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Review

γ-H2AX – A Novel Biomarker for DNA Double-strand Breaks

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Abstract. *When DNA damage, whether it is endogenous or exogenous, forms double stranded breaks (DSBs), it is always followed by the phosphorylation of the histone, H2AX. H2AX is a variant of the H2A protein family, which is a component of the histone octomer in nucleosomes. It is phosphorylated by kinases such as ataxia telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) in the PI3K pathway. This newly phosphorylated protein, γ-H2AX, is the first step in recruiting and localizing DNA repair proteins. DSBs can be induced by mechanisms such as ionizing radiation or cytotoxic agents and subsequently, γ-H2AX foci quickly form. These foci represent the DSBs in a 1:1 manner and can be used as a biomarker for damage. An antibody can be raised against γ-H2AX which can therefore be visualized by immunofluorescence through secondary antibodies. The detection and visualization of γ-H2AX by flow cytometry allow the assessment of DNA damage, related DNA damage proteins and DNA repair. γ-H2AX also has other applications in the detection of genomic damage caused by cytotoxic chemical agents and environmental and physical damage, especially in the context of cancer treatment and therapy.*

DNA damage occurs in all cells and the damage can be categorized into two types, endogenous and exogenous. Endogenous damage is caused by the cell itself and can arise from various pathways such as apoptosis, excision repair, oxidative damage or depurination. Exogenous damage occurs when cells are exposed to physical damage (radiation) or chemical agents (cytotoxic drugs). The DNA damage that ensues can be base damage, sugar damage, single stranded breaks (SSBs) or double stranded breaks (DSBs), the latter being lethal to cells.

Measuring DSBs has been of interest in the research community because of its predictable nature. These lethal nicks in the DNA allow the prediction of toxicity in cells or the death of cells. There have been many methods for detecting DSBs, such as neutral elution, pulse field electrophoresis (2-D gel electrophoresis) and comet assays, but the γ-H2AX assay is particularly precise. This recently developed assay is not only simple but is also more sensitive to DSBs than the other techniques. Furthermore, it can detect DSBs in intact cells, which allows fluorescent visualization and the physical localization of the DSBs. The simplicity, ease and sensitivity of this assay have been the cause of its increasing use as a popular method for detecting DSBs.

H2AX Protein

H2AX is a key factor in the repair process of damaged DNA. It is recruited to damage sites, which in turn recruit other DNA repair machinery (1). DNA is normally wrapped around a core histone molecule forming the nucleosome complex. Histone cores are made up of individual histone proteins called H2A, H2B, H3 and H4 (2). The H2A protein family has the greatest number of variants including H2A1, H2A2, H2AX and H2AZ plus many others (3). In human cells, H2AX constitutes approximately 10% of the H2A protein, but depending on the species of mammal, H2AX can range from between 2-25% of the total H2A protein (4). The H2A variants only differ by a few residues, but the H2AX protein is unique in eukaryotes due to its carboxy tail. Immediately before the stop codon, the tail has a highly conserved sequence comprised of one serine at position 139 and one glutamine residue at position 140, known as the SQ motif, followed by two more residues (4). H2AX has not been found to be concentrated at a specific region of the DNA, but instead is randomly incorporated into histones throughout the DNA (4).

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Phosphorylation of H2AX and its Related Molecules

H2AX can be phosphorylated on Ser 1, acetylated on Lys 5, and ubiquitinated on Lys 119, but it differs from other H2A molecules because it is phosphorylated on the 139th serine residue in the presence of DNA damage (4). This phosphorylated H2AX protein is termed γ-H2AX (5). Hundreds to thousands of H2AX are phosphorylated per DSB (6). H2AX proteins within a few megabases of the damage site are involved, but it is unclear how this phosphorylation propagates from the site of damage (4, 7). It is phosphorylated within minutes, from both SSBs and DSBs, but especially DSBs.

Phosphorylation of H2AX. In mammalian cells, ataxia telangiectasia mutated (ATM), ATM-Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) are all part of the PI-3K pathway and these proteins are responsible for the DNA repair response (8). Based on previous research, DNA-PK is the protein that senses DSBs during the non-homologous end joining (NHEJ) repair process (9), but ATM has been found to be the major protein that phosphorylates H2AX (8). There are background levels of phosphorylation, but upon DNA damage by radiation, ATM is the most active. Previously, it was suggested that DNA-PK was responsible for the background levels of γ-H2AX formation when ATM is absent (8). According to recent research, ATM and DNA-PK might not be the only contributors to low levels of γ-H2AX formation. Background levels of γ-H2AX in S-phase have been observed even when ATM was inhibited, implying that another kinase is also responsible for the phosphorylation of H2AX (10). ATR has been found to be the kinase responsible for forming γ-H2AX at sites of stalled replication forks and replication blocks (11, 12). Both kinases, ATM and ATR, may each be responsible for the phosphorylation of H2AX under varying circumstances. Since DNA-PK, ATM and ATR are responsible for low levels of γ-H2AX phosphorylation, there is further research needed to elucidate the function of each protein kinase. After H2AX is phosphorylated, γ-H2AX foci can be visualized using various techniques. These foci represent an accumulation of γ-H2AX, along with hundreds of other proteins that have localized at the site of damage (7).

