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Review Article

Phosphorylated H2AX: Prospective Role in DNA Damage Responses and a Credible Tool for Translational Cancer Research

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Abstract

Double Strand Breaks (DSBs) are the most deleterious DNA lesions among the first signs of cancer in eukaryotic cells. Occurring on exposure to one or more endogenous and/or exogenous factors and leading to fatal consequences like chromosome aberrations and genomic instability. Therefore, programmed and coordinated cellular processes function intra-cellular to stabilize the genomic information and repair damages. These processes are primarily a part of DNA replication and cell-cycle progression. However, various signalling factors also activate specialized DNA Damage Response (DDR) pathways for repairing DSBs. Lately, the phosphorylated histone variant H2AX (γH2AX) has been identified as a biomarker for DNA damage. Studies have shown a correlation between the concentration of cellular γH2AX and the extent of DNA damage. Hence, the quantification of DNA lesions can be done using simple spectroscopic and radiological techniques or immunofluorescent staining. For this reason, γH2AX has especially gained value as a biomarker in translational cancer research. Moreover, this approach may act as a boon in clinical trial studies for understanding the different phases of cancer and studying the pharmacodynamics of prospective drugs. Recently, γH2AX based studies have indicated the indispensable fate of DNA damages occurring during normal neurological development and in disorders like obesity. The current review focuses on the role of γH2AX in DDR pathways, and ways in which the correlation of γH2AX and DNA damage can be applied in monitoring the clinical response of DNA targeted therapies.

Keywords: H2AX, γH2AX, Double-strand Breaks, DNA Damage Response, DNA Repair, Genomic Instability, Translational Research
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Introduction

The measurement of DNA damage triggered as a result of aging, defects in DNA repair pathways and mutagenesis, forms the basis of clinical studies. These triggers are responsible for cellular fates leading to senescence, necrosis or apoptosis.[1] The continuous exposure of the cell cycle to endogenous factors (hydroxyl radical, nitric oxide and superoxide anion) and exogenous mutagens (UVB, ionizing radiation and chemicals) cause minor and repairable damage to DNA. However, the accumulation of these damages over a period of time may threaten the normal functioning of the cells. Sometimes, the chemical as well as physical factors may act together, and further, intensify the extent of DNA lesions. Several DNA repair mechanisms like Base Excision Repair (BER), Nucleotide Excision Repair (NER) and Mismatch Repair (MMR) are activated in these events to prevent the inheritance of damaged genetic material by the progeny. Several proteins including cyclins and cyclin-dependent kinases (cdk) are involved in the execution of these mechanisms. Thus, they act as regulatory proteins for proper transitioning of the cell through subsequent phases of the cell cycle.

Consequently, the Double-Strand Breaks (DSBs) are one of the deleterious DNA lesions which may occur due to several physical, chemical or biological factors. Exposure to ionising radiation is one of the common factors leading to DSBs. However, they may also occur, rarely, following an error during the replication or transcription processes of the cell cycle. The DSBs prompts the phosphorylation of a histone variant H2AX, producing γH2AX. This, in turn, acts as a predominant factor in activating the DDR pathway as a defence mechanism of a eukaryotic cell to detect and repair the damage. The repair phase begins with the assembly of DNA repair proteins at the site of chromatin damage and cell cycle arrest at several checkpoints.[2,3] The functional proteins of the DDR pathway i.e., MRE11/NBS1/RAD50, MDC1, 53BP1, and BRCA1 interact with γH2AX to form nuclear foci. These foci concentrate around the DSB, along with various DSB-repair factors,[4] and lead to the activation of other DNA repair pathways including:

- Non-Homologous End Joining (NHEJ)
- Phosphoinositide-3-Kinase–Protein Kinase B (PI3K-PKB)/AKT pathway
- Ataxia Telangiectasia and Rad3-related protein (ATR)
- Chromatin mediated DNA break recognition by ATM

(Ataxia Telangiectasia Mutated)
- p53/p21 pathway
- Proliferating Cell Nuclear Antigen (PCNA) inhibition pathway

These DDR pathways, thus mediate the genotoxicity and replication stress by arresting cell progression and proliferation and initiating DNA repair mechanisms. The ATM, ATR and DNA dependent protein kinases (DNA-PKs) are large serine or threonine kinases that belong to the PK family. They are the most upstream kinases and respond to DNA damage by phosphorylating at the serine/ threonine glutamine motifs. They also regulate several checkpoints following DNA damage. The DNA-PKs play a role in activating the preliminary NHEJ pathway. On the other hand, ATM and ATR proteins further activate the checkpoint kinases i.e., ChK1, ChK2 and MK2 by initiating their phosphorylation. Specifically, the DSBs activate the ATM, whereas ATR responds to a wider spectrum of DNA damage.[5] As a result, the γ-H2AX, which is evidently observed in almost all types of DNA damage, has been acknowledged as a sensitive biomarker for DSBs. The identification of specific biomarkers is the foundation of early diagnosis, better prognosis and management of different types of cancers.[6] The current review has focused on the role of H2AX in various DSB repair pathways and quantification of DNA damage. An overview of the use of H2AX as biomarkers in the diagnosis of various clinical disorders is also presented in this article.

