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Original Article

Role of γ-H2AX as a biomarker for detection of bladder carcinogens in F344 rats

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Abstract: Phosphorylation of histone H2AX at serine 139 (γ-H2AX) is known to be induced by direct DNA damage or cellular metabolic imbalances and malfunctions. Previous studies have reported that γ-H2AX is a useful biomarker for early detection of genotoxic bladder carcinogens in rats. The purpose of the present study was to determine the role of γ-H2AX as a biomarker for detection of non-genotoxic bladder carcinogens in rats. Six-week-old male F344 rats were treated with 15 different chemicals for 4 weeks. Immunohistochemical analyses revealed that all three genotoxic bladder carcinogens and six out of seven non-genotoxic bladder carcinogens significantly increased γ-H2AX formation in the bladder urothelium of rats. In addition, four out of five rat bladder noncarcinogens did not increase γ-H2AX formation in the bladder urothelium regardless of genotoxicity. These results suggest that γ-H2AX is a useful biomarker for detection of both genotoxic and non-genotoxic bladder carcinogens in rats. (DOI: 10.1293/tox.2020-0038; J Toxicol Pathol 2020; 33: 279–285)

Key words: γ-H2AX, carcinogen, urinary bladder, prediction, F344 rats

Introduction

Urinary bladder cancer is the fourth most common cancer and the eighth leading cause of cancer-related deaths among men in the United States[1]. GLOBOCAN 2018 estimated that approximately 549,000 new cases of bladder cancer and 200,000 deaths related to bladder cancer occurred in 2018[2]. The primary carcinogens implicated in the induction of bladder cancer are tobacco smoke, dye penetrants, arsenic, and aromatic amines, including benzidine, ortho-toluidine, and 2-naphthylamine[3]. The "Gold standard" for evaluating the carcinogenic potential of chemicals in rodents is the two-year bioassay (OECD Guidelines for the Testing of Chemicals; Test No. 451: Carcinogenicity Studies[4]). However, there has been an increase in the number of chemicals produced in recent years, and a large number of these chemicals have not been tested using the two-year bioassay. In addition, there is an increasing demand to reduce the use of animals in testing. Therefore, it is crucial to establish a short-term *in vivo* assay for prediction of carcinogenicity of different chemicals.

In response to double-strand breaks in the DNA, Ser139-phosphorylated histone H2AX (γ-H2AX) rapidly accumulates over a large region of chromatin surrounding double-strand break[5]. More recently, it has been reported that γ-H2AX accumulates in response to other types of DNA stress also[6, 7]. A 4-week study by Toyoda *et al.* showed that genotoxic bladder carcinogens significantly increased the number of γ-H2AX-positive urothelial cells in rats[8], indicating the importance of γ-H2AX as a biomarker for early detection of genotoxic bladder carcinogens in rats. Recently, it has been reported that certain non-genotoxic bladder carcinogens also increase the number of γ-H2AX-positive urothelial cells in rats[9,10]. These findings suggest that γ-H2AX may be a useful biomarker for detection of bladder carcinogens regardless of their genotoxicity. In the present study, to evaluate the usefulness of γ-H2AX as a biomarker for predicting bladder carcinogenicity of chemicals in rats, we

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examined the formation of γ-H2AX in the urothelium of rats treated with genotoxic bladder carcinogens, non-genotoxic bladder carcinogens, or rat bladder noncarcinogens for four weeks.

