Published in final edited form as:
Nat Rev Cancer. 2008 December ; 8(12): 957–967. doi:10.1038/nrc2523.

γH2AX and cancer

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Abstract

Histone H2AX phosphorylation on a serine four residues from the carboxyl terminus (producing γH2AX) is a sensitive marker for DNA double-strand breaks (DSBs). DSBs may lead to cancer but, paradoxically, are also used to kill cancer cells. Using γH2AX detection to determine the extent of DSB induction may help to detect precancerous cells, to stage cancers, to monitor the effectiveness of cancer therapies and to develop novel anticancer drugs.

The DNA double-strand break (DSB) is a serious lesion that can initiate genomic instability, ultimately leading to cancer[1,2]. It is no surprise that cellular genomic integrity is closely monitored by processes that detect and repair DSBs and that also halt cell cycle progression until repair is complete[3]. Human diseases with defects in these processes often exhibit a predisposition towards cancer[4]. A key component in DNA repair is the histone protein H2AX, which becomes rapidly phosphorylated on a serine four residues from the carboxyl terminus (serine c-4) to form γH2AX at nascent DSB sites[5]. During the 30 minutes after DSB formation, large numbers of γH2AX molecules form in the chromatin around the break site, creating a focus where proteins involved in DNA repair and chromatin remodelling accumulate[5] (FIG. 1a,f). This amplification makes it possible to detect individual DSBs with an antibody to γH2AX.

In addition to being a cause of cancer, DSB induction is paradoxically an effective treatment for cancer. Many therapeutic agents act by introducing sufficient DSBs into cancer cells to activate cell death pathways[6]. Some agents create DSBs directly, whereas others create various types of non-DSB DNA and cellular damage that can lead to DSB formation during attempted repair[7]. As DSBs contribute to both genomic instability and cancer treatment, monitoring their formation in a cell by detecting γH2AX focus formation may be a sensitive means to monitor cancer progression and treatment[8,9]. In this Opinion article, we discuss the known sources of DSBs and detail methods that use γH2AX to visualize and quantify DSBs. Finally, we illustrate possible clinical and medical roles for γH2AX detection in the

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DATABASES

National Cancer Institute Drug Dictionary:
http://www.cancer.gov/drugdictionary/5-azacytidine | batracylin | bleomycin | calicheamicin | camptothecin| cisplatin | clofarabine | cyclophosphamide | cytarabine | doxorubicin | etoposide | gemcitabine | hydroxyurea | imatinib mesylate | melphalan | mitoxantrone | SAHA | temozolomide | tirapazamine | trabectedin | UCN-01

OMIM:
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIMataxia-telangiectasia

UniProtKB: http://www.uniprot.org 53BP1 | ABRA1 | BRCA1 | CHK1 | H2AX | INO80 | KIT | MCPH1 | MDC1 | MYH | nibrin | p53 | PP4C | RNF8 | TOP2 | transcription intermediary factor 1β | UBE2N | UIMC1

FURTHER INFORMATION

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diagnosis of precancerous and cancerous cells, both in pharmacodynamic studies as a biodosimeter for optimizing drug treatment protocols, and in accelerating drug development, including phase 0 protocols. We do not discuss the various repair and signalling pathways involving γH2AX in any detail (FIG. 2) as these topics are covered in depth elsewhere[10–12].

H2AX

H2AX is a member of the histone H2A family, one of the five families of histones that package and organize eukaryotic DNA into chromatin. The basic subunit of chromatin, the nucleosome, consists of a core of eight proteins, two from each of the H2A, H2B, H3 and H4 families, with about 140 bp of DNA coiled around the core and the fifth histone family, H1, on the linker DNA acting as a bridge between nucleosomes[13] (FIG. 3). Each nucleosome contains two H2A molecules, of which ~10% are H2AX in normal human fibroblasts, a ratio that places an H2AX molecule in every fifth nucleosome on average (FIG. 3). In other cell types the percentage of H2AX has been found to be as low as 2% of total H2A (lymphocytes and HeLa cells) or as high as 20% (SF268 human glioma tumour cell line)[14]. The reasons for these different relative amounts of H2AX are unknown, although they may result from the unique regulation of H2AX synthesis. Most core histone species are synthesized in concert with DNA replication, being translated from small transcripts that terminate in a stem–loop structure rather than a poly(A) tail[15]. These replication-dependent histone species are encoded by intronless genes. In addition, a few replication-independent histone species are encoded by intron-containing genes and translated from poly(A) mRNAs[16]. The H2AX gene (*H2AFX*) contains features of both replication-dependent and replication-independent histone species. It is encoded by a small intronless gene and the transcript has the stem–loop structure that is characteristic of replication-linked histones; however, the *H2AFX* transcript is often read through to a poly(A) site about 1 kb downstream of the stem–loop. Therefore H2AX is synthesized in both replication-dependent and replication-independent manners[17]. The utility of this dual mechanism of translational regulation is unknown, but it may ensure the presence of sufficient H2AX molecules in the replicating genome for efficient DSB detection, whereas replication-independent synthesis ensures the continued presence of H2AX in G1 and G0 cells.

