DNA Damage Response Assessments in Human Tumor Samples Provide Functional Biomarkers of Radiosensitivity

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Abstract

Predictive biomarkers are urgently needed for individualization of radiation therapy and treatment with radiosensitizing anti-cancer agents. Genomic profiling of human cancers will provide us with unprecedented insight into the mutational landscape of genes directly or indirectly involved in the response to radiation-induced DNA damage. However, to what extent this wealth of structural information about the cancer genome will produce biomarkers of sensitivity to radiation remains to be seen. Investigators are increasingly studying the subnuclear accumulation (i.e., foci) of proteins in the DNA damage response (DDR), such as γ -H2AX, 53BP1, or RAD51, as a surrogate of treatment sensitivity. Recent findings from preclinical studies have demonstrated the predictive potential of DDR foci by correlating foci with clinically relevant endpoints such as tumor control probability. Therefore, pre-clinical investigations of DDR foci responses are increasingly moving into cells and tissues from patients, which is the major focus of this review. The advantage of using DDR foci as functional biomarkers is that they can detect alterations in DNA repair due to various mechanisms. Moreover, they provide a global measurement of DDR network function without needing to know the identities of all the components, many of which remain unknown. Foci assays are thus expected to yield functional insight that may complement or supersede genomic information, thereby giving radiation oncologists unique opportunities to individualize cancer treatments in the near future.

Introduction

It is evident that cancer patients display a wide range of treatment sensitivities with regard to both tumor and normal tissue responses. Predictive biomarkers are urgently needed for individualization of radiation therapy and DNA damaging systemic therapies, which includes chemotherapeutics, therapeutic antibodies, and molecularly targeted drugs such as poly(ADP-ribose)polymerase (PARP) inhibitors. Following exposure to ionizing radiation (IR) and many drugs, DNA double-strand breaks (DSBs) represent the principal lesion which, if not adequately repaired, can lead to cell death via the generation of lethal chromosomal aberrations or other mechanisms (reviewed in ref.¹). Alternatively, an inaccurately repaired or unrepaired DSB may result in mutations or genomic rearrangements in a surviving cell, thereby promoting genomic instability and malignant cell transformation. Complex DNA damage response (DDR) pathways have evolved, and are evolutionarily conserved, to protect the cell from the potentially deleterious effects of a DSB. Two principle pathways, homologous recombination repair (HRR) and non-homologous end-joining (NHEJ), remove DSBs by employing separate as well as overlapping protein complexes.

There is increasing evidence from functional studies and recent genomic analyses that alterations of DNA repair exist in human tumors that may impact treatment sensitivities ²⁻⁸. Therefore, identification of alterations in the DDR following DSB induction would be very useful for individualization of treatments. Possible treatment modifications include (de-)escalation of radiation dose or combining radiation therapy with targeted drugs that further exploit pre-existing DNA repair defects. It is possible that whole genome sequencing will ultimately provide us with sufficient information on the overall state of DSB repair in a given tumor to inform treatment approaches that produce DSBs. However, it is also possible, and in our opinion more likely, that genomic information will be in most cases insufficient to predict how tumors will respond to therapeutic DSBs (with some exceptions such as predicted synthetic lethality resulting from BRCA mutations and PARP inhibition ⁹).

Many investigators now believe that a functional assessment of the DDR in human tumor tissues can be used to predict treatment sensitivities^{2,4,5,8}. Such functional assays most often rely on the detection of subnuclear foci of DDR proteins (reviewed in ref.¹⁰). Foci represent multiprotein complexes that are organized into centers surrounding a DSB and can be visualized as “dots” using immunofluorescence microscopy in cells and tissues (**Figure 1A**). These complexes are highly dynamic structures in order to coordinate DNA repair and checkpoint responses. Many DDR proteins shuttle transiently in and out of the focus, or are degraded at the damage site. The composition of these repair centers depends on the type of DNA damage and the cell cycle phase of the damaged cell. The regulation of its components is ensured through complex post-transcriptional modifications and other mechanisms. The accumulation of DDR factors around a DSB is thought to serve multiple purposes including sheltering the broken DNA ends from decay, preventing undesired repair from occurring, and promoting chromatin relaxation to facilitate access of repair proteins. The increased concentration of many DDR proteins around the damage site likely stimulates their activities and results in an amplification of the DNA damage signal. Foci may also serve as a “toolbox” in which DDR proteins assemble to promote different repair pathways¹⁰.

There exists a vast literature on foci responses in established cell lines and a considerably smaller but emerging body of data on conducting foci assays on tissues and cells from patients^{4,5,8,11-14}. The focus of the current review is to provide an overview of the opportunities as well as technical challenges associated with the use of patient samples. The reader is also referred to a number of recent reviews on this and related topics^{10,15-19}. Overall, the analysis of DDR foci in patient-derived samples is feasible and reliable but correlations of foci data with clinical outcomes are urgently needed.

DDR Foci Endpoints: γ -H2AX and 53BP1

One of the earliest events in the DDR is the rapid phosphorylation of the histone variant H2AX at serine 139, four residues from its carboxyl terminus, in the vicinity of the DSB by members of the phosphatidylinositol 3-kinase family, i.e., Ataxia Telangiectasia Mutated (ATM), AT and Rad3-related