Materials and Methods

Test chemicals

The chemicals used in the present study included 1-amino-2,4-dibromoanthraquinone (ADBAQ; Alfa Aesar, Heysham, Lancashire, UK; purity, 97%), phenacetin (PNC; Tokyo Chemical Industry, Tokyo, Japan; purity, >99.0%), N-butyl-N-(3-carboxypropyl)nitrosamine (BCPN; Tokyo Chemical Industry; purity, >98.0%), N-nitrosodiphenylamine (NDPA; Tokyo Chemical Industry; purity, >98.0%), sodium o-phenylphenate (SOPP; Wako Pure Chemical Industries, Osaka, Japan; purity, >95.0%), 11-aminoundecanoic acid (AUDA; Sigma-Aldrich, St. Louis, MO, USA; purity, 97%), 1-naphthyl-N-methylcarbamate (Carbaryl; Sigma-Aldrich; purity, 97%), tributyl phosphate (TBP; Tokyo Chemical Industry; purity, >99.0%), saccharin sodium salt dihydrate (Na-Sac; Tokyo Chemical Industry; purity, 99.0%), pioglitazone (PGZ; Tokyo Chemical Industry; purity, >98.0%), 4-nitroquinoline-1-oxide (4NQO; Wako Pure Chemical Industries; purity, >98.0%), 8-hydroxyquinoline (8HQ; Tokyo Chemical Industry; purity, >99.0%), rosiglitazone (RGZ; Tokyo Chemical Industry; purity, >98.0%), sodium arsenite (iAs; Sigma-Aldrich; purity, 98%), and 1-nitropropane (1NP; Tokyo Chemical Industry; purity, >98.0%).

Based on genotoxicity and carcinogenicity to the urinary bladder of rats, the test chemicals were classified into three categories: genotoxic bladder carcinogens (ADBAQ, PNC, and BCPN)[11–13], non-genotoxic bladder carcinogens (NDPA, SOPP, AUDA, TBP, Carbaryl, Na-Sac, and PGZ)[14–20, (https://apvma.gov.au/node/14486)], rat bladder noncarcinogens (4NQO, iAs, 8HQ, RGZ, and 1NP)[19, 21–23].

Animals and experimental conditions

Five-week-old male F344 rats were obtained from Charles River Laboratories (Atsugi, Japan). Experiments 1 and 2 were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee of Nagoya City University School of Medical Sciences, and experiment 3 was approved by the Institutional Animal Care and Use Committee of Osaka City University Graduate School of Medicine. All experiments were conducted in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006). The rats were housed in plastic cages with hardwood chip bedding in an air-conditioned room maintained at a temperature of 23 ± 2°C, relative humidity of 55 ± 5%, and 12:12-h light:dark cycle. The rats were provided *ad libitum* access to basal diet (Oriental MF, Oriental Yeast Co., Tokyo, Japan) and tap water.

Experimental design

At the beginning of experiments 1 and 2, the animals were randomly allocated to groups of 5 to 6 rats each; and at the beginning of experiment 3, the animals were allocated to groups of 5 to 6 rats each based on their body weights (measured just before starting chemical treatment).

In experiment 1, the animals were fed diets supplemented with test chemicals at targeted doses of 1% ADBAQ, 0.5% PNC, 0.4% NDPA, 2% SOPP, or 1.5% AUDA for 4 weeks. In experiment 2, the animals were provided drinking water supplemented with 0.028% BCPN or 0.005% 4NQO in light-shielded bottles, or fed basal diets supplemented with 0.75% Carbaryl, 0.3% TBP, or 5% Na-Sac for 4 weeks. In experiment 3, the animals were administered 16 mg/kg body weight (bw) PGZ, 50 mg/kg bw RGZ, or 90 mg/kg bw 1NP dissolved in corn oil through gavage once per day, or fed diets supplemented with 173 ppm iAs or 0.6% 8HQ for 4 weeks. Owing to the reduction in the body weights of rats after treatment with 0.005% 4NQO and 90 mg/kg bw 1NP, the doses of 4NQO and 1NP were reduced to 0.0025% and 45 mg/kg bw, respectively (See results).