Immediately upon DSB formation, one or more of the PI3K-like kinases, a family including ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK), are activated and phosphorylate H2AX as well as many other DNA repair and checkpoint proteins (FIG. 2). γH2AX focal growth was first followed in fixed mitotic cells of the Indian muntjac (*Muntiacus muntjak*), nuclei of which contain six large chromosomes in females and seven in males (FIG. 1h). Muntjac mitotic chromosomes exhibit small γH2AX foci 3 minutes after exposure to ionizing radiation (IR). These become brighter and larger disc-shaped structures containing about 30 Mbp DNA at 9 minutes after IR, and reach maximal brightness and size 30 minutes after IR[5]. These findings suggest that H2AX molecules in a small region near the DSB site are phosphorylated first, and are followed by molecules at increasing distances from the break site[5]. However, other models are not excluded. Other mammalian cells, including the normal human fibroblasts WI38, respond to IR with similarly sized γH2AX foci (FIG. 1i). Many DNA repair and/or checkpoint protein species accumulate on the growing γH2AX focus, which may serve to open up the chromatin structure[18,19] and form a platform for the accumulation of DNA damage response and repair factors[20] (FIG. 1f; FIG. 2). Chromatin immunoprecipitation studies in yeast containing a single DSB revealed γH2AX over a region of approximately 30 kbp around the DSB[21] (FIG. 3d). Although much smaller than those found in mammalian cells, the foci in yeast cells contain about the same fraction of total H2AX per DSB as mammalian cells, which is ~0.03% of H2AX phosphorylated per

DSB[22]. The significance of this value is unknown, but it does indicate that the process can detect many simultaneous DSBs. It should be noted that H2AX can also be phosphorylated on serine 1 in a process independent of serine c-4 phosphorylation, but the role of this modification is unknown. Importantly, γH2AX refers to H2AX phosphorylated on serine c-4, irrespective of the phosphorylation status of serine 1.

As DSBs are repaired, γH2AX foci disappear. However, what constitutes complete repair is unclear. Is it simply when DNA is rejoined or is it when the chromatin proteins have returned to their pre-lesion positions? The neutral comet assay was used to show that γH2AX foci do generally disappear in concert with DSB rejoining, which suggests that the detection of γH2AX levels in cells is a reliable measure of the overall DSB rejoining[23]. However, other work[10] suggests that the quantity of γH2AX foci remain elevated well after most DSBs have been rejoined, suggesting that γH2AX removal may depend on other steps that follow DNA rejoining. Studies of the mechanism of γH2AX removal after repair have suggested two non-exclusive mechanisms: dephosphorylation of γH2AX or γH2AX removal from the chromatin. Dephosphorylation of γH2AX has been found to depend on phosphatases PP2A and PP4C[24–27]. Removal of γH2AX from the chromatin by histone exchange has been shown to occur in *Saccharomyces cerevisiae* during chromatin remodelling by INO80 and in *Drosophila melanogaster* by the TIP60-containing remodelling complex[28–32].

Although it is generally accepted that DNA DSBs induce the formation of γH2AX foci (a few exceptions are discussed below), H2AX may be phosphorylated in response to other DNA lesions. DNA single-stranded regions induced by ultraviolet C irradiation provide the best evidence of this. Ultraviolet C radiation induces the formation of γH2AX through ATR kinase activity[33]. However, γH2AX does not appear as foci in this case but rather as a diffuse pattern referred to as pan-nuclear staining (FIG. 1k). During TRAIL (TNF-related apoptosis-inducing ligand)-induced apoptosis, peripheral nuclear staining and pan-staining are also observed[107] (FIG. 1). Thus, it is important to distinguish the pattern of γH2AX staining when linking γH2AX to DSB formation. Controversies surrounding γH2AX and DNA lesions[34,35] are discussed in more detail in BOX 1.

Box 1

H2AX foci and DNA double-strand breaks

Although it is accepted that, with exceptions, almost every DNA double-strand break (DSB) forms a γH2AX focus, whether every γH2AX focus identifies a DSB remains controversial[86]. It is generally not possible to independently determine the presence of a DSB because visualization of γH2AX foci is several orders of magnitude more sensitive than other methods of DSB detection. It has been reported, for example, that γH2AX foci may persist over time in some tumour cells after the initiating DSBs have been rejoined[130]. However, as DSBs disrupt chromatin, complete repair also involves restoring the original chromatin conformation, which may also be facilitated by the presence of γH2AX foci. In addition, the formation of extremely small γH2AX ‘microfoci’ has been observed in the nuclei of senescent primary cells and in certain cancer cell types[87,131]. These microfoci differ from normal foci in that they do not increase in size and they do not contain other DNA repair factors. The function of these structures in the cell has yet to be determined. There are, however, several more definitive cases of γH2AX formation that are not due to DSBs, although in these cases γH2AX does not form a focal pattern. One example is found in the testis during spermatogenesis[79]. During pachytene the sex body (the condensed X–Y chromosome pair) remains completely covered with γH2AX independent of recombination-associated DSBs. Another example of γH2AX formation without focus formation occurs following cell exposure to ultraviolet C[34] and during

apoptosis[167]. The primary lesions induced by ultraviolet radiation can cause replication stress that may induce DSB formation leading to γH2AX foci formation[132]. However, during G1, pan-nuclear staining has been observed that seems to be dependent on nucleotide-excision repair machinery[34]. Additionally, a similar global γH2AX DNA staining has been observed during mitosis in some cell types[133]. Finally, a surprising recent report showed that γH2AX is localized — along with other DNA repair proteins — to the centrosome, an organelle that reportedly lacks DNA[134]. With these exceptions in mind, however, the γH2AX focus remains the most sensitive way to detect a DSB. Future work may elucidate the relationship of these other γH2AX patterns to γH2AX foci.

