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Histone γH2AX and Poly(ADP ribose) as Clinical Pharmacodynamic Biomarkers

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Abstract

Tumor cells are often deficient in DNA repair and damage response (DDR) pathways, and anticancer therapies are commonly based on genotoxic treatments using radiation and/or drugs that damage DNA directly or interfere with DNA metabolism leading to the formation of DNA double-strand breaks (DSBs), and ultimately to cell death. Since DSBs induce the phosphorylation of histone H2AX (γH2AX) in the chromatin flanking the break site, an antibody directed against γH2AX can be employed to measure DNA damage levels before and after patient treatment. Poly(ADP-ribose) polymerases (PARP1 and PARP2) are also activated by DNA damage and PARP inhibitors show promising activity in cancers with defective homologous recombination (HR) pathways for DSB repair. Ongoing clinical trials are testing combinations of PARP inhibitors with DNA damaging agents. Poly(ADP-ribosylation) (PAR) can be measured in clinical samples and used to determine the efficiency of PARP inhibitors. This review summarizes the roles of γH2AX and PAR in the DDR and their use as biomarkers to monitor drug response and guide clinical trials, especially Phase 0 clinical trials. We will also discuss the choices of relevant samples for γH2AX and PAR analyses.

Keywords

γH2AX; pharmacodynamics; indenoisoquinolines; poly(ADP-ribose); PARP; DNA repair

Background

There is a critical need in the field of cancer treatment to accelerate the validation of candidate drugs while ultimately reducing costs. One approach promising to improve the efficiency and speed of clinical trials utilizes biological markers to measure pharmacodynamic parameters in samples taken from the cancer patients themselves during drug protocols (1). Because many anticancer agents target DNA and the DDR pathways, biomarkers based on DNA damage endpoints. Biomarkers may also be useful for identifying individuals hypersensitive to radiotherapy in order to tailor treatments to minimize undesired side effects. The most desirable biomarkers would be those able to determine early in the course of treatment whether a drug is reaching its target and acting as intended. The last few years has seen the development of new biomarkers for clinical trials (2), especially in the context of Phase 0 trials (1). Here, we discuss two pharmacodynamic biomarkers, γH2AX

and PAR, currently being developed at the NIH medical center (Bethesda, MD, USA) by the National Cancer Institute.

γH2AX

H2AX belongs to the H2A family of histones, one of four families present in the nucleosomes that package eukaryotic DNA into chromatin (3). Upon DSB formation, hundreds of H2AX molecules in the chromatin are rapidly phosphorylated by members of the phosphatidylinositol-3 kinase (PI3K) family (4,5) forming a focus at the DSB site (see Fig. 1 for details). H2AX-deficient cells exhibit cell cycle checkpoint deficiency, increased genomic instability and sensitivity to genotoxic agents (6), observations corroborating findings of roles for γH2AX in multiple processes necessary to return the broken chromatin to its original state (see Fig. 1 for details). After completion of DNA repair and chromatin remodeling, dephosphorylation of γH2AX is carried out by several phosphatases including the p53-inducible phosphatase Wip1 (7,8), protein phosphatases 6 (9), 4 (10) and 2A (11).

Since its discovery in 1998 at the NCI (5), γH2AX has been a topic of basic research analyzing the DDR and of translational studies as a biodosimeter to measure the genotoxic effects of drugs and/or radiation exposure (4,5). γH2AX foci are found in almost all cell types after exposure to agents that directly induce DSBs--radiation therapy, and classical chemotherapy with DNA alkylating and radiomimetic agents. Importantly however, treatment with inhibitors of DNA topoisomerases and replication also generate a variety of DNA lesions that may result in the formation of DSBs (12), primarily in S-phase cells. It is important to note that γH2AX foci, usually in small numbers, may be present in cells even in the absence of exogenous damage (4) due to DNA damage that can occur during many common cellular processes, including replication, senescence, viral infection, exposure to endogenous reactive oxygen species (ROS), and carcinogenic adducts. DSBs may form during these processes or during DNA repair, producing genomic instability and increasing cancer risk, as exemplified by the increased cancer incidence in individuals with genetic defects in DNA repair genes, such as BRCA1, BRCA2, the FANC genes, ATM and CHEK2.

