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REVIEW ARTICLE

Validation of the γH2AX biomarker for genotoxicity assessment: a review

B. Kopp[1,2] · L. Khoury[3] · Marc Audebert[1,4]

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Abstract

The H2AX histone protein is rapidly phosphorylated at the serine-139 position (γH2AX) in response to a broad range of DNA lesions. γH2AX induction is one of the earliest events in the DNA damage response (DDR) and plays a central role in sensing and repairing DNA damage. Since its discovery, measuring γH2AX formation using numerous methods in in vitro and in vivo experiments has been an attractive endpoint for the detection of genotoxic agents. Our review focuses on validation studies performed using this biomarker to detect the genotoxicity of model chemicals using different methods. To date, nearly two hundred genotoxic and carcinogenic model chemicals have been shown to induce in vitro γH2AX in different cell lines by numerous laboratories. Based on 27 published reports comprising 329 tested chemicals, we compared the performance of the γH2AX assay with other genotoxic endpoints (Ames assay, micronucleus, HPRT and comet) regularly used for in vitro genotoxicity assessment. Notably, the γH2AX assay performs well (91% predictivity) and efficiently differentiates aneugenic and clastogenic compounds when coupled with the pH3 biomarker. Currently, no formal guidelines have been approved for the γH2AX assay for regular genotoxicity studies, but we suggest the γH2AX biomarker could be used as a new standard genotoxicity assay and discuss its future role in genotoxicity risk assessment.

Keywords γH2AX · pH3 · Genotoxicity · DNA damage · Micronucleus · Biomarker

Introduction

General mechanisms of γH2AX formation

H2AX is a member of the histone H2A family, one of the five families of histones that package and organize eukaryotic DNA into chromatin. DNA is wrapped around a core histone molecule forming the nucleosome complex consisting of eight individual histone 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 two nucleosomes (Flaus 2011). The H2A protein family has the most variants including H2A1, H2A2, H2AX and H2AZ and many others (Ausió and Abbott 2002; Kuo and Yang 2008). In human cells, 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 (Bártová et al. 2008; Bonner et al. 2008).

H2AX contains a single serine in its C-terminal tail that is highly conserved from plants to humans, suggesting a crucial role throughout evolution (Siddiqui et al. 2013). In 1998, Dr Bonner's group at NIH reported the phosphorylation of H2AX at serine 139 in human cells (γH2AX) a few minutes after DNA damage induced by ionizing irradiation (Rogakou et al. 1998). This phosphorylation event requires the activation of the phosphatidylinositol-3-OH-kinase-like family of protein kinases (PIKKs), ATM (ataxia telangiectasia mutated), ATR (ATM and Rad3-related) or DNA-dependent

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✉ Marc Audebert
marc.audebert@inra.fr

1 Toxalim, Université de Toulouse, INRA, INP-ENVT, INP-EI-Purpan, Université de Toulouse 3 Paul Sabatier, Toulouse, France

2 ANSES, French Agency for Food, Environmental and Occupational Health and Safety, Toxicology of Contaminants Unit, Fougères, France

3 PrediTox, Toulouse, France

4 INRA-UMR1331, Toxalim, 180 Chemin de Tournefeuille BP 93173, 31027 Toulouse Cedex 3, France

protein kinase (DNA-PKcs), and serves as a landing pad for the accumulation and retention of the central components of the DNA damage response (DDR). In accordance with the percentage of representation among H2AX variants in the chromatin, it was estimated that a single DSB causes H2AX phosphorylation to spread over up to two Mbp regions of chromatin, comprising nearly 2000 γH2AX molecules (Kinner et al. 2008; Rogakou et al. 1998).

Role of γH2AX in DDR

γH2AX is considered as a critical player in DDR (Fig. 1), able to induce signals for both the DNA damage sensitive cell cycle checkpoints and the DNA repair proteins (Niida and Nakanishi 2006). Today, how DNA damage is detected by the cellular machinery is still not fully understood, but the interaction between γH2AX and a mediator of DNA damage checkpoint protein 1 (MDC1) is recognized as one of the first steps during which the site of the DSB is prepared for DNA damage signaling and repair (Lamarche et al. 2010). γH2AX promotes stable accumulation of many other signaling and repair proteins including 53BP1 and BRCA1 at DSB sites (Yan et al. 2011). In this way, γH2AX could be considered as a sensor, present in the initial recognition checkpoints of DNA damage (Fig. 1).

The signals initiated by γH2AX and other sensors are transmitted to kinases, transducer proteins that regulate several effector molecules, notably the p53 tumor suppressor protein and the cdc25 family of phosphatases (Fig. 1) (Niida and Nakanishi 2006). Among transducers, ATM kinase is considered to be one of the main physiological mediators of H2AX phosphorylation in response to DSB formation. In turn, this induces the formation of a tri-complex called MRN complex (MRE11-RAD50-NBS1) that plays a critical role in the cellular response to DNA damage and in the maintenance of chromosomal integrity (Kobayashi et al. 2002; Zhang et al. 2006). H2AX can also be phosphorylated by ATR, particularly in response to DNA replication stress.

