Review Article

Repurposing DNA repair factors to eradicate tumor cells upon radiotherapy

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Abstract: Cancer is the leading cause of death worldwide. Almost 50% of all cancer patients undergo radiation therapy (RT) during treatment, with varying success. The main goal of RT is to kill tumor cells by damaging their DNA irreversibly while sparing the surrounding normal tissue. The outcome of RT is often determined by how tumors recognize and repair their damaged DNA. A growing body of evidence suggests that tumors often show abnormal expression of DNA double-strand break (DSB) repair genes that are absent from normal cells. Defects in a specific DNA repair pathway make tumor cells overly dependent on alternative or backup pathways to repair their damaged DNA. These tumor cell-specific abnormalities in the DNA damage response (DDR) machinery can potentially be used as biomarkers for treatment outcomes or as targets for sensitization to ionizing radiation (IR). An improved understanding of genetic or epigenetic alterations in the DNA repair pathways specific to cancer cells has paved the way for new treatments that combine pharmacological exploitation of tumor-specific molecular vulnerabilities with IR. Inhibiting DNA repair pathways has the potential to greatly enhance the therapeutic ratio of RT. In this review, we will discuss DNA repair pathways in active cells and how these pathways are deregulated in tumors. We will also describe the impact of targeting cancer-specific aberrations in the DDR as a treatment strategy to improve the efficacy of RT. Finally, we will address the current roadblocks and future prospects of these approaches.

Keywords: Ionizing radiation (IR); cancer; DNA damage; DNA repair; synthetic lethality; charged particle therapy (CPT); radiotherapy

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Introduction

Cancer is the leading cause of morbidity and mortality worldwide. According to the most recent estimation by the International Agency for Research on Cancer (IARC), in 2012, 8.2 million people worldwide died of cancer, with 14.1 million new cases being reported (1). The etiology of cancer is multifactorial and complex, and it varies widely from patient to patient. Hanahan and Weinberg identified uncontrolled proliferative potential, replicative immortality, and evasion of apoptosis as some of the major hallmarks of cancer cells (2). In the past decade, scientists have made considerable progress towards the treatment and understanding of these hallmarks, and when combined with advances in early detection and various treatment modalities, many cancers have become curable.

Since the discovery of X-rays in 1895, radiation therapy (RT) has constituted an important modality used in curative cancer treatment, in conjunction with surgery and chemotherapy (3). Its low cost and usefulness as a single-modality treatment for early stage cancer patients are the main reasons why RT is an important branch of medical oncology (4,5). Approximately 50% of all cancer

patients receive RT in some form during the course of their illness (6), and it is estimated that RT contributes to about 40% of curative treatments (7). In addition to conventional RT, the last decade has seen high energy proton and carbon ion beam radiotherapy [charged particle therapy (CPT)] gain in popularity. The theoretical advantages of CPT over conventional RT are focused dose distribution, high linear energy transfer (LET), and lower normal tissue toxicity. Also, proton and carbon particles have a higher relative biological effectiveness (RBE) than conventional X-rays. Hence, CPT has the potential to increase the efficacy of RT, particularly for controlling unresectable radioresistant tumors.

Cancer radiotherapy kills cancer cells mostly by inducing DNA damage. However, radiation exposure can also damage normal cells or tissues surrounding the tumor, leading to side effects. For example, radiation received by the heart during the treatment of pediatric, lung and breast cancer substantially increases the risk of developing cardiovascular diseases later in life (8). Similarly, studies indicate childhood cancer patients who received radiation to the brain develop cognitive impairment later in life, possibly because of damage to both hippocampal- and non-hippocampal-dependent domains (9,10). Therefore, increasing the therapeutic ratio, that is, maximizing the effects of radiation on cancer cells while minimizing damage to surrounding healthy tissue is the major goal of modern translational radiation oncology. Finding and exploiting genetic, epigenetic or microenvironmental differences between normal and malignant tissues in each individual patient is central to success.

Many studies show that as tumors become more mutagenic, they often become defective in one aspect of DNA repair but usually try to compensate by increasing the levels of certain repair proteins in the same pathway or in a different one. Since these molecular vulnerabilities are present in tumor cells but not in normal cells, targeting these alternative or backup pathways can render the tumor radiosensitive while leaving the normal tissue relatively resistant. Small-molecule inhibitors of DNA repair factors hold great promise for targeting these tumor cell-specific molecular vulnerabilities. These inhibitors can be honed to target a single step or a single protein in a DNA repair pathway that is vital for the survival of cancer cells but not for normal cells. Every tumor is different, and the goal of truly personalized radiation oncology is to understand the tumor genetics and selectively target the tumor-specific deregulated pathways in each cancer patient. However, the development of such a precise approach is offset by several real-world challenges. First, the mutagenic phenotype seen in tumors rarely results from the under- or over-expression of a single protein, and molecular pathogenesis is rarely linked to an isolated step in oncogenic progression (11). In addition, as tumors become increasingly mutagenic, they become more complex and heterogeneous, which often limits the usefulness of targeting a single protein or pathway. Also, the multifunctionality of many DNA repair proteins means inhibition of these factors sometimes cause unexpected toxicity to normal cells (12). For these reasons, despite the early promise and our extensive knowledge of tumor biology, many small molecule inhibitors of DNA repair factors failed in clinical trial and are not routinely used in clinic by the radiation oncologists (13).

Principles of RT

Depending on the type and stage of cancer, radiotherapy (RT) is used before or in combination with other treatment modalities (neoadjuvant and combination therapy). It is also often used for palliative purposes to relieve patients from symptoms caused by cancer. Radiation is delivered to the location of the tumor either externally (external beam radiation delivered to the location of the tumor from outside the body) or internally (brachytherapy delivered by directly implanting radioactive sources into the tumor site).