γH2AX and cancer

The human H2AX gene (*H2AFX*) maps to chromosome 11 at position 11q23, in a region that frequently exhibits mutations or deletions in a large number of human cancers[36,37], especially in haematopoietic malignancies such as acute myeloid leukaemia and acute lymphoid leukaemia[38]. Head and neck squamous cell carcinoma is often characterized by amplification of chromosomal region 11q13 as well as loss of distal 11q, the region containing *H2AFX*[39]. The increased chromosomal instability seen in these cells indicates that loss of 11q and *H2AFX* may contribute to tumour development, progression and resistance to therapy in this cancer subtype. These findings have led to the intriguing proposal that human *H2AFX* may be an excellent candidate gene to indicate susceptibility to lymphomas, leukaemia and other cancers[40,41]. A recent study showed a strong relationship between a single nucleotide polymorphism upstream of *H2AFX* and follicular lymphoma, a subtype of non-Hodgkin lymphoma, and mantle cell lymphoma, further supporting the contribution of H2AX to the risk of human lymphoma development[41]. Additional evidence of the tumour-suppressing role of H2AX comes from a study involving gastrointestinal stromal tumour (GIST) cell lines[42]. Imatinib mesylate, a clinically approved protein kinase inhibitor, has been shown to trigger apoptosis in GIST cell lines through upregulation of H2AX. Finally, a recent study of tumours from patients with breast cancer showed that 37% had altered *H2AFX* copy numbers[40].

Consistently, *H2afx*$^{-/-}$ mice survive well in unstressed conditions but they are less efficient at DNA DSB repair, leading to an increased incidence of chromosomal abnormalities[36,43]. *H2afx*$^{-/-}$ and *H2afx*$^{+/-}$ mice are not particularly cancer-prone; however, both are cancer-prone in a p53-null background, supporting the idea that H2AX has a role as a tumour suppressor[37,44].

DSB formation

DSBs can be caused by a variety of factors. These can be classified according to the underlying cause as follows: direct interaction with an agent, reactive oxygen species (ROS), metabolic processes, deficient repair, telomere erosion and programmed biological processes (FIG. 2; TABLE 1).

Before discussing the different sources of DSBs, it is important to note that some DSBs may be protected by chromatin proteins and may not induce γH2AX. Examples include the DNA ends in the topoisomerase II (TOP2)–DNA complex and the double-stranded DNA end in the telomere (see below). In addition, γH2AX foci are found at the periphery of heterochromatic regions but not within them[45], raising the possibility that heterochromatin may harbour DSBs that are concealed by chromatin proteins and hence do not form γH2AX foci. The profound sensitivity of cells from patients with ataxia-telangiectasia (AT, caused by defects in ATM activity) to IR appears to be due, at least in part, to their inability to

repair a subset of DSBs associated with heterochromatin, a subset that may involve up to 25% of cellular DSBs. The evidence suggests that, in normal cells, ATM signalling may temporarily perturb heterochromatin through KAP1 (also known as transcription intermediary factor 1β), a transcription repressor that is crucial for DSB repair within heterochromatin, but that this process is defective in AT cells[45]. These exceptions are explained by protein-mediated protection of DSBs. When these proteins are removed by repair processes, or by DNA erosion in the case of telomeres, the newly exposed DSBs induce γH2AX formation. With these caveats in mind, a γH2AX focus can be considered to represent a DSB.

Direct attack on DNA

DSBs can be induced directly by a variety of natural sources, including IR, radiomimetic chemicals and cosmic radiation[46,47]. A direct collision between a radioactive particle or γ-ray and a DNA double helix will lead to a cluster of multiple types of DNA damage, including single-strand breaks, base and backbone modifications, and DSBs[48]. As mentioned above, whereas IR and radiomimetic chemicals have been linked with increased cancer risk, they are also commonly used as chemotherapeutic agents[7]. IR and bleomycin both interact with DNA to directly produce DSBs and are used in cancer treatment[7].

ROS

Ionizing rays and particles interact not only with DNA itself but also with the other constituents of the cell, primarily water, to generate clusters of ROS[49]. When a cluster of ROS is sufficiently close to a DNA double helix, multiple lesions are formed on both strands, often leading to a DSB[50,51]. In addition to being caused by IR, ROS also arise from endogenous sources such as oxidative phosphorylation, cytochrome P450 metabolism, peroxisomes and inflammatory responses, and from exogenous sources such as chlorinated compounds, metal ions and phorbol esters[52]. ROS are estimated to be responsible for about 5,000 DNA single-stranded lesions per cell per day, mostly during replication, about 1% of which may lead to DSBs[53]. ROS are also implicated in ageing and the pathogenesis of human diseases including cancer and neurodegenerative disorders[54,55].