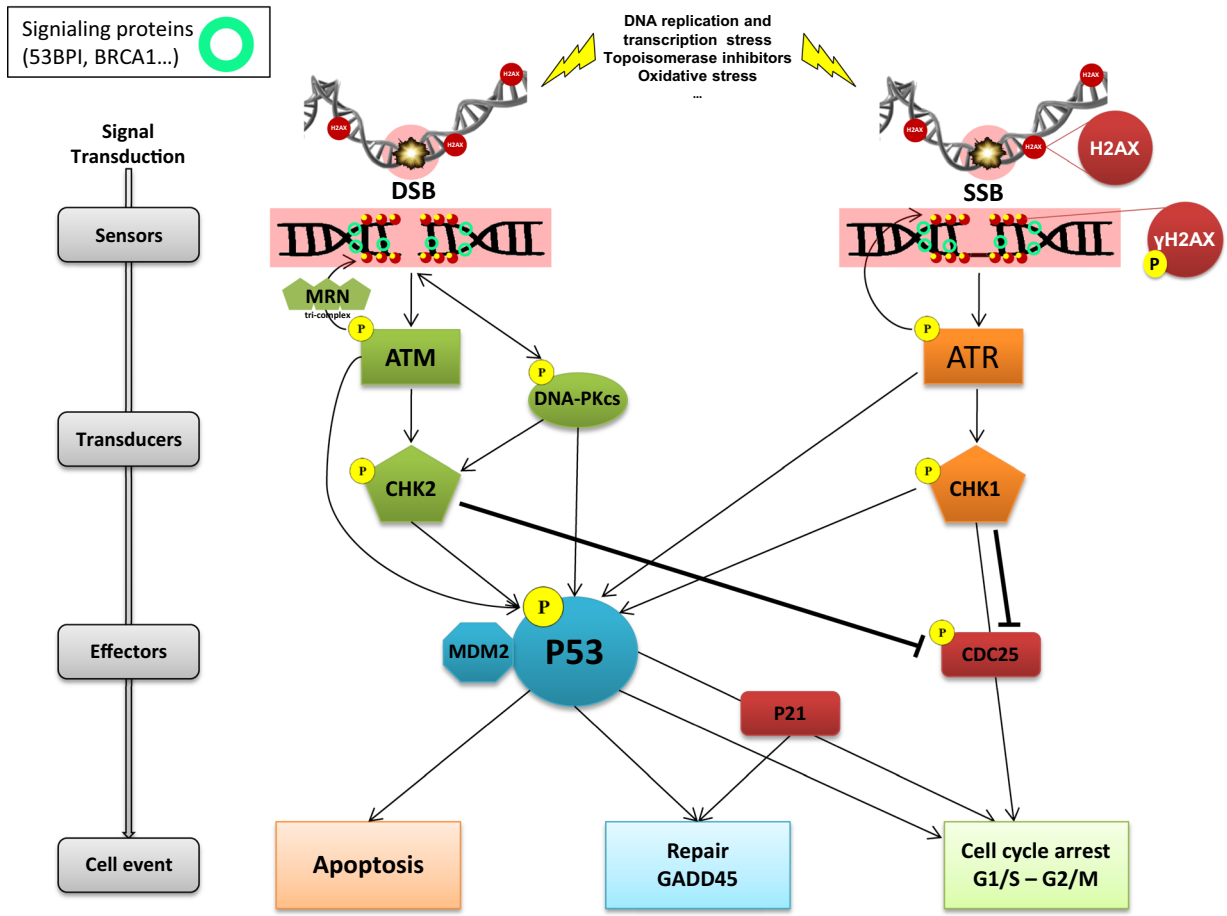

Fig. 1 Conceptual representation of the signal transduction and of the central role of γH2AX in DNA damage responses. DNA damage is rapidly recognized by sensors (H2AX) and transduced to transducers (ATM, ATR, Chk1, and Chk2). Checkpoint kinases transfer signals to effector molecules (p53, p21, cdc25) that play a central role in the cell's decision to undergo either cell cycle arrest, apoptosis or DNA repair

Finally, γH2AX accumulation attracts repair factors, increasing the concentration of repair proteins surrounding a DSB site and insures DDR (Kastan and Lim 2000; Mukherjee et al. 2006; Podhorecka et al. 2010; Ward and Chen 2001).

Usefulness of γH2AX in different scientific fields

Following spontaneous or induced double-strand breaks (DSBs) by a large type of DNA lesion, activation of the DDR has an evolutionary role in maintaining genome integrity and limits the occurrence of cancer (Bonner et al. 2008; Xiaofei and Kowalik 2014). Two major pathways for repairing DSBs are present in the cell: homologous recombination and non-homologous DNA end joining (NHEJ) (Lieber 2010). NHEJ DNA repair can result in misannealing of broken DNA ends and consequently in chromosomal changes (Audebert et al. 2004; Wang et al. 2005). Therefore, measuring the level of γH2AX is of particular interest to the research community because it can predict the response to genotoxic insult in cells. Moreover, universal conservation of the DNA damage response throughout eukaryotic evolution suggests that γH2AX could be useful for studies in many scientific fields (Redon et al. 2011). Since its discovery in 1998, the γH2AX biomarker has been widely used in many fields including cancer therapy, drug development, environmental and genetic studies (Bonner et al. 2008).