DNA damage is the primary effect of RT

Damage to the cellular DNA is the most well-known effect of radiation. Regardless of the mode of delivery, low LET ionizing radiation (IR), such as X-rays, gamma rays, and high LET charged particles used in CPT, deposits energy in the cells of the tissues it passes through. This energy deposition induces DNA lesions in the cells, ultimately forcing them to die, stop proliferating, or become senescent. Radiation-induced DNA lesions are multifaceted and can include base modifications such as 8-oxoG (14), single-strand breaks (SSBs), and double-strand breaks (DSBs) (15). Indeed, 1 Gy of low LET γ-radiation induces around 850 pyrimidine lesions, 450 purine lesions, 1,000 SSBs, and 20–40 DSBs in a mammalian cell (16). DNA lesions induced by IR can lead to cytotoxic, mutagenic, and carcinogenic effects if not repaired properly (17-19).

Repair of damaged DNA is of paramount importance

To ensure genomic integrity, cells have developed

Table 1 An overview of type of DNA lesions commonly encountered by the cell and the relevant repair pathways involved in the processing of these lesions

Type of DNA lesions	Source	DNA damage repair pathway
Bulky and helix-distorting DNA adducts	UV rays and chemical mutagens like cisplatin	NER, FA pathway
Damaged bases and non-helix-distorting DNA lesions	Ionizing radiation and reactive oxygen species	BER. In case of impaired BER, the SSBs are converted to DSBs, which are repaired by HRR during S and G2 phase. This synthetic lethal relationship is exploited by using PARP inhibitors to kill HRR deficient tumors
Single-strand breaks	Ionizing radiation and reactive oxygen species	Single-strand break repair
Base mismatches, incorrect insertion and deletion of bases	Replication errors	MMR
O6-methylguanine adducts	Alkylating agents like temozolomide, reactive oxygen and nitrogen species	Direct DNA repair. In absence of MGMT, the main enzyme in the DR pathway, MMR and NER can process these lesions
Double strand breaks	Ionizing radiation, reactive oxygen species and replication stress	DSBs are repaired either by NHEJ or by HRR in a cell cycle specific manner. Some overlap exists in between HRR and NHEJ pathways. DSBs which are not processed by NHEJ can be processed by HRR during S and G2 phase
DNA ICLs	Replication events and chemotherapeutic drugs like mitomycin C	NER and FA pathway

UV, ultraviolet; NER, nucleotide excision repair; FA, Fanconi anemia; BER, base excision repair; MMR, mismatch repair; MGMT, O6-methylguanine-DNA-methyltransferase; NHEJ, non-homologous end joining; HRR, homologous recombination repair; DSB, double-strand break; ICL, interstrand crosslink.

sophisticated mechanisms for repairing damaged DNA (*Table 1*). Base excision repair (BER) and SSB repair (SSBR) are essential for repairing damaged bases and SSBs. However, the most lethal types of lesions caused by IR are DSBs. Not only does IR cause DSBs directly, but unrepaired damaged bases and SSBs convert to DSBs when they encounter a replication fork (20). The two major cellular DSB repair pathways are non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ is an error-prone repair pathway that functions throughout all cell cycle phases, whereas HR is an error-free pathway that predominantly functions in the late S and G2 phases. Cells respond to DNA breaks by initiating a series of signaling pathways collectively known as DNA damage response (DDR) signaling. DDR contributes to the timely repair of DNA breaks, transient cell cycle arrest, transcriptional and post-transcriptional activation of a wide array of genes, and, under certain circumstances, programmed cell death. Faithful repair of DNA lesions and activation of DDR signaling are critical for cellular survival and prevention of genomic instability.

Dysfunctional DNA repair and cancer incidences

Deficiencies in DNA repair factors are a feature common to many cancers (21). Defects in specific DDR and DNA repair genes, such as ataxia telangiectasia mutated (*ATM*), Nibrin (*NBS1*), Werner syndrome (*WRN*), Fanconi anemia (FA), *BRCA1* , and *BRCA2*, give rise to cancer-prone disease syndromes (22-26). In addition to germ line mutations, many cancers show impaired DDR and defective checkpoints either because of somatic mutations (*ATM*, *TP53*, and cyclin-dependent kinase inhibitor 2A (*CDKN2A*), *BRCA1/2* being the most frequently mutated genes) or because of differential expression of DDR factors (6). Furthermore, DNA replication stress as a result of oncogene activation causes constitutive activation of the DDR and tumor progression (27). It has long been postulated that defects in both DNA repair and checkpoint responses in tumor cells affect the response to IR and can be exploited for targeted radiosensitization strategies (28) (*Table 2A,B*). However, RT also induces DNA damage in normal tissues surrounding the tumor, and using inhibitors

Table 2A An overview of most commonly altered DNA damage response signaling genes found in different types of cancer and relevant strategies for therapeutic intervention using small molecule inhibitors

Molecule	Function(s) in DNA repair	Type of abnormalities
ATM	Acts as the central activator of the DDR following DSBs	Loss of function mutation; increased copy number and autophosphorylation
MRE11	DNA damage sensor as part of the MRN complex and initial processing of double-strand DNA breaks prior to NHEJ and HR	Decreased expression
NBS1	DNA damage sensor as part of the MRN complex and initial processing of double-strand DNA breaks prior to NHEJ and HR	Polymorphism in NBS1 gene is most common; increased expression in some cases
DNA PKcs	Principle kinase in NHEJ pathway of DSB repair	Overexpression
Ligase IV/XRCC complex	Ligation of broken ends of DNA during the NHEJ pathway of DSB repair	Polymorphism in LIG4 gene
RAD51	Initiates DNA strand invasion and DNA strand exchange in HRR	Overexpression
RAD54	Facilitates RAD51mediated DNA strand exchange and promotes branch migration of Holliday junctions	Missense mutations at functional regions of RAD54
RPA	Protects and stabilizes ssDNA after resection step during the initial phase of HRR	Not known
BRCA1 and BRCA2	Initiates HRR in response to DSBs and stabilizes RAD51 binding to the DNA; transcriptional regulation of many DNA repair genes	Germline and somatic loss of function mutation; BRCA1 overexpression
c-Abl	Indirectly regulates HRR by phosphorylating RAD51 and catalyzing strand exchange	Overexpression
HSP90	Indirectly regulates HRR by facilitating correct folding and assembly of many proteins involved in HRR	Overexpression
APE1	Acts as the principle endonuclease in recognition and processing of AP sites in BER; also involved in transcriptional regulation of other genes	Overexpression
DNA polymerase β (pol β)	Synthesizes nucleotides and removes blocking residues during BER	Overexpression; missense mutations in pol β gene coding for variant proteins
FEN1	Removes 5' ssDNA overhangs in long patch BER; also important in microhomology-dependent alternative end joining of DSBs.	Overexpression
PARP	PARP 1 and PARP 2 are activated in response to SSB, cleaves NAD^+ generating nicotinamide and ADP-ribose scaffolds which facilitate recruitment of other factors to the damage site	Not clear
CHK1	Major checkpoint kinase responsible for the G2/M arrest in response to DNA damage	Decreased expression; increased expression and autophosphorylation
CDC25	Activates CDK and regulate entry into and progression through various phases of the cell cycle	Increased expression

ATM, ataxia telangiectasia mutated; DDR, DNA damage response; CDK, cyclin dependent kinases.