Metabolic processes

The replication machinery itself is another indirect source of DSBs. Typically, replication-linked DNA damage induces γH2AX through ATR, whereas IR-related DNA damage induces γH2AX through ATM and DNA-PK[7]. Many compounds, including the anticancer agents gemcitabine, melphalan, cisplatin and hydroxyurea, interfere with DNA replication and this may result in a DSB. Some of these compounds that cause replication stress may function by altering dNTP pools, by changing DNA replication frequency or by otherwise inhibiting DNA replication[56]. In addition, single-strand nicks can be converted to DSBs when encountered by the replication machinery[57]. Many cancer drugs act by interfering with the actions of TOP1 and TOP2 on DNA. TOP1 inhibitors include camptothecin, indolocarbazole and their pharmaceutical derivatives. These act by stabilizing the TOP1–DNA complex[58,59], giving more opportunity for collision with moving replication forks. Such collisions may result in a DSB, which can be repaired only after the trapped TOP1 is removed by proteolysis[60]. TOP2 is also a therapeutic target and many of the most widely used anticancer drugs, including etoposide, mitoxantrone and doxorubicin, act to stabilize TOP2–DNA complexes[61,62]. Similarly the transcription machinery can also be a source of DSBs[63].

Deficient repair

Deficient repair of other non-DSB DNA lesions may also lead to the formation of DSBs[1,3]. During base-excision repair (BER) several intermediates are formed that can lead to DSB formation and cytotoxicity if they persist[64]. Like BER, mismatch repair generates intermediate single-strand breaks that can result in DSBs[65]. Incomplete or inactive nucleotide-excision repair may leave persistent bulky lesions on the DNA. Although trans-lesion synthesis can bypass bulky DNA lesions, mutations can result[66]. Mutations in nucleotide-excision repair proteins have been linked to the cancer predisposing disorder xeroderma pigmentosum[67,68]. Likewise, defects in other DNA repair pathways may lead to increased genomic instability as seen by increased cancer risk. For example, mutations in MYH, a BER DNA glycosylase, have been shown to cause colorectal polyposis in humans, a syndrome that is associated with an increased risk of developing colon cancer[69]. Mutations in components of the mismatch repair machinery are associated with hereditary non-polyposis colorectal cancer or Lynch syndrome[70].

Eroded telomeres

DSB signalling is also associated with replicative senescence, a process that occurs after a certain number of cell divisions in normal mammalian cells and is characterized by irreversible cell cycle arrest accompanied by physiological and morphological changes. As senescent cells have irreversibly ceased division, senescence may have an important role in preventing tumorigenesis as well as promoting organismal ageing[71,72]. As most differentiated mammalian cell types lack telomerase, the enzyme that maintains telomere length, telomeres shorten with each cell division and ultimately fail to protect the end of the chromosomes. The uncovered DNA double-stranded end induces a γH2AX focus[73,74], making it an excellent marker of telomere erosion and hence replicative senescence. Cancer cells often escape senescence by activating telomerase, which enables them to replicate indefinitely, so telomerase is a putative target for anticancer drugs[75].

Programmed processes and other causes

DSBs are formed as an essential step during immune system development[76,77], meiosis[78,79] and apoptosis[80,81]. Retroviral integration also induces DSBs[82]. These processes are shown in FIG. 2.

Measuring γH2AX

As many cancers have increased numbers of cellular DSBs and many cancer treatments also induce DSBs, γH2AX has the potential to function both as a diagnostic tool and as an indicator of treatment efficiency[9,83,84]. Detection of γH2AX relies on antibodies raised to the H2AX phosphorylated C-terminal peptide CKATQAS(PO_4) QEY in humans[5]. Although γH2AX may be detected by mass using two-dimensional gel electrophoresis[14], immunocytochemical detection of γH2AX foci is several orders of magnitude more sensitive and allows the distinction between pan-nuclear staining and focus formation. Detection methods fall into two categories: those counting foci or other γH2AX-containing structures in images of cells and tissues, and those measuring overall γH2AX protein levels.

Counting γH2AX foci

Each focus contains at least several hundred γH2AX molecules, and the number of foci has been found to correlate closely with the number of DSBs, supporting the notion that the two are equivalent at least in the early stages of repair[5,85–87]. Such measurements can be performed by microscopy[85,88,89] or fluorescence-activated cell sorting (FACS)[90–92]. The lower limit of detection depends on how many cells can be examined and the background level of foci that is present in all cells and tissues. Responses to as little as 1.2 mGy,

equivalent to an average of 0.1 foci per cell in a population, have been reported[86]. Detection of γH2AX has been applied successfully to many human materials (including peripheral blood mononuclear cells (PBMCs), tissues and skin) to monitor DNA damage produced by low-level radiation exposure[88,89,93], subtle changes caused by radiation-induced bystander response[94] or by genomic instability[87,95]. Additionally, co-localization of γH2AX foci with other proteins involved in DNA damage repair allows spatial and temporal dissection of these processes, a valuable tool in analysing the mechanism of action of new anticancer agents[63,96–99]. It should be noted that there is a variable background level of γH2AX signals primarily associated with DNA replication and expressed mostly in S-phase cells[100]. S-phase cells can be discriminated from non-replicating cells by FACS on the basis of DNA content[90] (which has a sensitivity limit of 0.1–10 Gy (REFS 101,102)) or with microscopy by measuring PCNA-positive cells[103,104]. This background γH2AX level should be subtracted when analysing the induction of γH2AX by exogenous factors[88,90,105].