All chemicals were administered at carcinogenic or maximum doses reported in previous studies. Six non-treated rats served as controls for each experiment. Diets were changed once a week, and drinking water was changed twice a week. At the end of week 4, the animals were weighed, sacrificed by exsanguination under deep isoflurane anesthesia, and subjected to laparotomy for excision of liver, kidneys, and bladder. The liver and kidneys of each rat were weighed. Bladder tissues were inflated with 10% buffered formalin, fixed in 10% formalin, and routinely processed for paraffin-embedded sectioning and histopathological examination.

Immunohistochemical staining

For experiments 1 and 2, deparaffinized bladder sections were heated in Epitope Retrieval Solution (pH 9) for epitope retrieval, and incubated with rabbit polyclonal anti–γ-H2AX antibody (#2577; Cell Signaling Technology, Danvers, MA, USA) or rabbit monoclonal anti-Ki67 antibody (SP6, Abcam plc, Cambridge, UK), followed by staining with BOND-MAX (Leica Biosystems, Wetzlar, Germany) according to the manufacturer's instructions.

For experiment 3, deparaffinized bladder sections were heated in antigen retrieval buffer (sodium citrate, pH 6.0), treated with 3% H_2O_2, and incubated with rabbit polyclonal anti–γ-H2AX antibody or rabbit monoclonal anti-Ki67 antibody overnight at 4°C. Reactivity with primary antibody was detected by incubating the sections with biotin-labeled goat anti-rabbit IgG, followed by treatment with avidin-biotin-peroxidase complex (ABC kit; Vector, Burlingame, CA, USA) and 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution (Agilent Technologies, Santa Clara, CA, USA).

The number of γ-H2AX- or Ki67-positive cells in at least 1000 urothelial cells in morphologically normal urothelium of rats, except BCPN- and TBP-treated rats, was counted. In rats treated with BCPN and TBP, as morphologically normal urothelium was absent, the number of

γ-H2AX- or Ki67-positive cells in at least 1,000 urothelial cells in simple hyperplasia was counted.

Statistical analysis

Data are presented as mean ± standard deviation (S.D.). Homogeneity of variance was tested using the F-test. Differences in mean values between the control and treatment groups were evaluated using Student's *t*-test when the variance was homogeneous and using Welch's *t*-test when the variance was heterogeneous (two-group comparisons). $P<0.05$ was considered statistically significant.

Results

Body weight, organ weight, and food and water consumption

In experiment 1, the final body weights of rats treated with ADBAQ, SOPP, or AUDA were significantly lower than those of control rats (Table 1). Both absolute and relative liver weights were significantly higher in rats treated with ADBAQ, PNC, NDPA, or SOPP than those in control rats (Table 1). Absolute kidney weights were significantly higher in AUDA-treated rats than those in control rats (Table 1). Relative kidney weights were significantly higher in rats treated with ADBAQ, PNC, NDPA, SOPP, or AUDA than those in control rats (Table 1).

In experiment 2, the body weights of 4NQO group rats were lower after one week of treatment than those at the start of the experiment. Therefore, the dose of 4NQO was reduced from 0.005% to 0.0025% after week 1. The final body weights of rats treated with Carbaryl, TBP, or 4NQO were significantly lower than those of control rats (Table 1). Absolute liver weights were significantly lower in 4NQO-treated rats than those in control rats (Table 1). Relative liver weights were significantly higher in Carbaryl- and TBP-treated rats and lower in 4NQO-treated rats than those in control rats (Table 1). Absolute kidney weights were significantly lower in Carbaryl- and 4NQO-treated rats than those in control rats, but relative kidney weights were significantly higher in BCPN-, Carbaryl-, and 4NQO-treated rats than those in control rats (Table 1). Food and water consumption were higher in the Na-Sac group and lower in the 4NQO group as compared to those in the control group (Table 1).