(ATR), and DNA-dependent protein kinase (DNA-PK)²⁰⁻²³. H2AX is one of the most conserved H2A-variants, and within the H2A pool, the fraction of H2AX levels varies between 2 and 25%²⁰. Upon DSB induction, γ -H2AX, the phosphorylated form of H2AX, appears as subnuclear foci within minutes and reaches a maximum after approximately 30 minutes²⁴. Gamma-H2AX is dephosphorylated and foci disperse reflecting DSB repair^{24,25}. Although many repair proteins form distinct foci, γ -H2AX foci have the important feature to be a new protein species induced by physical DSB formation and are formed throughout all cell cycle phases²⁶. Recent studies have shown that NHEJ is the predominant pathway in the G1- and G2-phase of the cell cycle while HRR repairs only 15-20% of the DSBs in late S- and G2-phase²⁶. Residual foci which are detectable for more than 24 hours after IR likely represent misrepaired or incompletely repaired lethal DSBs²⁷. Thus residual γ -H2AX foci may be a useful surrogate of cellular radiosensitivity. Accordingly, for a variety of cell lines and for a broad range of IR doses, a linear correlation of the number of residual γ -H2AX foci with cell survival has been shown in most studies²⁷⁻³¹. Even for the low but clinically relevant dose of 2 Gy the correlation seems to hold although at this dose the assay may only be able to identify the most radiosensitive tumors (**Figure 1B**). With appropriate assay modifications, the clinical value of residual γ -H2AX will also lie in the prediction of radioresistance and in the prediction of radiosensitization caused by a molecular targeted agent. This is illustrated in **Figure 1C, D** where the addition of a heat shock protein 90 inhibitor clearly increases the number of residual γ -H2AX foci in some head and neck squamous cell carcinoma (HNSCC) cell lines which correlates with the ability of this agent to radiosensitize cells. In fact, a large number of molecularly targeted agents may radiosensitize through modulation of DSB repair as **Table 1** illustrates³²⁻⁵³. If this were to hold true in tumor biopsies from patients, γ -H2AX could be a very useful biomarker to guide the clinical administration of radiosensitizing targeted drugs.

Gamma-H2AX can be quantified by Western blotting, flow cytometry analysis, or by enumerating discrete foci using microscopy¹⁵. Although flow cytometry has the advantage of fast and high sample throughput, microscopy is the most sensitive method for γ -H2AX foci detection and,

therefore, is the predominant technique of γ -H2AX detection in current pre-clinical applications^{19,54,55}. Specifically in patient samples, several additional reasons account for microscopy as the method of choice to detect γ -H2AX foci: i) detection of individual DSB marked by foci, ii) heterogeneous responses in tumor tissue (e.g. oxic or hypoxic regions), iii) discrimination between foci from tumor cells or surrounding normal tissue, and iv) the degree of DNA damage (e.g. pan-staining in apoptotic, mitotic or necrotic cells, diffuse foci in S-phase cells).

The tumor suppressor TP53 binding protein 1 (53BP1) is an alternative marker for visualization of IR-induced DSBs⁵⁶ (**Figure 1E, Table 1**). Accumulating evidence suggests that it is located upstream in the DDR and involved in regulation of both HRR and NHEJ^{57,58}. Potentially, it is more specific for marking DSB than γ -H2AX which also stains areas of DNA not containing a DSB and apoptotic nuclei^{59,60}. However, more comparative studies of γ -H2AX and 53BP1 in human samples are needed^{61,62}.

Lastly, an imaging probe capable of revealing DSB *in-vivo* could provide useful information regarding treatment response and bypass the technical difficulties surrounding microscopy-based foci analysis on biopsy specimens as is further discussed below. The high abundance of γ -H2AX expression in tumor cells is most likely sufficient for molecular imaging. Cornelissen et al.⁶³ developed a radioimmunoconjugate that targets γ -H2AX, ¹¹¹In-DTPA-anti- γ H2AX-Tat, and is able to penetrate the cell and its nucleus. The radioimmunoconjugate was used to visualize radiation-induced accumulation of γ -H2AX with *in-vivo* fluorescence imaging and single photon emission computed tomography in a heterotopic breast cancer model. Although the probe warrants further validation such as dose response correlations, *in-vivo* imaging holds the promise of a non-invasive imaging tool for detecting treatment-induced DSBs in preclinical and clinical settings.

DDR Foci Endpoints: Focus on HRR

Of the two principal DSB repair pathways, NHEJ and HRR, the latter is more readily assayed owing to the nature of many HRR proteins to form visible foci. Foci of the main HRR recombinase,

RAD51, form in response to stalled/collapsed DNA replication forks or DSBs in the S- or G2-phase of the cell cycle (reviewed in ref ¹⁰). RAD51 foci are commonly used as a surrogate measure of local HRR activity based on genetic analysis and reporter substrate studies ⁶⁴⁻⁶⁶. The proper formation of RAD51 foci is controlled by a multitude of proteins, including BRCA2, RAD52, PALB2, and BRCA1, but also upstream regulators such as ATR or ATM. Thus, genetic or epigenetic defects anywhere in the HRR pathway can lead to an impairment or even complete abrogation of RAD51 foci formation. However, which defects in the Fanconi Anemia (FA) pathway impair FANCD2/BRCA2 function to compromise RAD51 foci formation remains controversial ^{4,67-71}. Some of the conflicting data may be related to variations in the type of DNA damage signal used and the ambiguity of what to consider a HRR “proficient” versus “deficient” cell. In addition, FA pathway defects are expected to only affect foci formation in S-phase, so that normal RAD51 foci formation in G2-phase can potentially mask any foci alterations in S-phase in an asynchronous cell population. This highlights the general need to assess cell cycle distributions and cell cycle specificity of RAD51 foci formation when conducting these studies.

BRCA1 and RAD51 co-localize in S-phase foci, consistent with a functional relationship between these two proteins⁷². BRCA1 foci are present in undamaged S-phase cells where they may be involved in processing stalled replication forks due to endogenous DNA damage. Following induction of exogenous DNA damage, these foci disperse and relocate at exogenous damage sites that contain RAD51⁷³. The recruitment of BRCA1 into foci is complex and involves several protein complexes with functions that extend beyond HRR control (reviewed in ref.⁷⁴). Mutations in the *BRCA1* gene will disrupt BRCA1 foci formation and impair RAD51 foci formation, while alterations in directly or functionally interacting proteins, such as MDC1, may attenuate the induction of BRCA1 foci in response to DNA damage^{75,76}.