Immunofluorescence microscopy

Tissues can be prepared for γH2AX focal analysis in several ways. Touch printing, a standard technique in diagnostic clinical cytology, involves pressing the freshly cut surface of a tissue repeatedly on a glass slide, a process that deposits cells on the slide. The slides are air-dried and stored at −80°C (REFS 87,106). Paraffin-embedded or frozen tissue sections are also suitable starting materials. Paraffin-embedded sections retain cellular and tissue morphology better than frozen sections, but peroxidase detection of γH2AX is not quantitative[83,84]. By contrast, frozen sections offer good sensitivity with fluorescent stains, but tissue morphology is less well retained. PBMCs isolated from blood samples can be cytospun onto slides and dried[88]. After samples are stained for γH2AX, images are acquired on epifluorescent or confocal microscopes. High throughput of samples is theoretically possible as the γH2AX foci can be detected with an air objective on a confocal microscope. The utility of high-throughput assays could be increased by combining a confocal microscope that has auto-focus capability with readily available image analysis software to automatically collect data on γH2AX foci, including such parameters as focal area, brightness and average number of foci per cell[89].

As γH2AX foci are sites of accumulation of many other proteins involved in DNA repair and chromatin remodelling, antibodies to these proteins can also be used as surrogates for DSB detection[18,107,108]. p53-binding protein 1 (53BP1), which quickly accumulates at γH2AX foci, has been used to detect DSBs[95,109,110]. Although immunostaining for other proteins in addition to γH2AX may yield important information, it cannot be assumed that DSB detection using other proteins is equivalent to using γH2AX. Accumulation of other DNA-damage proteins often depends on the phosphorylation of H2AX, but their rates of accumulation do not necessarily parallel that of γH2AX[18,95,111]. Additionally, γH2AX is a *de novo* species, whereas most other DNA repair proteins already exist in the nucleus before accumulating at a focus. Thus, although detection of accumulated DNA damage proteins such as ATM, components of the MRN complex and 53BP1 is useful in immunohistochemistry, background protein levels might be problematic[18,107,108]. If the protein of interest is also phosphorylated *de novo*, antibodies to the phosphorylated form may yield cleaner results. The most obvious example of this is the use of phospho-ATM antibodies to detect DNA damage[112].

Immunoblotting

Immunoblotting measures γH2AX amounts on a population basis and cannot discern whether γH2AX is in a focus or in another structure. With immunoblotting, the relative amount of H2AX compared with total H2A in different cells and tissues should be considered. As the amount of γH2AX formed per DSB is a percentage of total H2AX,

absolute γH2AX levels can vary considerably in different cell types containing identical numbers of DSBs[14], resulting in different signal strengths in immunoblotting assays. Thus, when comparing different cell types it is useful to measure total H2AX levels with an antibody to unphosphorylated H2AX to normalize for these differences. This is less of an issue with microscopy, as different cell types containing identical numbers of DSBs would be expected to have the same numbers of γH2AX foci, though the foci can differ in brightness. The same issue could arise if comparing different cell types by FACS.

Another issue with the detection of total γH2AX is that the DSBs that are induced during apoptotic cell death are themselves sufficient to induce the formation of γH2AX[80], and the apoptotic contribution to the total γH2AX signal may be greater than that from cells containing discrete γH2AX foci[91,167]. In population studies the signals from damaged but potentially viable cells cannot be differentiated from those of dying cells using immunoblotting, but can be differentiated using microscopy and FACS.

γH2AX in clinical research and therapy

Diagnostic uses

Replication stress increases levels of DNA DSBs not only in tumours but also in precancerous lesions[83,84]. The transcription factor p53, which is activated by DSBs, prevents cancer development by inducing senescence or apoptosis; however, many cancers have mutations in p53 that remove this barrier[84,113]. One proposed mechanism of cancer progression states that activated oncogenes induce the stalling and collapse of DNA replication forks, leading to the formation of DSBs in precancerous cells. Alone or in combination with other stresses, including hypoxia and inflammation, oncogene activation contributes to cancer-associated genomic instability and associated DNA damage[113,114]. Thus, γH2AX levels may reflect endogenous genomic instability in tissues and serve to detect precancerous lesions so that preventive measures can be taken or treatment options can be better informed.

Recent studies[9,115] have demonstrated increased levels of DSBs in tumour cells in clinical specimens from various tissues, as well as in tumour cell cultures. Other studies have demonstrated the possible utility of γH2AX measurements in clinical diagnostics: in the differential diagnosis of metastatic renal cell carcinoma[116], in monitoring ulcerative colitis (a chronic inflammatory disease that predisposes to colorectal cancer and in which shorter telomeres have been associated with chromosomal instability and tumour progression[117,118]) and in screening for patients with genomic instability syndromes such as AT[119] and radiosensitive severe combined immunodeficiency[4,120]. Measurements of γH2AX may be useful in detecting other perhaps undiscovered conditions that affect DNA repair and predispose to cancer.