Table 2B An overview of most commonly altered DNA damage response signaling genes found in different types of cancer and relevant strategies for therapeutic intervention using small molecule inhibitors

Molecule	Relevance in cancer	Therapeutic intervention strategies
ATM	Loss of function in lung, colorectal, pancreatic, breast, hematopoietic cancers. Hyperactivated in some prostrate, bladder and breast cancer	KU55933 (29,30)
MRE11	Breast, colorectal, gastric, ovarian, head and neck cancer	Mirin as an indirect inhibitor of HRR (31)
NBS1	Increased risk of breast, colorectal, lung, nasopharyngeal carcinoma, acute lymphoblastic leukemia with NBS1 polymorphism. NBS1 overexpression in esophageal, head and neck, lung cancer and hepatomas	Mirin as an inhibitor of MRN complex (31)
DNA PKcs	Glioblastoma, gastric, prostrate, lung, esophageal and oral squamous cell carcinoma	Wortmannin (32); LY294002 (33); NU7026 (34,35); CC-115 (12); CC-122 (36); NVP-BEZ235 (37)
Ligase IV/XRCC complex	Breast, ovarian and pediatric brain tumors	SCR7 as an inhibitor of NHEJ pathway (38)
RAD51	Pancreatic cancer, breast cancer, prostate cancer, soft tissue sarcoma, and leukemia	RI-I and its optimized version RI-2 as inhibitors of recombinase function of RAD51 (39,40). This can be of particular interest for radio-immunotherapy and carbon particle therapy
RAD54	Breast cancer, colon cancer, lymphoma	Streptonigrin as an inhibitor of RAD54 ATPase function, thus inhibiting HRR (41)
RPA	RPA has been identified as an auto antigen in breast cancer; increased RPA expression as a prognostic biomarker in some colon cancer	Compound 8 as a disruptor of RPA's N-terminal domain interacting proteins (42)
BRCA1 and BRCA2	Loss of function in breast and ovarian cancer; BRCA1 overexpression in some lung cancers	PARP inhibitors (e.g., iniparib, olaparib and 2nd and 3rd generation inhibitors) are used to treat BRCA deficient cancers using synthetic lethal approach (43-48)
c-Abl	Teratoid and malignant rhabdoid tumors	Tyrosine kinase inhibitors like imatinib, dasatinib and bosutinib as indirect inhibitors of HRR (12,49-51)
HSP90	Many solid tumors including breast, ovarian, uterine, oral and renal carcinoma	Different classes of HSP90 inhibitors e.g., 17-DMAG, tanespimycin, retaspimycin, PU-H71, ganetespib as indirect inhibitors of HRR (12,52,53)
APE1	Lung, breast, head and neck, bladder, and hepatocellular, cervical carcinoma. Also overexpressed in germ cell tumors, osteosarcoma, medulloblastoma and many solid tumors; associated with chemo and radio resistance	Methoxyamine (54); lucanthone (55); AR-03 (56); ML199 (57) as inhibitors of DNA repair function of APE1. E330 as an inhibitor of redox function of APE1 (12,58)
DNA polymerase β (pol β)	Pol β variant proteins in gastric, colorectal, prostrate, lung, breast, bladder, esophageal cancer. Overexpression in breast, prostate and colon cancer; also associated with chemo- and radio resistance	NCS-666715 and NSC-124854 (59,60)
FEN1	Lung, pancreatic, breast, prostrate, gastric cancer and neuroblastomas	N-hydroxy urea containing compounds (61)
PARP	Expression of PARP-1 protein in cancer and its associations with outcome are yet poorly characterized	PARP inhibitors (e.g., iniparib, olaparib and 2nd and 3rd generation inhibitors) are used to treat BRCA deficient cancers using synthetic lethal approach (43-48)
CHK1	Decreased expression in breast cancers; increased expression and activation in lung, liver, colon and cervical cancer. Cancer cells with impaired G1/S checkpoint are over-dependent on G2/M checkpoint to prevent premature mitotic entry and cell death	UCN-01 (62,63), XL-844 (64) and AZD7762 (65)
CDC25	Breast, ovary, thyroid, colorectal, prostrate liver, esophageal and brain cancer	Still under investigation. Some of the reported CDC25 inhibitors belong to various chemical classes including phosphate bioisosteres, electrophilic entities, and quinonoids (66,67)

ATM, ataxia telangiectasia mutated; DDR, DNA damage response; CDK, cyclin dependent kinases.

of important molecules in DSB repair, such as ATM or DNA-dependent protein kinase (DNA-PK), in conjunction with radiotherapy increases the radiosensitivity of normal cells. Nonetheless, some degree of specificity in treatment can be achieved because of the hyper-proliferative nature of cancer cells relative to normal cells, which allows inhibitors to sensitize cancer cells to radiation more than normal cells. Similarly, replicating cancer cells are more sensitive than normal cells to chemical agents that block replication-associated processes and HR. In addition, replication can further aggravate damage in cancer cells that results from the inhibition of repair. Thus, targeting tumor-specific molecular abnormalities or use of replication stress inducing agents which affects hyper-proliferative tumor cells more than normal cells offers the possibility of selective eradication of cancer cells. In the following section, we will discuss the DNA repair pathways commonly used to repair radiation-induced DNA damage and how the inhibition of tumor cell-specific deregulated DNA repair factors and pathways can potentially be exploited to sensitize cancer cells to radiation (*Figure 1*).

