide insightful tips of thoughts, but systematic analysis on the potential efficacy of PARPi and more generally DDRi in CRC is lagging behind. More experimental evidence from clinically relevant models is required to shed light on novel and potential CRC therapeutic strategies exploiting DNA repair vulnerability.

Hence, with the aim of defining potential predictive biomarkers to guide clinical development of PARP inhibitors in CRC, we have tested a large collection (n=99) of CRC cell lines carrying genomic defects responsible for resistance to EGFR-targeted therapy with the PARPi olaparib [19]. We found that up to 13% CRC lines were highly sensitive to clinically achievable concentrations of olaparib and that this response was positively correlated with susceptibility to oxaliplatin treatment *in vitro*. While no strong association between genomic defects in BRCA or other HR genes and olaparib sensitivity was observed, we found that functional assays based on detection of DNA damage response were able to pinpoint vulnerability to PARP inhibition. To increase the translational relevance of this study, we also exploited organoids derived from CRC patients and found a significant correlation between olaparib sensitivity and response to previously received oxaliplatin-based regimens. This observation brings to attention the potential applicability of PARPi as a maintenance therapy in those patients that have previously responded to platinum-based therapy, comparable to what is already approved in ovarian and recently pancreatic cancer [16, 66, 114]. Although we are aware of the limitation of this study, which was performed in a limited number of MSS patient-derived models and therefore not accounting for the whole CRC tumor landscape, we believe that systematic assessment of various preclinical models might unveil

a significant role for PARPi or DDRi to treat CRCs with currently no or very limited therapeutic options, such as those anti-EGFR resistant.

Clinical opportunities **-** The exploitation of DDR defects in CRC patients is at very early stage of development. We present here clinical evidence of targeting DDR in CRC from available studies in the literature.

- *Trials evaluating the role of PARPi*

We performed a systematic review of publications exploiting the use of PARPi in mCRC (**Table 2**). It was performed on 8th March 2020 according to PRISMA Criteria of 2009 (**Supplementary Figure 1**) [115, 116]. We reviewed MEDLINE/PubMed and ClinicalTrial.gov for published or ongoing clinical trials evaluating the efficacy of PARPi in CRC from January 2002 to December 2019. The Medical Subject Heading terms used for PubMed search were ((veliparib[Title]) OR (olaparib[Title]) OR (PARP[Title]) OR (niraparib[Title]) OR (rucaparib[Title]) OR (talozaparib[Title])) AND ((colorectal[Title]) OR (rectal[Title]) OR (colon[Title]) OR (solid[Title])). The Medical Subject Headings terms used for the search in ClinicalTrials.gov were ("Reruiting or not yet recruiting" as status), ("colorectal cancer" as condition/disease) and ("PARP", "olaparib", "veliparib", "niraparib", "rucaparib" or "talazoparib" as other terms). The inclusion criteria were the following: English language, specific treatment outcome report of CRC patients and at least 5 CRC patients included in the clinical trial. Gathering data on ongoing clinical trials, we selected only studies with specific indications for CRC patients. All publications not fulfilling these requirements were not included in the systematic analysis.

All published studies retrieved are phase I (n= 5) or II (n= 3) trials, with only one phase II randomized trial (**Table 2**). Five studies evaluate CRC patients only [101-104, 117], while three included also patients with other solid malignancies [118-120]. All but one [101] of the studies (87.5%) used PARPi in combination with other drugs, and in one of them (12.5%) PARPi was used with concomitant radiation therapy [103]. The number of patients included is low, ranging from 5 to 75 (**Table 2**). Importantly, none of these trials required specific alterations in DDR genes among inclusion criteria. ORR was 0% in the only one study evaluating PARPi monotherapy [101], while in studies combining PARPi with chemotherapy or radiotherapy ORR ranged from 0-57% (**Table 2**). Concerning treatment tolerability, G3-4 side effects vary between 12.5 - 76% (**Table 2**). However, toxicity data should be taken carefully since patients included were heavily pretreated. Leichman and coworkers investigated the efficacy of olaparib monotherapy treating CRC according to MMR

status but regardless of the presence of DDR alterations [101]. They conclude that olaparib alone is ineffective in both MSI and MSS CRC patients [101].