Genotoxicity and current in vitro regulatory assays

DNA is the central store of genetic information and constantly incurs damage caused by chemicals resulting from internal cell metabolism, but also by a range of exogenous compounds such as environmental contaminants, compounds produced during the heating and processing of foodstuffs, mycotoxins or certain secondary plant metabolites (Jeggo and Löbrich 2007; Kuo and Yang 2008). Genotoxicity depends on the ability of a chemical to damage the genetic information within a cell resulting in mutations that may lead to malignancy (Seukep et al. 2014).

Assessing genotoxicity is an essential component of the safety assessment of all types of substances (Corvi and Madia 2017). Although a variety of in vitro assays have been reported to successfully predict chemical genotoxicity in recent decades, only a small number of them have been accepted for regulatory purposes. These assays were validated by the Organization for Economic Co-operation and Development (OECD) guidelines that describe correct ways to conduct tests and are currently used to detect mutation and damage to DNA in a number of fields (OECD 2014). A wealth of literature is available on each of the standard in vitro genotoxicity assays, including the bacterial reverse mutation assay (OECD TG 471), the in vitro mammalian chromosomal aberration test (OECD TG 473), the in vitro mammalian cell gene mutation test (OECD TG 476 [Hprt] and TG 490 [MLA/tk]), and the in vitro mammalian cell micronucleus (MN) test (OECD TG 487). Although these regulatory assays have the power to detect potential genotoxic agents, they are not always sufficiently specific, due to the high incidence of "misleading" or "irrelevant" positive results that are not observed in in vivo studies (Kirkland 2011). In fact, increasing the specificity of in vitro assays currently used to assess genotoxicity is a major challenge that can be addressed either by optimizing conventional tests, or by including novel short-term assays (Kirkland 2011). Automation and miniaturization enabled by high-throughput technologies could provide more information on the genotoxic mode of action (MoA), and differentiate aneugen and clastogen genotoxic chemicals, which is mandatory in safety assessments.

Validation of the in vitro γH2AX genotoxicity assay

γH2AX is an early sensitive genotoxic biomarker induced by various types of DNA lesions: DNA double-strand breaks DNA bulky adducts, DNA single-strand breaks, DNA replication or transcription blocking lesions (DNA oxidation and alkylation), and is a powerful tool to monitor DNA damage in translational cancer research (Dudáš and Chovanec 2004; Kuo and Yang 2008; Mehta and Haber 2014; Sedelnikova et al. 2010). Consequently, some authors proposed the γH2AX biomarker as an alternative for genotoxicity screening in regulatory assessment (Audebert et al. 2010; Mishima 2017; Motoyama et al. 2018; Nikolova et al. 2014; Watters et al. 2009) (Table 1). The γH2AX assay was developed in the laboratory of the authors of this paper in a miniaturized format based on a 96-well plate for use in high-throughput systems (Audebert et al. 2010). Miniaturization of the technology for genotoxicity testing is particularly advantageous for screening or in the early stages of drug development because of small volume of samples and the limited availability of test compounds.

Original method and advanced techniques

Originally, Rogakou et al. (1998) used two-dimensional gel electrophoresis to detect γH2AX in mammalian cell cultures or cells from mice after exposure to ionizing radiation. Two-dimensional gel electrophoresis is one of the basic methods used to separate and quantify histone mixtures. Results demonstrated that γH2AX appears about 10 min after DNA damage in a quantifiable manner. After this initial development, the same team suggested using antibodies specific to

Table 1 In vitro genotoxicity validation studies with the γH2AX with or without the pH3 assay