The recruitment of FANCD2 into subnuclear foci is dependent upon mono-ubiquitination by the FA core complex as well as BRCA1 function ⁶⁹. To which extent FANCD2 is involved in the promotion of HRR remains poorly defined ^{77,78}. One role of FANCD2 in foci may be to facilitate HRR needed for replication fork restart ^{79,80}. Thus, attenuated or absent FANCD2 foci are expected to predict sensitivity

to a variety of DNA damaging agents including IR⁸¹. For clinical application, a challenge is to determine what exactly constitutes an attenuated FANCD2 (or BRCA1 or RAD51) foci response to DNA damage without knowing the normal kinetics of foci formation and the normal range of foci per nucleus across a range of different cancer cell lines and cancer tissues (see also below).

Even though HRR proteins such as RAD51, BRCA1, and FANCD2 are relatively straightforward to measure by cell-based microscopic analysis, it is quite possible that HRR defects do not contribute significantly to clinical radiosensitivity because NHEJ is the dominant pathway for the repair of IR-induced DSB in human cells¹. At a minimum, knowledge of HRR defects would be very useful for the selection of concurrent systemic therapies, including cisplatin or PARP inhibitors, which should yield at least additive tumor toxicity¹⁰. NHEJ proteins in contrast do not form discernible foci in cells (reviewed in ref. ¹⁷). This is likely due to high baseline expression in the nucleus and a requirement for a smaller number of molecules at the DSB site compared to HRR proteins. Phosphorylation of DNA-PKcs is required for NHEJ and can be detected as foci, but whether this can be used as a surrogate for NHEJ proficiency remains unknown⁸².

Requirement for a DNA Damage Signal to Induce Foci

It is obvious that measurement of therapeutic DSBs using γ -H2AX or 53BP1 foci requires preceding exposure to DNA damaging agents. It is also important to appreciate that the activity of the HRR pathway is similarly dependent on the ability to localize these proteins into foci, in order to coordinate and execute repair, but less dependent on protein expression levels¹⁰. For example, even in the presence of hypomorphic mutations in the HRR pathway BRCA1 and RAD51 foci may be visible in S- or G2-phase cells and the fraction of cells with foci and the number of foci per cells may increase post-irradiation^{65,83,84}. In contrast, completely BRCA1-deficient cells have an impaired ability to mount FANCD2 and RAD51 foci responses^{65,69}. This is illustrated in **Figure 2A** where BRCA1-mutant MDA-MB-436 breast cancer cells cannot mount any RAD51 foci response even though the nuclear expression level is normal. H1937 cells, which express a hypomorphic BRCA1 mutation, have retained

the ability to induce RAD51 foci. This functional difference is associated with a pronounced difference in sensitivity to mitomycin C (**Figure 2B**). This illustrates the importance of “functional biomarker” analysis (i.e., RAD51 foci) rather than assessment of “biomarker expression” (RAD51 expression).

Thus, the functional status of HRR (as an example of the DDR in general) is typically revealed only when cells are exposed to DNA damage. It should be possible to use the ability of cells to form DDR foci as a functional biomarker of the integrity of the DDR network, and vice versa interpret the absence of repair foci induction, coupled with a persistence of DSB markers γ -H2AX or/and 53BP1, as an indicator of treatment sensitivity. This is illustrated in **Figure 2C**.

DDR Foci Assays on Human Samples

Administration of radiation therapy with or without radiosensitizing agents will induce DNA damage not only in the tumor but also in the normal tissues. Although measurement of DDR foci is well established in *in-vitro* cell cultures, methods to translate these assays into patient-derived samples remain highly experimental. Assessing the foci response in live tumors would require a repeat biopsy following initial administration of treatment. Not only is such an approach limited to cancers that are easily accessible for a needle biopsy, such as head and neck, breast, extremity sarcoma, or cervix cancers, personalization of therapy may be difficult after it has been already initiated. DDR foci can also be assessed in circulating tumor cells (CTCs) or peripheral blood lymphocytes (PBL). The most promising alternative is the use of pre-treatment biopsies. Such samples can be interrogated for their ability to form DDR foci in order to select the appropriate treatment regimen for a given patient. This requires some form of *ex-vivo* approach to expose tumor tissues or other samples to DNA damage in the laboratory.

Several groups have used *ex-vivo* assays in recent years to monitor DDR foci responses following treatment with IR or drugs^{4,5,8,13,14,85}. This approach is based on the idea that tumor tissues can remain viable for several days if cultured properly following removal from the patient. The use of organotypic slice cultures is a method long established in the field of neurological science and only recently

expanded to the study of cancers⁸⁶⁻⁸⁹. Vaira et al.⁸⁹ established organotypical cultures from a variety of previously untreated epithelial cancers. Slices of 300-500 μm were cut from surgical samples with a vibratome and cultured on organotypical inserts to preserve the three-dimensional (3D) tissue architecture. Cell viability assays, gene expression profiling, and assessment of PI3K/AKT activity validated the *ex-vivo* culture approach for up to five days following removal from the patient.

Our own approach has been driven by the desire to obtain rapid read outs of treatment sensitivity using needle biopsies that can ultimately inform treatment decisions in real time^{13,90} (**Figure 3**). In brief, core biopsy materials are obtained, placed in chilled complete cell culture medium with 10% serum, and equilibrated in a humidified cell culture incubator at 37°C and 5% CO₂. Within 60-90 minutes after removal from the patient the specimen is divided into smaller samples and subjected to mock treatment, IR with 4-10 Gy, or drug treatments, depending on the amount of tissue available¹³. Following *ex-vivo* treatments, samples continue incubation for 5-48 hours depending on the particular experiment to allow for DDR foci formation. In cases where we do not extend incubation beyond ~24 hours we do not add antibiotics or antifungals to the medium. However, this may be considered when the microbial burden in the tumor specimen is high due to tumor location in the mouth or intestine. Samples are then snap-frozen in OCT (Optimal Cutting Temperature) for later analysis. Formalin fixation and paraffin embedding (FFPE) of samples is an alternative and economical long-term preservation method. Serial sections of approximately 4-5 microns are obtained. Every fifth section may be subjected to hematoxylin/eosin (H&E) staining for confirmation of viable tumor cells. The other slides are stained with specific antibodies for visualization of DDR foci and counterstained with DAPI for visualization of nuclei using immunofluorescence microscopy. Additional stains can be performed and are discussed below. Typically 8-10 random high-power (100x) images are obtained from at least two slides in order to count at least 200-400 tumor nuclei for each experimental condition (and up to ~1,000 depending on the particular experiment and presence of intra-tumoral heterogeneity).

