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Original Article

γH2AX is immunohistochemically detectable until 7 days after exposure of N-bis (2-hydroxypropyl) nitrosamine (DHPN) in rat lung carcinogenesis

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Abstracts: It is known that γH2AX, which is formed when there is a double-strand break in DNA, can act as a sensitive marker of genomic instability. In this experiment, the time-course manner of the expression of γH2AX in the lung was examined in the early phase after treatment with a lung carcinogen, N-bis (2-hydroxypropyl) nitrosamine (DHPN). The expression of γH2AX is expected to be one of the useful markers for lung carcinogenesis in early stages. Rats were separated into 10 groups of 5 rats. The DHPN groups were administered 0.1% DHPN in drinking tap water for two weeks, while the control group received drinking tap water. At 0, 1, 3, 7, and 14 days after finishing DHPN treatment, one group each from the DHPN and control groups was sacrificed. The removed lung tissues were examined for immunostaining of γH2AX and PCNA, and positive cells were counted. The γH2AX levels of the DHPN-treated groups were found to be increased significantly at 0, 1, 3, and 7 days (4.4 ± 1.4, 5.1 ± 2.7, 3.3 ± 1.0, and 4.1 ± 1.3%, respectively), and they dropped significantly on day 14 (1.1 ± 0.4%). The experiment showed that the γH2AX-positive score could be effectively measured for up to 7 days after exposure, as a significance difference was observed between the treated group and the control group. It can be deduced that γH2AX is an effective marker for DHPN-induced double-strand breaks in pulmonary epithelial cells. (DOI: 10.1293/tox.2017-0066; J Toxicol Pathol 2018; 31: 163–168)

Key words: γH2AX, rat, DHPN, lung, carcinogenesis, double-strand break

Introduction

Cancer is an international health problem and is currently one of the leading causes of mortality in all regions, albeit different types of cancer are more prevalent in different regions. The sites that are especially prone to cancer development are the lungs, colon, breasts, prostate, stomach, liver, esophagus, and cervix[1]. It was estimated that the approximately 14.1 million new cancer cases were reported worldwide in 2012; 1.8 million of those cases were lung cancer[2]. On a global scale, lung cancer is the leading and second leading cause of cancer deaths in men and women respectively[3]. Despite different treatments available for lung cancer, the rate of mortality is still high. It is estimated that there will be approximately 23.6 million cases of cancer diagnosed worldwide every year by the year 2030[4]. The increasing number of new cancer cases is a worrying trend and poses as a major health and economic burden globally. Therefore, early detection is crucial, as it can lead to better prognosis and early stage cancers may require less aggressive treatments. As such, it is crucial to identify effective biomarkers to detect early cancer development.

Cancer arises as a result of changes in the DNA, which can occur due to normal cellular processes that may lead to DNA damage. One of the more serious forms of DNA damage is the double-strand break, in which both strands in DNA are cleaved. Some sources of damage include cellular metabolites such as reactive oxygen species, irradiation, exposure to chemicals or certain drugs, and UV light[5]. Usually, damaged DNA is repaired by cellular pathways. However, if there are errors in the repair of a double-strand break, it can cause different genomic rearrangements due to deletions, translocations, and fusions of DNA, which are frequently found in the cells of malignant tumours[6]. Increased levels

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of double-strand breaks also increase the levels of structural chromosome aberrations, leading to overall genomic instability, which will progress into cancer[7]. Unrepaired DNA damage can also accumulate and lead to neoplastic transformation in the cell or even cell death[8, 9]. Therefore, the detection of double-strand breaks is crucial in monitoring the process of cancer formation and progression.

It is known that γH2AX, which is formed when there is a double-strand break, can act as a sensitive marker of genomic instability[10]. The unphosphorylated form of γH2AX is H2AX, which is a histone variant that makes up approximately 15% of total cellular H2A. A double-strand break induces a series of DNA damage repair reactions that lead to the phosphorylation of H2AX into γH2AX. The γH2AX accumulates at the site of damage in the nuclear foci, which can be visualized clearly when checked at the appropriate time[11]. In an experiment on the urinary bladder of rats, treatment with N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN) or other carcinogens, targeting the epithelium of the urinary bladder, was reported to increase the expression of several DNA repair enzymes and phosphorylation of histone H2AX, and γ-H2AX could have potential as a useful biomarker in the early detection of genotoxic bladder carcinogens[12, 13]. The expression of γH2AX is also reported to be increased 2–8 h after 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) treatment in vitro[14]. Therefore, the expression of γH2AX in pulmonary epithelial cells should be assessed in the early stages after carcinogen administration.