Role of H2AX in Non-Homologous End Joining (NHEJ) Pathway

The NHEJ is usually a first-line defence mechanism in mammalian cells for DSB repair (Figure 1). It is a quick and robust attempt of cell-cycle regulatory proteins to re-join the DNA ends. The recognition and binding of the KU heterodimer to the DSB initiates the NHEJ pathway. The dimer consists of Ku70 and Ku80 subunits which typically localises near the DSBs, forming a ring-shaped structure to encircle the DNA and allow KU heterodimer to slide on to the site of DNA break, within seconds of its formation. The interaction of KU heterodimer with DSBs has been validated through crystallographic images.[7] The DSB- bound heterodimer serves as a scaffold and actively recruits other factors of NHEJ to the damaged site. The X-ray repair cross-complementing protein 4 and DNA ligase IV (XRCC4-LIG4) complex then interacts with KU heterodimer to activate stabilizing factors, including DNA Protein Kinase C (DNA-PKC) and XRCC4 like factor (XLF), of NHEJ complex at the DSB site. Thus, closely aligned ligatable ends are created with a structural transition in the synaptic complex to resect the DNA ends, fill the gaps and remove blocking end groups. If necessary, DNA replication enzymes also participate in the above structural transitioning to initiate the DNA repair. During the last step of the NHEJ pathway, XRCC-4 stimulates the activity of Ligase IV by promoting the adenylation of DNA Ligase IV to join the broken ends of DNA.[8] The XRCC4-LIG4 complex also regulates the dissolution of the NHEJ complex from the DSB ends after ligation, by a currently unknown mechanism. However, the Small-Angle X-ray Scattering (SAXS) has revealed that auto-phosphorylation of DNA-PKCs induces a conformational change which is likely to release DNA-PKCs from DNA ends.[9] It has also been suggested that N-terminus keeps the DNA-PKCs activity in check. The perturbation of the N-terminal causes a conformational change leading to the opening of ATP binding pockets of the protein which results in higher kinase activity.[10] The fact that the inactivation of DNA-PKC activity via point mutations or small molecule chemical inhibition leads to defects in DSB repair and radio sensitivity, has led to a belief that the activity of DNA-PKC is essential for NHEJ.[11] A recent study also indicated that the ubiquitin E3 ligases RNF8 may be responsible for ubiquitylation and proteasomal degradation of Ku80.[12]

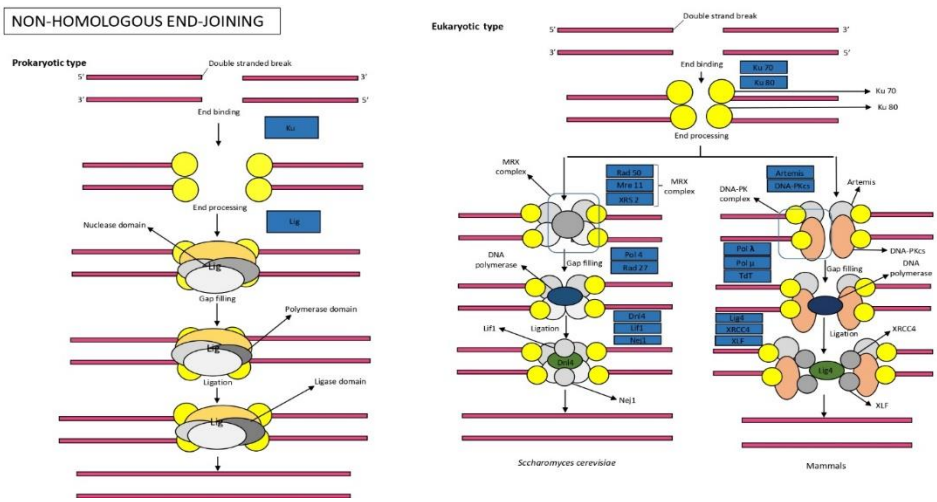

Figure 1. Non Homologous End Joining (NHEJ) Pathway.

In Eukaryotic and mammalian cells, the rapid phosphorylation of H2AX at serine 139 (S139) of the H2AX carboxyl-terminal SQEY motif, by ATM, DNA-PKC and ATR, initiates DSB repair by signalling a cascade of events. The H2AX chromatin domain, thus, may stabilize the broken DNA strands to prevent irreparable damage, suppress hairpin opening and subsequent end resection of Recombination Activated Gene (RAG) initiated DSBs.[13,14] It has also been indicated that the cells deficient in H2AX leads to decreased Class Switch Recombination (CSR), aberrant DSB response, genomic instability, elevated levels of chromosomal and chromatid breaks/ translocations or increased sensitivity to ionizing radiations. Also, the H2AX chromatin domain repairs over 10-15% DSBs induced by ionizing radiations. Therefore, it has been proposed that the H2AX chromatin domain exerts a controlled regulation over DSB repair apparent in Homologous Recombination (HR) and NHEJ pathways.[15,16] However, it was recently reported that the end resection during NHEJ in the G1 phase is distinct from that during HR.[17] In addition, the studies on the role of H2AX in DSB have been mostly focused on the V(D)J recombination and CSR in H2AX-deficient lymphocytes and mice models. Both models require induction of programmed DSBs by RAG endonucleases and Activation-Induced Cytidine Deaminase (AID) respectively and joining by NHEJ. These finding contradicts the above regulatory effect of γH2AX, and raises a question on its applicability, in NHEJ pathway. Hence, although H2AX is an important biomarker in DSB–repair pathways, its role is rarely understood in the NHEJ pathway.

Role of H2AX in PI3K-PKB (Phosphoinositide-3-Kinase-Protein Kinase B)/AKT Pathway

The PI3K-PKB/Akt is a highly conserved pathway that is regulated through multistep interlinked processes for its activation (Figure 2). The class 1A PI3Ks stimulate the activated receptors on binding with their regulatory subunit or adapter molecules like Insulin Receptor Substrate (IRS) proteins. This process triggers the activation of PI3K as well as the conversion of phosphatidylinositol (3,4)-bisphosphate (PIP2) lipids to phosphatidylinositol (3,4,5)-trisphosphate (PIP3). The PIP3 is then bound by PKB/Akt to initiate phosphorylation of T308 by PDK1 in the activation loop. In turn, the modified PKB/Akt activates mTORC1 by directly phosphorylating and inactivating proline-rich Akt substrate of 40 kDa (PRAS40) and Tuberous Sclerosis protein 2 (TSC2). The activated mTORC1 phosphorylates the ribosomal protein S6 (S6/RPS6) to promote protein synthesis and cellular proliferation. These mechanisms are dependent on mTORC1 substrates like eukaryotic translation initiation factor 4E Binding Protein 1 (4EBP1), and ribosomal protein S6 kinase 1 (S6K1). The above events, however, lead to partial activation of the PI3K-PKB/Akt pathway. The complete activation occurs on phosphorylation of Akt at S473 at the carboxy-terminal