Using the above approach, we conducted a pilot study in which core biopsies from seven women with previously untreated, locally advanced breast cancers (without *BRCA1/2* mutations) were

subjected to 8 Gy irradiation or mock treatment *ex-vivo*¹³. RAD51, BRCA2, and FANCD2 foci could be readily visualized in individual irradiated cells from three tumors with an intact foci response, while the other four tumors lacked foci induction. Yet, there was no difference in the baseline of foci numbers in non-irradiated tumor cells. Notably, three of the four foci-defective tumors were “triple-negative”, a phenotype associated with BRCA1 deficiency. There was reduced BRCA1 expression in only two of the four foci-defective tumors. Even though the number of subjects in this study was small, these data suggested that gene expression may correlate poorly with HRR pathway activity as measured by foci formation, and further, that a functional pathway defect is only revealed upon DNA damage induction, i.e., *ex-vivo* treatment.

Simon Powell's group has expanded on this approach and in a preliminary analysis identified six out of 30 breast cancers lacking a RAD51/BRCA1 foci response⁸⁵. Furthermore, instead of using core biopsies, an approach involving fine-needle biopsy aspirates was developed. Breast cancer aspirates were made into a cell suspension in ~1 ml of phosphate buffered saline, *ex-vivo* irradiated or mock treated, incubated for 4 hours, and fixed on glass slides after processing the cell suspension in a cytospin. It was felt that the staining process was generally easier compared to using intact tumor tissues, with less permeability problems and a better signal-to-noise ratio. The downside of this approach is loss of the 3D tissue architecture.

CTCs can be non-invasively isolated from the patient's blood and are therefore available for repeated measurements during treatment or for *ex-vivo* treatment studies. The γ -H2AX foci assay has been performed on CTCs in several clinical trials. Increased DSBs could be measured following treatment of a variety of solid tumors with the topoisomerase I inhibitor topotecan, of advanced breast cancers with platinum-based chemotherapy, of a panel of solid tumors and lymphomas with the PARP inhibitor veliparib in combination with topotecan, and of multiple solid cancers with a combined treatment of veliparib and cyclophosphamide⁹¹⁻⁹⁴. Although the advantages of a “liquid tumor biopsy” are obvious, the approach is limited due to technical challenges identifying CTCs, defining robust controls as well as turnaround time and costs of the isolation procedure⁹⁵. Similarly, establishing

patient-derived xenografts, primary cell cultures, or cell lines is a lengthy procedure that may not be suitable to inform real time treatment decisions.

γ -H2AX Foci for Predicting the Radiosensitivity of Human Tumors

Despite major improvements in cancer therapy for many tumor entities the overall cure rates are still unsatisfying and biomarkers for treatment individualization are needed⁹⁶. Soon after the discovery of the crucial functions of γ -H2AX in DSB repair it was hypothesized that the rate of γ -H2AX foci reduction due to repair and the relative residual DNA damage after exposure to IR would be a useful indicator for the intrinsic radiosensitivity of cells. A more rapid foci reduction and less retention were assumed to be associated with a more radioresistant cell type⁹⁷.

The possibility of using γ -H2AX foci as a potential predictor of tumor response to treatment was first explored in cohorts of advanced carcinomas of the uterine cervix^{98,99}. Comparison of residual (24 hours) γ -H2AX foci in 47 untreated and eight radiation/chemotherapy patients revealed that only 25% of tumor nuclei exhibited γ -H2AX foci before treatment and 74% after the start of treatment⁹⁸. Pre- and post-treatment biopsies from 26 patients showed an increased percentage of tumor cells containing γ -H2AX foci after therapy (i.e., 24 $\pm$ 19% and 38 $\pm$ 19%, respectively). Although small differences in delivered dose (1.8 vs. 2.5 Gy) could be quantified, local tumor control was unrelated to the fraction of cells that retained γ -H2AX foci⁹⁹. This study illustrates the difficulty of quantifying foci responses on post-treatment biopsy materials.

More than 50% of HNSCC patients with advanced stage disease develop a recurrence after primary radiation/chemotherapy, which in the majority of cases is not curable¹⁰⁰. Identification of patients with therapy-resistant tumors would potentially allow for early treatment adaptation and hold the promise of increased cure rates. In nine xenografted human HNSCC models, γ -H2AX foci were correlated with the necessary dose to control 50% of those tumors locally (TCD₅₀)^{2,101,102}. Foci evaluated 30 minutes or 24 hours after *in-vivo* irradiation were significantly correlated with TCD₅₀

values but residual foci were found to be a more robust marker for local control. In contrast to the above mentioned patient study, foci evaluation was performed here under consideration of the tumor oxygenation, a factor that critically impacts the IR-induced number of γ -H2AX foci *in-vivo*^{2,97,101}. Valid results for parameters of the tumor micromilieu can be gained in pre-treatment core needle biopsies, although accuracy is somewhat limited by intra-tumoral heterogeneity¹⁰³. The number of γ -H2AX foci *in-vivo* decreases linearly with increasing distance from the perfused vasculature¹⁰¹. Constant γ -H2AX foci values can be evaluated in viable, oxic tumor cells with a maximum distance of 45 μ m from the nearest perfused blood vessel indicated by the hypoxia marker pimonidazole and the proliferation marker BrdU². The micromilieu-corrected evaluation of foci *in-vivo* was successfully translated to *ex-vivo* irradiated tumor biopsies from xenografted tumors and patient-derived specimens (Menegakis et al., unpublished).

