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DNA Damage Response and Repair: Insights into Strategies for Radiation Sensitization

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1. Introduction

Glioblastoma is the most common form of primary brain tumors [1]. About 10,000 new patients each year in the US are diagnosed with GBM. Despite its comparatively low incidence of about 2-3 new cases per 100,000 people per year (for comparison, the incidence of colon cancer in the US is approximately 1 new case per 1,800 people per year), the total number of deaths per year attributable to glioblastoma rivals that of other major cancers. This disparity reflects the lethal nature of the disease. If untreated, patients with glioblastoma generally die within 3 months of their diagnosis [2]. Diffuse invasion into the surrounding normal cerebral parenchyma is a cardinal feature of glioblastoma, preventing surgical cure. In this context, it is not surprising that surgical resection alone does not significantly prolong patient survival [2]. When maximal surgical resection is combined with radiation and the chemotherapeutic agent, temozolomide, the median survival of patients improved to 14.6 months [3]. While this regimen achieves an approximate 10% five-year progression free survival [4], the majority of the afflicted patients succumb to the disease within a year of diagnosis. Effective therapeutic strategies are desperately needed.

Despite the abundance of strategies and agents that have been tested over the last half-century for the treatment of GBM, the single most efficacious modality and significant advance has been the use of post-resection radiation therapy. In 1966, the Montreal Neurology Institute published the first large case series that suggested a survival advantage in patients who received an average total post-resection IR dose of 5,000–6,000 cGy [5]. Over the next decade, other case series corroborated those data, suggesting an improvement in patient survival with post-resection IR [6-9]. Although interpretation of these case series is

confounded by their retrospective, non-randomized nature, and wide variations in radiation dose regimen, an overall trend of improved patient survival was seen.

The efficacious nature of radiation therapy in glioblastoma was subsequently confirmed in three subsequent randomized control trials. *BTSG 66-01*, the first of the initial three clinical studies that randomized patients with newly-diagnosed malignant gliomas to with Whole-Brain RadioTherapy (WBRT) with or without mithramycin [10]. While mithramycin did not significantly impact survival, the survival of patients who received adjuvant WBRT were extended to about 5 months on average. *BTSG 69-01* randomized patients post-surgical resection to best supportive care or chemotherapy (BCNU), with or without WBRT. Overall patient *survival* was improved with radiation therapy (with or without BCNU) when compared to supportive care alone. The median survival for the surgery only patient was 14 weeks. The median survival for patients who underwent surgery followed by radiation therapy was 36 weeks ($p<0.05$). Similar results were seen in another randomized trial, *BTSG72-01*.

A meta-analysis of *66-01, 69-01,* and *72-01* [11]showed that patients who did not receive radiation or were treated with less than 4,500 cGy exhibited poor survival (median survival of 4.2 and 3.1 months, respectively). On the other hand, patients treated with 5,000, 5,500, and 6,000 cGy showed improved survival (median survival of 6.5, 8.4, and 9.8 months, respectively). Subsequent dose escalation beyond 6,000 cGy revealed increased neuro-toxicity without significant therapeutic gain [12]. These studies collectively established the dosimetry for modern glioblastoma therapy.

It is important to note that, as a single agent, the efficacy of radiation therapy as a glioblastoma therapeutic has yet been matched by any chemotherapy [12]. For this reason, radiation therapy remains a universal component in clinical trials with curative intent for treatment of newly diagnosed glioblastomas. Identification of agents that augment the efficacy of radiation therapy remains a promising strategy for glioblastoma therapy. In this context, we will review the molecular mechanism underlying cellular response to radiation.

2. DNA repair and damage response following ionizing radiation

While Ionizing Radiation (IR) has been shown to induce a plethora of cellular effects, it is widely held that its tumoricidal activity is largely related its effect on DNA [13]. IR induces multiple types of DNA lesions, including damages to the nucleobase, sugar, and phosphate back bone [13]. Ultimately, these lesions, if unrepaired, are converted into Double Stranded DNA Breaks (DSBs). DSBs are highly cytotoxic as the presence of a single DSB in a cell is sufficient to induce death [14-19]. Our understanding of DNA damage response and DSB repair has expanded exponentially in the past decade. To make this vast information more accessible to the reader, we will organize the information into the following sections: 1) general mechanism of DSB repair, 2) mechanism by which DNA damage response activates DSB repair, and 3) effect of DNA damage on cell cycle progression.