In experiment 3, as decreased body weight and listlessness was observed in all 1NP group rats after the first gavage treatment, the dose of 1NP was reduced to 45 mg/kg bw since the second treatment. The final body weights of NP-treated rats were significantly lower than those of control rats (Table 1). Absolute liver weights were significantly lower but relative liver weights were higher in 1NP-treated rats as compared to those in control rats (Table 1). Absolute kidney weights were significantly lower in PGZ-, RGZ-,

Table 1. Body, Liver and Kidneys Weights, Food and Water Consumptions

Treatment	No. of rat	Body weight (g)	Liver		Kidneys		Food consumption (g/rat/day)	Water consumption (ml/rat/day)
			Absolute (g)	Relative (%)	Absolute (g)	Relative (‰)		
Experment 1								
Control	6	232.6 ± 10.3	8.2 ± 0.3	3.5 ± 0.1	1.6 ± 0.1	6.8 ± 0.1	13.0 ± 0.9	
ADBAQ	5	215.8 ± 4.5**	11.0 ± 0.3***	5.1 ± 0.1***	1.7 ± 0.1	7.7 ± 0.1***	13.0 ± 2.1	
PNC	5	224.2 ± 4.8	9.3 ± 0.3***	4.2 ± 0.1***	1.7 ± 0.1	7.4 ± 0.2***	13.3 ± 1.6	
NDPA	5	222.8 ± 3.3	9.7 ± 0.3***	4.4 ± 0.1***	1.7 ± 0.1	7.4 ± 0.2***	14.8 ± 2.1	
SOPP	5	209.8 ± 5.3**	9.0 ± 0.2**	4.3 ± 0.2***	1.6 ± 0.1	7.7 ± 0.3***	13.5 ± 2.6	
AUDA	5	214.5 ± 9.5*	7.9 ± 0.8	3.7 ± 0.2	1.9 ± 0.1***	8.7 ± 0.1***	12.2 ± 1.2	
Experment 2								
Control	6	240.0 ± 10.2	8.7 ± 0.6	3.6 ± 0.1	1.7 ± 0.1	7.0 ± 0.1	13.9 ± 0.7	20.0 ± 1.0
BCPN	5	244.5 ± 14.1	9.0 ± 0.7	3.7 ± 0.1	1.8 ± 0.1	7.2 ± 0.1*	14.1 ± 0.5	20.8 ± 1.3
Carbaryl	5	185.1 ± 4.2***	8.0 ± 0.2	4.3 ± 0.1***	1.5 ± 0.0***	8.3 ± 0.2***	12.9 ± 4.1	17.3 ± 2.5
TBP	5	223.8 ± 11.1*	9.2 ± 0.6	4.1 ± 0.1***	1.6 ± 0.1	7.2 ± 0.1	15.4 ± 1.1	22.6 ± 2.1
Na-Sac	5	234.9 ± 4.5	8.3 ± 0.3	3.5 ± 0.1	1.7 ± 0.1	7.1 ± 0.2	17.1 ± 2.6	26.5 ± 2.3
4NQO	5	175.5 ± 9.7***	5.8 ± 0.5***	3.3 ± 0.2**	1.3 ± 0.1***	7.7 ± 0.1***	11.6 ± 4.7	10.7 ± 4.2
Experment 3								
Control (ig)	6	237.5 ± 4.3	8.4 ± 0.2	3.5 ± 0.1	1.8 ± 0.0	7.4 ± 0.2	12.5 ± 0.4	20.4 ± 0.9
PGZ	6	245.0 ± 8.4	8.1 ± 0.4	3.3 ± 0.1	1.7 ± 0.0**	7.0 ± 0.1*	13.6 ± 0.5	22.6 ± 0.9
RGZ	6	246.4 ± 8.2	8.6 ± 0.4	3.5 ± 0.1	1.6 ± 0.1***	6.7 ± 0.2***	13.8 ± 0.7	22.5 ± 1.1
1NP	6	177.7 ± 12.1***	6.3 ± 0.7***	3.5 ± 0.3	1.6 ± 0.1***	8.8 ± 0.3***	9.3 ± 3.1	18.9 ± 4.4
Control (diet)	6	237.5 ± 10.5	8.7 ± 0.5	3.7 ± 0.1	1.8 ± 0.1	7.5 ± 0.2	13.4 ± 0.9	20.4 ± 1.2
iAs	6	233.3 ± 11.3	8.6 ± 0.6	3.7 ± 0.2	1.8 ± 0.1	7.6 ± 0.3	13.1 ± 0.9	24.0 ± 3.0
8HQ	5	240.0 ± 6.1	8.7 ± 0.4	3.6 ± 0.1	1.8 ± 0.0	7.6 ± 0.2	13.9 ± 0.7	21.6 ± 0.9