In this experiment, the time-course manner of the expression of γH2AX in the lung was examined in the early phase after treatment with a lung carcinogen, N-bis (2-hydroxypropyl) nitrosamine (DHPN). The expression of γH2AX is expected to be one of the useful markers for lung carcinogenesis in early stages. DHPN acts as a mutagen and carcinogen in the lung epithelium when orally administered[15]. In the lungs, DHPN first induces hyperplasia, followed by adenoma after a period of time and finally pulmonary adenocarcinoma[16].

Material and Methods

Chemicals

The DHPN (CAS: 53609-64-6) used as the lung carcinogen was purchased from Nacalai Tesque Inc. (Kyoto, Japan). A γH2AX antibody was purchased from Cell Signaling Technology (Danvers, MA, USA) and PCNA (Clone PC10, Code No. M0879) was purchased from Agilent (Santa Clara, CA, USA).

Animals

Fifty 6-week-old male F344 rats were purchased from Charles River Laboratories Japan (Kanagawa, Japan) and maintained in the Division of Animal Experiments, Life Science Research Center, Kagawa University, according to the Institutional Regulations for Animal Experiments. The regulations included the best considerations on animal welfare and good practice of animal handling contributing to the replacement, refinement, and reduction of animal testing (3Rs). The protocol of the experiment was approved by the Animal Care and Use Committee of Kagawa University. All of the animals were housed in polycarbonate cages with white wood chips for bedding and given free access to drinking water and a basal diet, CE-2 (CLEA Japan Inc., Tokyo, Japan), under controlled conditions of humidity (60 ± 10%), lighting (12-h light/dark cycle), and temperature (24 ± 2°C). The experiments were started after a 2-week acclimation period.

Following quarantine, all rats were randomly assigned to 10 groups of 5 rats each using a weight stratification-based computer program. Groups 1 to 5 were designated as DHPN experimental groups, while groups 6 to 10 were control groups. The DHPN groups were administered 0.1% DHPN in drinking tap water for two weeks, while the control group received drinking tap water. At 0, 1, 3, 7, and 14 days after finishing DHPN treatment, one group each from the DHPN and control groups was sacrificed.

Under deep anesthesia, the lungs, liver, and kidneys were removed. The lungs including the trachea and heart, the liver, and the kidneys were weighed. After that, the lungs were passed through 10% neutral buffered formalin and then injected with 10% neutral buffered formalin to inflate the structure. The lungs were separated into different lobes, and the trachea and heart were weighed. The final weight of the lungs was calculated by subtraction of the weight of the trachea and heart. All removed organs were immersed in 10% neutral buffered formalin for 3 days. The organs were then processed and embedded in paraffin to make slides.

Immunohistochemistry

All slides from lung tissues were deparaffinized and stained using an automated immunohistochemical stainer (Ventana HX Discovery System; Ventana Medical Systems, Tucson, AZ, USA) for γH2AX and PCNA. The slides were first treated with CC2 Buffer (Ventana Medical Systems) prior to immunohistochemical staining. The sections were then incubated in γH2AX (Cell Signalling Technology) under the following conditions: rabbit monoclonal, 1:10 dilution for 1 h. Finally, the sections were incubated in anti-rabbit IgG secondary antibodies (Vector Laboratories, Inc., Burlingame, CA, USA) for 32 min. The remaining sections were incubated in PCNA (Dako) under the following conditions: mouse monoclonal, 1:5,000 dilution for 16 min. These sections were then incubated in anti-mouse IgG secondary antibodies (Vector Laboratories, Inc., Burlingame, CA, USA).

The slides were viewed under ×400 magnification, and five fields were randomly selected from different lobes in each slide. The total number of cells in the fields were counted, omitting macrophages and other non-alveolar cells. Cells that were stained brown were also counted as positive cells for γH2AX. At least 1,000 cells were counted per slide, and the percentage of positive cells was calculated for each slide. The labelling index (%) was calculated with the formula % = (number of positive cells / number of total

Table 1. Mean Body Weight and Absolute Lung, Liver, and Kidney Weight of Rats in Their Respective Groups