The cancer stem cell (CSC) content and the intrinsic radiosensitivity of CSCs vary between tumors and affect their curability¹⁰⁴. The lack of reliable markers to discriminate between CSC and non-CSC impede the development of predictive tests for the clinic to optimize and individualize radiation therapy. There is evidence that the radioresistance of CSC is associated with altered γ -H2AX foci formation after irradiation. For example, glioblastoma cells expressing the CSC marker CD133 were shown to have fewer radiation-induced γ -H2AX foci than their non-CD133 expressing counterparts in an orthotopic model *in-vivo* but not in *in-vitro* cultured cells. Moreover, after irradiation a growth delay of seven days was associated with increasing percentage of CD133 positive cells *in-vivo*¹⁰⁵. Similar findings of reduced γ -H2AX foci in CSC models of lung cancer have been obtained in our laboratory (Willers et al., unpublished). These data emphasize the significance of *in-vivo* studies and tumor cell microenvironment to perform radioresistance studies by means of CSC and γ -H2AX foci.

γ -H2AX Foci and Radiation/Chemotherapy

The administration of chemotherapeutics can critically influence γ -H2AX foci results. Mumbrekar et al. reported that post-chemotherapy patients had significantly more γ -H2AX foci in PBLs than healthy donors which was very likely an effect of the doxorubicin- and cyclophosphamide-based regimen ¹⁰⁶. This is consistent with data showing that several chemotherapeutics induce DSB either directly or indirectly ¹⁰. The combined effect of IR and cisplatin on γ -H2AX formation was analyzed in *ex-vivo* and *in-vivo* irradiated PBLs of a group of 28 cancer patients. Cisplatin treatment led to a decrease in IR-induced γ -H2AX formation of about 30%. The foci variability was greater from patient to patient than for repeated measures within a patient ¹⁰⁷. Another commonly used agent, 5-fluorouracil (5-FU), is converted to the active metabolites fluorodeoxyuridine triphosphate and fluorouridine triphosphate which are incorporated into DNA and RNA. Although mechanistic details are still incomplete, base excision repair and mismatch repair seem to be the major pathways to repair 5-FU-induced DNA damage independent of DSBs ¹⁰⁸. Nonetheless, the γ -H2AX assay seems to have the potential to predict the individual sensitivity to 5-FU-based combination treatments ¹⁶. Interestingly, after *ex-vivo* irradiation of PBL, the γ -H2AX assay was very precise in terms of predicting radiosensitivity relative to *in-vivo* irradiated PBL ¹⁰⁹. However, concurrently administered chemotherapeutics, especially cisplatin, limit the precision of this method for predicting radiosensitivity ^{107,109}. In contrast, as suggested in **Table 1**, γ -H2AX assays will likely be useful to assess combinations with radiosensitizing targeted drugs that do not increase DSB levels unless they are combined with IR.

Identification of HRR Defects for Prediction of Treatment Sensitivity

The identification of HRR-defective tumors is important for several clinical applications such as prediction of radiosensitivity (including potential hypersensitivity to proton beam radiation ¹¹⁰), chemosensitivity (in particular platinum drugs), and many targeted agents (e.g., PARP inhibitors). At a minimum, this will involve assaying for key proteins such as γ -H2AX and RAD51 foci, but ideally also additional central DDR regulators such as BRCA1, FANCD2, and others.

Van Gent and colleagues obtained 54 fresh primary breast cancer samples from patients undergoing surgery ⁵. Organotypic slice cultures were prepared and subjected to 5 Gy irradiation. Incubation and irradiation of samples was performed within 6 hours after surgical resection. Two hours after irradiation, samples were fixed in formalin and paraffin embedded. Sections were stained for DAPI, geminin (to identify cells in S/G2-phase), and RAD51. A cell was considered positive for RAD51 foci if more than five nuclear foci were detected. Using this approach, 11% of tumors displayed a RAD51 foci defect. The foci defect was significantly associated with triple-negative breast cancer, and two of five HRR-deficient tumors did not show mutations in the *BRCA* genes but *BRCA1* promoter hypermethylation. The authors suggested that the RAD51 foci *ex-vivo* assay faithfully identifies HRR-deficient tumors and has clear advantages over gene sequencing. It is a relatively easy assay that can be performed on biopsy material, making it a powerful tool to select patients with an HRR-deficient cancer for PARP inhibitor treatment in the clinic.

The authors noted that the observed ~10% incidence of HRR-defective tumors was lower than prior estimates of ~25% of HRR defects in sporadic breast cancers ^{11-13,111}. However, conducting the assay with IR alone at a relatively short time point (2 hours) is likely to only capture severe HRR defects due to *BRCA1/2* disruption but will miss a number of other HRR defects. For example, our own *in-vitro* data indicate that tumor cells with an S-phase specific HRR defect, for example due to an alteration in the FA pathway, may display normal RAD51 foci induction after IR exposure but defective induction after replication-fork blocking treatments, such as cisplatin ⁹⁰. To detect this, a 24-hour time point would be required.

Another study performed the RAD51 foci assay on biopsies from 68 sporadic breast cancers that were obtained 24 hours after the first cycle of anthracycline-based chemotherapy ¹¹¹. The authors scored RAD51 and γ -H2AX foci on FFPE tumor tissues. A geminin-positive nucleus was scored as positive if it contained at least at one RAD51 focus. A low RAD51 foci score of < 10% positive nuclei was found in 26% of cancers. In a subset analysis it was shown that low RAD51 foci scores did not reflect lack of drug effect as there was a concomitant high γ -H2AX foci score as a marker of induced

DSB. Interestingly, a low RAD51 foci score was seen in 67% of the twelve triple-negative cancers, compared to 19% in the other cancers, and a low RAD51 score was correlated with pathological complete response at the time of surgery. The challenge of conducting an *in-vivo* approach such as this includes the logistics of obtaining a biopsy during treatment and uncertainties surrounding drug concentration in the biopsied tumor area.