*,**,***: $P<0.05$, 0.01 and 0.001 vs control, respectively. −: not examined. ADBAQ, 1-amino-2,4-dibromoanthraquinone; PNC, phenacetin; NDPA, N-nitrosodiphenylamine; SOPP, sodium o-phenylphenate; AUDA, 11-aminoundecanoic acid; BCPN, N-butyl-N-(3-carboxypropyl)nitrosamine; Carbaryl, 1-naphthyl-N-methylcarbamate; TBP, tributyl phosphate; Na-Sac, saccharin sodium salt dihydrate; 4NQO, 4-nitroquinoline-1-oxide; PGZ, pioglitazone; RGZ, rosiglitazone; 1NP, 1-nitropropane; iAs, sodium arsenite; 8HQ, 8-hydroxyquinoline.

and 1NP-treated rats than those in control rats; and relative kidney weights were significantly lower in PGZ- and RGZ-treated rats but higher in 1NP-treated rats than those in control rats (Table 1). Food and water consumption were lower in the 1NP group as compared to those in the control group (Table 1).

Histopathological examination of the urinary bladder

Simple hyperplasia was noted in two out of five AUDA-treated rats and in all BCPN-treated rats (Table 2). Simple, papillary, or nodular hyperplasia was observed in all TBP-treated rats (Table 2). No histopathological changes were observed among rats of the other groups.

Immunohistochemical analysis of the urinary bladder

γ-H2AX-positive cells with characteristic intranuclear dot-like foci were distributed throughout the bladder epithelium of rats treated with genotoxic or non-genotoxic bladder carcinogens (Supplementary Fig. 1: online only), as reported previously for genotoxic bladder carcinogens[8]. Labeling indices of γ-H2AX in the urinary bladders of rats treated with ADBAQ, PNC, NDPA, SOPP, AUDA, BCPN, Carbaryl, TBP, Na-Sac, or iAs were significantly higher than those of their respective controls (Fig. 1); in these rats, γ-H2AX-positive cells were mainly intermediate cells (Supplementary Fig. 2: online only). All three genotoxic bladder carcinogens, including ADBAQ, PNC, and BCPN, and six out of seven non-genotoxic bladder carcinogens, including NDPA, SOPP, AUDA, Carbaryl, TBP, and Na-Sac, increased γ-H2AX labeling in the urinary bladder of rats. The non-genotoxic bladder carcinogen PGZ did not increase γ-H2AX labeling in the urinary bladder of rats; and in PGZ-treated rats, γ-H2AX-positive cells were mainly umbrella cells (Supplementary Fig. 1: online only). Four out of five rat bladder noncarcinogens, including 4NQO, 8HQ, RGZ, and 1NP, did not increase γ-H2AX labeling; however, iAs significantly increased γ-H2AX labeling in the urinary bladder

Table 2. Chemical Information and Histopathology in Urinary Bladder

Treatment	Chemical information	No. of rat	Hyperplasia
Experment 1			
Control	-	6	0
ADBAQ	GTBC	5	0
PNC	GTBC	5	0
NDPA	NGTBC	5	0
SOPP	NGTBC	5	0
AUDA	NGTBC	5	2 (S)
Experment 2			
Control	-	6	0
BCPN	GTBC	5	5 (S)
Carbaryl	NGTBC	5	0
TBP	NGTBC	5	5 (S & PN)
Na-Sac	NGTBC	5	0
4NQO	BNC	5	0
Experment 3			
Control (ig)	-	6	0
PGZ	NGTBC	6	0
RGZ	BNC	6	0
1NP	BNC	6	0
Control (diet)	-	6	0
iAs	BNC	6	0
8HQ	BNC	5	0