2.1 General mechanism of DSB repair

DSBs are repaired by two major mechanisms: Non-Homologous End Joining (NHEJ) or Homologous Recombination (HR) [20, 21]. The simplest way of repairing DSB is by re-ligation. This process is termed NHEJ since no significant DNA sequence homology at the broken ends is required. Instead, the NHEJ enzymatic machinery relies on limited processing of the DNA ends followed by annealing short stretches of complementary DNA sequences.

Because short stretches of complementary sequences occur at some regularity in the human genome, NHEJ is prone to the generation of mutations, deletions, as well as chromosomal rearrangements[22].

The second major DSB repair process is termed HR. HR initiates with extensive 5′ to 3′ end processing of the broken ends into large regions of single stranded tails. The resultant 3′ single stranded tail invades a homologous donor sequence. This strand invasion is mediated by enzyme(s) termed "recombinase" that coats the single stranded tail. Subsequent strand extension and Holliday junction resolution result in restoration of DNA continuity. The resolution is mediated by specific enzymes(s) termed "resolvase" [21]. Because the process cannot proceed without extensive sequence homology, HR tends to be less prone to mutagenesis relative to NHEJ. Because of the requirement for a homologous donor sequence, HR occurs only in the S/G2/M phases of the cell cycle while NHEJ occurs throughout the cell cycle. Both HR and NHEJ contribute to the repair of RT induced DSBs, suggesting that these pathways may be functionally compensatory [23-25]. Given the critical role of these two processes, it is not surprising they are subject to complex regulation.

In mammalian cells, both are carried out by multi-step processes facilitated by a large number of proteins. The mechanistic details of these processes remain an active area of investigation. Working models are described as follows.

For NHEJ, upon DNA damage response activation (see below), the Ku70/Ku 80 heterodimer is recruited to the break site. This protein complex forms a ring shaped structure to protect the broken DNA ends [26]. Additionally, the heterodimer serves as a platform for binding of the critical kinase, DNA-PK [27], and the XRCC4-Ligase IV-XLF4 complex [28]to the site of damage. DNA-PK performs two important functions: 1) it phosphorylates the Ligase IV complex to facilitate the joining of DNA ends; and 2) in cases where DNA end processing is required before rejoining, DNA-PK binds to and recruits the Artemis endonuclease to perform this function. While other proteins also participate in NHEJ [21], *in vitro* reconstitution of NHEJ with these seven proteins (Ku70, Ku80, DNA-PK, XRCC4-Ligase IV-XLF4 complex, and Artemis) suggest that they are essential for this process.

In comparison, the genetics of HR is more complex and far less well understood. Upon DNA damage response activation, it is thought that the BRCA1 and BRCA2 protein are recruited to the site of DNA damage. These two proteins were cloned by virtue of their inactivation in familial breast cancer cohorts (BReast CAncer genes 1 and 2) [29-31]. Both proteins encode large molecular weight proteins that mediate multiple cellular processes to suppress tumor formation. One of these critical functions involves HR. It's proposed that BRCA1 and 2 bind to aberrant DNA structures related to DSB ends [32, 33]. Through BRCA2, the mammalian recombinase, RAD51, is recruited to the site of damage [34]. RAD51 coats the DNA and facilitates the strand exchange reaction in homologous recombination [35-37]. After strand invasion, resolution of the Holliday intermediate is mediated by a protein complex consisting of XRCC3 and RAD51C (two homologues of RAD51) [21, 38].

The mechanism by which HR and NHEJ is activated in response to DNA damage is discussed below.

2.2 DNA damage response

The DNA Damage Response (DDR) refers to the signal transduction cascades that are triggered by DNA damages. These cascades coordinate DNA repair, cell cycle progression, and cell death mechanisms to facilitate the faithful transmission of genetic material after

DNA damage. The process initiates with the recognition of DNA damage by specialized "sensor" proteins. These sensor proteins, in turn, recruit and/or activate "transducer" proteins required for subsequent signaling to "effector" responses, such as cell cycle arrest, apoptosis, transcription, and DNA repair [39]. Defects in DNA damage response have been associated with genomic instability, sensitivity to genotoxic agents, and cancer predisposition [40].

