Review

Early detection of urinary bladder carcinogens in rats by immunohistochemistry for γ-H2AX: a review from analyses of 100 chemicals

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Abstract: In safety evaluations of chemicals, there is an urgent need to develop short-term methods to replace long-term carcinogenicity tests. We have reported that immunohistochemistry for γ-H2AX, a well-established biomarker of DNA damage, can detect bladder carcinogens at an early stage using histopathological specimens from 28-day repeated-dose oral toxicity studies in rats. Given the markedly low level of γ-H2AX formation in the bladder urothelium of untreated rats, an increase in γ-H2AX-positive cells following chemical exposure can be relatively easy to identify. Among the 100 compounds examined to date, bladder carcinogens can be detected with high sensitivity (33/39; 84.6%) and specificity (58/61; 95.1%). As expected, γ-H2AX formation levels tended to be high following exposure to genotoxic bladder carcinogens, whereas nongenotoxic bladder carcinogens also increased the number of γ-H2AX-positive cells, probably through secondary DNA damage associated with sustained proliferative stimulation. γ-H2AX formation in the bladder urothelium reflects species differences in susceptibility to bladder carcinogenesis between rats and mice and shows a clear dose-dependency associated with the intensity of tumor development as well as high reproducibility. Some of the bladder carcinogens that showed false-negative results in the evaluation of γ-H2AX alone could be detected by combined evaluation with immunostaining for bladder stem cell markers, including aldehyde dehydrogenase 1A1. This method may be useful for the early detection of bladder carcinogens, as it can be performed by simple addition of conventional immunostaining using formalin-fixed paraffin-embedded tissues from 28-day repeated-dose toxicity studies in rodents, which are commonly used in safety evaluations of chemical substances. (DOI: 10.1293/tox.2022-0061; J Toxicol Pathol 2022; 35: 283–298)

Key words: alternative method, carcinogenicity, early detection, γ-H2AX, stem cell marker, urinary bladder

Introduction

The potential risk of carcinogenicity to humans has been evaluated for many chemicals, including pharmaceuticals, food additives, and pesticides, using long-term bioassays in rodents. However, there are numerous difficulties associated with standard carcinogenicity tests, including high-cost, time-consuming procedures and the necessity for a large number of animals1–3. In addition, although many new substances are developed annually, industrial chemicals, particularly those with low exposure and production, are exempted from long-term safety evaluations and are often used without determining their carcinogenicity. Accordingly, there is an urgent need to develop novel bioassays that can efficiently detect carcinogenicity in short-term studies to overcome these challenges and contribute to the improvement of animal welfare. Urinary bladder cancer is the sixth most common cancer in men worldwide, and smoking and occupational exposure are major risk factors underlying its development4. Accumulating epidemiological and experimental data have suggested that various chemicals, such as aromatic amines, are closely associated with the risk of bladder carcinogenesis5. More than 3,000 types of azo dyes synthesized from aromatic amines are employed in leather, clothing, food, toys, cosmetics, and other products worldwide and are essential for daily life6. Therefore, the development of an effective short-term method for detecting bladder carcinogens is expected to play an important role in the risk assessment of both existing and newly developed chemicals.

γ-H2AX, the phosphorylated form of the histone constituent protein H2AX at serine 139, is a well-established biomarker of DNA damage, particularly DNA double-strand breaks (DSBs)7. DSBs can be caused not only by direct interactions with ionizing radiation and certain genotoxic substances but also by various other factors, including reactive oxygen species, replication stress, and deficient DNA repair. Moreover, DSBs can also be derived from other non-DSB DNA lesions, such as single-strand breaks8.
γ-H2AX rapidly accumulates not only at the DSB site but also extensively in the surrounding chromatin region\(^4\), leading to the aggregation of repair proteins\(^5\); this process can be microscopically detected by immunofluorescence and immunohistochemistry using specific primary antibodies\(^6\). Therefore, immunostaining for γ-H2AX may be a useful tool to predict the genotoxicity and carcinogenicity of chemical substances\(^7-10\). The potential clinical applications of γ-H2AX immunostaining, such as prediction of cancer progression and response to radiotherapy, are also being investigated\(^11,12\). In bladder cancer, there may be a correlation between γ-H2AX formation in cancer cells and the risk of recurrence\(^13,14\), and γ-H2AX levels in peripheral leukocytes can be used as predictive factors for high-risk patients\(^15,16\).