ADBAQ, 1-amino-2,4-dibromoanthraquinone; PNC, phenacetin; NDPA, N-nitrosodiphenylamine; SOPP, sodium o-phenylphenate; AUDA, 11-aminoundecanoic acid; BCPN, N-butyl-N-(3-carboxypropyl)nitrosamine; Carbaryl, 1-naphthyl-N-methylcarbamate; TBP, tributyl phosphate; Na-Sac, saccharin sodium salt dihydrate; 4NQO, 4-nitroquinoline-1-oxide; PGZ, pioglitazone; RGZ, rosiglitazone; 1NP, 1-nitropropane; iAs, sodium arsenite; 8HQ, 8-hydroxyquinoline; GTBC: genotoxic bladder carcinogen; NGTBC: non-genotoxic bladder carcnogen; BNC: bladder noncarcinogen; S: simple hyperplasia; PN: papillary or nodular hyperplasia.

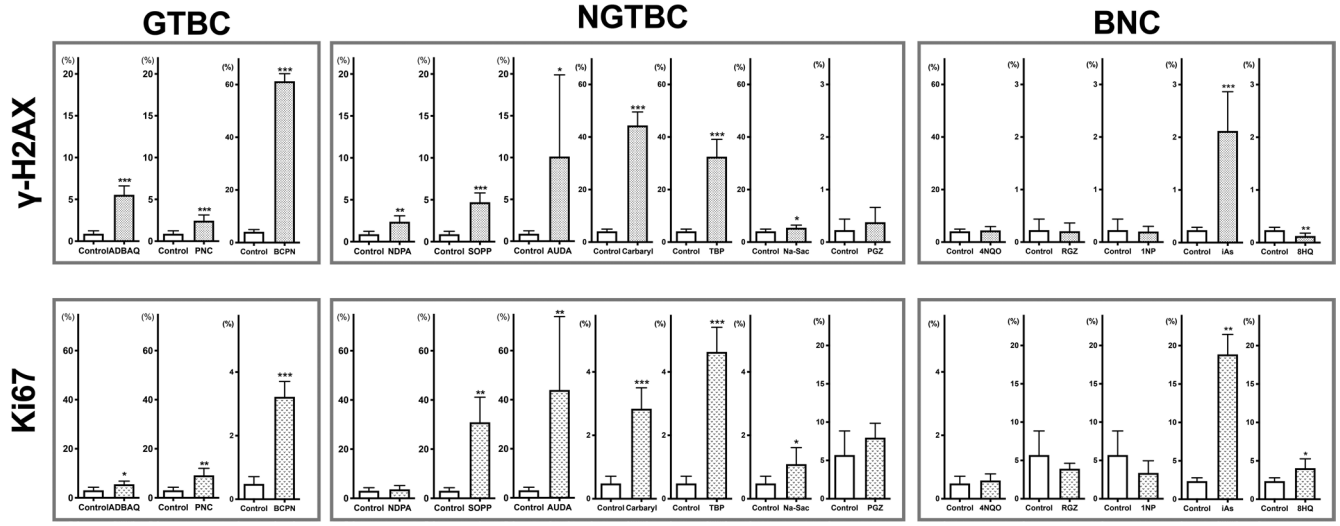

Fig. 1. γ-H2AX and Ki67 labeling in the bladder urothelium of F344 rats. *: $P<0.05$ vs. control, **: $P<0.01$ vs. control, and ***: $P<0.001$ vs. control. GTBC: genotoxic bladder carcinogen; NGTBC: non-genotoxic bladder carcinogen; BNC: bladder noncarcinogen.

of rats.