Poly(ADP-ribose) polymerases (PARPs) and Poly(ADP-ribose) polymers (PAR)

Poly (ADP-ribose) polymerases (PARPs) belong to a family of 17 structurally related mammalian enzymes [reviewed in (13-16)]. Only three PARPs have demonstrable poly(ADP-ribose) polymerase activity: PARP1, PARP2 (PARP1/2) and tankyrase 1. Other proteins containing all the structural features of functional PARP have been identified (i.e. PARP3, PARP4) but their ability to form poly(ADP-ribose) (PAR) chains still has to be demonstrated. PARP1 is the most abundant PARP and is normally associated with chromatin. Binding of non-PARylated PARP1 to chromatin increases chromatin compaction (14). PARP1 activation and PARylation are associated with transcription activation, in some cases in association with DNA cleavage by topoisomerase II beta (17,18). PARP1/2 are both strongly activated by DNA damage including DNA single- and double-strand breaks. The N-terminal region of PARP1 contains zinc binding domains that bind DNA (13-15). PARP1/2 complement each other. Single PARP1 or PARP2 knockout mice are viable though hypersensitive to radiation and DNA damaging drugs, whereas the double PARP1/2 knockout is an early embryonic lethal (15).

PAR was discovered approximately 50 years ago by Chambon and coworkers (19). Its formation is catalyzed by PARP1/2 in several steps (20). PARP is activated upon DNA binding by intramolecular folding and dimerization. The PARP homodimer then catalyzes the transfer of an ADP-ribose moiety from nicotinamide adenine dinucleotide (NAD+) to a lysine (or glutamate) residue of an acceptor protein, followed by the sequential addition of

multiple ADP-ribose units to the preceding ones forming linear and branched PAR chains containing up to 200 units. In response to DNA damage, PARP1 itself is the main PARylation acceptor (automodification) and over 90% of PAR is found on PARP1 (13). PARylation is a reversible reaction. Poly(ADP-ribose) glycosylase (PARG) acts as an endo- and exo-glycosidase and hydrolyzes the glycosidic linkages between ADP-ribose units of PAR producing free ADP-ribose, and ADP-ribosyl protein lyase hydrolyzes the remaining protein-proximal ADP-ribose monomers (14). Poly(ADP-ribose) hydrolysis via endo- and exo-glycosidase activities would restore PARP1's ability to identify DNA single strand breaks and activate a new DNA damage response if necessary. Another consequence of poly(ADP-ribose) hydrolysis would be the production of high cellular levels of ADP-ribose, which in turn, may be hydrolyzed to phosphoribose and AMP, increasing the AMP:ATP ratio and inducing an autophagic state (16). PAR functions as a docking polymer for a variety of chromatin and DNA repair proteins including histones, XRCC1, Ku, DNA-PKcs, condensins and DNA topoisomerases (13,15). PARylation acts as an early DDR post-translational modification that recruits repair proteins to the DNA damage site, including scaffolding protein, XRCC1, a factor involved in base excision repair (BER), DNA ligase III and DNA polymeraseβ (21-23) (see Fig. 2 for details). PARP along with XRCC1 has also been implicated in the alternative-NHEJ pathway of DSB repair (24,25), and in DSB repair during spermatogenesis (26). Because of PARP's involvement in SSB repair, its inhibition has been proposed to lead to DSB formation.

Since BRCA1 and BRCA2 are defective in many cancers and necessary for DSB repair, inhibition of PARP1 in such cancer cells results in enhanced and selective killing (27,28) (Fig. 2). PARP inhibitors provide a novel way to treat BRCA-deficient cells in combination with chemotherapy and radiation while sparing normal cells. Therefore, PARP is a valuable target for pharmacological strategies (16,29) and PARP inhibitors are now used in an increasing number of clinical trials (30).

Biosampling for γH2AX and PAR measurements

Ideally, the efficiency of a cancer therapy based on PARP inhibitors would be to measure both γH2AX formation and PARP activity (i.e. PAR levels) in tumor biopsies (31,32). However, issues of tumor accessibility, patient discomfort, and risk of infection prevent the widespread acquisition of tumor biopsies. Even if tumor biopsies were routinely available, other confounding issues might prevent straightforward interpretations of any results obtained from them. One issue stems from tumor heterogeneity (33), due to differing replicating fractions and to vascularization anomalies affecting oxygen, nutrient and drug delivery. Therefore, γH2AX and PAR amounts may differ among multiple biopsies of the same tumor. Perhaps comparability of tumor responses might be improved by characterizing the extent of hypoxia and/or replication status along with γH2AX levels in the cells being analyzed. Another confounding factor involves decisions to repeatedly sample the same nodule with consequent influence on the biomarker or to sample a different nodule with questions of biological variability. One unique solution to obtain multiple tumor samples that may yield useful information involves the isolation of circulating tumor cells (CTC) from the patient's blood (34), which is much more feasible than obtaining multiple tumor biopsies (35).