References	Cell lines	Compounds tested			Performance assessments			Technique used
		Genotoxic		Non-genotoxic	Specificity (%)	Sensitivity (%)	Accuracy (%)	
		Aneugens	Clastogens					
Banath and Olive (2003)	V79	0	6	0	nd	100	100	Flow cytometry
Zhou et al. (2006)	FL and CHL	0	5	1	100	80	83	Immunofluorescent microscopy
Watters et al. (2009)	L5178Y, MEFs	0	3	2	100	100	100	Flow cytometry and microscopy
Matsuzaki et al. (2010)	CHL, CHO and V79	6	8	1	100	100	100	ELISA
Smart et al. 2011	L5178Y	0	26	10	90	96	94	Flow cytometry
Magkoufopoulou et al. (2011)	HepG2	0	4	3	100	100	100	Flow cytometry
Audebert et al. (2012)	HepG2, LS174T	0	10	3	100	100	100	Flow cytometry
Tsamou et al. (2012)	HepG2	0	34	30	93	62	76	Flow cytometry
Garcia-Canton et al. (2013)	BEAS-2B	0	12	8	100	92	95	HCS (immunostaining and detection by automated fluorescence) microscopy
Khoury et al. (2013)	HepG2	2	21	38	95	74	87	In-Cell Western (ICW)
Nikolova et al. (2014)	CHO-9	0	14	10	100	100	100	Flow cytometry and microscopy
Ando et al. (2014)	HepG2	3	9	14	86	92	88	HCS
Bryce et al. (2014)	TK6	11	18	11	100	90	92	Flow cytometry
Cheung et al. (2015)	TK6	1	1	1	100	100	100	Flow cytometry
Bernacki et al. (2016)	TK6	0	11	37	97	82	94	Flow cytometry
Quesnot et al. (2016)	HepaRG	0	6	1	100	100	100	ICW
Khoury et al. (2016b)	HepG2, LS174T, ACHN, Hep3B	0	43	2	0	93	89	ICW
Bryce et al. (2016)	TK6	14	31	39	97	93	95	Flow cytometry
Khoury et al. (2016a)	HepG2, LS174T, ACHN	16	8	8	88	96	94	ICW
Chevereau et al. (2017)	V79 and V79 derived	0	13	3	100	100	100	ICW
Ji et al. (2017)	HT29	0	4	3	100	100	100	ELISA

Table 1 (continued)

References	Cell lines	Compounds tested			Performance assessments			Technique used
		Genotoxic		Non-genotoxic	Specificity (%)	Sensitivity (%)	Accuracy (%)	
		Aneugens	Clastogens					
Wilde et al. (2017)	TK6	9	11	11	100	85	90	Flow cytometry
Bryce et al. (2017)	TK6	13	33	38	97	98	98	Flow cytometry
Bryce et al. (2018)	TK6	4	36	14	93	92	93	Flow cytometry
Kopp et al. (2018a)	HepG2	1	9	0	nd	100	100	ICW
Takeiri et al. (2019)	TK6	0	9	4	100	100	100	HCS
Dertinger et al. (2019)	TK6	12	14	14	93	92	92	Flow cytometry

γH2AX to study the biomarker (Rogakou et al. 1999). Today, quantification of the γH2AX biomarker relies on antibodies raised against the histone H2AX phosphorylated at serine 139, enhancing the sensitivity and visibility of γH2AX foci (Table 1). Many manufacturers provide specific antibodies raised against γH2AX.

Western blotting can detect γH2AX, but is limited to distinguishing subtle differences in γH2AX levels and is not ideal for large numbers of samples (Reddig et al. 2018). In 2006, Zhou et al. were the first to evaluate genotoxicity with the γH2AX assay using immunofluorescence microscopy. These authors evaluated the ability of a set of model chemicals to induce γH2AX, including direct and bioactivated genotoxins in human amnion FL cells and Chinese hamster CHL cells. The formation of γH2AX foci was found to be a good indicator of genotoxicity, and their results correlated well with those of the neutral comet assay. The authors then assessed the sensitivity of γH2AX compared to that of the comet assay, and the best cell line to use to evaluate DNA damage.

High-throughput evaluation of γH2AX

Flow cytometry makes it possible to rapidly quantify γH2AX in large cell populations (Albino et al. 2004; Smart et al. 2011). Additional methods using flow cytometry that facilitate simultaneous quantitative analysis of cytotoxicity, perturbations of the cell cycle and induction of aneuploidy are also described in the literature (Bryce et al. 2007, 2014, 2016, 2017, 2018). Flow cytometry detects the fluorescence of γH2AX per nucleus (Bryce et al. 2007, 2014, 2016, 2017, 2018; Smart et al. 2011; Tsamou et al. 2012). To evaluate the effectiveness of flow cytometry for γH2AX detection, Smart et al. (2011) tested 31 compounds including genotoxic chemicals with different modes of action. To confirm the sensitivity of the method, γH2AX results were compared with those obtained with the Ames bacterial assay, the mouse lymphoma assay and/or chromosome aberration assay using the L5178Y cell line. Results of the validation set showed that the quantification of γH2AX by flow cytometry was highly predictive of Ames positive compounds (94% agreement). Smart et al. also demonstrated that the sensitivity (96%) and specificity (90%) of the γH2AX assay by flow cytometry were comparable with other in vitro mammalian genotoxicity assays. In another study, Tsamou et al. (2012) also concluded that γH2AX quantification by flow cytometry is a promising genotoxicity assay. In their study, HepG2 cells were exposed to 64 selected compounds with known genotoxic properties. Their results demonstrated that γH2AX quantification was more accurate than that achieved with other combinations of in vitro and in vivo assays (Ames test, mouse lymphoma assay and chromosomal aberration/micronucleus test) and that the γH2AX assay is thus a useful, rapid and cost-effective cell-based tool for early screening of compounds for in vivo genotoxicity. The relatively low sensitivity of the method (62%) reported by these authors may be linked to the fact that they only tested one concentration of each compound.