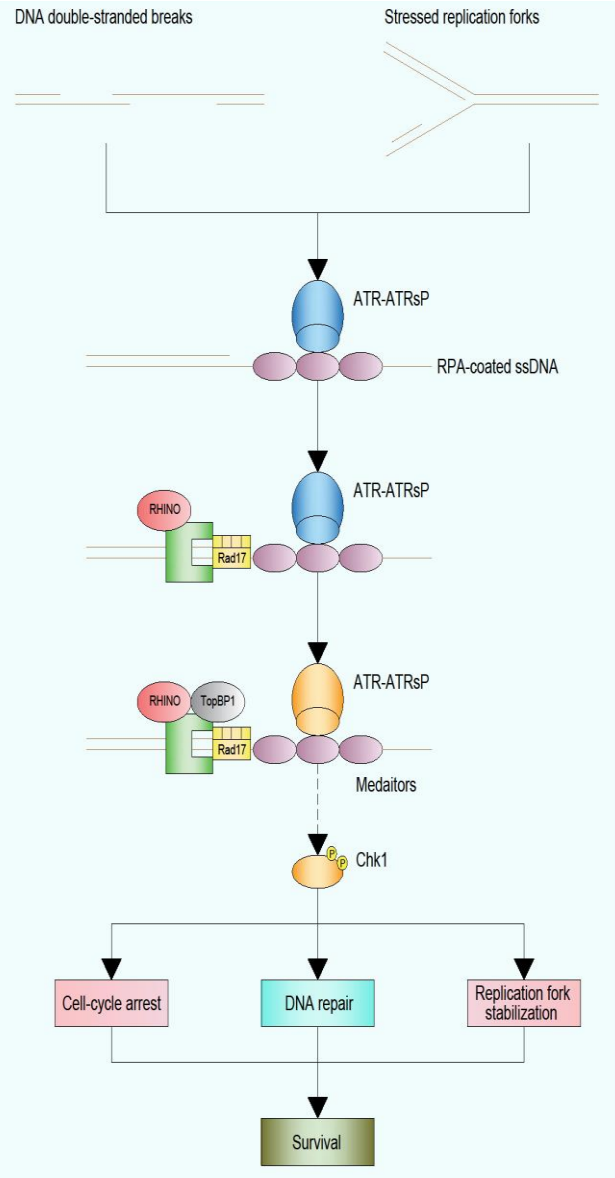

Figure 2. PKB/AKT Activation Downstream of RTKs Via the PI3K Pathway.

hydrophobic motif, either by mTOR or DNA-PK and mediates several cellular functions like angiogenesis, metabolism, growth, proliferation, survival, protein synthesis, transcription and apoptosis. It also inhibits the pro-apoptotic FOXO proteins. Here, every cellular process is initiated by substrate-specific phosphorylation events occurring in the cytoplasm and nucleus.[18,19,20] The above events following DNA damage due to ionizing radiations or other mutagenic factors enhance the DNA damage-induced transcription to aid in cell survival. The suppression of the Akt signalling pathway can occur as a result of dephosphorylating mechanisms of various phosphatases acting at different sites. The functional phosphatases like PHLPP1 and PHLPP2, dephosphorylate Atk at its hydrophobic motif site- serine 473 (S473). The dephosphorylation of Akt at its activation loop (T308) and the hydrophobic motif is caused by PP2A. The lipid phosphatase PTEN dephosphorylates PIP3 to convert it into PIP2 and thus removes the upstream

signal for Akt activation.[21,22]

The DNA-PK has been identified as a putative S473 Akt kinase and ATM as its subset.[23] They are accumulated and localised along with γ-H2AX and S1981 p-ATM at the site of chromatin damage. Yet the induction of Ser p-473 Akt has been reported to be independent of both PI3K and DNA-PK. Instead, it occurs downstream of Meiotic Recombination 11 (MRE11)-dependent ATM activation and Ring Finger Protein 168 (RNF168)-dependent histone ubiquitinylation. Independently, the MRE11 complex (consisting of MRE11, RAD50 and NBS1) and RNF168 (an E3 ubiquitin ligase) function as DSB sensor and γ-H2AX non-proteolytic poly-ubiquitinylation of the DSB-flanking chromatin respectively, to restore the genetic structure.[24,25] Hence, although the function of H2AX is not completely deduced in the PI3K-PKB/Akt pathway, it clearly plays a major role.

Role of H2AX in ATR Signalling Pathway and Chromatin Mediated DNA Break Recognition by ATM

Like Akt, the ATR signalling is a multistep process but can be induced by a variety of DNA lesions leading to the formation of single-stranded DNA. The Pre-mRNA Pocessing factor 19 (PRP19)-assisted ubiquitylation of RPA has been suggested to facilitate ATR Interacting Protein (ATRIP) binding to damaged chromatin site. The ATR forms a stable complex with ATRIP and promotes ATR localization at the site of replication stress and DNA damage. The ATR-ATRIP heterodimer interacts with the nucleofilament formed between single-stranded DNA and Replication Protein A (ssDNA-RPA) complex with DNA damage specific RAD9-RAD1-HUS1 clamp, since it is unable to directly interact with DNA. Studies have shown that DSBs with blunt dsDNA ends as well as dsDNA ends with ssDNA overhangs can activate ATM signalling.[26,27] The DNA damage-specific clamp is loaded at the damaged site with the help of the clamp loader complex (RAD17-RFC) and facilitated by RPA. This is followed by phosphorylation of subunit RAD9 on residue S387 leading to binding of DNA topoisomerase 2-binding protein 1 (TopBP1) with the FATC domain of ATR and activation of the ATR pathway.[28] A recent study confirmed the function of RHINO (Rad9, Rad1, Hus1 interacting nuclear orphan, encoded by RHNO1) in ATR activation by mediating DNA damage specific clamp with TopBP1 and initiating checkpoint signalling in the absence of DNA damage.[29] Contradictions to the above signalling model first came to light when the dispensable nature of RPA was highlighted for ATR activation and subsequent Chk1 phosphorylation following genotoxic stress.[30,31] Moreover, the cells depleted of RAD9 and HUS1 did not completely recapitulate defective ATR signalling indicating the redundancy of these factors in ATR signalling or an alternate function of ATR.[26,32,33]