Because of these confounding issues concerning tumor biopsies, procedures to assess other more accessible, surrogate, patient tissues by less invasive means are being developed. The advantages and inconveniences of different tumor and surrogate tissues for γH2AX detection are summarized in Figure 3. The most suitable surrogate sample should be selected considering the type of treatment received by the patient (see Fig. 3 for details).

Circulating blood lymphocytes and leucocytes are terminally differentiated (post-mitotic) cells which respond consistently to irradiation with robust γH2AX signals proportional to the radiation doses (36,37); however, their use for assessing the action of anticancer drugs that interfere with DNA replication is problematic. In contrast, PAR measurements in lymphocytes can be performed and reduction in PAR levels can readily be observed following PARP inhibitor treatment in most patients (32).

Buccal cells can be collected very simply by swabbing the patient's inner cheeks, but collection of numbers of living cells sufficient for testing can be problematical. In addition, buccal cells, a type of terminally differentiated stratified squamous epithelium, can present a challenge in procedures involving cell lysis or permeabilization when using microscopy (38). In addition, buccal cells appear to have very high background levels of DNA damage (38,39). Nevertheless, significantly increased γH2AX formation was detected in patients undergoing routine dental radiographic examination, indicating that the use of γH2AX in oral cells could serve as sensitive indicators of low-dose radiation exposure (40).

Another minimally invasive procedure consists of collecting hairs plucked from the scalp or eyebrows. A substantial portion of the hair bulb may stay attached to the hair shaft and contain dividing and stem cells (41) which can be used to measure γH2AX levels and PARP inhibition (42). However, considering that a patient's hair follicles are in various stages of growth, plucked hairs containing replicating cells (i.e. in anagen phase) should be favored for chemotherapy assessments (Redon C. and Bonner W.M., unpublished).

Finally, since skin contains proliferative cells, such a tissue would be valuable to determine DNA damage (i.e. γH2AX levels) following treatment with drugs that interfere with DNA replication. However, like tumor biopsies, multiple sampling would result in patient discomfort and may lead to possible complications, such as infections.

A major issue is the relationship of the responses of γH2AX and PAR in surrogate tissues vs. the tumors and to the relationship between those responses and the treatment outcome. While performing these comparisons for solid tumors is challenging because of the difficulty of biopsy collection, blood malignancies may present a more accessible situation. γH2AX and PAR levels could be directly measured in malignant cells collected in blood samples. However, the use of γH2AX in surrogate tissues can help determine both a drug's genotoxicity and its pharmacokinetics in vivo. Such comparisons could be extended to multiple drug combinations.

Assays for γH2AX and PAR detection

Several immunological techniques can be used to quantify γH2AX and PAR (PARP activity is deduced from changes in PAR levels). They may consist of counting antigen intensities in cells and tissues [i.e. microscopy and fluorescence-activated cell sorting (FACS)] or quantifying their overall levels (i.e. Western blotting and ELISA) (4,32,42-44) (Figs. 3,4). In recent years, the Pharmacodynamic Assay Development and Implementation Section (PADIS - National Cancer Institute (NCI)), in collaboration with Abbott Laboratories and the National Clinical Target Validation Laboratory at the NCI has developed an ELISA for PAR levels in tissue samples. With this assay, the in vivo effect of a PARP inhibitor could be measured (45) (Fig. 4). Finally, a method using an electrochemoluminescent detection system (an assay derived from the Meso Scale Discovery Technology or MSD assay) was recently reported. This ELISA-based methodology allows γH2AX measurements in tumors after irradiation (46).

Although each of these methods can be effective and gives important information, they are not currently usable for high throughput screening (HTS) and still involve extensive human

labor. Recently, Garty et al. reported the development of HTS system as a radiation biodosimetry tool for radiation triage and using γH2AX immunofluorescence assay, called as the RABIT (Rapid Automated Biodosimetry Tool) that would allow the screening of 6,500 samples a day (47).