Targeting DNA repair as a cancer therapy

Utilizing synthetic lethality in cancer treatment

In the context of DNA repair, synthetic lethality between two repair pathways arises when the loss of either pathway individually is tolerable, but the simultaneous loss of both pathways is lethal to the cell. It has long been postulated that a synthetic lethal approach could be useful for cancer treatment (68), and utilizing the poly (ADP-ribose) polymerase (PARP)-BRCA interaction is the best example of exploiting the synthetic lethal relationship that has proven clinically useful to control certain types of cancer (43). PARP1 facilitates repair of damaged bases and SSBs by acting as a scaffold for other BER proteins. Inhibiting PARP1 impairs BER, which results in the accumulation of DSBs that have converted from unrepaired SSBs during replication. Normal cells, with intact HR, are not substantially affected (69,70). In contrast, studies have shown that HR-impaired *BRCA1/2*-deficient tumors are highly sensitive to DNA damage in the presence of PARP inhibitors, both in tumor models *in vivo* and in the clinic (44-47,71). Since then, different PARP inhibitors, such as olaparib (AZD2461), and iniparib (BSI-201), have been subjects of intense investigation, either as mono- or combination therapies to treat breast cancer patients. However, a phase III trial of iniparib (BSI-201) failed to show major advantages in the treatment of metastatic triple negative breast cancer (TNBC) patients (72), and phase III development of olaparib (AZD-2281) to treat hereditary *BRCA1*- and *BRCA2*-associated breast cancer was halted in 2011 (73). These failures can be explained in part by the multifunctionality of the PARP1 protein, insufficient inhibition of PARP1 DNA repair activity, as well as heavily pretreated patient cohorts and the phenotypical heterogeneity of some cancers (12). Despite these setbacks, 115 different PARP inhibitors are currently in the clinical trial phase (12), and efforts are underway to find more synthetic lethal relationships between DNA repair proteins that can be therapeutically exploited. In addition to *BRCA1/2*-deficient cancers, *PTEN1*- and *ATM*-deficient cancers and colorectal cancers containing microsatellite instabilities are also sensitive to PARP inhibitors (74).

Inhibition of BER in cancer treatment

BER corrects SSBs and non-helix-distorting small base lesions that arise from IR. There are two sub pathways: short-patch BER, where only one nucleotide is replaced, usually during the G1 phase; and long-patch BER, where 2–13 nucleotides are replaced in the S/G2 phase of the cell cycle. The repair mechanism consists of five key enzymatic steps that involve excision of the damaged base by DNA glycosylase, nicking of the damaged DNA strand by AP endonuclease, removal of the sugar fragment by phosphodiesterase, filling of the gap by DNA polymerase, and finally, sealing of the nick by DNA ligase (75,76).

Among the many factors involved in BER, apurinic/apyrimidinic endonuclease 1 (APE1) accounts for over 95% of the total AP endonuclease activity in human cell lines and, besides DNA repair, also performs functions such as redox regulation (mediated through a separate redox domain) and transcriptional regulation of other genes. APE1 dysregulation or upregulation is a common feature in many solid cancers (77,78). Furthermore, overexpression of APE1 is linked with chemo- and radio-resistance of tumors, rapid progression, and overall poor prognosis of the disease (79). Inhibition of APE1, when combined with radiotherapy or DNA damage-inducing chemicals, increases tumor cell killing (80,81). Further, blocking the redox activity of APE1 has been shown to negatively attenuate tumor cell growth and angiogenesis (82,83). Additionally, inhibition of APE1 redox activity and STAT3 activation together causes