The γH2AX plays an important role as a docking site for chromatin remodelling in events of DNA damage to prevent further damage. Hence, they form the basis of DDR pathways as well as V(D)J recombination.[34] It is believed that γH2AX acts as an anchor for P53 Binding Protein 1 (53BP1), Nuclear Factor with BRCT Domain 1 (NFBD1, also known as MCD1), and Mre11-Rad50-Nbs1 (MRN) complexes to facilitate DNA-protein interactions and prevent further dissociation of broken DNA ends. Subsequently, it prevents error-prone repair of DSBs.[35] However, these specific anchoring at DSB sites may suppress defective translocations by inhibiting irreversibly the dissociations of broken DNA ends. This may result in abnormal re-joining of chromatin fragments and occurrence of cancer phenotypes.[34,36] During the above cellular events, ATR and ATM phosphorylate various DDR and DNA repair proteins, at single or multiple Ser/Thr-Gln (S/T-Q) sites, including H2AX at S139. ATR and ATM predominantly phosphorylate Chk1 and Chk2, resulting in checkpoint activation, which in turn phosphorylate Cdc25 phosphatases to delay the progression of the cell cycle.[37] Most commonly, studies have suggested both ATM and ATR activation in response to UV irradiation-induced DNA damage.[5,38,39,40]

Role of H2AX in the p53/p21 Pathway

The p53 is a tumour suppressor protein that is activated as a result of oncogene stimulation; nucleotide depletion and DNA damage.[41] It is a trans-activator protein that responds to the above stimuli by preventing the proliferation of damaged DNA through the mechanisms of senescence, apoptosis or cell-cycle arrest.[42] The repression of p53 occurs through direct or indirect mechanisms. During the direct mechanism, p53 is directly recruited to the promoter region of its target genes and transcribed. Hundreds of transcriptional factors have been identified so far. The direct mechanism is reported to regulate BIRC5, CDC25C, CDC25B, CHK2, cyclin B, CKS1B, RECQL4, and CDC20 genes.[43] The indirect mechanism is mediated through the activation of p21 and regulates hTERT, EZH2, and CHK1 genes to represses the activation of the cell cycle.[44] Specifically, the transcription of p21 inhibits cyclin-dependent kinase (cdk) activity and/or binds to PCNA to cease the cell at the G1 phase.[45,46] The G2 arrest is also reported in the p53/p21 pathway to prevent damaged cells to undergo mitosis.[47] On DNA repair, the cell cycle is progressed to the S phase by the proteosome-dependent degradation of p21. Interestingly, the H2AX deficient cells have not shown such degradation resulting in caspase-dependent mitotic catastrophe in damaged cells. In contrast, H2AX containing cells carry out the p53/p21 pathway efficiently in events of DNA damage.[48]

Role of H2AX in the PCNA Inhibition Pathway

The PCNA is a conserved, multifunctional and non-oncogenic protein essential for cell growth and survival and is usually

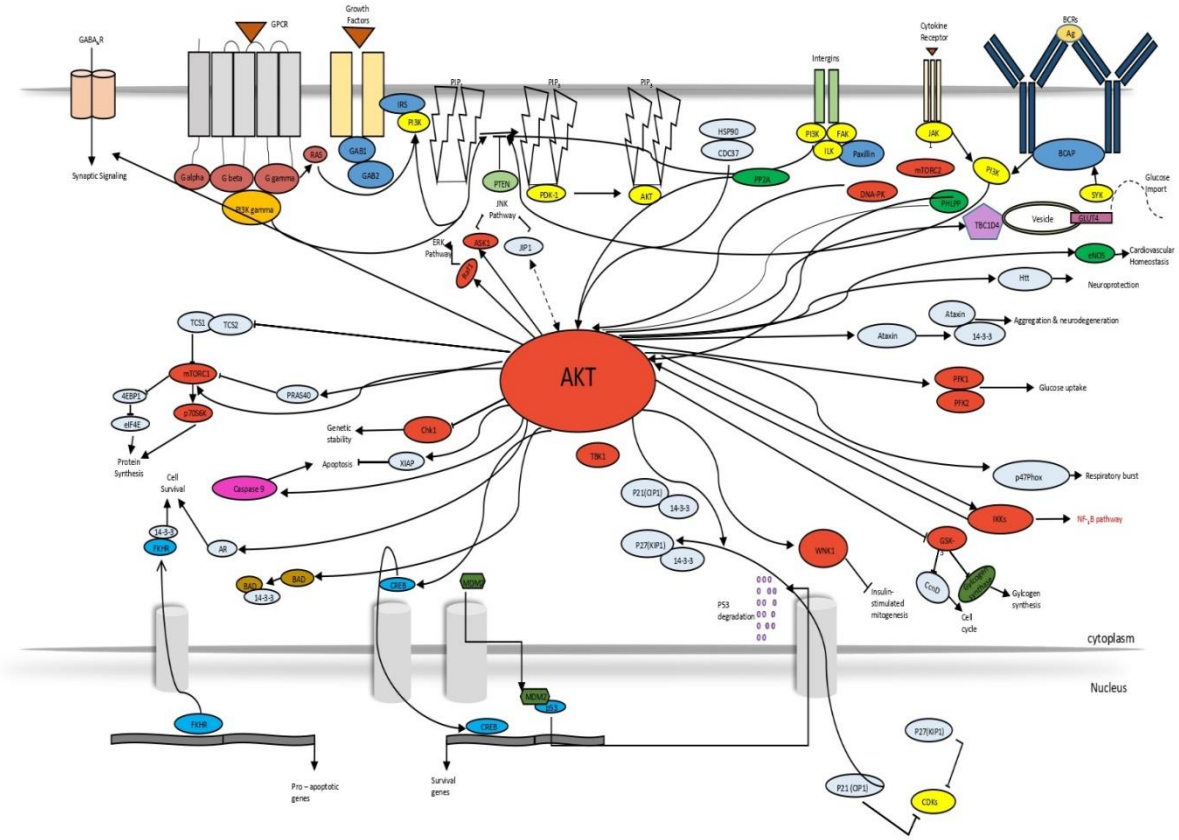

Figure 3. Signalling Events Activating PKB/AKT and Cellular Functions Regulated by PKB/AKT.