Associated proteins. Many repair proteins co-localize with γ-H2AX; some directly bind to γ-H2AX while others are associated with binding proteins. γ-H2AX interacts with specific proteins such as the breast cancer 1 protein (BRCA1) through the C-terminal domain (BRCT) or a fork head associated (FHA) domain. It has been found that NBS1 (the defective gene responsible for Nijmegen breakage syndrome) associates with γ-H2AX through the FHA and BRCT domains. NBS1 is necessary for the sequential recruitment of the NBS1/hMRE11 (human meiotic recombination 11 protein)/hRAD50 (N/M/R) repair complex, which forms around the break site and is responsible for the recruitment and activation of ATM (1).

BRCA1, 53BP1 (p53 binding protein 1) and MDC1 (mediator of DNA damage checkpoint) all contain the BRCT domain and they all co-localize with γ-H2AX (1). MDC1 has also been shown to be a bridging factor between γ-H2AX and the N/M/R complex. Counter arguments suggest that NBS1 is not necessary for the recruitment of the N/M/R complex and that only MDC1 is necessary, so further research may be necessary to fully elucidate the relationships between these proteins (2). It is speculated that redundant recruitment occurs. Additionally, cohesion complexes are found to localize with γ-H2AX through binding hMRE11 (13).

After DNA is repaired, γ-H2AX is dephosphorylated. PP2A is the phosphatase that regulates γ-H2AX levels in human cells (14). This protein also co-localizes with γ-H2AX in various assays. The dephosphorylation of γ-H2AX is useful for the assessment of DNA repair discussed later.

γ-H2AX antibody. An antibody can be raised against γ-H2AX, or more specifically against the last nine residues, including the phosphorylated serine (8). This antibody has been used in the most recent experiments to measure the amount of γ-H2AX formed. When this antibody is labeled with fluorescence, it allows the visualization of H2AX, but it also has been found to disrupt normal processes. When the antibody is introduced, it can disrupt NBS1 foci formation under ionizing radiation (1).

Assessment/detection methods. γ-H2AX foci are commonly detected and quantified by fluorescence, which involves primary γ-H2AX antibodies and fluorescent secondary antibodies. Immunofluorescence requires the use of epifluorescent microscopes, which allow the naked eye to count cells with DNA damage (7, 10). The use of fluorescence can be extended to other assays, like the Western immunoblot (8), 2-D gel electrophoresis (4) and flow cytometry. Flow cytometers have lasers and detectors that allow the active sorting of cells based on a cell's particle density or the color of fluorescence (15, 16).

To assess γ-H2AX foci, the results of immunofluorescence have been tested against the comet assay. This is a method used in laboratories to detect DNA damage in single cells (17). Under alkaline conditions, the comet assay can detect both SSBs and DSBs (18). This assay has been deemed quick, simple and reliable, but recently, it has been established that the γ-H2AX assay (flow cytometry method) is 100 times more sensitive to detecting damage levels than the comet assay (19). Flow cytometry can also show how DNA damage is correlated with drug cytotoxicity (20).

Ionizing Radiation-induced γ-H2AX Formation

After irradiation with ionizing radiation (IR), H2AX is rapidly phosphorylated and there is always a constant number or percentage of γ-H2AX formed per DSB. For an X-ray dosage of 1Gy, there is always around 1-2% of H2AX that becomes γ-H2AX, regardless of the H2AX to H2A ratio (4). γ-H2AX form not just in cell cultures, but in whole living organisms treated with lethal and non-lethal doses of IR (4). Cell lines may have various background levels of γ-H2AX, for example the cancer cell lines Caski and SW756 both show high background levels of γ-H2AX foci (21). Since various cell lines may not have the same background level of γ-H2AX, the number of γ-H2AX foci formed are different. According to recent research, the difference between cell lines is not foci number, but instead foci intensity (22). In another study, H2AX-deficient mouse embryonic stem cells were subjected to IR. Not only were the cells highly sensitive to IR, but they also showed higher genomic instability compared to controls (23).

γ-H2AX Induced by Drugs/Cytotoxic Agents

Many existing drugs and cytotoxic agents, particularly agents that cause DNA DSBs, are associated with the formation of γ-H2AX. Various classes of cytotoxic agents can be defined such as DSB-causing drugs, SSB-causing drugs, or enzyme inhibiting drugs, which do not directly damage the DNA. Enzyme inhibiting drugs inhibit enzymes upstream from H2AX in their respective pathways thus causing γ-H2AX formation. Topoisomerase I and Topoisomerase II inhibitors, such as topotecan, camptothecin and mitoxantrone, induce cuts in the DNA (10). These lesions produce γ-H2AX and the foci can be visualized.

