

-H2AX assay: a technique to quantify DNA double strand breaks

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Introduction

Nucleosomes are the individual unit of hereditary material, condensed to form the chromatin filaments in eukaryotic cells. The nucleosome consists of deoxy-ribo nucleic acids (DNA) wrapped around an octamer of the four core histone proteins namely the H2A, H2B, H3, H4 and H1; of which H1 acts as linker to connect adjacent nucleosomes. The histones are highly conserved proteins involved in the packaging of DNA. Recent studies have documented evidence that histone variants are present in many mammalian cells, which have specialized biological functions in cellular metabolism (Fernandez-Capetillo *et al.*, 2004).

H2AX histone

The histone H2A family contains three members named H2A1-H2A2, H2AZ, and H2AX. Originally, it has been reported that H2AX is an isoform of H2A and present only in lower eukaryotes. However, recent evidence showed that H2AX constitutes a major H2A species and its level varies from 2-25% of the mammalian histone H2A pool, depending upon the cell line and tissue examined. H2AX is highly conserved in eukaryotes and contains a distinguishing C-terminal extension carrying a SQ (E/D)(I/L/F/Y) consensus motif (Mannironi *et al.*, 1989). Similar to other members of H2A family, H2AX can undergo phosphorylation, acetylation and ubiquitination to regulate the cellular events (Rogakou *et al.*, 1998). The H2AX protein is reported to be involved in DNA repair and repair defect syndromes, regulation of cell division, cell growth and immuno-receptor rearrangement. In view of

its multiple functions, it has been implicated that H2AX is an important player in cancer causing genomic instability in humans (Fernandez-Capetillo *et al.*, 2004).

-H2AX Phosphorylation

Rapid phosphorylation (within a second) of a serine-139 residue located 4 aminoacids from the COOH terminus is reported as a unique feature of the H2AX upon cellular exposure to DNA damaging agents. H2AX is phosphorylated at an evolutionary conserved phosphatidylinositol-3-OH kinase related kinase (PI3KK) motif. H2AX phosphorylation due to radiation is predominantly mediated by ataxia telencetesia mutated (ATM) protein. Due to replication stress is mediated by ataxia telencetesia related (ATR) proteins, hypertonical stress and DNA fragmentation are mediated by DNA dependent protein kinases. The amount of H2AX that was -phosphorylated per double strand break (DSB) corresponded to a region of about 2MB in chromatin or thousands of nucleosomes (Rogakou *et al.*, 1999). Mutation in the PI3KK-C terminal of *Saccharomyces cerevisiae*, histone H2AX resulted in hypersensitivity to agents that leads to DSB, strongly suggesting H2AX is required for the recognition and repair of DNA DSB. The development of phosphor-specific anti -H2AX antibody confirmed that, indeed, H2AX is massively phosphorylated in chromatin surrounding DSBs, known as nuclear foci. The phosphorylated H2AX can be quantified using western blotting or *in situ* immuno-fluorescence. Phosphorylation of H2AX is induced in response to DNA double strand breaks originated from diverge origins

including external damage, replication fork collision, apoptosis and dysfunctional telomeres (Lobrich *et al.*, 1999).

Significance of -H2AX assay

Wide variety of markers has been reported as an indicator of exposure to therapeutic agents to normal as well as tumor cells both *in vitro* and *in vivo*. Markers based on the chromosomes abnormalities and /or gene mutations are suitable to quantify the residual damage and not the actual amount of damages induced by therapeutic agents. Ample evidence has been generated that therapeutic agents induced wide spectrum of DNA damages; it has been generally assumed that DSB are considered as a lethal event, as it can result in the formation of stable chromosomal aberrations and leads to late consequences like genomic instability and carcinogenesis. In mammals, non-homologous end joining and homologous recombination repair appears to be the major mechanism of rejoining DNA ends (Sancar *et al.*, 2004). Whereas much is known about the rejoining of DNA DSBs, much less is known about how these breaks are initially recognized and its exact quantity. -H2AX assay has been widely used to quantify the exact amount of double strand breaks produced by radiation and ionizing agents and the repair kinetics.