overexpressed in all tumour cells. It interacts with eukaryotic cell cycle proteins and acts as a link between Cdk2 and its substrates, such as RFC and DNA ligase 1, which are phosphorylated by the Cdk2 kinase. However, the down-regulation or inhibition of PCNA function is observed in normal cells, as compared to tumor cells, as they are more vulnerable to stress. In a native form, PCNA occurs in the nucleoplasm in the form of ring-shaped homotrimer. However, its functional form i.e., a linear monomer is relocalized in the cytoplasm. On loading at the primer-template junctions of DNA, PCNA encircles the DNA and initiates DNA replication and DNA repair.[49,50,51,52] At the same time, it also interacts with pro-caspases to inhibit apoptosis and promotes glycolysis. In contrast, one of the important characteristics of PCNA is that it interrupts the recognition of tumor cells by natural killer cells, on the cell membrane. It has been observed that H2AX is phosphorylated in the S-phase of cells in events of DNA damage. The γH2AX foci then co-localizes with PCNA to initiate further responses of DSB repair. A recent study also reported elevated expression of γH2AX in cells treated with cisPt plus PCNA-I1S, suggesting an additive effect of PCNA-I1S and cisPt on DNA damage.[50]

Standard and Advanced Techniques for Quantification of DSBs

Most commonly, the extent of DSBs is quantified using electrophoresis or labelling techniques. The neutral comet assay (alternatively known as single-cell gel electrophoresis) is the most commonly used DSB quantification technique due to its sensitivity to detect DNA damage at the cellular level. In this technique, the cells are embedded on an agarose gel and the damaged DNA migrates in the electrical field forming a tail-like comet whereas the undamaged DNA remains at the head of the comet.[53] The Pulse Field Gel Electrophoresis (PFGE) is used to separate large DNA molecules after digestion with restriction enzymes and application to a gel matrix under the influence of an electrical field that changes directions periodically.[34] Typically, relatively small DNA fragments are accumulated in this process that migrates in the agarose gel, whereas intact DNAs remain at the site where they are embedded. The quantification of results is easier in case of the PFGE as compared to immunoblots and Southern blot techniques. The DNA damage can also be quantified using Terminal deoxynucleotidyl Transferase (TdT) dutp nick-end labelling (tunel) assay. It has been designed to detect apoptotic cells that undergo extensive DNA degradation during the late stages of apoptosis. The method is based on the ability of TdT to label blunt ends of DSBs, independent of a template.[54]

The comet assay is being widely used as it is more sensitive and versatile; however, initially, the quantitative assessment of DSBs was mostly based on PFGE. Over time,

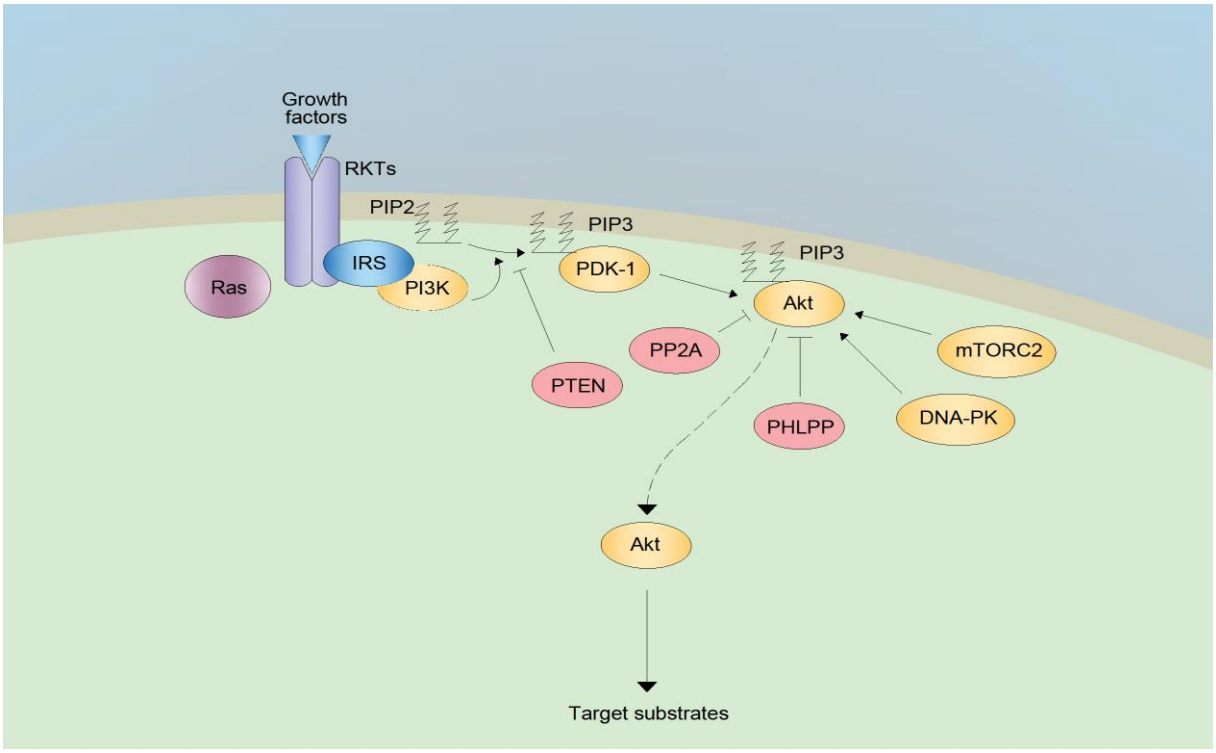

Figure 4. Activation of the ATR Pathway by DNA Damage and DNA Replication Stress.