Both γH2AX and PAR assays are specific and very sensitive. A single DSB (corresponding to one γH2AX focus) can be visualized by microscopy and responses to radiation doses as low as 1.2 mGy and 100 mGy can be detected by microscopy and FACS respectively (4). The ELISA-based measurements of PAR has a lower limit of detection (LLD) <6 pg/ml with a dynamic range of 7.8 to 1000 pg/ml (Dr. J. Ji, personal communication).

γH2AX in clinical oncology

γH2AX in cancer chemotherapy

Many cancer therapies rely on agents that preferentially kill tumor cells by generating DNA damage (48) including DSBs [reviewed in (4)] (Table 1). Methods to detect DSB levels directly in patient material cells could be invaluable for optimizing treatment, particularly with chemotherapeutic agents whose efficacies could vary among individuals with different genetic backgrounds. Because biosampling tumors is not always possible, alternate surrogate tissues have been developed depending on availability (i.e. hair sampling could be problematic in patients with alopecia due to prior treatments) or on the agent (i.e. terminally differentiated surrogate cells may not respond well when using a drug interfering with replication). For this reason, biosampling of several tissues would be preferable during drug development.

Clinical studies with different methods of γH2AX analysis are described in Table 2. In a phase I clinical study for patients with refractory leukemia, flow cytometry revealed increased γH2AX levels in circulating leukemia cells in 12 of 13 patients after treatment with a novel deoxyadenosine analog (clofarabine) combined with an alkylating agent (cyclophosphamide) (49). The same group also reported that the peripheral blood mononuclear cells from 23 patients with myelodysplasia (MDS), and high-risk acute myelogenous leukemia (AML) exhibited increased γH2AX levels after sequential administration of a DNA methyltransferase inhibitor (5-azacytidine) and a histone deacetylase (HDAC) inhibitor (Ectinostat) (50). However the latter data did not show any correlation between the patients' responses and γH2AX induction. Another clinical study demonstrated the ability of the minor groove binding agent SJG-136 to increase γH2AX levels in both the patients' lymphocytes and tumor biopsies (51). Fong et al. described the use of plucked-eyebrow hairs to follow patients treated with a PARP inhibitor. Their study showed a correlation between γH2AX levels and PARP inhibition (42). Finally, a trial testing the combination of a farnesyltransferase inhibitor (Tipifarnib) and a topoisomerase II inhibitor (etoposide) for individuals diagnosed with AML used a γH2AX assay on AML marrow blasts (52). This drug combination demonstrated genotoxicity by both the use of γH2AX and increased subdiploid DNA content.

γH2AX use in radiotherapy

In contrast to chemotherapeutic agents, ionizing radiation can induce DSBs regardless of cell cycle phase, thus, radiation induced-γH2AX could in principle be detectible in all tissues or cells including G0 lymphocytes and oral cells. Counting γH2AX foci has been used successfully for biodosimetry (36,53-56). After exposure γH2AX foci levels in patient lymphocytes and skin biopsies exhibited a good correlation with the radiation doses.

Additionally, γH2AX measurements might provide information useful in improving patient outcome during radiotherapy. Although treatment with radiation is commonly used, some

patients develop severe, possibly lethal, side effects. Patients with DSB repair deficiencies, such as ataxia telangiectasia caused by mutation in ATM gene, are highly sensitive to radiation exposure (57). Thus, γH2AX measurements may allow the identification of hypersensitive individuals in order to improve the efficacy of radiotherapy and to avoid therapeutic accidents (58,59).

It is important to note that therapeutic and accidental radiation exposures are rarely uniform and therefore can result in subpopulations of lymphocytes with different exposures, a phenomenon that should be taken into account for dose estimation. Moreover, because hypoxic microenvironments in solid tumors result in the decrease of the number of DSBs by radiotherapy (33), variations in γH2AX levels could occur in different tumor samples for a same irradiation dose.

γH2AX use in diagnostics

γH2AX also has potential uses in medical diagnosis (Table 2). Since both pre-cancerous and cancerous cells were found to exhibit increased genomic instability (60,61), a biomarker able to identify such cells in patients would be a useful diagnostic tool. In fact, it was shown that γH2AX can be used as a biomarker for cancer as tumor biopsies show increased γH2AX levels (62,63). γH2AX measurements also established high levels of DNA damage in colon biopsies of patients with ulcerative colitis, an inflammation disease linked to higher risk for colorectal cancer. A recent study showed the dependability and accuracy of γH2AX in the diagnosis of metastatic renal cell carcinoma (64).