Ongoing trials are listed in **Table 3**. Of interest, one of these trials required alterations in DDR among inclusion criteria (NCT04171700).

- *Trials evaluating the role of other DDR inhibitors*

We also systematically reviewed clinical trials published, or currently ongoing, targeting other DDR alterations in CRC, for which inhibitors are currently in clinical testing in phase I and II studies. A systematic review was performed on 8th March 2020 according to PRISMA Criteria of 2009 (**Supplementary Figure 2**) [115, 116]. We reviewed MEDLINE/PubMed and ClinicalTrial.gov for published or ongoing clinical trials evaluating the efficacy of PARPi in CRC from May 1978 to December 2019. The Medical Subject Heading terms used for PubMed search were (ATM[Title]) AND ((colorectal[Title]) OR (colon[Title]) OR (rectal[Title]) OR (solid[Title])); (ATR[Title]) AND ((colorectal[Title]) OR (colon[Title]) OR (rectal[Title]) OR (solid[Title])); (CHK[Title]) AND ((colorectal[Title]) OR (colon[Title]) OR (rectal[Title]) OR (solid[Title])); (WEE1[Title]) AND ((colorectal[Title]) OR (colon[Title]) OR (rectal[Title]) OR (solid[Title])); (DNA-PK[Title]) AND ((colorectal[Title]) OR (colon[Title]) OR (rectal[Title]) OR (solid[Title])). The Medical Subject Headings terms used for the search in ClinicalTrials.gov were ("Reruiting or not yet recruiting" as status), ("colorectal cancer" as condition/disease) and ("ATM", "ATR", "CHK", "WEE1" or "DNA-PK" as other terms). The inclusion criteria were the following: English language, treatment outcome report of CRC patients and at least 5 CRC patients included in the clinical trial. Gathering data on ongoing clinical trials, we selected only study with specific indications for CRC patients. All publications not fulfilling these requirements were not included in the analysis.

We retrieved one phase I published study describing the efficacy of a WEE1 inhibitor (AZD1775) combined with chemotherapy [121]. In this study, 16 patients with CRC were enrolled with an 6.3% ORR and a considerable number of G3-4 events as side effects [121].

We also retrieved a currently ongoing study exploiting the same WEE1 inhibitor but combined with irinotecan to treat *RAS* or *BRAF* mutant CRC in the setting of second-line treatment (NCT02906059).

Apart from these trials with specific indication for CRC, many studies are currently recruiting for targeting DDR alterations across histologies. Many of them are evaluating the efficacy of combining DDR inhibitor (PARPi such as olaparib or talazoparib, ATRi or others) with checkpoint

inhibitors such as pembrolizumab (NCT04123366) or avelumab (NCT03565991) in patients affected by tumors harboring various DDR alterations according to different NGS panels. Based on DDR prevalence data available [25], between 10 – 30% of CRC patients could be potentially enrolled in these trials and results are awaited.

Discussion

The exploration of DDR alterations as biomarkers for cancer therapy in CRC is at the beginning of a complex path, and still largely based on analogies observed in other solid tumors according to a tissue-agnostic paradigm. However, there are peculiar features that should be taken into consideration for performing successful translational research in this tumor type. While it is known that BRCA1/2 defects can predict for vulnerability to PARP-inhibitors and platinum-based agents in both gynecological and CRC, this may not be true when referring to genetic alterations in other genes of the DDR pathway, reflecting potential tissue-specific dependencies for DNA repair mechanisms. As an example, defects in ATM can confer susceptibility to both PARP inhibitors and oxaliplatin in CRC [106, 122, 123], while their role in response to platinum agents in ovarian cancer is still debated [124, 125].