the development of an antibody specific to γH2AX further simplified the detection and quantification of DNA damage. Similarly, the techniques like flow cytometer or western blotting based on microscopic measurement of nuclear foci present added advantage, along with high sensitivity, to correlate γH2AX expression with DNA damage.[34] A recent study has reported comprehensive characteristics of γH2AX and pH3 on exposure to genotoxic chemicals like clastogens and aneugens with the help of stable isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS). They reported that this technique can not only differentiate the mechanistic information quantitatively but also provide detailed profiles of DNA damage and repair processes, on intoxication with clastogens and aneugens. The significance of ID-LC-MS/MS was based on the observed profiles of these genotoxins. Clastogens induce an increase in γH2AX and a decrease in pH3, whereas aneugens induce either an increase or a decrease in pH3 with no obvious effect on γ-H2AX.[55] Another recent study used lanthanide-coupled secondary antibody with time- resolved fluorescence and adapted the assay to a 96-well plate technique screening and differentiating low numbers of DNA DSBs. It was reported that the fluorescence intensity of cells stained with Alexa-488 fluorophore for γ-H2AX expression increased with the number of DNA DSBs and was comparable to counting foci at the tested time interval.[56]

Applications of γH2AX

The phosphorylated protein "H2AX" is a histone variant of the H2A family and has been recently recognized as a powerful biomarker for DSBs. H2AX contains a unique SQ motif within its C-terminal tail. The interaction of proteins and signalling factors of DDR pathways and the SQ motif serves as a mediator for its localised retention- an important parameter to trigger several cellular mechanisms.

As indicated previously in this study, deletion of H2AX is associated with radiation sensitivity, chromosomal instability, growth retardation, defects in DDR signalling and immune-deficiencies. Hence, it is used in translational cancer research as a tool to monitor natural and induced DNA lesions, and gain better insights into the cellular mechanisms in various DDR pathways. In the current decade, more conclusive insights to the biological effects of DNA damaging agents has been presented by quantifying H2AX in damaged cells. Several such DNA damaging agents are continuously used in chemotherapy, radiotherapy, drug discovery and in vitro testing. The importance of H2AX can also be understood from its necessary interaction with proteins of the DDR pathway for its induction. Several previously known and novel proteins as well as signalling factors form a complex with γH2AX, by co-localizing at the site of DNA damage, in order to trigger the DDR pathway. This step not only induces DDR response but also facilitates amplified signal transductions. The γH2AX is also known to be involved in the regulation of checkpoints in DDR pathways.[57]

Biomarker in Cancer Therapy

As a means of cancer therapy, the γH2AX detection and

quantification are used for improving the efficiency of drugs, radiations, and chemotherapies. Commonly, γH2AX was used as a biomarker in basic translational research. However, its application in molecular pharmacology, to study the pharmacodynamics in clinical trials, is comparatively a recent development.[58,59] Lately, the first three out of four phases of clinical trials use experimental designs involving the use of γH2AX to evaluate drug response in cancer patients. During phase I, γH2AX assays are used to study drug safety and pharmacokinetics in events of specific cancer phenotypes or generalized DNA damage. This is done simply by evaluating the level of γH2AX in response to different doses of drugs and/or comparing it with other known proteins or biomarkers of DNA lesions like topoisomerase I inhibitors, PARP inhibitors and DNA alkylators.[60] The establishment of drug tolerance concentrations and a lethal dose is also possible using these approaches in the treatment of onset and advanced cancers. However, most studies have investigated γH2AX in evaluating drug combinations like PARP inhibitor with a DNA damaging agent.[61,62,63] When compared with standard techniques that detect DNA lesions, γH2AX assays are more sensitive, efficient and reproducible. Moreover, its central position in DSB detection and repair can effectively result in cancer drug development and treatment.

In general, elevated levels of γH2AX are observed in different types of benign and metastatic cancer. This suggests its possible use as screening as well as a monitoring tool for early detection of cancers. Moreover, the designing of personalised doses of chemotherapy, radiation or combination therapies may be possible sooner due to advances in γH2AX based studies. This is highly advantageous because every individual responds differently to similar treatment strategies, due to differences in immunological, exogenous and endogenous factors. Ionizing radiation-induced DNA damage is the most common source of DNA damage and hence effective assessment protocols are more critically required for the same. Hence, numerous studies and advances in the treatment of ionizing-radiation induced DNA damage have been published in the literature.[64,65] The first evidence of the practical implementation of γH2AX in clinical studies was provided by Qvarnstrom et al.[66] They analysed skin punch biopsies collected from prostate cancer patients undergoing radiotherapy and assessed the radiation doses in exposed individuals using γH2AX based techniques. Soon after, another study reported the formation and disappearance of γH2AX foci in lymphocytes of individuals subjected to small radiation doses during computed tomography examinations.[67] A recent study has reported an advanced tool to study dose-response relationships in animal organs and human tissue biopsies after internal exposure to radiation using γH2AX immunofluorescence staining and Confocal Laser Scanning Microscopy (CLSM). The above study reported the efficiency of CLSM

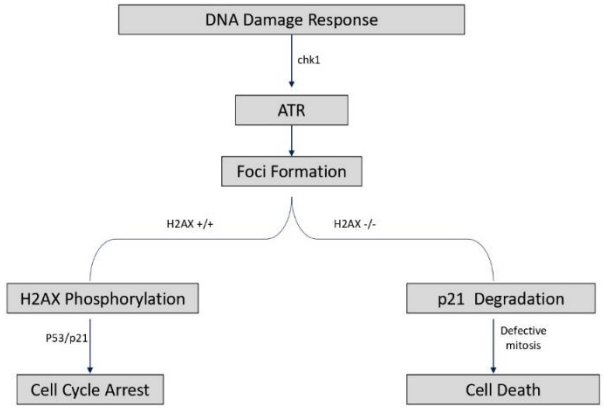

Figure 5. H2AX is Required for Cell Cycle Arrest Via p53/p21 Pathway.