High content screening (HCS) is a new automated microscopy method currently under development. HCS enables the rapid and highly accurate analysis of large numbers of experimental data points (Hopp et al. 2017). After acquisition of cell images, image analysis software examines different endpoints. Ando et al. (2014) treated HepG2 cells with a panel of compounds, including several known genotoxic agents, and examined γH2AX formation using the HCS method. Their results showed the sensitivity of the assay 24 h after exposure to be 100%. In parallel, other ways of optimizing γH2AX detection using the HCS method were underway. The results obtained with a panel of

well-characterized genotoxic and non-genotoxic compounds in the human bronchial epithelial cell line BEAS-2B indicated high accuracy (95%), sensitivity (92%) and specificity (100%) of the in vitro γH2AX assay by HCS (Garcia-Canton et al. 2013). A recently published study using metabolically competent HepaRG cells for HCS for genotoxicity with automated detection of γH2AX demonstrated the genotoxicity of aflatoxin B1, benzo[a]pyrene (BaP), 7-12-dimethylbenzanthracene (DMBA), fipronil and endosulfan and confirmed the suitability of γH2AX assay for genotoxicity screening in this cell line (Quesnot et al. 2016).

A variation of the γH2AX assay with HCS was developed and the first validation of this biomarker in HepG2 cells with the 61 compounds recommended by the European Center for the Validation of Alternative Methods (ECVAM) was performed by Khoury et al. in 2013. Their high-throughput genotoxicity assay, named γH2AX In-Cell Western (ICW), is highly sensitive and specific, and allows the simultaneous examination of cytotoxicity and genotoxicity (Audebert et al. 2010). Using an anti-γH2AX antibody and DNA labeling, both DNA and γH2AX fluorescence in each well of the microplate were simultaneously quantified using an Odyssey Infrared Imaging Scanner (Audebert et al. 2010; Graillot et al. 2012a, b; Khoury et al. 2013, 2016a, b; Kopp et al. 2018a, b). The advantage of this technique over HCS is that cell analysis software is not required (notably avoiding the problem of nuclear segmentation) and is less expensive than flow cytometry and HCS techniques.

The γH2AX In-Cell Western method has the advantage of allowing high throughput (thanks to the use of multi-well plates) and can be used with numerous in vitro cell models (Audebert et al. 2010; Graillot et al. 2012a, b; Khoury et al. 2016a, b). In contrast to flow cytometry, which is generally performed with cells in suspension like the TK6 cell line, the ICW technique can be easily performed with either adherent cells or cells in suspension, which is very useful when screening compound genotoxicity in different cell line models (Graillot et al. 2012b). The use of several human cell lines originating from different tissues involved in chemical biotransformation, including the liver and the intestine, is highly recommended to properly investigate the role of metabolic activation without the need to use the rodent liver S9 fraction (Guengerich 2000; Kirkland et al. 2016). HepG2 and HepaRG cells (from liver) or the LS-174T human cell line (from the colon) are known to exhibit significant functional phase I and II bioactivation capabilities and can be used in the γH2AX ICW assay (Khoury et al. 2013, 2016a, b; Quesnot et al. 2016).

γH2AX and cell apoptosis

Because γH2AX induction can also result from apoptosis (Luczak and Zhitkovich 2018), cytotoxicity could be a confounding factor in genotoxicity analysis with the γH2AX test, as is the case in some other regular in vitro assays (MN or comet). It is recommended to limit the cytotoxicity to 50% to differentiate true genotoxicity from false-positive genotoxic results due to apoptosis (Fellows and O'Donovan 2007; Khoury et al. 2013). Indeed, the results of many studies performed using flow cytometry or ICW techniques with non-genotoxic chemicals that induce apoptosis identified no false-positive compounds with the γH2AX biomarker at sub-toxic concentrations. Notably, with the ICW technique, Khoury et al. 2013 observed no false-positive classifications using the second ECVAM list of compounds that includes cytotoxic chemicals. Likewise, using flow cytometry analysis, and excluding highly fluorescent γH2AX-positive events caused by apoptotic cells from the analysis, Bryce et al. observed no false-positive results for non-genotoxic chemicals at sub-toxic concentrations in several studies (Bryce et al. 2007, 2014, 2016, 2017, 2018; Dertinger et al. 2019). A very recent study by Moeglin et al. (2019) demonstrated that uniform widespread nuclear phosphorylation of histone H2AX was an indicator of lethal DNA replication stress. Moreover, as H2AX phosphorylation is an indicator of early DNA damage, damage can be assessed at an early time point, thereby avoiding false-positive results due to apoptosis induction (Dertinger et al. 2019).

Comparing the performance of γH2AX with that of other genotoxicity assays

Westerink et al. (2011) also evaluated the compounds recommended by ECVAM in HepG2 cells using the in vitro MN assay. Their comparison of the sensitivity and specificity of the MN assay and of the ICW γH2AX assay showed that the γH2AX assay was more efficient in detecting genotoxic compounds. Khoury et al. (2013) demonstrated that five compounds (ENU, 2-AAF, cadmium chloride, hydroquinone and AZT) were not correctly detected as genotoxic by the MN assay, whereas the chemicals induced γH2AX. Moreover, for eight compounds shown to be positive in both assays, the γH2AX assay had a lower observed effect concentration (LOEC). In particular, the LOECs for DMBA and BaP determined by the γH2AX assay were 100-fold lower than those determined by the MN assay.