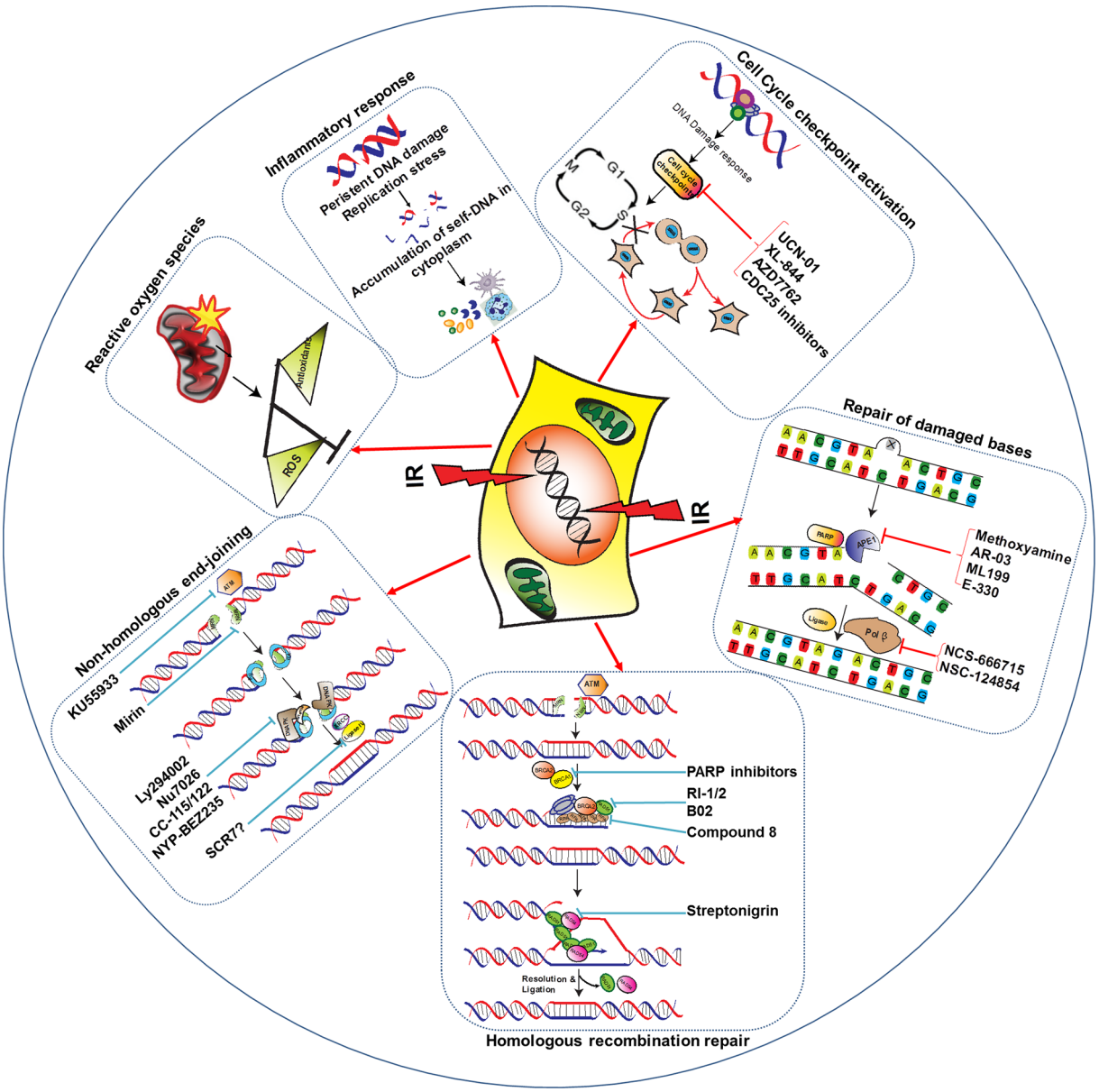

Figure 1 Schematics of direct and indirect effects of ionizing radiation and strategies for the therapeutic intervention. ROS, reactive oxygen species; PARP, poly (ADP-ribose) polymerase; IR, ionizing radiation; ATM, ataxia telangiectasia mutated.

synthetic lethality in human pancreatic and glioblastoma cell lines (84). Thus, inhibition of APE coupled with radio- or chemo-therapy is suitable for targeting multiple DNA repair and signaling pathways that promote tumor growth and survival. Several inhibitors of APE1 are in various stages of pre-clinical development. A broad spectrum BER inhibitor, methoxyamine (MX, or TRC102), is used in combination treatment for different types of cancer (54). In addition, several non-specific APE inhibitors act as topoisomerase II poisons and can potentially be clinically useful for treating cancers with high topo II expression (54). E3330, a selective inhibitor of APE's redox function, has shown promise for treating pancreatic cancer and acute lymphoblastic leukemia (12,58). In addition, several direct inhibitors of APE1, like ML199 (57), AR03 (56), and compounds containing 2-methyl-4-amino-6,7-dioxolo-

quinoline structure (85), have shown promise *in vitro*.

DNA polymerase β (pol β) synthesizes nucleotides and removes blocking residues during BER. Like APE1, pol β is often upregulated in many cancers and is linked with resistance to RT and chemotherapeutic agents. Two new inhibitors of pol β, NCS-666715 and NSC-124854, have been shown to sensitize colon cancer cells to DNA damage *in vitro* and are currently under evaluation in animal models (59,60). Inhibition of another endonuclease, Fen1, which is involved in long-patch BER and overexpressed in many cancers, has shown promise in a cell culture system (12,62). Finally, PARP1 and PARP2 are important sensors for SSBs and facilitate recruitment of additional BER factors, such as XRCC1, to the SSB site (86). As discussed earlier, many inhibitors of PARP1, are already in clinical use, and trials are ongoing for second- and third-generation PARP inhibitors.

Inhibition of DSB repair in cancer treatment

DSBs are the most harmful type of DNA damage induced by IR. One Gy of low LET IR is capable of producing 20–40 DSBs in a cell, as well as around 1,000 SSBs, many of which are converted to DSBs during replication (16). In addition, the reactive oxygen species (ROS) produced as a secondary effect of IR also induces DSBs. In mammalian cells, DSB are repaired by two main pathways: NHEJ and HR.

Inhibition of NHEJ in cancer treatment

NHEJ is the predominant DSB repair pathway in G0/G1 cells but remains active throughout the cell cycle. The main goal of NHEJ-mediated repair is fast rejoining of two broken DNA ends with minimal end processing, regardless of sequence homology. However, NHEJ-mediated repair results in the loss of up to 20 nucleotides from either side of the DSB junction, making it error-prone (87). Classical NHEJ-mediated DNA repair consists of four steps: recognition and binding of broken DNA ends by Ku70/Ku80 heterodimer; recruitment and stabilization of the NHEJ factors, like DNA-PK catalytic subunit (DNA-PKcs) and Artemis, at the DSB for bridging the DNA ends; processing of the DNA ends to make them suitable for ligation, which is mediated by different exo- and endo-nucleases; synthesis of new nucleotides by DNA polymerases μ and λ; and finally, ligation of the two severed DNA ends by ligase IV/XRCC4/XLF complex (88). Many studies have shown that defective NHEJ-mediated DSB repair is associated with increased risk of carcinogenesis, and functional NHEJ is linked with better treatment outcomes (12). On the other hand, overexpression of NHEJ factors or increased NHEJ activity is known to make cancer cells resistant to chemo- or radio-therapy (6,89). Despite our extensive knowledge about the NHEJ pathway, only DNA-PKcs has been exploited as a target for anticancer therapy so far. DNA-PK is overexpressed in many different types of cancer, including gastric cancers, oral squamous cell carcinoma, lung carcinoma, and esophageal cancer (90-92). Recent discoveries also identify DNA-PKcs as a master regulator of cancer metastases (93-95). Two small-molecule pan-PI3K inhibitors, wortmannin and LY294002, have been used to inhibit DNA-PKcs activity *in vitro* but have toxic off-target effects and are too pharmacologically unstable to be used in human patients (32,33). NU7026 is a more selective and potent DNA-PK inhibitor and has been shown to radiosensitize cancer cells *in vitro* (34,35). Three other small molecules, CC-115 (dual inhibitor of DNA-PK and mTOR), CC-122 (pleiotropic pathway modulator), and NVP-BEZ235 (oral pan-class I PI3K inhibitor), are currently in different phases of clinical trial (12,36,37). In addition to DNA-PK, ligase IV has also been investigated as a target for its anticancer therapeutic value. SCR7, an inhibitor of ligase IV DNA binding activity, has been shown to inhibit NHEJ both *in vitro* and *in vivo*. It also potentiates the effect of radiation or chemotherapy by promoting the accumulation of DSBs and inducing apoptosis of cancer cells (38).