Groups	Recovery days	Treat-ment	No. of rats	Body weights (g)	Lung		Liver		Kidney	
					Absolute (g)	Relative (%)	Absolute (g)	Relative (%)	Absolute (g)	Relative (%)
1	0	DHPN	6	181.6 ± 10.0*	0.87 ± 0.07*	0.48 ± 0.04	7.69 ± 0.38	4.24 ± 0.25*	1.33 ± 0.07*	0.73 ± 0.03
6	0	Control	5	199.0 ± 8.0	0.97 ± 0.05	0.49 ± 0.02	7.75 ± 0.57	3.89 ± 0.14	1.42 ± 0.05	0.71 ± 0.03
2	1	DHPN	6	184.7 ± 7.1*	0.91 ± 0.07	0.49 ± 0.02**	8.04 ± 0.40	4.35 ± 0.17**	1.36 ± 0.03*	0.74 ± 0.02*
7	1	Control	5	205.0 ± 7.5	0.86 ± 0.10	0.42 ± 0.05	7.74 ± 0.52	3.77 ± 0.13	1.43 ± 0.07	0.70 ± 0.02
3	3	DHPN	5	190.8 ± 8.1*	0.86 ± 0.04*	0.45 ± 0.03	7.95 ± 0.23	4.17 ± 0.08**	1.34 ± 0.06*	0.70 ± 0.01
8	3	Control	5	207.6 ± 8.5	0.95 ± 0.06	0.46 ± 0.04	7.87 ± 0.51	3.79 ± 0.10	1.49 ± 0.09	0.72 ± 0.03
4	7	DHPN	5	206.4 ± 10.1	0.94 ± 0.09	0.45 ± 0.03	8.13 ± 0.46	3.94 ± 0.11**	1.43 ± 0.16	0.69 ± 0.05
9	7	Control	5	219.7 ± 9.4	0.95 ± 0.03	0.43 ± 0.03	8.05 ± 0.39	3.66 ± 0.13	1.20 ± 0.08	0.68 ± 0.03
5	14	DHPN	5	225.4 ± 3.8	1.02 ± 0.06	0.45 ± 0.02	8.17 ± 0.13	3.62 ± 0.08*	1.51 ± 0.03	0.67 ± 0.02
10	14	Control	5	235.3 ± 10.9	0.94 ± 0.09	0.40 ± 0.05	8.26 ± 0.39	3.51 ± 0.06	1.52 ± 0.08	0.65 ± 0.02

*Significantly different from the control group at P<0.05. **Significantly different from the control group at P<0.01.

cells) × 100.

Data for body and organ weights and labelling index of γH2AX were analyzed using Student *t*-test.

Results

Table 1 shows body weights and the lung, liver, and kidney weights at 0, 1, 3, 7, and 14 days after finishing DHPN treatment. Significant differences of P<0.01 could be seen between the average body weights of the DHPN group rats and the control rats after a recovery period of 0 days, 1 day and 3 days. In respect to purely lung weight, only recovery days 0 and 3 showed any significant differences, while recovery day 1 did not show significant differences despite the difference in body weight.

Macroscopically, it was found that no lesions developed and that there were no significant differences between the lungs of the different groups, whether they were the DHPN-treated groups or the control groups.

No morphological differences were found in the lungs between DHPN-treated and control groups. Immunohistochemically, the groups treated with DHPN showed an increased number of prominent positive cells for γH2AX that are visualised as dark brown nuclei (Fig. 1). It was possible to see a clear difference in the number of positive cells between the DHPN and control groups even prior to cell counting.

Regarding the results of cell counting, a significant difference of at least P<0.01 was observed in the γH2AX labelling indexes between the DHPN-treated groups and control groups at recovery days 0, 1, 3, and 7. Recovery day 1 showed the highest significance of P<0.05. However, the labelling indexes on day 14 were not significantly different.

Table 2 shows the mean positive scores for γH2AX and PCNA for each respective group. Stastically significant differences in γH2AX levels were found between the DHPN-treated groups and control groups from recovery day 0 up to day 7, with a slight increase between days 3 and 7. However, the level of γH2AX in the DHPN groups dropped significantly by day 14. A statistically significant difference in PCNA level was found between the DHPN-treated groups on day 3 (Fig. 1), whereas the PCNA level did not significantly differ between the DHPN-treated groups and control groups on any other days (Table 2).