We hypothesized that DNA repair-related factors could be used as biomarkers for the early detection of bladder carcinogens and examined the expression of 12 DNA repair-related factors in bladder epithelial cells of rats orally treated with N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN), a genotoxic bladder-specific carcinogen, by immunohistochemistry\(^17\). The results revealed that although most factors showed similar expression levels in the control and BBN-treated groups, γ-H2AX formation, rarely detected in the control group, was significantly elevated not only in BBN-induced bladder tumors but also in the surrounding normal-appearing mucosa. Moreover, we found that several bladder carcinogens increased the number of γ-H2AX-positive urothelial cells in rats after 28 days of treatment, whereas carcinogens that are not targeted to the urinary bladder did not cause this change\(^18-20\). Subsequently, we have focused on the development of a novel method for the early detection of the bladder carcinogenicity of chemicals using γ-H2AX immunostaining in the rat urinary bladder.

In this review, we describe this method and the analytical results for 100 chemicals examined to date. Furthermore, we discuss the current issues underlying the system and practical applications for elucidation of the mechanisms of bladder carcinogenesis.

**Protocol**

A 28-day repeated-dose oral toxicity study in rodents, including histopathological examination of all organs, is widely used for evaluating the safety of chemical substances. This method has been standardized by the OECD Test Guideline No. 407 (TG 407)\(^21\) and is required in many countries, particularly in the early-phase of development of industrial chemicals and pharmaceutical drugs. The early detection of bladder carcinogenicity by γ-H2AX immunostaining can be performed using formalin-fixed paraffin-embedded histopathological specimens obtained from these 28-day studies without the need for additional groups or special sampling processes, such as frozen tissues.

**Animal experiment and processing of bladder samples**

Male and female specific pathogen-free rats (6 weeks old) are divided into control and multiple treatment groups consisting basically of 5 animals each, as in the normal 28-day test. The animals are orally administered a test compound in the basal diet, drinking water, or via gavage for 28 days. In the validation experiments, we selected Fischer and Sprague-Dawley rats as strains widely used in toxicity studies. Given that male rats are typically more susceptible to bladder carcinogenic agents\(^22\), we used male F344/ DuCrICrj or CrI:CD(SD) rats (Charles River Laboratories, Yokohama, Japan). The formation of γ-H2AX in response to DNA damage is a common mechanism that remains highly conserved in many eukaryotes and is unlikely to have strain differences within itself\(^23\). The dose of each substance was based on the carcinogenic dose in the carcinogenicity test or the maximum tolerated dose for 28-day studies from the published data (Table 1). During necropsy, for rapid fixation of the urothelium for histopathological and immunohistochemical analyses, the urinary bladders should be inflated with 10% neutral-buffered formalin. Although the evaluation can be more easily performed by ligating the urethra while the bladder is inflated and preparing strip-shaped specimens of the bladder mucosa, standard half-sections are also sufficient for evaluation. In both cases, the bladder should be sliced along the longitudinal axis, and both the area close to the trigone of the bladder and the dome should be included in the specimen. As with conventional immunostaining, prolonged storage in a fixative solution may reduce the reactivity of specimens to antibodies. Sectioning and paraffin embedding after the completion of fixation should be performed as soon as possible.