PAR in clinical oncology

Several PARP inhibitors (at least eight: Iniparib/BSI-201, Olaparib/AZ2281, Veliparib/ABT-888, AG014699, MK-4827, CEP-8933/CEP-9722, INO-1001, GPI 21016) have been developed and are now used alone or combined with other drugs in several clinical trials (30). Because of the high homology between PARP1 and PARP2, most PARP inhibitors can target both enzymes and future studies will probably look for specific inhibitors (65). The first report of a PARP clinical trial using the drug BSI-201 alone in a phase I study came out in 2008 (66). Since, this drug has been used in more phase I trials in combination with other drugs, in phase II clinical studies and is now entering phase III study (67,68). Olaparib and veliparib/ABT-888 are also undergoing clinical development in several phase I and phase II trials. The National Cancer Institute conducted the first clinical pharmacodynamic trial (Phase 0) of ABT-888, an orally available small-molecule inhibitor of PARP in patients with advanced malignancies. PAR levels in tumor biopsies and peripheral blood mononuclear cells were measured by using a validated ELISA (32). The data obtained could be used to guide the design of several phase I combination studies of veliparib, including ongoing trials with topotecan and cyclophosphamide, each of which include measurement of PAR as a pharmacodynamic endpoint.

In these studies, it is crucial to take measurements at the in vivo cellular level, both for the drug effect on PARP activity (i.e. PAR levels) and the consequences of this inhibition (i.e. DNA damage induction via γH2AX detection). Recent years have seen the development of ELISA for PAR detection that can now replace the less reliable immunoassays (32,69) (Fig. 4D). Thus, PAR levels can be measured in both peripheral blood mononuclear cells (PBMCs) (mostly lymphocytes) and tumor biopsies. These measurements show a significant correlation between the effects of the PARP inhibitor in PBMCs and the tumor samples (32) raising the possibility that blood samples could be used as tumor surrogates to follow PARP inhibition.

Conclusions

The availability of biomarkers of DNA damage offers the opportunity to evaluate clinical cancer samples and determine their DDR status prior and during therapy. The systematic use of γH2AX and PAR in tumor samples in the absence of treatment may identify groups of patients with a particular prognosis. γH2AX and PAR may also be useful to evaluate the activity of novel drugs in the tumor and normal tissues in response to treatment, which could accelerate the drug triage process -- aiding go - no-go decisions, or lead compound selection, for example. Hopefully, incorporation of such biomarkers will eventually eliminate a substantial fraction of drugs that fail in Phase II-III clinical trials.

The related question is whether γH2AX and PAR could be developed beyond that of a tool for clinical trials, to a marker with utility in cancer therapy. Following treatment, assessment of γH2AX and PAR could allow identification of patients who had insufficient evidence of DNA damage so that different drugs could be selected, or strategies to increase drug exposure, change schedule, or improve activity could be identified. It is likely that γH2AX and PAR only represent the first generation of DDR biomarkers and that more sensitive and convenient biomarkers will be developed in the near future. Ultimately, one of the major challenges remains to sample tumors and to develop non-invasive detection procedures. In the meantime, it will be interesting to take advantage of analyses in circulating tumor cells.

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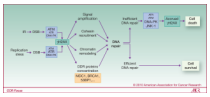

Figure 1. γH2AX following DSB formation

H2AX is phosphorylated by members of the phosphatidylinositol-3 kinase (PI3K) family; which one is involved depends on the type of genotoxic stress (4). While ATM and ATR are primarily involved in H2AX phosphorylation following IR and replication stress respectively, DNA-PK and JNK-1 were shown to be responsible for γH2AX formation during apoptosis (97,98). γH2AX foci are known to be involved in the recruitment and stabilization of DDR proteins including Mre11, Rad50, Nbs1 (the MRN complex), MDC1, 53BP1, BRCA1, ATM, and RNF8 (6,99-101). DSB repair is performed by the homologous recombination (HR) and non-homologous end joining (NHEJ) pathways. HR, driven by the *BRCA2, RAD51* and *RAD52/54* genes, is the more accurate because it utilizes a homologous DNA segment to act as a template for the damaged DNA region. Repair is also performed by sister chromatid-dependent recombination repair via cohesin recruitment (102,103). In contrast, NHEJ is faster, does not require a homologous DNA segment, and can operate in non-replicating cells. However, it is error-prone. The classical effectors of NHEJ are the end-binding proteins Ku70/80, DNA-dependent protein kinase (DNA-PKcs), the nuclease Artemis, the scaffolding protein XRCC4 and ligase IV. Recently, a slow DNA-PK-independent NHEJ pathway involving PARP1, histone H1, XRCC1 and ligase III has been proposed (24,25). The γH2AX foci are also involved in chromatin alteration via recruitment of remodeling complexes and in signal transduction (accrued ATM activation, G2/M cell cycle checkpoint). In addition, γH2AX foci, through their recruitment of the cohesions and the MRN complexes, are involved in binding and tethering the broken DNA ends may help prevent the dissociation of the broken chromosome ends (104).