In a recent study, Kim et al. (2016) examined the DNA-damaging effect of four direct/indirect genotoxins in the HepG2 cell line using the comet assay and the γH2AX assay. Results indicated that the two assays assessed DNA damage caused by ethyl methanesulfonate (EMS), *N*-methyl-*N*-nitrosourea (MNU), ultraviolet C (UVC) irradiation and benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE) with different sensitivity. Lower EMS and MNU concentrations induced γH2AX foci in a dose-dependent manner with tenfold higher sensitivity than the alkaline comet assay. Likewise, Nikolova

et al. in 2014 concluded that the γH2AX assay is more reliable, sensitive, and robust than the comet assay for detecting genotoxic chemicals. Chevereau et al. (2017) investigated the genotoxic potential of several heterocyclic aromatic amines using the ICW γH2AX assay in V79 cells and three V79-derived cell lines. For seven HAAs, it was possible to compare the results of the γH2AX assay with those of the Hprt mutagenicity assay previously performed using the same cell lines (Glatt 2003). The comparison demonstrated that ICW γH2AX was more sensitive than the gene mutation assay. Very recently, Dertinger et al. (2019) demonstrated a perfect correlation between γH2AX and MN inductions for clastogenic compounds.

Compared with the in vitro MN test, like comet, the γH2AX assay does not need to wait for an entire cell cycle to be revealed. γH2AX was consequently detected as early as few minutes after DNA damage induction (Rogakou et al. 1998). The γH2AX assay can also be easily performed in non-cycling cells (like liver HepaRG cell line), because DNA lesions that induce DNA transcription arrest also cause γH2AX induction (Quesnot et al. 2016).

Complementarity of γH2AX with other endpoints

Recently published recommendations for the assessment of chemical genotoxicity suggested that new in vitro assays should be developed to enable more rapid and effective determination of genotoxic MoA (Kirkland 2011; Kirkland et al. 2016). Clastogens (DNA damage leading to point mutations and/or structural chromosomal mutations) and aneugens (chromosome loss or gain) are key classes of genotoxic agents. In chemical risk assessment, differentiating aneugenic and clastogenic effects is essential to assess the genotoxic properties of compounds (Hernández et al. 2013). The comet assay cannot efficiently detect aneugenic chemicals (Sykora et al. 2018). In contrast, the in vitro MN assay and the γH2AX biomarker are able to efficiently detect clastogenic and aneugenic compounds, in the case of γH2AX, linked to the chromothripsis phenomenon (Khoury et al. 2016a, b).

Following the study by Bryce et al. (2014), Khoury et al. (2016b) proposed a method to efficiently differentiate aneugenic and clastogenic chemicals using the γH2AX biomarker combined with the phosphorylated histone H3 at the serine 10 (pH3) biomarker in different cell lines. Histone H3 is phosphorylated at Ser 10 by the aurora kinase family to enable chromosome condensation and segregation during mitosis and is a specific biomarker of mitotic cells (Prigent and Dimitrov 2003). Like for γH2AX, specific antibodies from different manufacturers can be used for the quantification of pH3. In the study by Khoury et al., three human cell lines (HepG2, LS-174T and ACHN) were exposed to model chemicals: 10 aneugenic, 5 clastogenic, 5 non-genotoxic chemicals and a test set of 13 chemicals whose genotoxic potential is the subject of debate. The study was based on the combination of γH2AX and pH3 biomarkers and not only efficiently differentiated all genotoxic chemicals but also gained insight into the genotoxic MoA and metabolic activation requirements (Khoury et al. 2016a). In the same way, 67 model chemicals were tested in TK6 cells over a 4 h and a 24 h period using the MultiFlow™ DNA Damage Kit comprising γH2AX and pH3 biomarkers by flow cytometry (Bryce et al. 2016). Results demonstrated that 95% of chemicals were correctly classified in agreement with a priori prediction of their genotoxic potential. Finally, the complementarity of γH2AX/pH3 biomarkers has been further demonstrated in different studies and qualified as a rapid and efficient method for screening the genotoxic potential of chemicals (Bernacki et al. 2016; Bryce et al. 2017, 2018; Dertinger et al. 2019; Wilde et al. 2017).

Kopp et al. (2018a) investigated γH2AX and several other proteins involved in the DNA damage response signaling pathway after DNA damage to find out if a specific biomarker or combination of biomarkers could differentiate mechanisms of clastogen action in the HepG2 cell line. Most of the results obtained with the biomarkers tested were linked to the specific DNA-damaging properties of the individual compounds tested and their repair. Notably, data confirmed that the combined analysis of γH2AX, pH3 and the phosphorylated form of the p53 protein at Ser 15 (p-p53) is an efficient way to differentiate aneugens, clastogens, and non-genotoxic chemicals. However, the panel of cellular biomarkers did not make it possible to find specific biomarker signatures of the mechanism of clastogenic action.

