Bringing DNA Repair in Tumors into Focus

□□ *Commentary on Bañuelos et al. p. 3344*

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Biomarkers are of crucial importance in optimizing cancer therapies. In this edition of *Clinical Cancer Research*, Bañuelos and colleagues assess H2AX phosphorylation as a predictive biomarker of response to DNA damaging agents. We discuss these results as well as the impact that double strand break repair biomarkers may have in cancer therapy.

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In this issue of *Clinical Cancer Research*, Bañuelos and colleagues assess H2AX phosphorylation as a predictive biomarker of response to DNA damaging agents that cause double strand breaks (DSBs) (ref. 1). DSBs, where breaks in both strands of DNA occur in close vicinity, are especially lethal to replicating cells. This sensitivity has long been exploited in cancer therapy; radiotherapy and a wide range of chemotherapies (e.g., cisplatin, temozolomide, camptothecin, etoposide, cytarabine, gemcitabine etc.) all cause DSB formation. This occurs either directly, as is the case with ionizing radiation (IR) and bleomycin, or by indirect routes such as replication fork stalling, an event that can lead to fork collapse and DSB formation. Even some of the novel highly selective/less toxic classes of anticancer therapies, such as the PARP inhibitors (e.g., Olaparib) rely, in part, on the tried and tested approach of causing DSB formation (2).

Despite the widespread use of these therapies, robust biomarkers of DSB formation that could be used to predict and monitor drug efficacy and response are not available. Moreover, intertumoral and interindividual variation in these responses might explain the variability in the clinical efficacy of therapies. One potential biomarker of DSB formation and repair is phosphorylation of the histone H2AX. H2AX is a member of the histone H2A family, one of the five histone types that package DNA into chromatin. Each nucleosome, the basic subunit of chromatin, contains two H2A molecules, of which approximately 10% are H2AX. Shortly after DSB formation, PI3-family kinases such as ATM, ATR, and DNAPK phosphorylate a number of proteins, including H2AX. Via a series of protein-protein interactions, the phosphorylated form of H2AX, γH2AX, initiates DNA repair processes and the stalling of the cell cycle while DNA is repaired (3). In cells in culture, γH2AX can be detected by immunofluorescence as distinct nuclear foci, as early as 30 minutes after damage. As DSBs are repaired, the number of γH2AX foci present per nucleus gradually decreases and thus the elevation and decline of γH2AX foci reflects the activation of the DSB repair apparatus, as well as the eventual repair of DSBs (Fig. 1).

Because of the role of H2AX and the ability to detect γH2AX by approaches such as flow cytometry (FACS) or immunohistochemistry, Bañuelos and colleagues and others have previously investigated the potential of γH2AX as a biomarker. For example, γH2AX levels can predict the response to chemotherapeutics *in vitro* (4, 5) and pilot studies have assessed the potential of γH2AX as a nontumor tissue pharmacodynamic (PD) marker of DNA damage in radiotherapy and PARP inhibitor trials (3, 6). γH2AX has also been assessed as a diagnostic biomarker, both in rectal cell carcinoma and melanocytic lesions (7, 8).

In this new article (1), Bañuelos and colleagues extend their studies to assess the potential of γH2AX as a predictive biomarker of tumor response to radio- or chemotherapy *in vivo*. As an experimental model, Bañuelos and colleagues used human tumor cell lines xenografted into mice that were subsequently treated with either cisplatin, IR, or a cisplatin-IR combination mirroring a clinical protocol used for advanced cervical cancer. To assess predictive power, γH2AX foci from treated xenografts were quantified and compared to the *in vitro* clonogenic survival of cells derived from the same xenografts. As in previous studies by these authors (9), a reduced γH2AX response 24 hours after treatment predicted higher survival rates of tumor cells *in vitro*, especially when γH2AX was assessed by immunohistochemistry, rather than FACS. The greater power of immunohistochemical detection perhaps reflects the consensus opinion that γH2AX foci most likely represent the response to DSB formation, whereas the total amount of γH2AX, as detected by FACS, also includes phosphorylation not foci related nor necessarily caused by DSB formation (3).