Interestingly, HRR defects seem to be common in a variety of epithelial tumor types with incidences that appear to be in the order of 15-25% in many cases (reviewed in ref. ¹⁰). A similar figure was observed when we interrogated a panel of lung cancer cell lines and tissues for the ability to induce RAD51 in response to cisplatin ⁴. Tissue samples from surgical resections were exposed to 8 μ M cisplatin or IR as a control and snap frozen after 5 hours. Nuclei with at least two foci were scored as positive. It is noted in this context that there are variations between reports with regard to the number of foci per nucleus in a cross section that signifies a “positive” cell. More studies on the question as to how many foci constitute a repair-defective versus –proficient cell in tissues are needed.

Cells in S-phase were identified by staining for PCNA. Similar to the cell line data, there was a range of RAD51 foci induction with numbers lower than in cell lines, i.e., on average only 6% of cisplatin-treated cells in each tumor scored positive for RAD51 foci compared to ~19% in cell lines. This lower fraction of cells with RAD51 foci in tumor tissues was explained by a several-fold lower fraction of cells in S-phase. In 15% of tumors, very few cisplatin-induced RAD51 foci (< 1% of cells) were seen, indicating a putative HRR defect and suggesting cisplatin sensitivity. *In-vitro* analysis, however, indicated that γ -H2AX foci were actually superior to RAD51 foci in terms of predicting cell kill by cisplatin, likely because mechanisms other than loss of RAD51 function can also cause cisplatin sensitivity. In addition, HRR defects can manifest themselves not only as a failure to induce RAD51 foci but also a failure to resolve foci, presumably due to defects in resolvase proteins such as SLX4 or FAN1 ^{90,110}. Detection of this phenomenon would require more than one time point and extending tissue incubation to at least 48 hours, which may be difficult to carry out on limited amounts of biopsy

materials. Thus, foci scores or signatures encompassing at least several DDR proteins will have to be designed to accurately assess the DDR after treatment at an early time point.

These data also indicate that anti-cancer drugs will penetrate tumor tissue samples *ex-vivo* and elicit a DDR as illustrated in **Figure 1A and 4A**. With this type of assay one can envision a rapid assessment of drug sensitivities on biopsy material *ex-vivo* and results reporting within a few days. This is of particular interest for current efforts to identify predictive biomarkers of PARP inhibitors which can be employed in monotherapy or in combination therapy, for example with IR. While some data suggest that IR-based *ex-vivo* assays will accurately predict PARP inhibitor sensitivity^{5,112}, we are not aware of any studies that have exposed organotypic 3D cultures to PARP inhibitors. To this end, Curtin and colleagues established cell cultures from malignant pleural effusions obtained from 13 patients with various cancers⁸. Cultures were exposed to a PARP inhibitor, and RAD51 and γ -H2AX foci were scored, in parallel to next-generation sequencing of DNA repair genes. The authors determined that one third of cultures had a HRR defect. No mutations in DNA repair genes were associated with HRR status in this small cohort.

Technical Challenges of Foci Studies in Tumor Tissues *In-Vivo* or *Ex-Vivo*

There exist numerous but not insurmountable logistical and technical challenges with regard to functional assessment of foci responses in tumor tissues *in-vivo* and *ex-vivo*. Collection of fresh biopsy materials or other tissues for *ex-vivo* treatments requires considerable resources. Biopsies of tumors during treatment carry the additional issue of timing with regard to the prior treatment and potential uncertainty with regard to concentration of drug, if it was administered, within the biopsied tissue. Needle biopsies always carry the risk of not knowing whether the biopsy location is representative of the tumor as a whole. Surgical specimens obtained before initiation of therapy typically provide an ample source of tissue for analysis. However, absence of gross tumor post-surgery precludes direct correlation of therapy outcomes with the results of the *ex-vivo* foci assay.

Intra- and inter-tumoral heterogeneity of foci formation within tumor specimens is of considerable concern. This is already well established for γ -H2AX foci where it may relate to genomic instability and other factors⁹⁹. **Figure 1F** shows immunohistochemistry images of a HNSCC sample stained for tumor cell identification (panel a) and assessment of oxygenation status (using pimonidazole) and cell viability (BrdU) (panel b). This type of co-staining is important because hypoxia can affect γ -H2AX foci formation as well as other DSB repair pathways^{113,114}. In this context, it is unknown how potential changes in hypoxia/reoxygenation upon removal of the tumor tissue from the patient and incubation in the laboratory at ~20% oxygen will affect foci readouts. Studies to compare *in-vivo* irradiated tumors with *ex-vivo* irradiated biopsies are currently being conducted in Dresden to determine the possible influence of *ex-vivo* culturing on foci formation.

Figure 1F also illustrates that some tumors can have an increased γ -H2AX signal at baseline, i.e., without any treatment (panel c). Elevated endogenous γ -H2AX foci levels are suggested to be a characteristic of carcinogenesis and also are associated with tumors positive for the human papilloma virus (HPV)^{2,115,116}. The latter is likely related to the observation that HPV-positive human HNSCC cell lines and tumors are more radiosensitive than HPV-negative cancers due to compromised DSB repair¹¹⁷⁻¹¹⁹.

Similarly, foci of HRR proteins may vary considerable as they will only occur in cells that are in S- or/and G2-phase. The fraction of cells in these cell cycle phases is much lower in tissues than in established cell lines *in-vitro*. In addition, there can be considerable heterogeneity with regard to the presence of cycling tumor cells within a biopsy specimen. This is illustrated in **Figure 4B** which shows the quantification of RAD51 foci responses following *ex-vivo* treatment of lung cancer samples with cisplatin. In some tumors there is considerable variation of RAD51 foci counts across different parts of the sample, and in untreated tumors this variation could mask any treatment-induced foci increase. In another example, FANCD2 foci induction by IR is predicted to be S-phase specific and thus can be

clearly heterogeneous (**Figure 1G**). These problems may be overcome by simply increasing the number of analyzed images or/and co-staining with S/G2 markers such as geminin or PCNA.