The labeling indices of Ki67 in the urinary bladders of rats treated with ADBAQ, PNC, SOPP, AUDA, BCPN, Carbaryl, TBP, Na-Sac, and iAs were significantly higher than those of their respective controls (Fig. 1). All three genotoxic bladder carcinogens, including ADBAQ, PNC, and BCPN, and five out of seven non-genotoxic bladder carcinogens, including SOPP, AUDA, Carbaryl, TBP, and Na-Sac, increased Ki67 labeling in the urinary bladder of rats. However, the non-genotoxic bladder carcinogens NDPA and PGZ did not increase Ki67 labeling in the urinary bladder of rats. Three out of five rat bladder noncarcinogens, including 4NQO, RGZ, and 1NP, did not increase Ki67 labeling; but iAs, and 8HQ increased Ki67 labeling in the urinary bladder of rats.

As described above, the labeling indices of γ-H2AX and Ki67 were similar in 13 of the 15 treated groups. Significant increases in both γ-H2AX and Ki67 labeling indices were observed in the urothelium of rats treated with the bladder carcinogen ADBAQ, PNC, BCPN, SOPP, AUDA, Carbaryl, TBP, or Na-Sac; however, neither of these indices were increased in rats treated with the bladder noncarcinogen 4NQO, RGZ, or 1NP, and in rats treated with the non-genotoxic bladder carcinogen PGZ. On the other hand, both the indices were increased in rats treated with the noncarcinogen iAs.

Discussion

To evaluate the usefulness of γ-H2AX as a biomarker for prediction of carcinogenicity of chemicals to the rat urinary bladder, we assessed γ-H2AX formation using immunohistochemistry in a 4-week toxicity study. Although histological changes were observed only in rats treated with the genotoxic bladder carcinogen BCPN and in rats treated with the non-genotoxic bladder carcinogen AUDA or TBP, significant increases in γ-H2AX formation were observed in rats treated with the genotoxic bladder carcinogen ADBAQ, PNC, or BCPN and in rats treated with the non-genotoxic bladder carcinogen NDPA, SOPP, AUDA, TBP, Carbaryl, or Na-Sac. There was no significant increase in γ-H2AX formation in rats treated with the bladder noncarcinogen RGZ, 4NQO, 1NP, or 8HQ. However, rats treated with the bladder noncarcinogen iAs did exhibit a significant increase in γ-H2AX formation, but did not display simple hyperplasia in our study that included only male rats. In previous reports, female rats treated with iAs for 4 weeks developed simple hyperplasia; however, these lesions did not progress to cancer over the course of time[24]. Therefore, an increase in γ-H2AX formation was expected in these rats. In contrast, rats treated with the bladder carcinogen PGZ did not exhibit an increase in γ-H2AX formation. Overall, the sensitivity and specificity of γ-H2AX as a biomarker for prediction of bladder carcinogenicity of chemicals were 90% (9/10) and 80% (4/5), respectively. Previous studies reported that seven out of nine bladder carcinogens were positive for γ-H2AX formation and six bladder noncarcinogens were negative for γ-H2AX formation[8, 9, 25]. The results of previous studies together with our results indicate that γ-H2AX labeling index predicted bladder carcinogenicity of chemicals with a sensitivity of 84% (16/19) and specificity of 91% (10/11) (Table 3).

Increased expression of Ki67, a cell proliferation marker, is potentially related to carcinogenesis; as induction of cancer often involves cytotoxicity and regenerative proliferation, the carcinogenicity of a test chemical can be defined by its capability to induce a proliferative response in the tissue[26]. In our study, the sensitivity and specificity of Ki67-based prediction of bladder carcinogenicity of chemicals were 80% (8/10) and 60% (3/5), respectively. Combined with the results from the previous studies[8, 9, 25], Ki67 labeling index predicted bladder carcinogenicity of chemicals with a sensitivity of 74% (14/19) and specificity of 82% (9/11). Therefore, the Ki67 labeling index may also serve as a biomarker of carcinogenicity in the urinary bladder.