The correlations between γH2AX focus formation in xenografts and the *in vitro* clonogenic survival of cells are persuasive but it is critical that this biomarker is assessed in patient samples. To achieve this, Bañuelos and colleagues examined γH2AX foci in tumor material from cervical cancer patients treated with cisplatin and IR. Importantly, the authors show that with the correct sample preparation procedure, it is feasible to measure γH2AX foci in the formalin-fixed-paraffin-embedded biopsies that are commonly used in clinical pathology. By examining biopsies both before and after treatment, they also provide vital proof-of-principal data suggesting that treatment-induced increases in γH2AX can be detected in biopsy material. Despite the small

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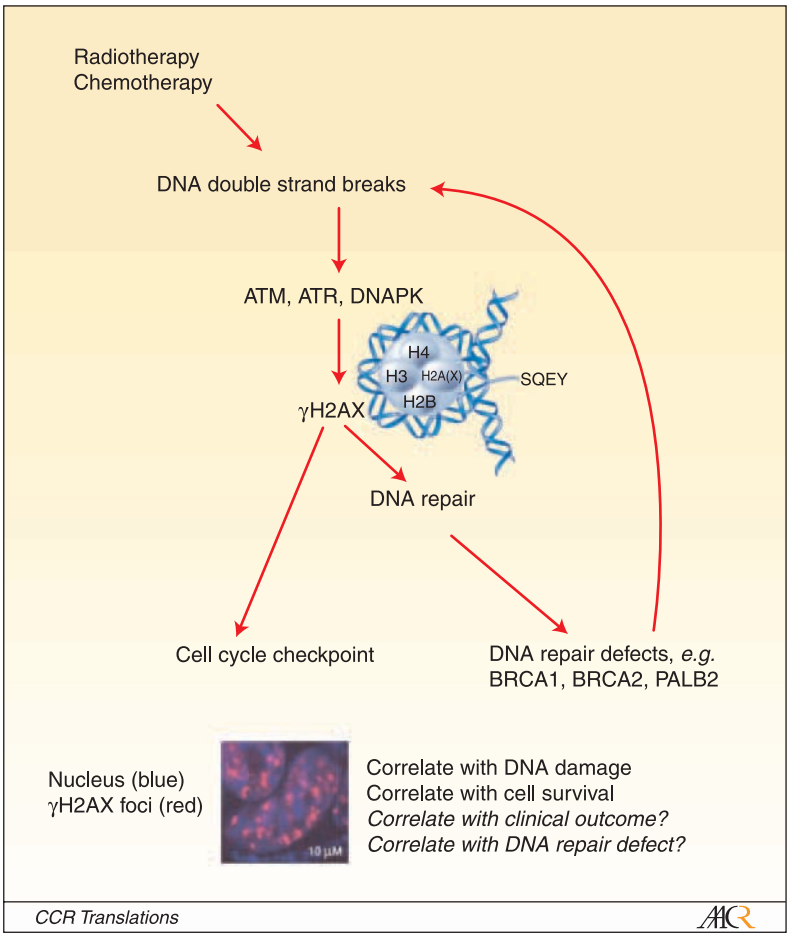