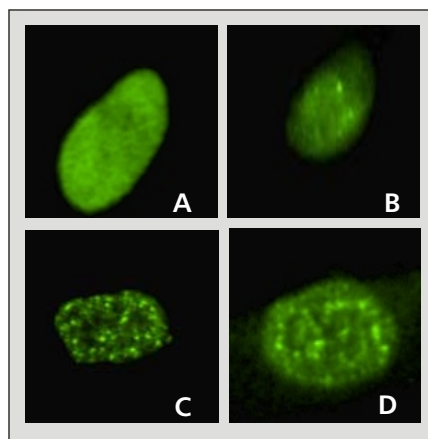
-H2AX is specifically bond by MDC-1 that assist in the assembly of several proteins, including ATM, the MRE 11 / Rad 50 / nibrin complex and 53 PB1 that subsequently participates in the spreading of -H2AX formation in a 2-Mbp region from the DSB. This amplification of the locally generated

DSB signal later assist in the globalization of the DSB response through massive ATM mediated chk2 activation. H2AX is rapidly -phosphorylated after exposure of cells to ionizing radiation, with half-maximal amounts reached at 13 min. At the maximum, 1030 min after irradiation, the stoichiometry suggests that hundreds to several thousand -H2AX molecules are present per DNA double-strand break in mammals. In the present study, we report the functional significance of H2AX a variant of H2A histone protein induced by bleomycin (a widely used chemotherapeutic drug, which mimics radiation) and its role in maintaining the genome stability and its implications.

Cell line maintenance and bleomycin exposure

The WI38 cells were obtained from national centre for cell sciences, Pune, and maintained as a monolayer culture in our laboratory. Cells were grown in plastic tissue culture flasks using Dulbecco's modified essential medium supplemented with 10% fetal bovine serum and antibiotics (Penicillin 100IU/ml, Streptomycin 100 g/ml and Gentamycin 2.5 g/ml). The cells grown for 24 hours (50-75 thousand cells/p60 dish) after subculture were treated with bleomycin (10 to 80 g/ml) for 3 hours at 37°C. At the end of 3 hours bleomycin was removed from the cells by washing with PBS for 3 times. Then the cells were fixed by adding 2ml of ice cold methanol, incubated at 37°C for 5 mins and 3 ml of 1% BSA-PBST (37°C for 30 mins). Then 1 ml of primary antibody of H2AX (rabbit polyclonal IgG) at a concentration of 1 g/ml was added and incubated at 4°C for 16 hours in humid chamber. Then the primary antibody was discarded and the cells were washed with phosphate buffer (thrice for 5 mins each). Finally, 2 g/ml of secondary antibody (goat polyclonal IgG with FITC conjugate) was added, incubated at room temperature for an hour in a dark area and the cells were washed with phosphate buffer (thrice for 5 mins each). About 15µl of counter stain (DAPI II) was added, cove-slip was placed carefully and observed under fluorescent microscope using FITC filter. For each concentration, about 100 cells were scored randomly (the analysis was restricted to well distinguished with clear nuclear

structured cells as shown in figure-1) and the number of H2AX foci was recorded using image analysis system (Cytovision, Version 3.2 from Applied Imaging, UK). The mean frequency of H2AX foci from the cells scored.



A = WI-38 cells without any H2AX foci
B, C & D = WI-38 cells one or many H2AX foci

Figure-1: WI-38 cells with H2AX foci exposed to bleomycin

H2AX foci obtained in WI-38 cells exposed bleomycin

The H2AX foci obtained for various concentrations of bleomycin are given in table-1. The obtained results shows, that bleomycin induced a significant increase in DNA damages in all concentrations (p=0.0001). However the increase in DNA damage is not concentration dependent. Further, the distribution of foci among the cells also moderately increased when the dose increased, in the mock treated cells most of the counted cells showed 1-3 foci. Whereas in the bleomycin treated cells the number of foci

varies between 3 and 20 per cells. The number of foci increased at higher concentration of drug treatment, where majority of the cells carries more than 4 foci per cells and this increase in foci is concentration dependent.

As it is known that the induced DNA damage induced by radiation interferes with cell cycle pertubarations (activation of check points, cell cycle arrest, DNA damage repair, or apoptosis), we would like to know the fate of DNA damages induced by the bleomycin. This was achieved and was carried out by holding the cells at contact inhibited stage for 1 hour after bleomycin treatment. The DNA damage measure after 1 hour liquid holding was measured and that is shown in table-2. Similar to the DNA damage measure immediately after bleomycin exposure the DNA damage remain higher when compare to control in all the concentrations studied. However for the same concentrations of drug the cells were further incubated for one hour after drug treatment, the foci frequency reduced significantly (p=0.0001) (figure-2). After the 1 hour time gap the cells with the foci also moderately reduced when compare to the immediately fixed cells after the exposure to bleomycin. The number of cells without foci also increases in cells that were incubated for one hour after the bleomycin treatment. (Average for various concentrations of bleomycin treatment = 10%).