imaging in the detection of intracellular cell-specific γH2AX foci as well as its quantification. Thus, it can be suggested that the application of γH2AX based quantification can be suitably extended to nuclear medicine dosimetry research.[68] The γH2AX has also provided better insights into cancer development in blood malignancies such as multiple myeloma, where the disease progression is usually accompanied by the clonal expansion of malignant plasma cells in the bone marrow. The precursor cells of multiple myeloma i.e., Monoclonal Gammopathy of Undetermined Significance (MGUS) show low levels of γH2AX as compared to its higher level in plasma cells.[69] A similar association between γH2AX expression and BRCA1 mutation has also been reported commonly in breast cancers, which is most prevalent among cancer disorders worldwide.[70,71,72] However, the association of γH2AX in ovarian cancer could only be predicted, till now, based on generalised assumptions. The first report of H2AX as a novel prognostic biomarker in screening of ovarian cancer was reported recently.[73] The gene set enrichment analysis of the ovarian cancer cells demonstrated that the gene sets involved in the G2/M checkpoint and DNA repair mTORC1 signalling were enriched in the H2AX. The second leading site of cancers is the stomach (gastric cancers) which is characterized by atrophic gastritis, intestinal metaplasia or low-grade dysplasia. Recent studies have correlated the γH2AX expression with morphological variations in superficial and atrophic gastritis and gastric cancer.[74,75]

Radiation Hypersensitivity

The drug-based therapy in cancer treatment is still underway and the search for novel anticancer agents is actively in progress. Meanwhile, radiotherapy remains a dependable strategy for the management and treatment of different types of cancers, with over 60% of patients receiving the therapy at some stage of treatment. Radiotherapy may be given alone or in combination with surgical procedures. It is also a part of chemo- and hormonal therapies in patients. However,

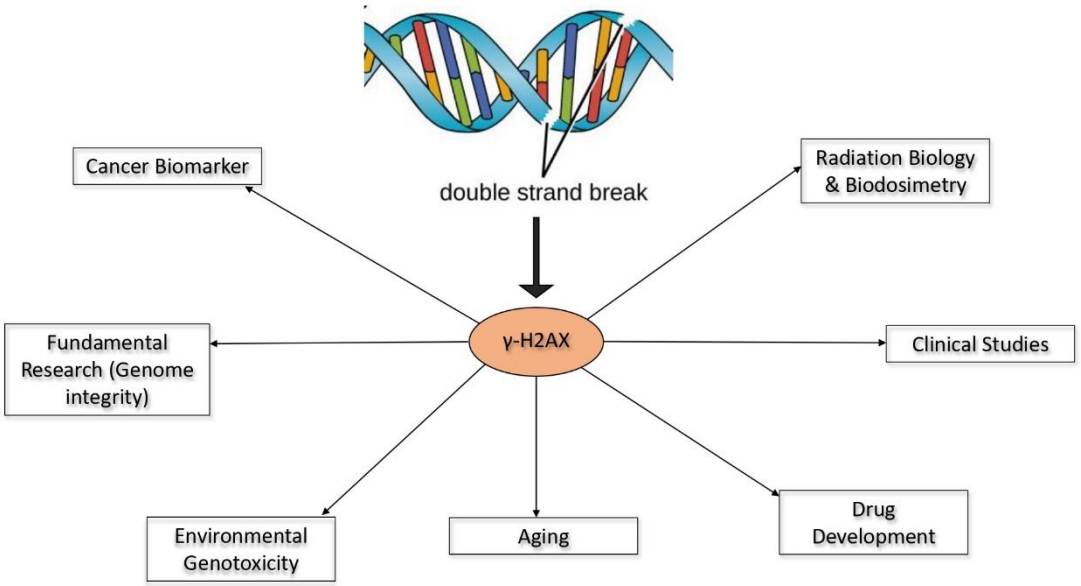

Figure 6. Applications of Gamma-h2ax.

over 5% of radiotherapy patients develop severe side effects like mucositis, erythema, edema and fibrosis.[76,77] These side effects occur mostly as a result of radiation hypersensitivity that is developed along with the increasing DNA damages caused during the progression of cancer. The most challenging factor in radiotherapy is the prediction of Normal Tissue Toxicity (NTT) during and after radiotherapy. This is because, in certain cases, patients develop delayed NTT and show excessive reactions even to low doses of radiotherapy which sometimes have irreversible and lethal effects.[78] In general, the defects in DDR or DSB repair pathways, similar to those observed in patients with ataxia- telangiectasia, predispose them to neurodegenerative and cancer disorders. In such cases, the use of radiotherapy is avoided to prevent any complications that can be logically anticipated. The use of γH2AX can predict radio-sensitivity in patients using modified irradiation protocols and this can tremendously help in preventing extreme incidences of delayed NTT.[79] Based on γH2AX observations, a study has indicated the presence of twice as many foci at high scanner settings as compared to lower doses. They also suggested that lowering the scanner settings will result in a reduction of X-ray-induced DNA damage in patients.[76] The dose of radiation, however, may not be quantified by correlating DNA damage level with H2AX in peripheral blood lymphocytes. A recent study reported the biological effects of radiation exposure due to the Fukushima Daiichi Nuclear Power Plant (FNPP) accident on cattle within a 20 km radius of the disaster that occurred in Japan. Their study design involved the measurement of DNA damage by estimating H2AX in peripheral blood lymphocytes of farm animals. However, the radiation dose could not be evaluated possibly due to changes in metabolism and environmental conditions that lead to the different speeds of natural recovery in these animals.[80]

Chronic Inflammations and other Disorders with Elevated γH2AX

Apart from cancer disorders and radio sensitivities, chronic inflammatory diseases also result in DNA damage due to overproduced reactive oxygen species along with other factors. Hence γH2AX has been used as a biomarker in Chronic Obstructive Pulmonary Disease (COPD). Increased DSBs and their linkage to oxidative DNA damage have been confirmed in patients with COPD and these factors are believed to trigger nuclear hypermutability and lung cancer. High levels of γH2AX have been reported in alveolar wall cells of lung tissues and pre-neoplastic lesions collected from patients with COPD and lung cancer respectively, but not in normal lung tissues. High levels of γH2AX can also be stimulated by replicating viral proteins during adenovirus infection that results in chronic inflammatory conditions. Similarly, lifestyle choices like smoking can also trigger excessive inflammatory responsiveness, which, over time, may result in the development of lung cancer. Hence, γH2AX based evaluation may be effective in the management of COPD and prevent further progression towards complicated diagnosis in patients.[81,82,83,84]

Chronic and low-grade inflammation and genome instability have also been associated with obesity, which in turn presents an increased risk of developing some types of cancer later in life. Significantly higher levels of γH2AX have been identified in obese children as well as grown individuals.[85] A study also reported differential expression of H2AX along with HMGB1 between human subcutaneous and omental visceral adipose tissue depots. They suggested that the DNA methylation and genetic variations mediate fat depot- specific regulations,

and levels of H2AX and HMGB1 are related to lipid parameters.[86] Certain disorders (Dyskeratosis congenital and Schizophrenia) and infections (caused by HBV) are also known to be predisposed to cancer.[87,88,89] Increased γH2AX levels have also been recorded in the lymphocytes of these patients.