Upon DNA damage, the strand discontinuities trigger complex changes in DNA topology secondary to histone acetylation and phosphorylation of chromatin proteins [41]. The unveiled strand break is recognized by the Mre11-Rad50-Nbs1 (MRN) complex. In addition to serving as an exo/endonuclease to process the DSBs into single stranded DNA tails [42], the MRN complex also recruits the Ataxia Telangiectasia Mutated (ATM) protein kinase to the site of the DSB [43, 44]. When recruited to DSBs, ATM – normally existing in an inactive dimeric form – dissociates and autophosphorylates on multiple residues that are thought to be important for activation of ATM's kinase activity [45]. The activated ATM phosphorylates the histone protein, H2AX, over a region of megabases surrounding a DSB [46, 47]. The phosphorylated H2AX (also known as γ-H2AX), in turn, recruits the Mediator of DNA Checkpoint (MDC1) protein [48, 49]. The MDC1 protein serves as a scaffold protein for docking of the E3 ubiquitin ligase complex, UBC13-RNF8 [50], which serves to poly-ubiquitinate H2AX. Completion of this poly-ubiquitination reaction requires a second ubiquitin ligase, RNF168 [51]. RNF168 is recruited to the site of DNA damage through its interaction with HERC2 and RNF8 [52]. The poly-ubiquitination reaction alters local chromatin structure as well as provides docking site for the ubiquitin binding protein, RAP80. RAP80, in turn, recruits the BRCA1/BRCA2/RAD51 repair complex by direct physical interaction[53]. This complex initiates DSB repair by HR as well as arrests cell cycle progression in a process known as DNA damage checkpoint activation (see ensuing section).

It is important to note that while the above damage response is described in a linear manner, parallel interactions occur at each step. For instance, MDC1, in addition to recruiting UBC13-RNF8, also interacts with ATM [48] and MRN [54] to stabilize the repair complex. The aggregate effect of these other complex interactions induces chromatin state changes surrounding the DSB and the localization of numerous proteins required for coordinating DNA repair and checkpoint regulation.

Similar to HR, the NHEJ process can be initiated by the MRN complex upon DDR activation. The Mre11 protein in the complex can directly interact with the Ku70 subunit [55]. Moreover, the RAD50 protein in the MRN complex encodes a high-affinity DNA binding domain and a second domain that facilitates homodimeric interactions that holds DNA ends in close proximity [56] to facilitate subsequent NHEJ.

Since the MRN complex may initiate either HR or NHEJ, a central question in the field of DNA repair involves the mechanism of this regulation. Inappropriate activation of HR in the G1 phase of the cell cycle could lead to cell death. Similarly, activation of NHEJ during the S/G2 phases of the cell cycle could increase the rate of mutagenesis. One of the key mediators of this regulatory process involves the protein CTBP Interacting Protein (CTIP). In a landmark study [57], CTIP was found to interact with the MRN complex to promote its exo/endonuclease activity and process DSBs into single stranded DNA ends. Importantly, this activity is regulated by cell cycle dependent phosphorylation events mediated by Cyclin-Dependent Kinases (CDKs). In the S/G2 phase of the cell cycle, CTIP is phosphorylated. Thus, the MRN complex processes DSBs into single stranded tails required for the initiation of HR. On the other hand, in the G1 phase of the cell cycle, CTIP remains

unphosphorylated, and the MRN complex remains inactive as an exo/endo-nuclease. Without this processing, HR cannot be initiated. Thus, NHEJ becomes the predominant repair process.

2.3 Effect of DNA damage on cell cycle progression

In addition to the assembly of repair complexes as above described, DNA damage triggers signaling to proteins required for cell cycle progression, such as the CDK/cyclin complex. Generally speaking, DNA damage checkpoint regulation occurs at three distinct phases of the cell cycle: the G1-S transition, the intra-S-phase, and the G2-M transition. Most of what we understand of this transduction process involves protein phosphorylation cascades, though the importance of other types of reversible modifications, such as ubiquitination and sumoylation, are become increasingly apparent [58]. Here, we will review an illustrative example of signal transduction between DNA damage sensors and cell cycle regulation. Upon recognition of DSB, the MRN complex recruits and activates the critical ATM kinase. The ATM kinase, in turn, phosphorylates the tumor suppressor p53 and another kinase termed Chk2 (Checkpoint Kinase 2) [59]. ATM phosphorylation of Chk2 activates its kinase activity which, in turn, phosphorylates both p53 and MDM2. These phosphorylation events stabilize p53 by interrupting its association with its negative regulator, MDM2 [60]. Activated p53 then induces the transcription of its target genes, which include the critical regulator of the G1-S transition, p21 [61]. The binding of p21 to the CDK-cyclin complexes and prevents phosphorylation of the retinoblastoma protein (pRb). When in a hypophosphorylated state, pRb blocks cell proliferation by sequestering and altering the function of E2F transcription factors that control transcription of genes required for progression from G1 into S phase. Disruption of the pRb pathway – as occurs with mutant p53 or p21 – liberates E2Fs and allows cell proliferation, which renders cells insensitive to DNA damage-induced anti-growth signals that normally operate to inhibit passage through G1 phase of the cell cycle [62]. With regards to the G2-M checkpoint, ATM release inhibition of p53 additionally results in the transcriptional induction of 14-3-3σ in addition to p21. The 14-3-3σ protein sequesters the cyclinB-cdc2 kinase complex in the cytoplasm and prevents nuclear phosphorylation events required for G2/M progression [63]. Additionally, p21 binds to any residual cdc2 that enters the nucleus to prevent its activation. These and other ATM events prevent progression through the G2/M transition and afford time for DSB repair [64].