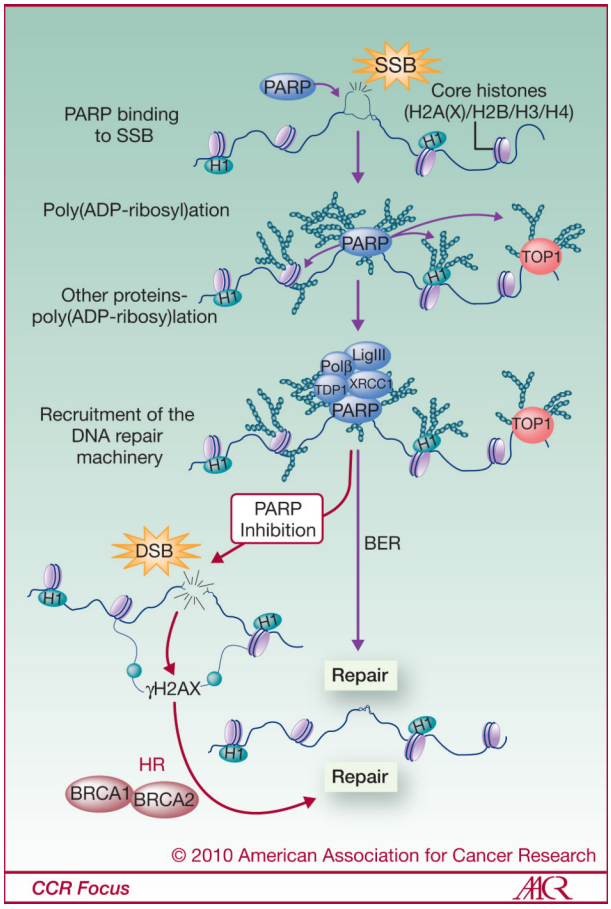

Figure 2. Schematic representation of PARP1's role in single-strand break repair

Upon detection of the DNA single-strand break (SSB) lesion, PARP1 is activated and, in turn, synthesizes PAR polymers attached to itself and other acceptor proteins at the DNA lesion site (Histone H1, other core histones, TOP1,...). These accrued post-translational modifications favor the recruitment of other factors involved in DNA repair, especially those involved in the base excision repair (XRCC1, Tdp1, Ligase III, Polβ). If left unrepaired, for example with PARP inhibition (red arrows), SSBs can lead to DSBs and γH2AX foci formation. DSB repair requires BRCA1/2, proteins that are deficient in many, including breast and ovarian, cancers.

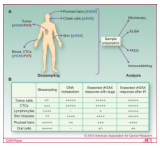

Figure 3. Human biopsy analysis

(A) Biopsy sites and methods used to analyze γH2AX levels and PARP activities. (B) Biosample accessibility, state of DNA metabolism, and expected γH2AX response. Note that the most accessible tissues for analysis are not always the most appropriate for measuring a drug response. For example, lymphocyte and oral cells, two highly differentiated cell types, may exhibit poor responses to chemotherapeutic drugs targeting DNA replication. In contrast, tumor cells, while clearly appropriate, are often poorly accessible particularly for repetitive sampling. γH2AX formation is independent of the cell cycle state, occurring in cancer cells as well as in lymphocytes and oral cells after irradiation (37,40,70). CTCs: circulating tumor cells.