Pharmacodynamic uses

How cells respond to therapeutic agents may differ between individuals for a number of reasons, including genetic makeup, undetected inflammatory processes and subclinical infections. DSB measurements may be more pertinent for anticancer agents that depend on the patient's metabolism for drug activation and/or effect than for those that damage DNA directly (FIG. 2; TABLE 1), but the ability to obtain immediate feedback on how a particular patient responds to a given agent could enable clinicians to tailor treatment to the individual.

Several studies have looked at γH2AX levels in patients to help determine whether and how such measurements might be used in the clinic[88,89,121] (TABLE 2). For agents such as IR and radiomimetics, taking a blood or skin sample at various times after or during the

treatment could provide information on patient sensitivity (that is, of normal tissue). Other uses may include examining skin punches to compare calculated doses with received doses of IR, or measuring the level of radiation exposure after a nuclear accident. Leukocytes were taken from different patients and irradiated *ex vivo* in order to compare γH2AX responses between patients[101]. *In vivo* measurements of γH2AX in leukocytes have also yielded strong linear correlation between the mean number of γH2AX foci per PBMC and integrated total-body radiation dose after site and time dependence are considered[88,93,121,122]. Skin punch biopsies gave linear responses after consideration of the local radiation dose[89]. These results suggest that a standard technique could be developed to monitor received radiation doses in exposed individuals.

Although measuring the level of DNA DSB damage by determining γH2AX amounts in blood or skin cells may give information on how a treatment is affecting normal cells in the body, tumour cells may respond differently depending on unique factors such as altered gene expression, the proportion of cells in S-phase and the amount of tumour vasculature. For example, overexpression of ATP-binding cassette transporters may increase drug export from the cells, making tumour cells resistant to a drug that causes ample DNA damage elsewhere in the body[123]. Therefore, direct analysis is still required to establish how a drug is affecting the tumour. Analysis of blood or skin can determine the extent of damage caused by a drug to normal cells in that individual. Coupling that information with that obtained from tumour biopsies may permit clinicians to tailor treatment to the individual patient. The DSB repair kinetics after drug administration could also be monitored in this manner, and may yield useful information for treatment decisions.

Drug development and phase 0 protocols

The γH2AX assay may be useful as a pharmacodynamic biomarker to aid the development of novel anticancer compounds in both patients and model systems. As DSBs are a sign of genotoxic stress, following γH2AX formation may help determine within a few hours the genotoxic potential of a compound administered to cells in culture or in mice. Notably, γH2AX focus formation is being used in phase 0 studies to determine whether a compound results in a response in patients[124]. Lymphocyte, skin and tumour biopsies are taken before and after administration of the compound to help determine the extent of DNA damage. The aim of such studies is to facilitate the development of more efficacious cancer treatments and to increase the number of potential drugs in development.

Conclusions

This Perspective has concentrated on γH2AX as a potentially useful tool to further human health. The above discussions indicate that monitoring DSB responses through γH2AX formation is already showing excellent potential for judging therapeutic progress and cancer progression[89,93,101,122,125]. Quick and inexpensive methods using γH2AX formation for DSB detection in blood, skin or other tissues that are obtained by minimally invasive means could be a valuable tool, permitting clinicians to monitor whether an agent is causing the desired level of damage in a patient. Quicker assays, such as an enzyme-linked immunosorbent assay (ELISA) for γH2AX, could be developed and automated to permit almost real-time monitoring of DNA damage levels in the clinic.

In addition, it may be that the level of ongoing DNA damage and repair is an extremely sensitive indicator of organismal stress. A number of recent studies have used DNA damage as an output to determine overall cell health, including examining DNA-damage effects from air pollution[126], handling chemotherapeutic agents[127], mobile phone use[128] and eating organic versus regular apples[129]. Thus, γH2AX may be useful in determining whether a particular environmental agent is stressful to an animal or person. As genome integrity is

central to cellular health and γH2AX focus formation is currently the most sensitive assay for genome integrity, being able to routinely monitor DSB levels in individuals could provide useful tools for improving human health.

Acknowledgments

We thank K.W. Kohn for continuous insights during the course of our H2AX studies. We thank B.J. Baird, National Cancer Institute, for critical reading of the manuscript and J. Doroshow for his commitment to the development of γH2AX as a clinical biomarker. The authors are funded by the Intramural Research Program of the National Cancer Institute, Centre for Cancer Research, National Institutes of Health.