Interlaboratory evaluation of the γH2AX assay

The γH2AX assay had been shown to be a rapid, robust and semi-automatic assay to detect DNA-damaging agents, and γH2AX can be quantified using several methods (Table 1). Different teams have tested the genotoxicity of many compounds using the γH2AX assay in the last decade (see Supplementary data). After comparing all these experiments, we concluded that, independently of the experimental design (cell type, detection method, etc.) γH2AX induction observed with model chemicals was similar. For example, in numerous independent experiments, very similar time and concentration responses of γH2AX induction have been observed for two model genotoxic chemicals, (BaP and etoposide), whatever the methods or the cell lines used for quantification (Audebert et al. 2010; Matsuzaki et al. 2010; Smart 2008; Smart et al. 2011; Watters et al. 2009; Yan et al. 2011; Zhou et al. 2006). However, as expected, some differences between cell lines have been observed with compounds that require bioactivation to induce genotoxicity

(Audebert et al. 2010; Chevereau et al. 2017; Khoury et al. 2013, 2016a).

In a recent study, Bryce et al. 2017 reviewed experiments conducted in 7 independent laboratories using a list of 84 chemicals representing clastogenic (33), aneugenic (13), and non-genotoxic chemicals (38) with the γH2AX/pH3 biomarkers. All the experiments were conducted with TK6 cells exposed to chemicals in 96-well plates at a wide range of concentrations for 24 h. First, the ensemble of all 231 experiments was ≥ 98% in agreement with a priori MoA groupings, thereby confirming the high specificity and sensitivity of this assay. Second, the efficiency of the multiplex genotoxicity assay based on γH2AX and pH3 was transferable across laboratories, thereby attesting to the reproducibility of results between participating laboratories. All these data confirm both the robustness and the usefulness of γH2AX/pH3 assay for the rapid screening of chemicals.

We performed a detailed analysis of all the results described in the 27 studies (listed in Table 1 (and in Supplementary data), including 329 independent compounds: 35 aneugenic, 152 clastogenic, 7 aneugenic/clastogenic and 135 non-genotoxic chemicals. We demonstrated a sensitivity of 95% (128/135), a specificity of 87.5% (170/194) and a predictivity of 91% (300/328) of the γH2AX/pH3 assay. We noted that eight compounds (out of 24) that were not correctly classified as genotoxic had been tested in only one study and at only one concentration (Tsamou et al. 2012). When these uncertain data were excluded, the overall specificity of the assay increased to more than 92%. Moreover, we noted that in the case of four chemicals out of seven, i.e., diazinon, ethyl acrylate, gefitinib and WY-166443, incorrectly classified as non-genotoxic, different studies did demonstrate their carcinogenic potential. These last results highlight the fact that this assay may permit to detect non-genotoxic carcinogen.

In vivo γH2AX genotoxicity studies

Despite the fact that numerous studies have demonstrated the expression of γH2AX in different precancerous and cancerous cells in human (Bonner et al. 2008), few studies have validated this biomarker in experimental animals. The most frequently validated in vivo tests to detect genotoxicity in rodents are MN and the comet assay (Table 2). Using standard and novel genotoxic assays, such as γH2AX, micronucleus, comet and PigA, the genotoxicity of the carcinogenic 4-chloro-ortho-toluidine (4-CloT) was consequently evaluated in different male rat tissues (Guerard et al. 2018). Immunohistochemistry of γH2AX was performed on liver sections collected from all the animals and γH2AX positive nuclei were observed. Similar results were obtained with the γH2AX and the comet assay. Another recent study with mitomycin C (MMC) rat treatment concluded that γH2AX is an appropriate biomarker to quantify DNA damage in vivo, but further studies are required for optimization and validation (Motoyama et al. 2018).

Studies were also performed using the kidney carcinogenic mycotoxin ochratoxin A (OTA). In the first study, exposure to OTA-induced DNA damage was followed by an increase in mutant frequencies of the *red/gam* gene at the renal outer medulla, the carcinogenic target site in *gpt* delta rats via oral contamination (Hibi et al. 2011). Other studies clearly revealed γH2AX induction by OTA in a dose-dependent manner in *gpt* delta rats and in mice (Kuroda et al. 2014, 2015).

To give an example of in vivo evaluation of the performance of γH2AX assay, four studies were recently published that included quantitative analysis of the γH2AX biomarker in multiple rodent organs after exposure to model carcinogenic compounds (Table 2). In the first study, ICR mice were treated with MMC and EMS (Matsuda et al. 2018). In the second study, male Wistar rats were exposed to EMS, ENU and doxorubicin (Plappert-Helbig et al. 2019). In the third study, male F344 rats were administered monocyclic aromatic amines (Toyoda et al. 2019). In these studies, all the carcinogenic compounds were correctly predicted as being genotoxic in multiple organs with the γH2AX biomarker. Very interestingly, Sone et al. (2019) reported that the γH2AX biomarker was associated with urinary carcinogenesis in a species-specific manner. These studies concluded that for the evaluation of in vivo genotoxic effects, γH2AX is an ideal test to supplement the existing package for genotoxicity testing. Further studies are required with more model chemicals with different MoA to assess the predictivity of the γH2AX biomarker as complement to the standard battery of in vivo tests.