Inhibition of HR repair (HRR) in cancer treatment

HR is the predominant DSB repair pathway in the S and G2 phases of the cell cycle, where a homologous sequence of the sister chromatid acts as a template for DNA synthesis and gap filling in an error-free manner. Unrepaired radiation-induced damaged bases and SSBs are converted into DSBs during replication and require HRR to repair (96,97). Furthermore, *in vitro* studies suggest that HRR is essential for processing clustered DSBs induced by charged particles.

Mechanistically, HRR consists of three steps: first, during the pre-synaptic step, a DSB is recognized and resected to generate single-strand DNA overhangs. These are bound by the protective replication protein A (RPA) to prevent self-annealing. RAD51 and other associated proteins then bind to the DNA ends. Next, during the synaptic step, the resultant nucleoprotein filament invades the sister chromatid or homologous chromosome in search

of homologous regions to form heteroduplex DNA. New nucleotides are added to the 3' end of the invading strand by polymerase η using the sister strand as a template. An extended D-loop is formed which captures the second end of the break resulting in formation of Holliday junction (HJ). Once formed, the HJ undergo branch migration generating varying lengths of heteroduplex DNA. The final post-synaptic step involves resolving the HJ and migration of repaired ends migrate toward each other to restore the duplex DNA (98).

Deregulated HR activity is observed in many cancers, either due to defects in one of the HRR proteins or as a result of compensatory upregulation of HRR proteins following the loss of another DNA repair pathway. Germ line or somatic *BRCA1* and *BRCA2* mutations, which account for breast and ovarian cancers, have been studied comprehensively and can be sensitized to DNA damage by treatment with PARP inhibitors. Elevation of the HRR pathway has also been correlated with incidences of sporadic breast cancer (99). Deregulated expression of MRN complex proteins, which act as HR damage sensors, is observed in melanoma, ovarian, colorectal, and head and neck cancers (89). Mirin, a selective small-molecule inhibitor of MRE11, sensitizes cancer cells to DNA damage, but its mechanism as an inhibitor of HRR is unclear, since MRN complex also acts upstream of NHEJ (31). RPA complex and RAD51 are two essential proteins involved in the HRR process. Targeted inhibition of RPA has been difficult because of its multiple functions beside DNA repair. Recently, efforts have been made to block RPA's N-terminal domain interaction with other proteins, which are essential for DNA repair, without compromising its DNA binding activity (42). RAD51 is upregulated in pancreatic cancer, breast cancer, soft tissue sarcoma, and leukemia (100), and tumor response to radiation correlates directly with RAD51 expression levels (101). Additionally, evidence suggests that the RAD51-mediated HRR pathway is critical for processing the complex DSBs induced by charged particles, including protons and carbon (12C) ions used in treatment of aggressive and unresectable tumors (102-104). Thus, RAD51 is a valid target for sensitizing cancer cells to radiation. RI-1, a small-molecule inhibitor of RAD51's recombinase activity, has shown promise *in vitro* and *ex vivo* conditions, but further *in vivo* studies regarding its pharmacologic evaluation and efficacy are needed (39,40,105). Recent studies suggest that the inhibition of RAD52 and RAD54, which are RAD51 paralogs, can be clinically useful for killing *BRCA2*-deficient tumors (41,106,107). Despite our knowledge of deregulated HRR pathways in many cancers, only inhibitors of c-Abl and HSP90, two proteins that are known to regulate HRR activity only indirectly, are currently in clinic or in advanced clinical trials as anticancer agents (49,52). The non-receptor tyrosine kinase c-Abl is activated by ATM in response to DNA damage and phosphorylates RAD51, and treatment with tyrosine kinase inhibitor imatinib sensitizes the cancer cells to radiation (50). Furthermore, inhibiting RAD51 has proven effective in killing imatinib-resistant CML cells (51). HSP90 is a molecular chaperone that corrects folding of many proteins including RAD51. An HSP90 inhibitor, 17-allylamino-17-demethoxygeldanamycin, radiosensitizes human tumor cell lines by inhibiting RAD51-mediated HRR pathway (53). However, better small molecule inhibitors of HRR factors with higher specificity and improved efficacy are need to be developed to take full advantage of deregulated HRR pathway seen in many tumors.

Inhibition of cell cycle checkpoints in cancer treatment

Besides DNA repair, activation of cell cycle checkpoints is a vital part in the DDR signaling that has been investigated as a potential target for cancer therapy. Unlike normal cells, cancer cells often show impaired G1/S cell cycle checkpoint activation and depend heavily on S and G2 checkpoints to prevent premature mitotic entry with unrepaired DNA damage. Thus, inhibition of the G2 block using checkpoint inhibitors will augment DNA damage in cancer cells and ultimately result in selective killing of tumor cells (108). Among checkpoint inhibitors, UCN-01 (a dual CHK1/CHK2 inhibitor) showed promise in pre-clinical models but was not clinically successful because of poor bioavailability and unacceptable side effects (62,63). Similarly, other CHK1/CHK2 inhibitors, like XL-844 and AZD7762, failed to show substantial improvement in clinical trials (63-65). Recently, inhibitors of CDC25 phosphatase, a downstream substrate of ATM kinase and a regulator of the cell cycle, are being tested as therapeutic targets in oncology (66,67).

ROS as secondary effects of radiation

In addition to directly inducing nuclear DNA damage, IR exposure leads to increased production of ROS that may damage nucleic acids, proteins, and lipids (*Radiobiology for the Radiologist*, 7th Edition by Eric J. Hall and Amato J. Giaccia), leading to persistent cellular oxidative stress even after initial radiation exposure. Radiation also results in excess production of ROS by mitochondria, which can

result in cell death (109). Thus, IR induces damage in nuclear and extranuclear DNA (e.g., mitochondria) not only by direct deposition of energy but also by excess production of ROS. Enhanced ROS levels induce cellular oxidative stress even long after radiation exposure, often limiting cellular proliferation and inducing cell death or apoptosis.