γ-H2AX formation is known to be induced by direct DNA damage, such as damage caused by radiation. Recently, it has been reported that γ-H2AX formation is also induced by cellular metabolic imbalances and malfunctions due to certain factors, including oxidative stress, DNA replication stress, and telomere attrition[6, 7, 27]. A previous study suggested that γ-H2AX might serve as a useful biomarker for early detection of genotoxic bladder carcinogens[8]. Our results indicate that γ-H2AX may serve as a useful biomarker for the detection of not only genotoxic bladder carcinogens but also non-genotoxic bladder carcinogens. However, the sensitivity of γ-H2AX for detection of non-genotoxic rat bladder carcinogens (70%) was lower than that for detection of genotoxic rat bladder carcinogens (100%). This result might be attributed to the mechanisms of γ-H2AX formation[6, 7, 27].

In the present study, no increase in γ-H2AX and Ki67 indices was observed in PGZ-treated F344 rats. PGZ is a

Table 3. Summary of γ-H2AX Detection on Rat Bladder Carcinogens and Noncarcinogens

Chemicals	Sensitivity			Specificity
	GTBC	NGTBC	BC (GTBC+NGTBC)	BNC
The present study	3/3 (100%)	6/7 (86%)	9/10 (90%)	4/5 (80%)
Previously reports	6/6 (100%)	1/3 (33%)	7/9 (78%)	6/6 (100%)
Total	9/9 (100%)	7/10 (70%)	16/19 (84%)	10/11 (91%)

GTBC: genotoxic bladder carcinogen; NGTBC: non-genotoxic bladder carcnogen; BC: bladder carcinogen; BNC: bladder noncarcinogen.

peroxisome proliferator-activated receptor γ agonist, and its mode of action involves increased formation of urinary solids, resulting in urothelial cytotoxicity and regenerative cell proliferation[20, 28]. We demonstrated that PGZ induces cytotoxicity, cell proliferation, and hyperplasia in SD rats in our previous 4-week study[29]. In the present study, no increased cell proliferation or histopathological changes were observed in the urothelium of PGZ-treated F344 rats, which suggests that F344 rats are less susceptible to PGZ-induced bladder carcinogenesis than SD rats. Therefore, PGZ did not increase γ-H2AX formation in F344 rats, at least in part, due to the low susceptibility of F344 rats to PGZ-induced bladder carcinogenesis.

In the present study, iAs treatment significantly increased γ-H2AX and Ki67 labeling indices in the urothelium of F344 rats. Though iAs does not cause bladder cancer in rats, it is known to induce pre-neoplastic changes in the urinary bladder of rats[24]. Moreover, in our previous study, we demonstrated that iAs induced cytotoxicity, cell proliferation, and hyperplasia in the urinary bladder of F344 rats[30]. It is known that γ-H2AX formation is also associated with oxidative stress[7], and iAs induces oxidative stress in the bladder urothelium of F344 rats[31]. Therefore, increased γ-H2AX formation in the bladder of iAs-treated rats might be related to events, including oxidative stress, tissue damage and subsequent regenerative cell proliferation, and formation of preneoplastic lesions, which are likely associated with bladder carcinogenesis. Notably, though iAs is generally negative in rodent carcinogenicity studies[32, 33], it is a known human bladder carcinogen[22].

In conclusion, combining the results of previous studies with our results, γ-H2AX can predict bladder carcinogenicity of both genotoxic and non-genotoxic chemicals with a sensitivity of 84% and specificity of 91%. These results suggest that γ-H2AX is a useful biomarker for prediction of bladder carcinogenicity of test chemicals.

Disclosure of Potential Conflicts of Interest: The authors declare that they have no conflicts of interest.

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