In CRC, understanding whether DDR defects could potentially represent a new biomarker for selecting CRC patients as candidates for platinum-based chemotherapy and specific DDR targeting agents (i.e. PARPi) represents an urgent medical need. To this regard, preclinical studies can contribute to understand disappointing results of clinical trials with DDR inhibitors in this tumor. Several issues should be indeed taken into consideration and might have hampered interpretations of clinical results.

First, molecular selection was not performed in any of the trials with published results. Second, no consensus on which is the optimal panel of DDR genes to be tested has been reached. In addition, the role of functional testing, instead of genomic scars or NGS analysis, has not been assessed. Third, optimal compounds to be tested in the clinical setting (PARPi or other DDR targeting drugs) have not been defined. Indeed, only one currently ongoing study (NCT03983993) is considering PARP inhibition as an option for those patients not progressing on an oxaliplatin-based line of treatment. Finally, no correlation between DDR alterations and benefit from platinum-based treatment is available in the literature.

In order to address these clinical issues, preclinical and translational research is expected to be helpful. Some recent works on cell lines and patient-derived models have provided evidences

of efficacy of PARPi, but systematic analysis on a large cohort of CRC models is still missing. We recently reported promising results from PDX and patients-derived organoids, suggesting that maintenance with olaparib might represent a valid therapeutic opportunity also in CRC [19]. Accordingly, we suggest the development of specific clinical trials to verify this hypothesis in the clinic, broadening the investigation to a larger number of DDR inhibitors.

In clinical practice, oxaliplatin rechallenge --even though based on limited evidence-- is often considered for patients in later lines of treatment with adequate performance status. This option is essentially based on a retrospective trial including 23 patients and reporting 18% response rate (RR) and 65% disease-control rate (DCR) [126]. Remarkably, there are no molecular criteria to select patients for this strategy. In this regard, given the well-known sensitivity to alkylating agents of tumors with DDR alterations, a better understanding of DDR in CRC might help to identify patients more likely to benefit from oxaliplatin rechallenge. This approach might also allow better selection and minimize the risk of severe toxicities such as oxaliplatin immune-induced syndrome, which has been shown to be associated with the use of this drug in the rechallenge setting [127, 128].

It is also intriguing to speculate that various DDR alterations might not equally impact on sensitivity to different platinum compounds. While the role of oxaliplatin in CRC is well established, cisplatin has been less investigated, even if ORR was around 35% in clinical trials in the advanced setting [129]. Since a study showed different mechanisms of action between oxaliplatin and cisplatin [130], it might be hypothesised that different DDR alterations might have a role in conditioning a different sensitivity to oxaliplatin or cisplatin. More preclinical testing followed by specific trials should be set to test this hypothesis.

Finally, the potential role of liquid biopsy identifying DDR alterations at baseline and during treatment might offer new insights on this topic. Liquid biopsy has been demonstrated capable of monitoring clonal evolution and to identify genetic alterations occurring in CRC during anti-EGFR treatment [131, 132]. However, unlike prostate cancer in which liquid biopsy can identify resistance mechanisms to PARPi [133], its capability to identify DDR alterations has never been explored in CRC and might be helpful to recognize those alterations already known to be associated to DDR deficit.

In summary, we believe that a better understanding of DDR alterations might have potential transformative implications in CRC treatment. Indeed, these alterations could identify new subsets of patients both likely benefitting from PARPi or other DDRi (i.e. WEE1 inhibitors) and

achieving most benefit from platinum-based regimens in frontline treatment as well as in the reintroduction setting. Although very promising, the exploitation of DDR deficiency in CRC is still at the very beginning, and preclinical and clinical data integration based on functional testing stands as a fundamental step for clinical translation.