Inhibition of direct DNA repair (DR) in cancer treatment

Clinical alkylating agents like temozolomide (TMZ) or radiation-induced ROS generate adducts that can compromise genomic integrity (110). O6-meG adducts formed in DNA are mutagenic, block DNA polymerase activity, and are considered cytotoxic. DR of O6-meG adducts is mediated by a single enzyme, O6-methylguanine-DNA-methyltransferase (MGMT), using a non-reversible chemical reaction that does not require a nucleotide template, breakage of the phosphodiester backbone, or new DNA synthesis (110). Increased expression of MGMT negatively affects cancer prognosis. Almost 90% of recurrent gliomas show overexpression of MGMT and are resistant to chemotherapy (111). Conversely, targeted inhibition of MGMT increases cancer cells' sensitivity to alkylating agents. O6-benzylguanine (O6-BG) and its derivative, O6-(4-bromothenyl) guanine, are currently being investigated for the treatment of TMZ-resistant gliomas in pre-clinical and clinical settings in combination with RT (111).

Charged particles in cancer treatment

Advantages of CPT over conventional RT

Charged particle radiation using protons and nuclei of carbon, neon, helium, and silicon has greater potential to control tumors than X-ray radiation. The theoretical advantages of CPT are better spatial selectivity in dose deposition and better efficiency in cell killing. This is because charged particle radiation has a Bragg peak, which maximizes the dose distribution within the target volume (clinical tumor volume or CTV) while minimizing the effect on the surrounding normal tissue (112). Thus, proton and carbon particles have a higher RBE than conventional X-rays. Depending on the biological endpoint used to measure RBE, proton beams have RBE values between 1.0 and 1.2 (113), and carbon ion beams have RBE ranging from 1.5 to 5 (114). Carbon and proton beams are better equipped to kill cancer stem cells (CSCs) associated with colon, pancreatic, and mammary carcinoma (115-118), which are responsible for making tumors radio- and chemo-resistant (119). Despite these advantages, only protons and, to some extent, carbon ions have been used clinically for treating cancer patients because of the high capital and operational costs associated with CPT. Currently, only 49 proton (14 in the United States) and 6 carbon ion (none in the United States) treatment centers operate worldwide, mostly in Asia and Europe.

Use of proton beams in cancer treatment

As of 2013, 93,895 patients have received proton therapy during cancer treatment. A significant proportion of these patients are children. The National Cancer Institute reported 10,380 new cases and more than 1,200 deaths from pediatric cancer (age 0–14) in 2016 (https://www.cancer.gov/types/childhood-cancers). Leukemia, brain and central nervous system (CNS) tumors, soft tissue sarcoma, and neuroblastoma are the most common types of childhood cancers. Although pediatric cancer patients are relatively few in number (1% of total cancer), there is great concern regarding the long-term effects of high dose radiation on surrounding normal tissues that are still developing in children (120). Because of its efficacy in sparing normal tissue, proton therapy has been used to treat pediatric cancers, including Hodgkin's lymphoma, tumors of the skull base, medulloblastoma, rhabdomyosarcoma, ependymomas, and low-grade astrocytomas. Although the full effect of proton therapy will not be clear for many years, initial studies and dosimetry analyses show that proton therapy is well tolerated, shows better local control of tumors, and has a lower risk of normal tissue toxicity and secondary cancers than conventional RT (121-123). Proton therapy has also been used extensively to treat ocular melanomas with favorable treatment outcomes (95% local control rate and a 90% eye retention rate); it has been particularly useful in the treatment of large ocular melanomas not approachable via brachytherapy (124). Proton therapy has also proven effective in controlling spinal cord tumors, including chordomas and meningiomas, with 80–90% local control and tolerable toxicity (124). Proton therapy has also been used to treat prostate cancer patients with treatment outcomes similar to intensity-modulated RT (IMRT) but with less normal tissue toxicity (124). Initial results also indicate that proton therapy can be clinically useful for treating head and neck cancers (125-127) and breast cancer (128), achieving the same local control of the disease

as conventional RT but with lower toxicity to surrounding normal tissues.

Use of carbon beams in cancer treatment

In recent years, carbon ion radiotherapy has gained immense popularity because it provides sharp lateral penumbra, sharp distal dose fall off, less dependence on hypoxic conditions, less dependence on cell cycle phase, and higher RBE, which may be advantageous for treating radioresistant tumors that require superior dose conformity while reducing the collateral effect on surrounding normal tissues (129,130). As of 2014, almost 15,000 cancer patients have been treated with carbon ions (131), and new carbon ion treatment centers are in different stages of development in the United States, Australia, South Korea, Taiwan, and China (132). Two independent studies performed among pediatric cancer patients with chordoma, chondrosarcoma, osteosarcoma, skull base, head and neck, and CNS tumors found carbon ion radiation to be effective in disease control and well tolerated by patients (133,134). Carbon ion radiotherapy has also demonstrated efficacy in treating bone and soft tissue sarcomas, including large, unresectable sarcomas and sacral chordomas, which are normally radioresistant and have a poor prognosis (135-138). Two different studies conducted among advanced, radioresistant head and neck cancer patients also concluded that carbon ion RT substantially improved patient survival (139,140). Another study conducted among 64 patients found carbon particle radiation to be an effective and safe treatment option for hepatocellular carcinoma, which can only be partially treated with surgery and has low tolerance for conventional RT (141). Pancreatic cancer is the fourth most common cause of cancer death in the United States, with a survival rate of less than 10%. A study conducted by National Institute of Radiological Sciences (NIRS), Japan found that preoperative, short-course carbon ion radiotherapy followed by surgery substantially increased 5-year survival rate in pancreatic cancer patients without any increase in toxicity (142). Excellent local control of the disease and a 59% 5-year survival rate were also obtained when locally recurrent, radioresistant renal cancer patients were treated with an escalating dose of carbon ion radiation (143). Lung cancer is the leading cause of cancer-related death worldwide (144). Hypofractionated carbon ion radiation of unresectable non-small cell lung tumors resulted in almost 90% local control of the disease and improved 5-year survival rate (71,145). More clinical trials assessing the efficacy of carbon ion therapy in lung cancer patients are ongoing (132).