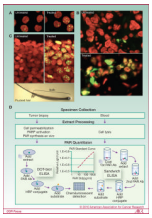

Figure 4. γH2AX and PAR detection

γH2AX detection in lymphocytes (A), tumor needle biopsies (B), and plucked hairs (C) from patients undergoing chemotherapy. The white box in the lower panel (C) marks the region of active γH2AX formation in a typical plucked hair. Green, γH2AX; red, DNA. (D) Pharmacodynamic assay developed at the National Cancer Institute to measure PAR as a biomarker for PARP inhibition in both tumor biopsies and peripheral blood mononuclear cells.

Table 1

Anticancer drugs that produce γH2AX

Note that all the anticancer drugs listed can also induce delayed γH2AX activation by apoptosis.

DRUGS	MECHANISM OF INDUCTION	REFERENCES
Bleomycin	Direct DSB – iron mediated oxidative cleavage	(70)
Camptothecins and Indenoisoquinolines	Indirect: conversion of SSB to DSB by replication	(12,71,72)
Doxorubicin, Etoposide, Mitoxantrone Batracylin	Direct DSB by trapping topoisomerase II cleavage complexes. Also indirect: ROS formation	(73-75)
Cytarabine, Gemcitabine, Hydroxyurea	Indirect: replication fork collapse (chain termination; deoxyribonucleotide pool depletion)	(76-78)
Cisplatin, Temozolomide Aminoflavone Trabectedin	Indirect: DNA alkylation	(79-82)
Imatinib (Gleevec®)	Indirect: apoptosis induced by Kit/PDGF tyrosine kinase inhibition	(83)
5-azacytidine SAHA (vironostat)	Indirect: epigenetic modifications	(84,85)
PARP and DNA-PK inhibitors) (see ref. 1 for PARPi)	Indirect: interference with SSB and DSB repair	(27,86,87)
SJG-136	Indirect: DNA alkylation	(53)
Tirapazamine	Indirect: ROS production in hypoxic cells	(73,88)
TRAIL	Indirect: Death receptors-mediated activation of DNA-PK	(89,90)
UCN-01 and AZD7762	Indirect: Checkpoint inhibitor potentiating IR- and replication-induced DNA damage by topoisomerase I inhibitors, cytarabine and gemcitabine.	(77,91,92)

Table 2
Some published Examples of clinical studies using γH2AX detection *in vivo*

Studies are listed in three separate groups: A. γH2AX used in clinical trials, B. γH2AX used for radiation biodosimetry, C. γH2AX used for diagnostics.

APPLICATION	TISSUE	STUDY DETAILS	METHOD	REF.
(A) γH2AX used in clinical trials				
Chemotherapy	PBMCs	Phase 1 study of clofarabine followed by cyclophosphamide for adults with refractory acute leukemias	FACS	(49)
Chemotherapy	PBMCs / tumor biopsies	A phase 1 study of SJG-136	Microscopy	(51)
Chemotherapy	AML marrow blasts	A phase 1 study of the combination of tipifarnib and etoposide for patients with AML	FACS	(52)
Chemotherapy	PBMCs	A phase 1 study of 5-azacytidine and entinostata for patients with MDS, chronic myelomonocytic leukemia, and AML	Immunoblotting	(50)
Chemotherapy	Plucked eye-brows	A phase 1 study of olaparib	Microscopy	(42)
(B) γH2AX used for radiation biodosimetry				
Computed tomography	PBMCs	DNA damage measured after multi-detector row CT	Microscopy	(93)
radiotherapy	PBMCs	DNA damage measured in cancer patients after local radiotherapy to different sites of the body	Microscopy	(54)
radiotherapy	Skin	DNA damage measured in prostate cancer patients undergoing radiotherapy with curative intent	Microscopy	(53)
X-ray examination	PBMCs	DNA damage measured after cardiac catheterization	Microscopy	(94)
X-ray examination	PBMCs	DNA damage measured after coronary CT angiographic procedure	Microscopy	(95)
X-ray examination	PBMCs	DNA damage measured after angiographic procedure	Microscopy	(56)
X-ray examination	PBMCs	DNA damage measured after percutaneous transluminal angioplasty	Microscopy	(55)
(C) γH2AX used for diagnostics				
Diagnosis	Tumor biopsies	Diagnosis of metastatic Renal Cell Carcinoma	Microscopy	(64)
Diagnosis	Tissue biopsies	Monitoring DNA damage in Ulcerative Colitis	Immunoblotting	(96)
Radiosensitivity diagnosis	T-cells and lymphoblast oid cell lines and/or PBMCs	Confirmation of radiosensitive A-T patients	FACS	(58)