Occasionally it is difficult to identify tumor cells based on DAPI morphology alone, which requires additional stains such as cytokeratin or H&E. Foci counting is labor-intensive, indicating a need for automation of foci scoring. Appropriate software applications are available for foci analysis in cell cultures but sophisticated solutions to semi-quantify foci in tumor specimens are missing^{8,120}. However, we find that manual scoring by a trained investigator or technician is more reliable in terms of distinguishing foci from unspecific stains or artifacts, which in a tissue context are quite common. To this end, it must be emphasized that staining of tumor tissue for DDR foci requires extensive optimization especially when co-staining is involved, and antibody performance can differ quite dramatically from *in-vitro* experiments. Nonetheless, solid tumor specimens with preserved 3D architecture have a major advantage: they are the closest model of the actual tumor and should therefore predict the clinical treatment outcome most precisely.

γ -H2AX Assays for Biodosimetry and Prediction of Normal Tissue Toxicity

While the focus of this review is on foci assays in tumor tissues we will also briefly address data on the value of γ -H2AX for the measurement of IR exposure and prediction of normal tissue toxicity. Particularly the excellent correlation of γ -H2AX foci and IR exposure doses in lymphocytes support the promise of using γ -H2AX in tumors¹⁹, assuming that intra- and inter-tumoral heterogeneity with regard to foci expression can be overcome.

Isolation of PBL from patient blood samples is a non-invasive method which guarantees easy access of biomaterial and repeated measures during treatment. These liquid biopsies are widely used in biodosimetry to estimate the delivered dose to an exposed individual¹²¹. For example, the analysis of γ -H2AX foci in PBL from a group of cancer patients 30 minutes after *in-vivo* irradiation showed a linear dependency of γ -H2AX foci formation and mean body dose. The steepness of the foci dose response

curve for PBLs increased with the partial blood volume of the irradiated organ limiting the applicability and precision for individual prediction of applied mean dose ¹²².

The γ -H2AX foci assay also has been used on *ex-vivo* irradiated blood lymphocytes as a clinical biomarker to identify small subsets of patients at risk of severe normal tissue toxicity. Microscopic foci analysis was confirmed as the method of choice by comparing three different methods (foci, comet, and neutral filtration assay) to detect a γ -H2AX signal in *ex-vivo* irradiated (2 Gy) PBLs from 54 HNSCC patients receiving radiation/chemotherapy. The foci assay could best detect a correlation of γ -H2AX signal and acute oral mucositis and dermatitis (grade ≥ 3) ¹²³. In two studies with 41 and 47 pediatric solid tumors and leukemia patients receiving DNA-damaging therapy, γ -H2AX foci were evaluated in *ex-vivo* irradiated PBL which enabled the identification of patients at risk for high-grade acute and late toxicities and allowed for detection of DSB repair deficiencies, although not all treatment-associated normal-tissue toxicities could be explained by DSB repair insufficiencies ^{124,125}. Similar observations were made with regard to severe late effects in prostate cancer patients and acute (grade ≥ 2) dermatitis in breast cancer patients ^{106,126}. In contrast to these findings, a number of studies which also used blood lymphocytes for γ -H2AX evaluation could not find a correlation with acute side effects ^{109,127,128}. However, caution must be taken with the interpretation of these results. None of the above studies was randomized, all were performed at single institutes, and the recruited patient numbers are generally low.

Conclusions

Over the next few years, genomic profiling of human cancers will provide us with unprecedented insight into the mutational landscape of genes directly or indirectly involved in the DDR. However, to what extent this wealth of structural information about the cancer genome will reveal biomarkers of radiosensitivity and radiosensitization by anti-cancer agents remains to be seen. The concept of highly ordered and regulated protein machineries at the DSB has profound implications for the development of

biomarkers to predict the functional status of DSB repair in a given cancer. Even if the entire genome and epigenome of a cancer was deciphered, a lack of in-depth knowledge of the functional interactions of DDR gene products and other regulatory components would complicate predictions of DSB repair proficiency and cellular sensitivity to anti-cancer agents including IR. These considerations stress the value of functional DDR assays that can provide a measure of DSB repair without needing to fully understand the functional consequences of all genomic alterations.

The advantage of using DDR foci as functional biomarkers is that they can detect repair defects due to several mechanisms such as gene mutations, epigenetic events, or alterations in signal transduction pathways which are increasingly recognized as modulating DDR ¹⁰. Moreover, they provide a global measurement of DDR network function without needing to know the identities of all the components, many of which are still unknown. One can envision developing mechanism-based “DDR foci signatures” that reflect nodal points in the network. Such signatures may include γ -H2AX, 53BP1, BRCA1, FANCD2, RAD51, and other proteins. Research into monitoring these foci responses is now increasingly moving into cells and tissues from patients which should produce functional insight that can complement or supersede genomic information, thereby giving radiation oncologists unique opportunities to individualize cancer treatments.