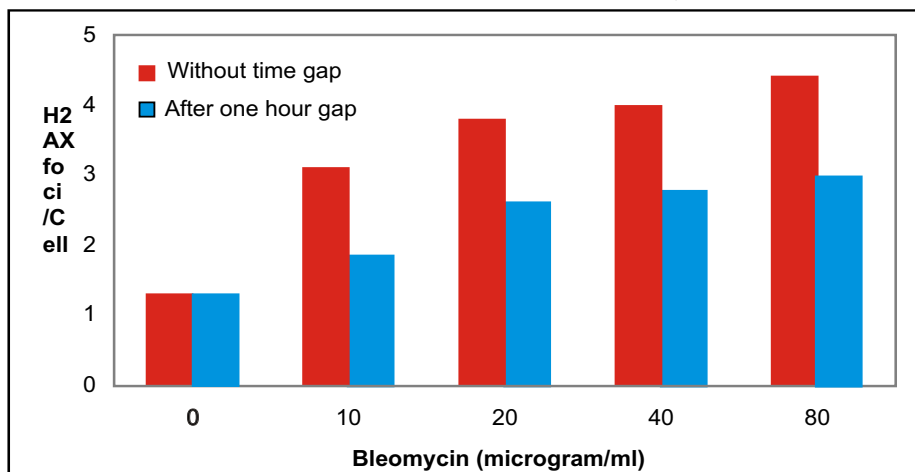
Implications of H2AX foci obtained in WI-38 cells after bleomycin treatment

The radiomimetic agent bleomycin is a free radical-based DNA damaging agent, which induces double-strand, breaks by highly

Table 1: Frequency of H2AX foci in WI-38 cell line exposed to bleomycin and analyzed without repair time.			
Bleomycin concentration (µg/ml)	Number of -H2AX foci	Cells without foci	Frequency of foci ± Standard Error
0	131	45	1.31 ± 0.066
10	310	42	3.10 ± 0.176
20	383	35	3.83 ± 0.195
40	396	32	3.96 ± 0.199
80	436	27	4.36 ± 0.208

Table 2: Frequency of H2AX foci in WI-38 cell line exposed to bleomycin and analyzed after 1 hour repair time.

Bleomycin concentration (µg/ml)	Number of -H2AX foci	Cells without foci	Frequency of foci ± Standard Error
0	131	49	1.31 ± 0.066
10	192	51	1.92 ± 0.135
20	265	50	2.65 ± 0.163
40	276	40	2.76 ± 0.168
80	303	34	3.03 ± 0.174

**Figure-2 Comparison of -H2AA foci frequency obtained WI-38 cell line exposed to bleomycin and analyzed with and with out time gap**

specific, concerted free radical attack on deoxyribose moieties in both DNA strands. The lesions induced by this 'radiomimetic' drug is thus actually only a small subset of the myriad lesions induced by ionizing radiation, yet effects of such radiomimetic agent on cells are remarkably similar to those of radiation. Wide variety of markers has been reported as an indicator of exposure to therapeutic agents to normal as well as tumor cells. Majority of the markers based on the chromosomes abnormalities or gene mutations are widely used to quantify the residual damage and not the actual amount of damages induced by therapeutic agents. Double strand breaks are believed to be a most important lesion produced in chromosomes because, as these breaks can lead to chromosomal aberrations during repair, which are lethal to the cells (Hall, 2005). To quantify exact amount of DSB's, we have adopted a recently developed H2AX foci assay as a tool and bleomycin, as a representative chemotherapeutic drug as it is radiomimetic in nature. The obtained results showed that bleomycin induced a significant amount of DSB's (table-1) and repaired to

certain extent (table-2). The types of lesions and repair to the DNA are different among individuals and among tissues and response of patients to therapy. Further, repair processes, which act in response to attack by cytotoxic agents profoundly, influence survival, growth, development and transformation of cells and may increase a risk for secondary cancer. In the present study cells given an hour repair time followed by bleomycin treatment showed a significant reduction in H2AX frequency indicates a repair of damages induced by bleomycin (figure-2). It was further supported from the results that not only the number of H2AX foci is reduced but also a reduction of 10% cells without any H2AX foci.

It has been reported that when ever cells exposed to DNA damaging agents, the cells may either activate check points and cell cycle arrest, repair of DNA damages or cell death. Thus the outcome depends on the extent of DNA damage, cell type and sensitivity to damaging agent (Sancar *et al.*, 2004). As WI38 is the normal human diploid fibroblast

cells, we assumed the reduced amount H2AX foci in bleomycin treated cells given an hour time is mainly due to repair. However, after repair, many cells showed existence of DSB's as H2AX foci. When the cells enters into cell cycle, the DSB's may get repaired either by non homologous end joining or homologous recombination repair; the repair might be a normal or mis-repair ends up in deleterious chromosomal aberrations and results in genomic instability. We suggest that, a genome unstable cell may alter/stimulate cell signaling pathways responsible for second malignancy.

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