Other drugs that cause the formation of γ-H2AX must in some way induce DSBs. Cisplatin is a drug that crosslinks the DNA and upon repair by NHEJ, DSBs are created (24). Gemcitabine and hydroxyurea can both independently trigger the phosphorylation of H2AX due to collapsed replication forks (8, 25). Drugs such as neocarzinostatin, doxorubicin, bleomycin, tirapazamine and etoposide all cause DSBs and therefore γ-H2AX foci form (8, 21). These drugs have all been used in the study of radiotherapy and cancer treatments.

γ-H2AX in Cancer Cells

Recently, H2AX has been investigated in regards to cancer. In one study, both copies of H2AX were knocked out in mice and these mice quickly developed lymphomas and solid tumors (26). H2AX has been described as a dosage-dependent tumor suppressor in mice and humans (26). Other studies have shown that the H2AX gene location, 11q23, is frequently altered in cancer cells which may cause a 3-fold increase in γ-H2AX formation (21). As a marker for DNA damage, H2AX will be higher in cancer cells due to mutated and unchecked cell cycles.

Radiotherapy target. Radiation therapy is one of the main modalities for the treatment of cancer patients. The radiation disrupts DNA processes and causes DNA breaks. X-rays are now sufficiently sensitive to localize the DNA damage to a spot only 2.5 micrometers across (27). Any post modifications of chromatin after DNA damage, such as γ-H2AX, can be used as a therapeutic target for radiotherapy (28). The persistence of γ-H2AX after IR is particularly significant since it can be used as a marker of radiosensitivity (29). Maximizing radiosensitivity in cancer cells is extremely important to radiation therapy. By increasing the effectiveness, the dosage and frequency of radiation treatments can be decreased and it may be possible to target the more sensitive cells specifically. For example, when the tumor suppressor p53 is knocked out in a cell line, those cells show longer persistence of γ-H2AX due to increased damage. Cell lines that are wildtype for p53 show faster γ-H2AX loss than p53 deficient cell lines (21). Using γ-H2AX foci to assess radiosensitivity, further radiotherapy developments may improve cancer treatments.

Uses of γ-H2AX Foci

The use of γ-H2AX in cancer therapy is currently being investigated. γ-H2AX is an excellent target for improving radiotherapy effects. The H2AX tail peptides can be synthesized and they inhibit γ-H2AX formation by competing against H2AX for phosphorylation by the upstream kinases or for binding downstream targets (29). These tail peptide antagonists may also enhance cell death by inhibiting normal DNA repair and checkpoint responses. Since most DSBs are limited to tumor cells, these tail peptides exhibit no deleterious effects on unirradiated tissue (29).

Residual γ-H2AX can be used to identify the radiosensitivity of cells or their ability to recover from damage and the efficiency of the cellular repair process (30). The percentage of tumor cells that still have γ-H2AX foci 24 hours after radiation doses proves useful for quantifying cell responses (30). The rate of γ-H2AX loss is dependent on the cell type but has been measured to be faster for radioresistant cell lines (24). Additionally, radiation treatment has been used in combination with cytotoxic agents, such as Vorinostat, to enhance the effect of radiotherapy and to prolong the presence of γ-H2AX (31).

As discussed, γ-H2AX is a sensitive and early indicator of DSBs in vitro and *in vivo* (32). γ-H2AX has also proved useful for detecting low-levels of DNA damage (21). It is 100 times more sensitive than other methods and can be used to detect doses of radiation down to 1 mGy (33). This could

be useful in the future for improving the dosage of radiation therapy on an extremely localized target. γ-H2AX can also be useful for elucidating the pharmacodynamics of cytotoxic drugs and tumor killing drugs. The foci can be measured to elucidate whether or not drugs reach the tumor, whether or not drugs reach their activated forms, and whether or not the drugs affect the DNA (25).

Summary and Prospects

Overall, H2AX has been widely used by many researchers as a tool to measure induced DNA damage. The protein itself, as well its associated proteins, has many potential applications for clinical use. The H2AX assay is widely employed, and other assessment methods for novel γ-H2AX applications have been developed. For example, computational methods and its related software have been created to measure correlations between the foci and related proteins (34).

H2AX may even be a potential assessment method for detecting tumors. Pharmacodynamically it could be a sensitive test for clinical drugs in their testing stages. It can also be used to test the rate and efficiency of the DNA repair process (24). Additionally, as a dosage-dependent tumor suppressor, since the over expression of tumor suppressors can lead to induced senescence of tumor cells, it could become a potential cancer treatment (35). With the numerous applications of H2AX in the treatment and diagnosis of cancer, further research may one day provide a more effective cancer treatment.

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