Discussion

γH2AX is a very sensitive marker for DNA double-strand breaks as it plays a crucial role in the repair of double-strand breaks. This process is described by Ozaki *et al.*[11]. When a double-strand break or DNA damage occurs, the kinase ataxia telangiectasia mutated (ATM) is autophosphorylated at Ser 1981 to form the active p-ATM. This leads to the phosphorylation of H2AX into γH2AX at Ser 139. γH2AX then accumulates in at a region called the nuclear foci in order to flank the damage site. DNA damage response proteins such as the MRN complex are then recruited to the nuclear foci by γH2AX working together with NFBD1. Meanwhile, it is currently thought that the histone variant H2AX and its phosphorylation on Ser 139 (γH2AX) cannot be simply considered a specific DNA double-strand-break (DSB) marker with a role restricted to the DNA damage response but rather should be considered a "protagonist" in different scenarios[17]. Reactive oxygen species (ROS) are known to induce oxidative DNA damage, which also induces γH2AX12. Apoptotic DNA fragmentation can also cause γH2AX induction[14].

There are several methods to detect γH2AX expression, with the most common methods being immunostaining, flow cytometry, western blot, and ELISA. As the rate of cancer morbidity rises, advances in the field of screening and diagnostics has seen an increase in research to make techniques simpler, faster, and less invasive to the patients. A paper published by Reddy *et al.*[18] described a new method for the detection and quantification of γH2AX called Dual Acid Extraction that is economical and time efficient. The method is a liquid-biopsy based monitoring tool that can isolate circulating histones in the serum of tumors, making it less invasive to patients. Its application in cancer monitoring can potentially reach a wider audience and improve the prognosis of many patients. There is a report indicating that the expression of γH2AX can be used as a means of assessing the prognosis of patients suffering from non-small

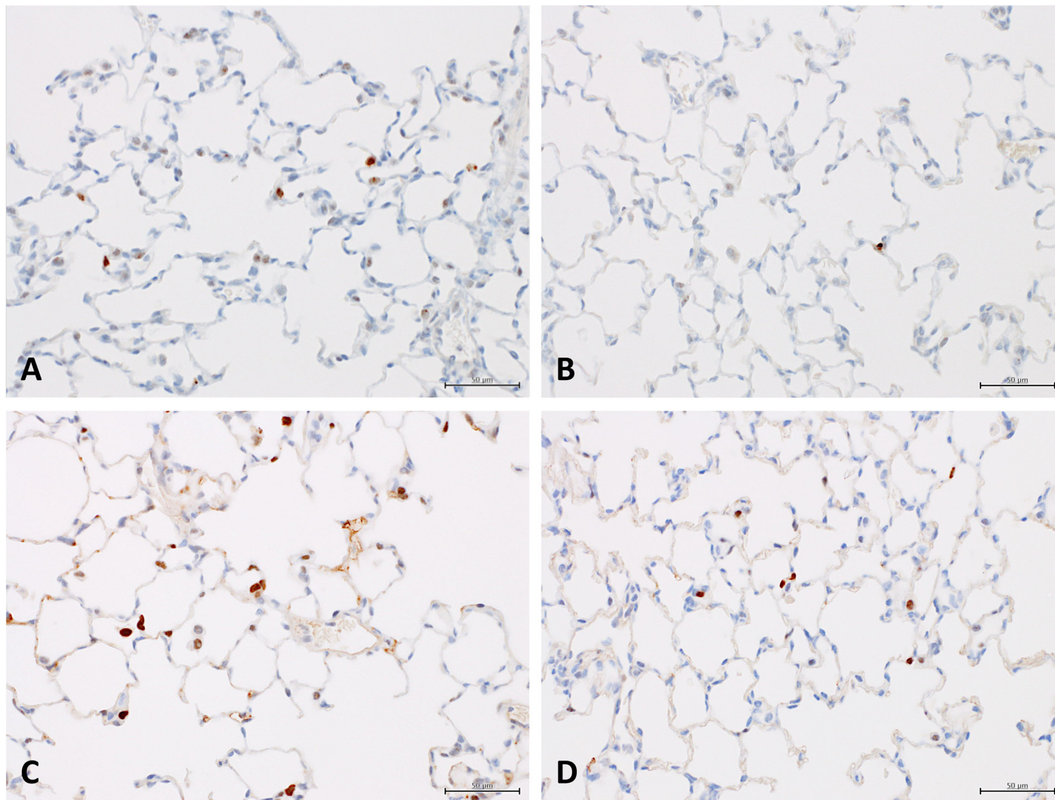

Fig. 1. Immunohistochemical findings of the lungs at 3 days. The images in A and B are for γH2AX, and those in C and D are for PCNA. Positive cells are indicated by brown staining of the nucleus. Randomly selected slides of lung sections under ×400 magnification. A DHPN-treated group is shown in A and C, and a control group is shown in B and D.