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Highlights

- In colorectal cancer (CRC) the therapeutic application of DNA Damage Response (DDR) alterations has to be elucidated.
- Results from clinical trials exploiting DDR alterations as a target are disappointing and hampered by several limitations.
- A subset of CRC is vulnerable to PARP inhibition as suggested by recent preclinical evidences.
- Currently, there is no consensus on methods to assess the functional role of DDR alterations in CRC.
- A better understanding of DDR alterations based on functional testing might have a transformative impact on CRC treatment.

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Tables

Table 1. DNA damage response (DDR) molecular targets and corresponding drugs in colorectal cancer (CRC), together with potential vulnerabilities that could be exploited to treat metastatic CRC harboring DDR defects.

Table 2. Published studies investigating the potential role of poly (ADP-ribose) polymerase-inhibitors (PARPi) and other DNA Damage Response inhibitors in colorectal cancer.

Table 3. Ongoing clinical trials specifically assessing the potential role of poly (ADP-ribose) polymerase-inhibitors (PARPi) and other DNA Damage Response inhibitors in colorectal cancer.

Figures

Figure 1. Schematic representation of DNA Damage Response and pathway activation.
Different types of agents can trigger DNA damage and specific types of DNA damage (bulky adducts, single base mismatch/indels, single-strand DNA breaks (SSBs) or double-strand DNA breaks (DSBs)) can activate definite downstream signalling pathways known as the DNA damage response (DDR) pathways, that through a cascade-like activation (sensor-mediator-effector proteins) can promote cell cycle control and DNA repair. PARP inhibitors (PARPi) can increase number of SSB, leading to conversion to DSB. Following DSB formation, the Ku70/Ku80 dimer binds and stabilizes the DSB ends, and recruits DNA-PK that will in turn activate the NHEJ effectors LigaseIV-XRCC4. DSBs can alternatively activate the HR system through the MRN complex that will activate ATM modulating CHK2 and cell cycle checkpoint control. ATR is triggered by RPA-ATRIP coated ssDNA that can derive from resected DSBs or stalled replication forks. The ATM and ATR pathways have multiple points of interactions; for practical reasons only few are shown. Keys: DSB: double-strand break. SSB: single-strand break. NER: Nucleotide Excision Repair; HR: Homologous Recombination; NHEJ: Non-Homologous End Joining; BER: Base Excision Repair; MMR: Mismatch Repair.

Figure 2. DNA damage response (DDR) deficiency assessment methods in colorectal cancer.
* In cancer types other than colorectal cancer, “Signature 3” identify HRD and classify DDR alterations missense BRCA1/2 mutations; associated with BRCA1 and RAD51C silencing by promoter methylation; germline/somatic mutations of BRCA1/BRCA2; BRCA1 promoter hypermethylation; RAD51C hypermethylation or biallelic loss of PALB2 (Staaf et al, Nat med 2019).
° ”Static” defines methods able to capture DDR deficiency in a single time lapse. °° “Dynamic” defines methods potentially capable to describe DDR deficiency alongside with tumor growth and evolution during time/under drug treatment. Keys: PDX = patient-derived xenograft; CRC = colorectal cancer; HGSOC = high-grade serous ovarian cancer; OIIS = oxaliplatin immune-induced syndrome; HRD = homologous recombination deficiency; PARPi = PARP inhibitors; TNBC = triple negative breast cancer; DDR = DNA damage repair; VUS = variants of unknown significance.

Supplementary figures

Supplementary figure 1. Flow diagram representing the systematic review process performed according to PRISMA Statement to collect publications exploiting the use of PARP-inhibitors in metastatic colorectal cancer.

Supplementary figure 2. Flow diagram representing the systematic review process performed according to PRISMA Statement to collect trials published, or currently ongoing, targeting other DNA damage response alterations in metastatic colorectal cancer.

Table 1. DNA damage response (DDR) molecular targets and corresponding drugs in colorectal cancer (CRC), together with potential vulnerabilities that could be exploited to treat metastatic CRC harboring DDR defects.