3. Strategies for sensitization

A prediction of the above presented model is that inhibition of any of the proteins required for DDR or DSB repair should lead to radiation sensitization. In general, this prediction has been confirmed [65]. However, therapeutic agents that directly inhibit these critical proteins are still years from reaching clinical trial. Encouragingly, several FDA-approved agents have recently been shown to modulate DNA damage response. This property may be explored as therapeutic strategy.

3.1 Molecular rationale for therapeutic window

Before considering the strategy of radiation sensitization, one must first consider the molecular rationale for therapeutic window. After all, if normal and tumor cells were equally sensitized by the agent, then no therapeutic efficacy is gained.

A large body has yielded data suggesting that oncogene activation creates a tumor state that increases the accumulation of DNA damage [66-69]. This damage, if unrepaired, can be converted into DSBs that eventually lead to cell death. To compensate for this increased DNA damage, the tumor cells require increased utilization of DNA repair processes [69]. In this context, the administration of radiation introduces additional DNA damage that further taxes the already over-utilized repair process. This situation, in turn, increases the likelihood of an unrepaired DSB causing cell death. The same effect can be achieved by inhibition of DSB repair. The following sections will review FDA-approved agents with such properties. It is important to note that these agents induce pleiotropic effects beyond DSB repair inhibition.

3.2 DNA damaging agents

Conventional chemotherapy involves DNA damaging agents that are often used in conjunction with radiation. In this context, these FDA-approved agents often sensitize the tumoricidal effects of radiation. The mechanism of this sensitization is thought to be related to the generation of DNA damages that sequester critical DNA repair proteins. For instance, lesions generated by cisplatin bind to and sequester the Ku70/80 heterodimer and thereby compromise the efficiency of NHEJ [70]. Further, most DNA damages induced by conventional chemotherapy are ultimately converted to DSBs [71]. These DSBs will titrate away the repair proteins available to repair the DSBs induced by subsequent radiation. These types of mechanisms likely account for the increased glioblastoma patient survival observed in the context of concurrent radiation/ temozolomide treatment relative to radiation treatment alone [3, 4].

3.3 Proteasome inhibitors

As a result of extreme aneuploidy, copy-number variation, and transcriptional alteration that are present in many cancer cells, there is increased stress on the chaperone pathways (such as heat shock proteins) to maintain folding of over-expressed proteins. When the capacity of these chaperone proteins becomes saturated, the unfolded proteins require degradation by the proteasome complex [72]. Thus, tumor cells exhibit increased dependency on proteasome function. Indeed, proteasome inhibition has been demonstrated to selectively ablate cancer cells both *in vitro* and *in vivo* [73]. The proteasome inhibitor bortezomib has attained FDA-approval as a treatment for multiple myeloma and mantle cell lymphoma.

Recent studies implicate proteasome function in DSB repair. The yeast Sem1 protein is a subunit of the 19S proteasome that is required for efficient HR [74]. The human Sem1 homologue, DSS1, physically interacts with the HR protein, BRCA2, and is required for its stability and function [75-77]. Using the DR-GFP assay to directly assess HR efficiency, Murakawa et al. demonstrated that HR efficiency is significantly reduced by proteasome inhibition [78]. As a whole, these studies suggest proteasome inhibition as a means to target HR in cancer therapy.