Fig. 1. γH2AX as a potential biomarker in cancer. DNA double strand breaks (DSBs) can be caused by radiotherapy or a number of different chemotherapeutics. Other endogenous stresses, such as oncogene-induced replication can also lead to DSB formation, most likely owing to replication fork stalling and collapse. The formation of DSBs triggers the activation of the PI3-kinase family members, ATM, ATR, and DNAPK, and the subsequent phosphorylation of the histone H2AX, which can be detected by immunohistochemistry, as distinct nuclear foci at the site of DSBs (as shown). This histone, whose phosphorylated form is known as γH2AX, forms part of the nucleosome complex around which DNA is normally wound. In response to phosphorylation, γH2AX activates components of the cell cycle checkpoint machinery as well as components of the DSB repair apparatus. Failure to repair DSBs, as in tumor cells bearing *BRCA1* or *BRCA2* mutations, also results in elevated DSBs and γH2AX levels. The increases and subsequent decreases in γH2AX levels mirror DSB formation and repair and thus detection of γH2AX foci allow this process to be followed. Studies using in vitro cell culture show that the formation of γH2AX foci correlate well with exposure to DSB-forming agents as well as tumor cell survival. Work from Bañuelos and colleagues in this issue of *Clinical Cancer Research* (1) shows that γH2AX foci formation in tumor biopsies correlates with exposure to cisplatin and ionizing radiation. The shape of the nucleosome is adapted by permission from Bonner et al. (3). The picture of the cells is reprinted with permission from Farmer et al. (13).

sample size of this pilot study precluding any analysis of the predictive power of this approach, this study has highlighted many of the issues that require further investigation. For example, in tumor samples, γH2AX was more frequently detected in regions that stained positive for CA9, a marker of hypoxia. There is a growing body of evidence suggesting that hypoxia leads to the coordinated suppression of particular DNA repair pathways, including homologous recombination, a major mechanism for the repair of DSBs (10). It is therefore possible that the elevation of γH2AX foci in CA9 positive tumor cells represents a failure to repair DSBs, and could ultimately predict tumor cell death. Whether this is the case, these data argue the case for combining γH2AX and CA9 markers in larger studies in which comparisons with outcome data can be made.

Bañuelos and colleagues also describe significant heterogeneity of γH2AX staining in some tumors but little in others (1). This observation highlights the fact that, despite a great deal being known about DNA repair processes in cells in culture, very little is understood about how these act in real tissues and in pathological conditions such as cancer. Consequently, well-designed and suitably powered prospective clinical studies are now required that will not only test the predictive power of a γH2AX biomarker but also the extent of intratumoral and inter-individual variation. These studies will also need to address a more practical issue; the gold standard method for looking at DSB-induced γH2AX, at least in the experimental laboratory, is confocal immunofluorescent microscopy. This technique, if converted into a routine, medium-throughput test, will require an element of computational image analysis. Our own studies, using some of the commercial high-throughput confocal microscopes and image analysis software packages suggest that, with some optimization, γH2AX foci can be measured in a relatively high-throughput fashion.

Regardless of these challenges, γH2AX has clear potential as a biomarker, both in terms of pharmacodynamics and in estimating the ability of tumors to repair damaged DNA.

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The recent observation that precancerous lesions exhibit elevated levels of γH2AX foci, most likely caused by an increase in oncogene-induced replicative activity (11), also suggests that γH2AX may have potential as a surveillance biomarker, an idea supported by the increase in γH2AX in colonocytes from patients with ulcerative colitis, a condition predisposing to colorectal cancer (12). γH2AX may also have potential as a biomarker when used in combination with additional measurements of DNA damage and response. For example, it is entirely possible that combining measurements of γH2AX with those of other DNA damage proteins may give a better prediction of the response to DNA damage. From a functional perspective this makes sense; although important for the response to DNA damage, γH2AX is not the only determinant. For example, it is clear that the ability to form nuclear RAD51 foci, a surrogate marker of DSB repair by homologous recombination, can predict survival after treatment with agents such as cisplatin and PARP inhibitors (2). Finally, it is important to remember that γH2AX foci do not directly indicate DSB formation, merely the cellular response, and thus combining direct measurements of DSBs with γH2AX quantification may be informative.

In conclusion, markers such as γH2AX that estimate the extent of DNA damage and repair have considerable potential but still require substantial validation. Eventually though, these biomarkers may allow the refinement of the use of particular cancer therapies.

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