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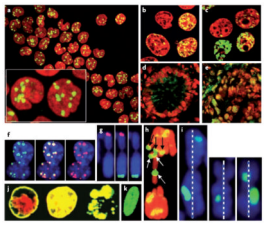

Figure 1. γH2AX staining patterns observed in mammalian cells

a | Human peripheral blood mononuclear cells 30 minutes after exposure to 1 Gy of ionizing radiation (IR). **b** | Colon cancer cells treated with campthotecin. **c** | Leukaemic cells treated with etoposide. **d** | Frozen sections of normal human colon. **e** | Frozen sections of colon adenocarcinoma. **f** | γH2AX foci (left) and p53 binding protein 1 (right) co-localize (middle) in mouse fibroblasts 30 minutes after exposure to 1 Gy of IR. **g** | Eroded telomeres in ageing cells. Telomeric DNA (left, red), γH2AX (right, green) and merged (middle). The putative eroded telomere (bottom) exhibits a γH2AX focus but is too short to bind the telomeric DNA probe, whereas the putative functional telomere (top) binds telomeric DNA and lacks a γH2AX focus. **h** | Broken metaphase chromosomes of Indian muntjac cells 90 minutes after exposure to 0.6 Gy of IR. The white arrows denote γH2AX foci on broken chromatid ends and the black arrows denote foci on the remaining metaphase chromosomes. **i** | Selected chromosomes of a WI38 normal human fibroblast metaphase spread 30 minutes after exposure to 1 Gy of IR. The left panel shows the largest chromosome in the metaphase, assumed to be chromosome 1 or chromosome 2, with a length about 245 Mbp (dotted white bar). The foci are approximately 40 and 16 Mbp. The middle and right panels show two other chromosomes from the same metaphase at the same scale. The chromosome in the middle panel has an estimated length of 169 Mbp and a focus about 20 Mbp. The chromosome in the right panel has an estimated length of 149 Mbp with foci of 45 and 22 Mbp. **j** | Colon cancer cells treated with TRAIL (TNF-related apoptosis-inducing ligand). From left to right: peripheral nuclear staining (1 hour treatment), pan-staining and apoptotic bodies fully stained with γH2AX (3 hour treatment)[167]. **k** | A normal human fibroblast after being exposed to an acute dose of ultraviolet C, exhibiting a pan-nuclear staining pattern. DNA is red for images **a–e**, **h** and **j** and blue for images **f**, **g** and **i**. γH2AX is green for all images. Part **h** is reproduced, with permission, from REF. 5 © The Rockefeller University Press (1999).

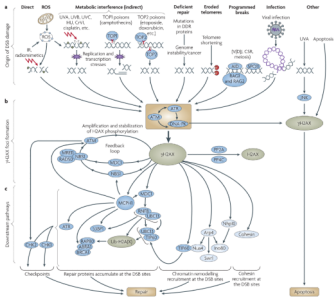

Figure 2. H2AX is a central component of numerous signalling pathways in response to DNA double-strand breaks (DSBs)

a | There are several categories of origins of DSBs. Direct: H2AX phosphorylation occurs after treatment with agents including ionizing radiation (IR) or radiomimetic drugs[14,101]. ROS are ions or small molecules (produced by normal metabolism and exogenous agents) that can cause DSBs. Indirect: Drugs, chemicals and DNA modifications induce replication and transcription stress, leading to DSBs[7,58,63]. Ultraviolet (UV) radiation can cause DSBs in S-phase [34,132]. Deficient repair: Mutations in DNA-damage repair (DDR) proteins result in genomic instability, leading to DSBs[115]. Eroded telomeres: Critically short telomeres reveal double-strand ends[73,74]. Programmed: H2AX phosphorylation occurs in V(D)J recombination, class switch recombination (CSR) and meiosis[76,77,135]. Infection: Retroviral integration induces DSBs[136]. **b** | γH2AX focus growth. Three kinases, ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK), respond to DSBs, resulting in initial H2AX phosphorylation[7,33,137]. A signal amplification loop involving H2AX, nibrin (also known as NBS1) and mediator of DNA damage checkpoint protein 1 (MDC1) stimulates ATM and increases H2AX phosphorylation[138]. Protein phosphatases PP2A and PP4C bind to and dephosphorylate γH2AX[24,26,27] **c** | Downstream signalling pathways. NBS1 (REFS 139,140) and MDC1 (REF. 141) bind to γH2AX, which allows the accumulation of DDR proteins including the MRN (MRE11–RAD50–NBS1) complex, RNF8, BRCA1 and p53-binding protein 1 (53BP1)[18,20,43]. RNF8 catalyses the ubiquitylation of H2AX, which then recruits RAP80 (also| known as UIMC1) and BRCA1 to DSBs through ABRA1 (REF. 18). Accumulation at DSB sites of MCPH1 (also known as BRIT1) also depends on γH2AX[142]. MCPH1 is involved in chromatin condensation and cell-cycle arrest, by interacting with BRCA1, NBS1, CHK1, 53BP1, MDC1, ATR, RPA (replication protein A) and ATM[143,144]. Accumulation of 53BP1, BRCA1, CHK1 and NBS1 on accrued γH2AX through MDC1 and/or MCPH1 could explain the role of γH2AX in checkpoints[145]. Chromatin remodelling complexes participate in DNA repair[32]. The human histone acetyltransferase TIP60 complex (in yeast, Nua4 complex) and the yeast ino80 and Swr1 complexes interact with H2AX or γH2AX[29,146,147]. DSBs facilitate the association between TIP60 and UBC13 (also known as UBE2N), regulating the H2AX acetylation that is necessary for H2AX ubiquitylation (Ub) and its release from chromatin[146]. γH2AX is crucial for sister-chromatid homologous recombination[148], probably by facilitating the interaction between sister chromatids[148,149]. H2AX has multiple roles during apoptosis[80,150,151,167]. Human proteins are represented in dark blue, yeast proteins in light blue. AID, activation-induced cytidine deaminase; CrVI, hexavalent chromium; HU, hydroxyurea; RAG, V(D)J recombination-activating protein; ROS, reactive oxygen species; TOP, topoisomerase.