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Figure Legends

Figure 1 Examples of DNA damage response (DDR) foci assessment in human cancer tissues and cells. A) Representative immunofluorescence microscopy images (100x) investigating the DDR of patient-derived lung cancer tissues treated with ionizing radiation (IR) or cisplatin *ex-vivo* ⁴. B) Correlation of clonogenic survival fraction at 2 Gy with the percentage of cells displaying at least 10 γ -H2AX foci 24 hours (h) after 2 Gy irradiation for 18 non-small cell lung carcinoma (NSCLC) cell lines. The number of foci at baseline is subtracted. Results of a linear regression analysis are shown (Willers et al., unpublished). C) Left panel, representative microscopy images illustrating the increase in residual γ -H2AX foci 24 h after irradiation with 6 Gy plus the HSP90 inhibitor (i) 17-AAG at 20 nM compared to IR alone. Right panel, fraction of head and neck squamous cell cancer (HNSCC) cells with at least 20 γ -H2AX foci after treatment with IR with or without 17-AAG (Liu et al., unpublished). D) Radiosensitization factor for 2 Gy IR +/- 17-AAG using a previous IR/drug screening approach ¹²⁹. E) Representative images illustrating the persistence of 53BP1 foci following *ex-vivo* irradiation of tumor tissues from a bladder cancer patient. F) Immunohistochemistry and immunofluorescence images of consecutive sections of a HNSCC patient sample. Panel a, Differential staining with haematoxylin/eosin for tumor cell identification; Panel b, Definition of tumor oxygenation status: hypoxia marker pimonidazole in dark red cytoplasmic stain, marked with white asterisk. Definition of cell viability: proliferation marker BrdU in brown nuclear stain, marked with black arrows. Tissue is counterstained with hematoxylin. Box indicates position of panel c; Panel c, Unexposed control sample with high background level of γ -H2AX foci in green. DNA is stained with DAPI in blue. Scale bar and original image magnification in a and b 200 μ m 10x, in c 40x. G) Representative images of IR-induced FANCD2 foci in only 5% of cells in a tissue sample from a lung cancer patient ⁴.

Figure 2 Importance of DNA damage-induced foci formation. A) RAD51 protein expression by Western blot (upper image) and nuclear staining using immunofluorescence microscopy (lower images) in two breast cancer cell lines, MDA-MB-436 cells with no BRCA1 expression due to mutation and HCC1937 cells, which also have a BRCA1 mutation but express a protein with residual function. MMC, mitomycin C. (Willers et al., unpublished). B) Clonogenic survival fractions following MMC treatment at the indicated concentrations for 1 hour. C) Schematic illustrating how defects in the BRCA/Fanconi Anemia (FA) pathway could be identified through monitoring of foci responses.

Figure 3 Principle of *ex-vivo* foci assay. See text for details. IF, immunofluorescence; H&E, hematoxylin and eosin stain; IHC, immunohistochemistry

Figure 4 Penetration of drug into tumor tissue and heterogeneity of RAD51 foci response. A) Example of RAD51 foci responses 5 hours after exposure of breast cancer tissues to cisplatin (8 μ M) or ionizing radiation (IR) (10 Gy). Cells with at least two foci were scored as positive. Horizontal lines represent the median (Willers et al., unpublished). B) Analogous to panel A, example of RAD51 foci responses 5 hours after exposure of lung cancer tissues to cisplatin (8 μ M). Data points represent foci counts from random high-power images ⁴.

Figure 1

Figure 1 (2 columns)

Figure 2

Figure 3

Figure 3 (1.5 columns)

Figure 4

Table 1. Examples of radiosensitization by molecular targeted agents correlating with increased number of residual DSB

Target	Drug	IR	γ -H2AX	53BP1	Cancer cell lines	Reference (no.)
AKT	API-59CJ-OH	1-5 Gy	X		A549, H460 (NSCLC)	Toulany et al. 2008 (32)
AMPK	Metformin	6 Gy	X		MiaPaCa-2, Panc1 (PDAC)	Fasih et al. 2014 (33)
ATR	VE-822	6 Gy	X		MiaPaCa-2 (PDAC)	Fokas et al. 2012 (34)
EGFR	Erlotinib	2 Gy	X	X	A549, H1299 (NSCLC)	Mylynen et al. 2011 (35)
	Erlotinib & cetuximab	8 Gy	X		multiple NSCLC cell lines	Wang et al. 2011 (36)
HDAC	NDAC1054	6 Gy	X	X	A549 (NSCLC)	Hehlhans et al. 2013 (37)
	MS-275	5 Gy	X		U251 (GBM), DU145 (Prostate Ca)	Camphausen et al. 2004 (38)
HER2	Trastuzumab	4 Gy	X		A549, H661 (NSCLC)	Toulany et al. 2010 (39)
HSP90	17-DMAG	2 Gy	X		MiaPaCa-2, ASPC1 (PDAC)	Dote et al. 2006 (40)
	PU-H71	5 Gy	X		A549 (NSCLC)	Segawa et al. 2014 (41)
IGF1R	CP-751,871	10 Gy	X		H460 (NSCLC)	Iwasa et al. 2009 (42)
MEK	PD98059	2 Gy	X		A549 (NSCLC)	Kriegs et al. 2010 (43)
MET	MP470	4 Gy	X		multiple GBM cell lines	Welsh et al. 2009 (44)
mTOR	INK128	2 Gy	X		PSN1 (PDAC)	Hayman et al. 2014 (45)
	Rapamycin & RAD001	8 Gy	X		MCF7 (Breast Ca)	Chen et al. 2011 (46)
PARP	ABT-888	4 Gy	X		HCT116 (CRC)	Shelton et al. 2013 (47)
PI3K	BEZ235	4 Gy	X		FaDu (HNSCC)	Fokas et al. 2012 (48)
	LY294002	4 Gy	X		U251 (GBM)	Kao et al. 2007 (49)
SRC	Dasatinib	4 Gy	X	X	multiple HNSCC cell lines	Raju et al. 2012 (50)
STAT3	NSC74859	1 Gy	X		ECA109, TE13 (ESCC)	Zhang et al. 2014 (51)
TGF β RI	LY2109761	2 Gy	X		U87MG (GBM)	Zhang et al. 2011 (52)
WEE-1	PD0166285	4 Gy	X		several osteosarcoma cell lines	PosthumaDeBoer et al. 2011 (53)

Ca, carcinoma; ESCC, esophageal squamous cell cancer; GBM, glioblastoma multiforme; HNSCC, head and neck squamous cell carcinoma;

NSCLC, non-small cell lung carcinoma; PDAC, pancreatic duct adenocarcinoma