Conclusions

This review shows that today the γH2AX biomarker is widely used by many researchers to measure DNA damage in in vitro genotoxicity screening, because it is a sensitive and specific tool. Moreover, compared to the other regulatory in vitro genotoxic assays (Ames, MN or comet), the combination of the γH2AX and pH3 biomarkers is more predictive and is the only assay that can enable a rapid and effective identification of genotoxic MoA (i.e., to differentiate clastogenic/gene-mutagenic and aneugenic action). In addition, the assay appears to be robust and reproducible irrespective of the technique (ICW, HCS, flow cytometry) or the standard cell lines used for genotoxicity assessment (e.g. HepG2, TK6, L5178Y, HepaRG). These criteria comply with OECD guidelines (concurrent negative controls, positive

Table 2 In vivo genotoxicity validation studies with the γH2AX assay

Compound	CAS number	Rodent model	Organs	Results	References
N-butyl-*N*-(4-hydroxybutyl)-nitrosamine (BBN)	3817-11-6	Rat (F344)	Urinary bladder	+	Toyoda et al. (2013)
N-butyl-*N*-(4-hydroxybutyl)-nitrosamine (BBN)	3817-11-6	Rat (F344)	Urinary bladder	+	Toyoda et al. (2015)
2-Nitroanisole (2-NA)	100-17-4			+	
2-Acetylaminofluorene (2-AAF)	53-96-3			+	
p-Cresidine	120-71-8			+	
2,2-Bis(bromomethyl)-1,3-propanediol	3296-90-0			+	
Phenethyl isothiocyanate	2257-09-2			+	
Dimethylarsinic acid	75-60-5			+	
Melamine	108-78-1			+	
Uracil	200-621-9			+	
Glycidol	556-52-5			–	
N-nitrosodiethylamine (DEN)	55-18-5			–	
Acrylamide	79-06-1			–	
Aminomethylphenylnorharman (AMPNH)	nd	Rat (F344)	Urinary bladder	+	Toyoda et al. (2018)
Aminophenylnorharman (APNH)	219959-86-1			+	
4-Chloro-ortho-toluidine (4-CloT)	3165-93-3	Rat (Wistar)	Liver	+	Guerard et al. (2018)
Mitomycin C	50-07-7	Rat (RccHan)	Testes	+	Motoyama et al. (2018)
Mitomycin C	50-07-7	Mice (ICR)	Bone marrow	+	Matsuda et al. (2018)
			Stomach	+	
			Kidney	+	
			Spleen	+	
			Liver	+	
			Lung	+	
			Testis	–	
Nicotine-derived nitrosamine ketone (NNK)	64091-91-4	mice (A/J)	Lung	+	Elisia et al. (2019)
N-butyl-*N*-(4-hydroxybutyl)-nitrosamine (BBN)	3817-11-6	Mice (B6C3F1)	Urinary bladder	+	Sone et al. (2019)
2-Acetylaminofluorene (2-AAF)	53-96-3			+	
p-Cresidine	120-71-8			+	
Uracil	200-621-9			+	
2-Nitroanisole	100-17-4			–	
Glycidol	556-52-5			–	
N-nitrosodiethylamine	55-18-5			–	
Acrylamide	79-06-1			–	
Dimethylarsinic acid	75-60-5			–	
Melamine	108-78-1			–	
2,2-Bis(bromomethyl)-1,3-propanediol	3296-90-0			–	
Acetoaceto-o-toluidide (AAOT)	93-68-5	Rat (F344)	Urinary bladder	+	Okuno et al. (2019)
o-Toluidine	95-63-4	Rat (F344)	Urinary bladder	+	Toyoda et al. (2019)
o-Anisidine	90-04-0			+	
2,4-Xylidine	95-98-1			–	
p-Toluidine	106-49-0			–	
Aniline	62-53-3			–	
Diethylnitrosamine (DEN)	55-18-5	Mice (C57BL/6)	Liver	+	Ge et al. (2019)

controls, cell proliferation, adequate number of cells, etc.) like the in vitro MN TG 487. The possibility to define a test guideline for the use of the γH2AX/pH3 assay in regular genotoxicity studies requires further discussion. We also recommend further investigation to confirm the robustness of the γH2AX/pH3 biomarkers for in vivo chemical evaluation of genotoxicity and carcinogenicity.

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Author contributions All the authors contributed to writing the manuscript.

Compliance with ethical standards

Conflict of interest MA and LK are co-founders of Preditox SAS. LK is CEO of Preditox. MA serves as consultant to Preditox SAS.

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