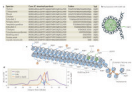

Figure 3. H2AX and γH2AX foci

a | H2AX is an H2A histone with a core sequence conserved with other H2A species and a tail conserved through evolution connected by a linker of variable length. **b** | The SQEY tail extends from the core nucleosome near the entry and exit point of the DNA. **c** | The nucleosomes form a 30 nm fibre with H2AX molecules in every fifth nucleosome on average in mammals and every nucleosome in yeast. Approximately 10% of the H2AX molecules are phosphorylated at any one time in a focus. **d** | In yeast, chromatin immunoprecipitation studies show that H2A, the functional analogue of H2AX, is not phosphorylated near the site of the double-strand break (DSB), although histones (H2B) are present. Two repair proteins, Mre11 and Rad51, accumulate very near the break site. Part **d** modified, with permission, from REF. 21 (2004) © Elsevier Ltd.

Table 1

Anticancer agents that produce γH2AX

Agent	Mechanism of induction and remarks	Staining pattern	Refs
IR	Direct and mostly indirect: ROS SSB conversion by replication and DNA processing	Focal (FIG. 1a,f,h,i)	152
Bleomycin	Direct: metal ion-mediated oxidative cleavage	Focal	153,154
Camptothecins, indenoisoquinolines	Indirect: conversion of SSB (resulting from TOP1 cleavage complexes) into DSBs by replication	Focal co-localized with replication foci (FIG. 1b)	58,104,155
Doxorubicin, etoposide, mitoxantrone, batracylin	Indirect: production of DSBs by trapping TOP2 cleavage complexes; doxorubicin also induces ROS formation	Focal (FIG. 1c)	98,156,157
Cytarabine, gemcitabine, hydroxyurea	Indirect: replication fork collapse (due to chain termination or deoxyribonucleotide pool depletion	Focal	158–160
Cisplatin, temozolomide, aminoflavone, trabectedin	Indirect: DNA alkylation	Focal	63,161–163
Tirapazamine	Indirect: ROS production in hypoxic cells	Focal	156
5-Azacytidine, SAHA	Indirect: epigenetic modifications (DNA methylation and histone deacetylation inhibition)	Focal	164,165
PARP and DNA-PK inhibitors	Indirect: interfere with the repair of SSBs and DSBs that are induced by other agents	Focal	97,166
UCN-01	Indirect: checkpoint inhibitor potentiating replication-associated DNA damage induced by TOP1 inhibitors cytarabine and gemcitabine	Diffuse	159
TRAIL	Indirect: death receptor-mediated activation of DNA-PK	Peripheral and diffuse (FIG. 1j)	167
Imatinib	Indirect: apoptosis induced by KIT and PDGF tyrosine kinase inhibition	Diffuse (FIG. 1j)	42

All the anticancer agents listed can also induce delayed γH2AX activation by apoptosis. See FIG. 1j for apoptotic patterns. DNA-PK, DNA-dependent protein kinase; DSB, double-strand break; IR, ionizing radiation; PARP, poly(ADP-ribose) polymerase; PDGF, platelet-derived growth factor; ROS, reactive oxygen species; SAHA, suberoylanilide hydroxamic acid; SSB, single-strand break; TOP, DNA topoisomerase, TRAIL, TNF-related apoptosis-inducing ligand.

Table 2

Studies using γH2AX detection in humans

Tissue	Study	Method	Refs
PBMCs *ex vivo*	Used IR and calicheamicin to make frank DSBs in blood cells *ex vivo*	γH2AX formation determined by FACS	101
PBMCs *ex vivo*	Correctly identified the four AT patients out of a pool of 19 people presented for testing	γH2AX formation determined by FACS	119
PBMCs *in vivo*	A phase 1 study of clofarabine followed by cyclophosphamide for adults with refractory acute leukaemias	γH2AX formation determined by FACS	125
PBMCs *in vivo*	Assessed leukocyte DNA damage after multi-detector row CT — a quantitative biomarker of low-level radiation exposure	γH2AX foci counted after CT scans	93
PBMCs *in vivo*	Assessed γH2AX focus formation in tumour patients after local radiotherapy at different sites of the body	γH2AX foci counted after radiotherapy	122
Skin *in vivo*	Studied skin biopsies from two patients with prostate cancer who were undergoing radiotherapy with curative intent	γH2AX foci counted in skin sections	89
Tumour biopsy samples	Differential diagnosis of metastatic RCC, which induced, but was not limited to, HCC and ACC	γH2AX foci counted in tumour biopsy samples	116
Tissue biopsy samples	Explored relationships among telomere lengths, γH2AX intensities, age, disease duration and age of disease onset	Correlated telomere lengths and γH2AX focal incidences	118

ACC, adrenocortical carcinoma; AT, ataxia-telangiectasia; CT, computed tomography; FACS, fluorescence-activated cell sorting; HCC, hepatocellular carcinoma; IR, ionizing radiation; PBMC, peripheral blood mononuclear cell; RCC, renal cell carcinoma.