Clinical Trial Studies Based on γH2AX

In cancer patients, the response to a treatment may be unknown for several weeks unless assessed alongside an imaging technique. A much more practical approach for cancer treatment may be possible if more information is available on a patient's early response, using non-invasive and non- radio-therapeutic approach.[90] Besides tumors, various markers and signature sets of gene sequences /mRNAs have been examined for its correlation with cancer. However, not all markers correlate with the prognosis of cancer, unless carefully vetted.[91] Lately, a microscopy-based γH2AX assay was described as the most sensitive tool to measure DSB induction. Consequently, new intensive high throughput molecular methods to detect circulatory γH2AX expression, as a marker of response to treatment for patients, could potentially be utilized as a predictive biomarker for early prognosis of cancer. This approach will substantially speed up the analysis while maintaining sensitivity. For this purpose, several clinical trials have been conducted to investigate a diagnostic approach to detect cancer/tumor at an early stage. In 2012, Redon et al. conducted a study to develop a new molecular method for analysis, such as the use of a novel ELIZA based assay, for the quantification of γH2AX levels in comparison to total H2AX expression in cells and tissues. The assay readily detected DNA damage induced by ionizing radiation or DNA-damaging agents and provided a precise reading regardless of sample composition when compared to traditional microscopy-based approaches. Besides, they also reported the increased sensitivity of microscopic analysis over flow cytometry for quantification of γH2AX levels. This is because the latter uses single-cell suspension such as Peripheral Blood Mononuclear Cells (PBMCs) for analysis whereas tissue, tumor and biopsies can be analysed easily using a microscope. Hence the flow cytometry may be quicker but it is not as sensitive as the microscopic method for quantification of γH2AX levels. The ELISA assay, therefore, gained popularity considering its performance, high-throughput screening and an internal control (for total H2AX) for reliable measurements.[92] Sooner, the use of pre- and post-treatment circulating tumor cells count as a prognostic and predictive biomarker of response to treatment of cancer affecting the breast, prostate, colorectal, and lungs.[93] A clinical trial study was carried out using human colorectal adenocarcinoma cell line HT29. The findings confirmed that ELISA results correspond to the findings of a microscopy-based assay for the assessment of cell responses to DNA-damaging agents.[94]

In contrast, another study validated the use of PBMCs treated with DSB inducing agents as a suitable analytical technique exhibiting sensitivity and precision. This study suggested that the flow cytometry detects DNA repair deficiency in PBMCs treated with DNA repair inhibitors, as well as the deficiency in DNA repair signalling in PBMCs.[95] Most recently, a study proposed a suitable ex vivo method to detect γH2AX foci by immune-labelling the internal irradiated tissues and combining this method with CLSM to provide a quantitative digital analysis of the intra-nuclear γH2AX foci. By using this technique, they were able to observe a dose-dependent elevation of γH2AX foci even in different liver tissues with major differences in proliferation rate and radio-sensitivity.[68]

An endogenous γH2AX expression in breast cancer tissue has been evaluated in a cohort reporting display of γH2AX in triple-negative, BRCA1 and p53 mutated breast cancer cell lines previously.[96] A recent study reported higher expression of γH2AX in patients with HER2/neu+2, who had positive Chromogenic In-Situ Hybridization (CISH) compared to patients with HER2/neu+2, who had negative CISH.[71] The endogenous expression of γH2AX was advanced to evaluate and predict the effectiveness of pre-operative radiotherapy in colorectal adenocarcinoma cell lines, which showed a higher expression of γH2AX in WiDr cell line compared to DLD-1 cell line without any therapy. Similarly, the suppression effect on cell viability was higher in DLD-1 than WiDr cells implying that γH2AX plays an important role in radio sensitivity. Their finding also suggested that the CRC cells have an elevated expression of γH2AX and possess a certain degree of tolerance to irradiation.[97] Hence, so far, the potential of γH2AX as a sensitive biomarker can be well established. Although, further investigations are needed to evaluate the most sensitive analytical technique for detecting γH2AX levels in response to DNA damage. The widespread use of these assays will help us to study DDRs more comprehensively which, in turn, will support the development of more effective anti-cancer agents.

Conclusion

The determination of γH2AX levels is a sensitive biomarker for DDR and DSBs. This information has been potentially transformed into clinical opportunities in cancer assessment and therapy. The interaction of γH2AX with several proteins has been determined and these studies have immensely contributed to translational cancer research. The sensitivity of γH2AX to DNA lesions enables the evaluation of the biological effects of various anticancer drugs and radiations in low doses. In addition, γH2AX assays allow continuous monitoring to evaluate immediate as well as long term effects of these agents. Moreover, the threatened DNA damage anticipated on exposure to environmental pollutants

and surrounding conditions can also be assessed based on the level of γH2AX. Hence early detection of unknown mutagens and DNA lesions can be treated at an early stage. One obvious disadvantage of γH2AX based studies is that anticancer treatment (with agents that induce apoptosis and DNA fragmentation of cancer cells) may result in increased stress and production of reactive oxygen species. This, in turn, stimulates the phosphorylation of H2AX. Although, cell death and anticancer treatment-related to H2AX phosphorylation can be discriminated against based on H2AX signals, they require the use of appropriate diagnostic techniques (fluorescence microscopy, cytometry) with suitable modifications to adapt to the different characteristics of cancer disorders.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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