Drug target	Drug name	Potential synergy
PARP1	Olaparib Veliparib Talazoparib Rucaparib Niraparib	HR pathway
ATM	AZD0156	ATR, PARP1 and XRCC1
ATR	M6620 AZD6738	ATM, PARP1, ERCC1, XRCC1, CHK1
CHK1	LY2603618 SRA-737	ATR, PARP1 and WEE1
WEE1	AZD1775	ATR and HR pathway
DNA-PK	M3814	ATM, ATR and DSB inducers (ChT or RT)
PD-1 PD-L1 CTLA-4	Nivolumab Ipilimumab Pembrolizumab Atezolizumab Durvalumab	Defects in MMR (endogenous in MSI CRC, or induced by Temozolomide in MSS CRC)

KEYS: HR=Homologous recombination. ChT=Chemotherapy. RT=Radiotherapy. CRC=Colorectal cancer. MMR= Mismatch repair. MSS=Microsatellite stable. MSI=Microsatellite instability. DSB=Double-strand break.

Table 2. Published studies investigating the potential role of poly (ADP-ribose) polymerase-inhibitors (PARPi) and other DNA Damage Response inhibitors in colorectal cancer.

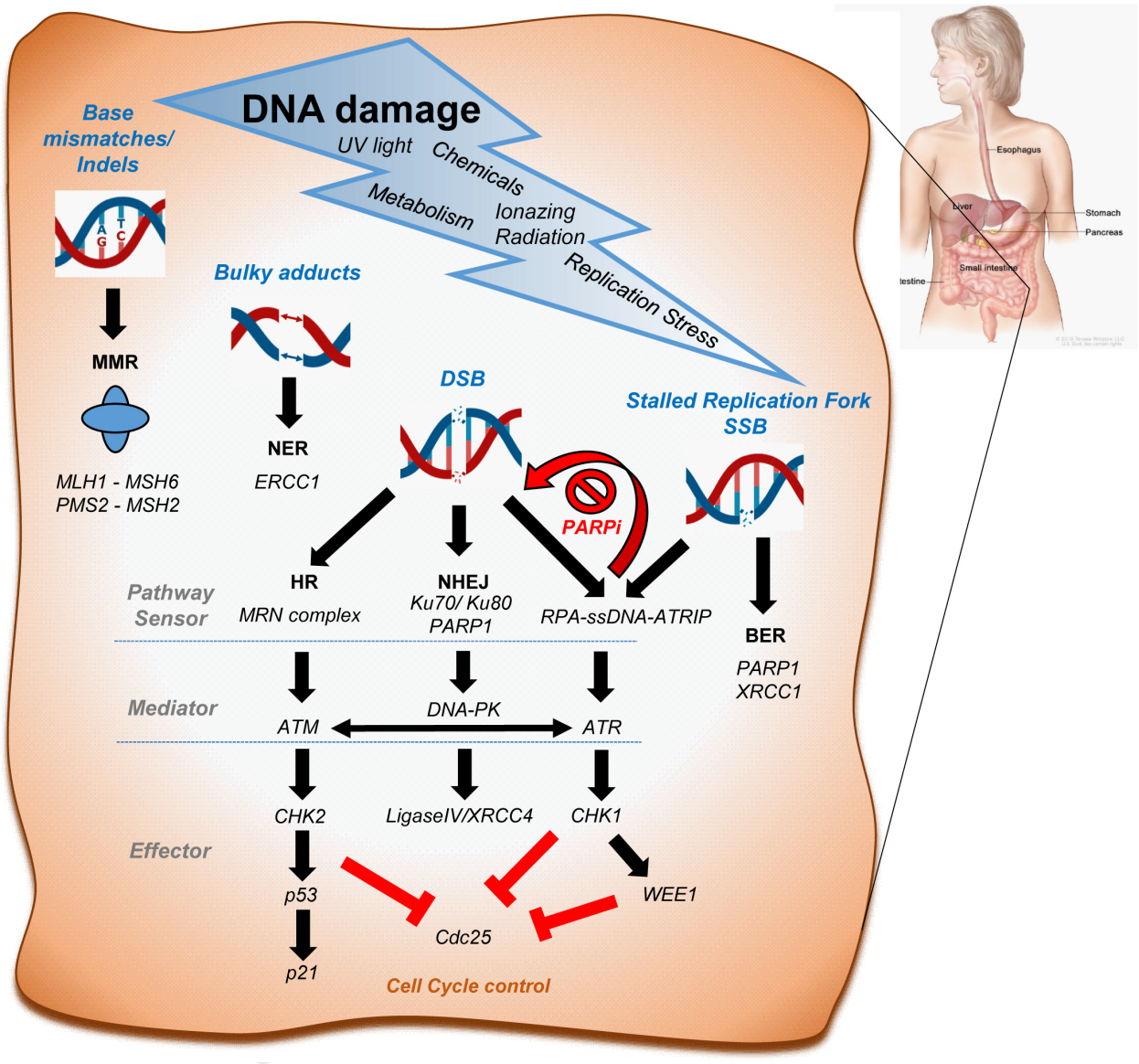
Authors and Ref	Phase	Interventional Drug Regimen	CRC Patients Enrolled	Molecular Selection	ORR (%)	SD (%)	mPFS (m.)	HR	G3-4 Side Effects (%)
Berlin 118	I	FOLFIRI + veliparib	10	No	2/10 (20.0)	NA	NA	NA	38.0 *
Chen 117	I	Irinotecan + olaparib	25	No	0/25 (0.0)	9/22 (40.9)	NA	NA	76.0 **
Gorbu-nova 102	II R.	FOLFIRI + veliparib ± bevacizumab	65 (each arm)	No	37/65 (57%) ***	20/65 (31.0)	12.0	0.91 (0.6-1.4)	59.0 **
Kummar 120	I	Topotecan + veliparib	5	No	0/5 (0.0)	1/5 (20.0)	NA	NA	70.0 */**
Leichmann 101	II	Olaparib	33	No	0/33 (0.0)	5/33 (15.2)	1.8	NA	48.5
Pishvaian 104	II	Temozolide + Veliparib	75	No	2/75 (2.7%)	16/75 (21.3)	1.8	NA	18.7
Samol 119	I	Topotecan + olaparib	8	No	0/8 (0.0)	3/8 (37.5)	NA	NA	47.4 *
Czito 103	Ib	Capecitabine + RT + veliparib	32 °	No	9/31 (29.0) °°	NA	NA	NA	12.5
Leijen 121	I	AZD1775 (WEEi) + ChT °°°	16	No	1/16 (6.3)	NA	NA	NA	65.8 *