Table 2. Mean Positive Scores for γH2AX and PCNA for Each Respective Group

Groups	Days	Treatment	No. of rats	Labelling index (%)	
				γH2AX	PCNA
1	0	DHPN	6	4.4 ± 1.4**	12.0 ± 2.0
6	0	Control	5	1.6 ± 1.2	9.9 ± 2.3
2	1	DHPN	5	5.1 ± 2.7*	8.4 ± 1.7
7	1	Control	5	1.7 ± 0.7	8.5 ± 1.4
3	3	DHPN	5	3.3 ± 1.0**	11.3 ± 0.9**
8	3	Control	5	1.8 ± 0.4	5.9 ± 1.3
4	7	DHPN	5	4.1 ± 1.3**	6.1 ± 0.6*
9	7	Control	5	1.0 ± 0.6	4.5 ± 1.2
5	14	DHPN	5	1.1 ± 0.4	5.3 ± 1.1
10	14	Control	5	1.0 ± 0.5	5.2 ± 1.0

*Significantly different from the control group at $P<0.05$. **Significantly different from each respective control group at $P<0.01$. At least 1,000 cells were counted per slide, and the percentages of positive cells were calculated for each slide.

cell lung carcinoma. A high expression of γH2AX increased the chances of mortality by 2.15 fold[19]. Other than acting as a screening tool for cancer formation and its prognosis, γH2AX can also be used to screen for genotoxicity of different chemicals or drugs that require metabolic activation to assess the extent of their effects on the pulmonary system of living organisms[14].

DHPN was chosen as a potent carcinogen of the pulmonary system in this experiment. It is an alkylating agent with two propyl chains and a very potent mutagen and can potentially target several organs, causing malignant tumors in the lungs, liver, kidney, thyroid, and ovaries over a long period of exposure while causing benign tumors in all the previously mentioned organs in addition to the pancreas over a shorter period of time[20]. It is a useful tool for assessing the tumorigenic activities of other potentially hazardous chemicals and substances that can increase the rate of cancer formation after initiation[16]. DHPN is one of the nitrosamines, and the nitrosamines induce DNA adducts[21]. DHPN is reported to be associated with activation of mutations of the *Kras* gene at codon 12 in 47% of rat lung neoplastic lesions[22]. These mechanisms for the carcinogenesis of DHPN can induce DNA damage without double-strand breaks and raise the possibility that increases in γH2AX are not derived from double-strand breaks. Further research needs to be done using other known lung carcinogens as a source of double-strand break induction to compare the optimal period of screening.

In this experiment, the γH2AX levels of the DHPN-treated groups were found to be increased from recovery day 0 up to day 7 compared with the control groups. In a previous report in the rat urothelium at 2 weeks after administration of a carcinogen for 4 weeks, γ-H2AX-positive cells

were markedly reduced in number, suggesting the progression of DNA repair and removal of damaged cell by apoptosis[13]. On the other hand, γ-H2AX expression induced by some genotoxic bladder carcinogens (i.e., BBN, 2-NA, and 2-AAF) remained significantly higher than that in control rats, even after 2 weeks of recovery. The maintenance of high expression of γ-H2AX after the recovery period may indicate that severe DNA damage occurred after short-term administration of a crucial bladder carcinogen[13]. The cause of the difference between the present results in the lung and the results in the urinary bladder needs to be considered by further examination.

The PCNA levels in this experiment showed a tendency to decrease with time in both the DHPN-treated and control groups, though the mechanism of this tendency is unknown. The synthesis of PCNA fluctuates during the cell cycle, with a specific increase during the S phase, and this protein is an important component in pathways leading to DNA synthesis and cell proliferation[23]. A noteworthy increase in PCNA-positive cells was not seen compared with γH2AX in the present experiment. This supports the suggestion the expression of γH2AX exhibits a more sensitive reaction to carcinogens in early stages.

The present experiment showed that the γH2AX-positive score could be effectively measured for up to 7 days after exposure, as a significance difference of $P<0.05$ was observed between the DHPN-treated and the control groups. It can be also deduced that γH2AX is an effective marker for DHPN in pulmonary epithelial cells. Research using other lung carcinogens should be carried out in the future in order to assess the feasibility of using γH2AX as a double-strand break marker.

Disclosure of Potential Conflicts of Interest: We have no conflicts of interest to be declared.

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