The mechanism by which proteasome inhibition modulates HR remains an area of investigation. One hypothesis frequently put forth is the following. The proteins destined for proteasome degradation are typically modified by attachment of multiple ubiquitin moieties [74]. Processing of the tagged protein releases the tagged ubiquitin to replete the intracellular pool. Proteasome inhibition, thus, leads to accumulation of ubiquitinated proteins. This accumulation, in turn, depletes the intracellular ubiquitin pool. Since free ubiquitins are required to activate HR, the repair process is compromised by proteasome inhibition.

3.4 Epidermal Growth Factor Receptor (EGFR) inhibitors

EGFR is frequently amplified or mutated in several cancer types, including Non-Small Cell Lung Cancer (NSCLC) and glioblastomas [79-81]. As aberrant EGFR signaling is required to sustain tumor survival and proliferation in some cancers, targeted inhibition has led to selective tumor ablation [79]. Clinical trial success has led to FDA-approval for treatment of NSCLC.

Several studies have demonstrated that EGFR inhibition sensitized tumor cells to radiation [65]. Insights into the mechanism underlying this sensitization have been provided by several recent studies. One series of studies demonstrate that a subset of EGFR travels to the nucleus where it binds to and enhances DNA-PK activity to enhance NHEJ [82, 83]}. Indeed, glioblastomas over-expressing an over-active form of EGFR (termed EGFRvIII) exhibit radiation resistance that can be abridged by treatment with DNA-PK inhibitors [84]. Another series of studies reveal that EGFR inhibition leads to retention of BRCA1 in the cytoplasm, thereby causing defective HR [85]. Finally, other downstream effectors of EGFR, including the Extracellular signal Regulated Kinase (ERK1/2) also modulates HR efficiency [86]. It is likely that the radiation sensitization effect of EGFR inhibition represents a culmination of these individual effects.

3.5 Other late stage clinical trial agents

There are several other agents that are in mid- to late- clinical trial testing that have also been shown to inhibit DNA damage response. For instance, Histone DeACetylase (HDAC) inhibitors have been shown to down regulate the transcript level of BRCA1 [87]. These agents have also been shown to disrupt the chromatin re-organization required for ATM activation [41]. As another example, Heat Shock Protein 90 (HSP90, the prototypical chaperone protein) inhibitor treatment inhibits ATM autophosphorylation upon DNA damage [88] and destabilizes the MRN complex [89, 90], thereby inhibiting HR. Finally, CDK1 inhibition causes the loss of a critical phosphorylation event on BRCA1 required for its HR function [91].

There are additional modulators of DDR and DNA repair not described here [65]. Indeed, the number of pharmacologic inhibitors that either directly or indirectly inhibit DSB repair is being uncovered at a rapid pace. Careful consideration should be given for combination with radiation therapy in clinical trial design.

4. Closing remarks

Radiotherapy is the most effective post-surgical treatment modality in the management of glioblastoma. Adjuvant radiotherapy alone provides a more than doubling of median survival. Incremental gains with additional medical therapy have proven elusive, with most agents showing moderate activity *in vitro* or with encouraging early clinical experience only to demonstrate a lack of benefit in larger trials. Attempts at treatment intensification with radiotherapy have been similarly disappointing. Molecular understanding of DNA damage response and repair, on the other hand, has now afforded novel therapeutic targets. These targets are particularly attractive in the context that oncogenes induce increased DNA damage accumulation and cause tumors to become hyper-dependent on DNA damage response pathways. Encouragingly, several FDA-approved agents modulate critical proteins in DNA damage response/repair, including conventional DNA damaging agents, proteasome inhibitors, and EGFR inhibitors. Clinical trials involving these and other agents modulating DNA damage response should be designed with this consideration.

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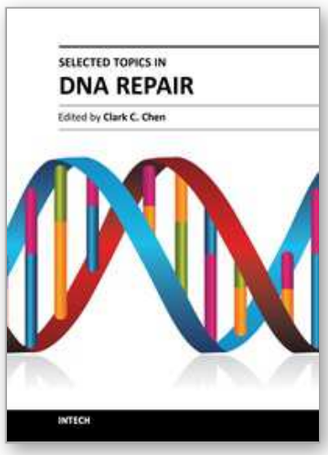

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This book is intended for students and scientists working in the field of DNA repair, focusing on a number of topics ranging from DNA damaging agents and mechanistic insights to methods in DNA repair and insights into therapeutic strategies. These topics demonstrate how scientific ideas are developed, tested, dialogued, and matured as it is meant to discuss key concepts in DNA repair. The book should serve as a supplementary text in courses and seminars as well as a general reference for biologists with an interest in DNA repair.

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