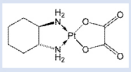
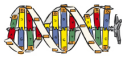
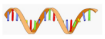
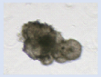
KEYS: REF=Reference. CRC=Colorectal cancer. ORR=Overall response rate (PR+CR). mPFS=Median Progression-free survival. M=months. HR=Hazard ratio for PFS. SD=Stable disease. R=Randomized. NA=Not assessable. RT=Radiotherapy. WEEi=WEE inhibitor. ChT=Chemotherapy. *Data regarding all patients enrolled in clinical trial, not only CRC. **No data on how many patients experienced ≥1 G3-4 side effects; data presented in the table refers to overall prevalence of neutropenia which was the most common G3-4 event. ***Data reported in the table describe ORR in veliparib arm; in control arm ORR was 62% (40/65 patients). °=Only patients with stage II/III rectal cancer were enrolled. °°=Data presented in the table refers to pathological complete response rather than ORR given the setting of locally advanced disease. °°°=Different chemotherapy agents were combined to AZD1175 (gemcitabine or cisplatin or carboplatin).

Table 3. Ongoing clinical trials specifically assessing the potential role of poly (ADP-ribose) polymerase-inhibitors (PARPi) or other DNA Damage Response (DDR) inhibitors in colorectal cancer.