Inhibition of DNA repair coupled with charged particle RT

Recent pre-clinical studies demonstrated that inhibition of NHEJ using NU7026, a potent DNA-PK inhibitor, can further sensitize non-small cell lung cancer cells to carbon ion radiation (34,146). However, carbon ions' high LET induces clustered DNA damage, a class of lesions comprising DSBs, SSBs, and abasic sites within one or two helical turns of DNA, induced by a single radiation track (147). Evidence suggests that HRR, but not the NHEJ pathway, is critical for processing DSBs induced by heavy ions, including carbon ions (102-104). Thus, inhibition of HRR factors can potentially be used to sensitize tumors to carbon particle therapy. Further understanding of the radiobiology of carbon ion particles, including the nature of the DNA lesions induced, the pathway used for repairing DNA lesions, and the fidelity of the DNA lesions repaired, is needed to achieve the greatest benefits of carbon particle therapy.

Combining radiation and immunotherapy approaches for cancer treatment

In 1980, Stone and colleagues observed that the host's immune competence often determines tumor response to RT. Yet, deliberate efforts to channel RT to hone the immune system against tumor cells have only started in the last decade. Tumor cells are known to thwart the immune system by attracting immunosuppressive cells or factors in the microenvironment. Ipilimumab and pembrolizumab, two monoclonal antibodies that have been used clinically to treat metastatic melanoma, target the immune suppressive factors cytotoxic T-lymphocytes-associated protein 4 (CLTA4) and programmed cell death protein 1 (PD1), respectively (148). Limited availability of dendritic cells (DCs) to present the tumor antigens also contributes to the immune system's tolerance of tumors (149). Recent pre-clinical studies have found that dying tumor cells exposed to RT accumulate cytosolic DNA, which activates the stimulator of interferon genes (STING) pathway (150,151). STING activation is critical for the expression of inflammatory cytokines and chemokines, specifically type I interferon (IFN), which in turn recruits DCs to the tumor and activates them. Activated DCs are capable of presenting the tumor antigens to T cells,

which primes the T cells to selectively kill tumor cells (150,151). Thus, radiation can activate the cell-mediated immune response against tumors by enhancing tumor DNA delivery to DCs. Recently, Matsunaga and colleagues showed that carbon particle radiation coupled with DC-based immunotherapy resulted in control of primary tumors in immunocompetent mice (152). Further, these challenged animals developed long lasting $CD8^+$ T-cell-mediated anti-tumor immunity, which prevented recurrence of the tumors. They could not replicate these results in immune-deficient nude mice, however, thus highlighting the importance of the immune system in controlling the tumor (152). In addition to priming DCs, RT also activates a specific subset of neutrophils, which induces oxidative stress on the tumor cells and eventually activates tumor-specific cytotoxic T cells (153). In addition to the adaptive immune response, studies have suggested that DNA repair and replication factors play a role in the innate immune response. For example, ATM-deficient cells were found to have increased cytosolic self-DNA, leading to increased inflammation (154). Similarly, MRE11, a DSB sensor protein, recognizes cytosolic DNA and initiates innate immune response signaling (155). Moreover, RPA2 and RAD51 were shown to protect the cytosol from the accumulation of self-DNA (156). Our recent study found a link between DNA replication, repair, and immune response after exposure to radiation (157). Our *in vitro* studies indicate that, in the absence of RAD51, exposure to radiation leads to increased replication stress, which results in the accumulation of cytosolic DNA, triggering a STING-mediated immune response. However, further *in vivo* studies are necessary to determine the efficacy of using small-molecule HRR inhibitors coupled with RT as an immunotherapeutic approach for killing tumor cells.

Current limitations and future direction

Abnormalities of the proteins involved in DDR and repair are common in cancers and may be induced by both genetic and epigenetic causes. Thus, the potential clinical utility of DNA repair inhibitors is encouraging. However, the greatest obstacle to developing inhibitors against DNA repair proteins is tumor heterogeneity, as levels of DNA repair proteins vary widely not only between different types of tumors, but also among patients with the same type of tumor. Registering the deregulated pathways and the cause of tumorigenesis in a given patient is necessary to determine the optimum treatment for that patient. However, analyzing the massive genomics and proteomics data to determine whether mutations in a particular gene or abnormal expression of a protein are clinically useful as a prognostic or predictive biomarker remains a daunting task. Also, both the specificity and the efficacy of small-molecule inhibitors should be taken into account when choosing the right course of treatment. Many DNA repair inhibitors that sensitize cancer cells to radiation are not specific to tumors, as these factors also affect normal tissue survival and function to some degree. Thus, applying small-molecule inhibitors with a low to moderate degree of specificity carries an inherent risk to clinical success. Therefore, utilizing our knowledge of synthetic lethal interactions, which make cancer cells overly dependent on one pathway in the absence of other functional pathways, and translating these cancer cell-specific molecular vulnerabilities into therapies can be pivotal for increasing the therapeutic ratio for radiation oncology. Even then, the evolution of resistance often limits the efficacy of the treatment. For example, *BRCA1/2* mutant tumors treated with PARP1 inhibitors often generate a secondary mutation that makes them resistant to PARP inhibition (158-160). Additionally, many factors affect the tumor response to DNA damage and RT, including the tumor microenvironment. For instance, most cancer cells are hypoxic and show increased expression of HIF-1. Hypoxia is known to induce tumor radio resistance through the activation of HIF-1, which is entwined with molecules that are central to DDR and checkpoint control; thus, is rightly considered an attractive target molecule for cancer therapy (6).

In the last decade, research and clinical trials have proved the therapeutic importance of using DNA repair inhibitors can be beneficial for cancer treatment. The advent of databases like REPAIRtoire (http://repairtoire.genesilico.pl/) is a valuable resource, which lists known diseases correlated with mutations in genes encoding DNA repair proteins. Further understanding of the basic biology underlying the DDR and the mechanisms responsible for its dysregulation in cancer will provide exciting opportunities for new and effective cancer therapies. However, to take full advantage of the discoveries made in the laboratories, biomarker tests in the clinics need to be reliable, not cost prohibitive and able to be run with existing clinical facility in a timely manner. When combined with new discoveries of improved DNA repair inhibitors, patient specific biomarker detection will enable earlier diagnosis and better treatment of cancer, which is the ultimate goal of 21st century precision medicine.

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Footnote

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