Study Number	Phase	Drug Regimen	Molecular Selection
NCT02484404	I/II	MEDI4736 (anti PDL-1) + olaparib ± cediranib (anti-VEGF)	• No
NCT03875313	Ib/II	Telaglenastat (glutaminase inhibitor) + talazoparib	• No
NCT03851614 (DAPPER)	II	Durvalumab + olaparib (Cohort A)	• MSS CRC
NCT04171700 (LODESTAR)	II	Rucaparib	• Deleterious mutation (germline or somatic) in *BRCA1, BRCA2, PALB2, RAD51C, RAD51D, BARD1, BRIP1, FANCA, NBN, RAD51 or RAD51B*
NCT04166435	II	Temozolomide + olaparib	• *MGMT* promoter hypermethylation
NCT03251612	II	Olaparib	• Drug sensitivity testing
NCT03983993 (NIPAVect)	II	Panitumumab + niraparib	• *RAS* wild-type • Maintenance olaparib after first line oxaliplatin-containing chemotherapy allowed on the trial if SD, PR or CR for at least 4 months
NCT03337087	I/II	Nal-IRI + leucovorin + fluorouracil + rucaparib	• No
NCT02906059	Ib	AZD1775 (WEE1 inhibitor) + irinotecan	• *RAS* or *BRAF* mutant CRC • 2nd line of treatment

KEYS: CRC=Colorectal cancer. MSS=Microsatellite stable.


DNA damage
UV light
Chemicals
Metabolism
Ionazing Radiation
Replication Stress
Base mismatches/ Indels
Bulky adducts
DSB
Stalled Replication Fork
SSB
MMR
MLH1 - MSH6
PMS2 - MSH2
NER
ERCC1
PARPi
HR
MRN complex
NHEJ
Ku70/ Ku80
PARP1
RPA-ssDNA-ATRIP
BER
PARP1
XRCC1
Pathway Sensor
Mediator
ATM
DNA-PK
ATR
CHK2
LigaseIV/XRCC4
CHK1
Effector
p53
p21
Cdc25
WEE1
Cell Cycle control
Esophagus
Liver
Stomach
Pancreas
Small intestine

Level	Method		Strengths	Weaknesses
Clinical	Sensitivity to Platinum Agents		• Standard treatment in CRC • Easy to evaluate • Low cost	• No common definition of platinum sensitivity in CRC (≠ HGSOC) • Limited number of cycles allowed due to drug tolerability (i.e. neurotoxicity, OIIS) • Not fully predictive of "PARPness"
Molecular	DNA gene panels		• Availability of data on genetic alterations found in DDR genes deposited in public databases • Customizable	• High number of VUS • Poorly predictive for PARPi and platinum sensitivity • "Static" assessment°
	Genomic Scars		• Commercially available tests ("MyChoice HRD" and "FoundationFocus CDx $_{BRCA\ LOH}$") • Predictive of clinical benefit with PARPi in ovarian cancer and with platinum in TNBC	• Not fully predictive of PARPi and platinum sensitivity in CRC • "Static" assessment°
	Mutational signatures		• "Signature 3" and HRDetect successfully identifies homologous-recombination-repair deficiency associated with germline and somatic *BRCA1* and *BRCA2* mutations in breast, pancreatic, and ovarian cancers *	• No solid data related to HRD in CRC • "Static" assessment°
Functional	Organoids		• Could be directly derived from patient tumor biopsy • "Dynamic" and functional assessment°° • Potentially predictive for drug sensitivity • Short-term drug testing • Possibility of testing multiple drugs as single agents or combinations (High-throughput)	• High costs • Difficulties for long-term propagation • Absence of microenvironment
	PDXs		• "Dynamic" assessment°° • Representative of patient intra-tumor heterogeneity • Potentially predictive drug sensitivity	• Animal manipulation required • High costs of maintenance • Long time required for drug testing • Low-throughput