**Immunohistochemistry**

The immunostaining procedure is essentially the same as conventional procedures. Most commercially available anti-γ-H2AX antibodies require heat-mediated antigen retrieval for staining of paraffin-embedded specimens. Testes may be used as positive controls because γ-H2AX formation constantly occurs during spermatogenesis\(^24\). The immunohistochemical method used in the validation study is briefly described below. The sections were deparaffinized, hydrated, and autoclaved in 10 mM citrate buffer (pH 6.0) for 15 min at 121°C for antigen retrieval. To inactivate endogenous peroxidase activity, all sections were immersed in 3% H₂O₂/methanol solution for 10 min at room temperature. After blocking nonspecific reactions with 10% normal goat serum (Nichirei Corp., Tokyo, Japan), the sections were incubated with primary antibodies for γ-H2AX (diluted 1:1,000; anti-phospho-histone H2A.X [Ser139] rabbit polyclonal antibody or anti-phospho-histone H2A.X [Ser139] [D7T2V] mouse monoclonal antibody; Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. Visualization of antibody binding was performed using a Histofine Simple Stain Rat MAX PO Kit (Nichirei Corp.) and 3,3’-diaminobenzidine. All sections were counterstained with hematoxylin.
| Chemicals Used in the Early Detection of Bladder Carcinogens by Immunohistochemistry for γ-H2AX |
|-----------------|---------------------------------------------|--------|-----------------|-----------------|
| **Bladder carcinogen (38)** | **Abbreviation** | **CAS No.** | **Reference for carcinogenicity** | **Experimental conditions in this evaluation** |
| 2-Acetylamino-1-methyl-6-azacytosine | AA | 53-96-3 | | |
| o-Aminoazotoluene | o-Az | 2298-13-7 | | |
| 1-Amino-2,4-diamino-6-dimethylnitrosamine | ADDBAQ | 81-49-2 | | |
| 4-Amino-2-nitrophenol | ANP | 119-34-6 | | |
| Aminophenylhydrazine | APNH | 219959-86-1 | | |
| o-Antiside hydrochloride | o-Ams | 134-29-2 | | |
| Aristolochic acid I | AA1 | 313-67-7 | | |
| 2,2-Disulfobenzy| B | 0.015% | | |
| N-Benzyl-N-(3-carboxypropyl) | BCPN | 38252-74-3 | | |
| N-Benzyl-N-(4-hydroxybutyl) | BBN | 3817-11-6 | | |
| 4-Chloro-o-phenylenediamine | COP | 95-83-0 | | |
| p-Cresidine | p-Cre | 120-71-8 | | |
| Cyclophosphamide monohydrate | CPA | 6055-19-2 | | |
| 3,3'-Dimethylbenzidine | DXB | 119-81-4 | | |
| 2,2'-Dimethyl-4-aminoazobenzene | DMAB | 58109-32-3 | | |
| Disperse blue I | DIB | 2475-45-8 | | |
| N-Ethyl-N-(4-hydroxybutyl) | ETHB | 54897-62-0 | | |
| 2-Nitroaniline | 2-NA | 91-23-6 | | |
| N-Nitosobis(2-oxopropyl)amine | BOP | 60599-38-4 | | |
| N-Nitroso-N-methylurea | MNH | 684-93-5 | | |
| N-Nitrosomorpholine | NMOR | 59-89-2 | | |
| 2-Nitrosotoluene | 2-NT | 611-23-4 | | |
| Phenacetin | PNC | 62-44-2 | | |
| Phenethyl isothiocyanate | PEITC | 2257-09-2 | | |
| o-Toluidine hydrochloride | o-Tol | 636-21-5 | | |
| 11-Aminouracil | AU | 2432-99-7 | | |
| m-Cresidine | m-Cre | 102-50-1 | | |
| Dimethylarsinic acid | DMA | 75-60-5 | | |
| Melamine | Mel | 108-78-1 | | |
| 1-Naphthyl-N-methylcarbamate | MNC | 63-25-2 | | |
| N-Nitrosodiphenylamine | NDPA | 86-30-6 | | |
| Pyrrolizino hydrochloride | PGZ | 112529-15-4 | | |
| Saccharin sodium salt dihydrate | Na-Sac | 6155-57-3 | | |
| Sodium o-phenylenediamine | SOP | 132-27-4 | | |
| Sulfasalazine | SSZ | 590-78-1 | | |
| Tributyl phosphate | TBP | 126-73-8 | | |
| Trisodium nitrotriacetate monohydrate | TATA | 18662-53-8 | | |
| Uracil | URA | 66-22-8 | | |

**Non-bladder carcinogen (44)**

| Chemicals Used in the Early Detection of Bladder Carcinogens by Immunohistochemistry for γ-H2AX |
|-----------------|---------------------------------------------|--------|-----------------|-----------------|
| 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine | PhIP | 105650-23-5 | | |
| Azoxyimethane | AOM | 25843-45-2 | | |
| 2,4-Diaminoanilino sulfate hydrate | DAA | 6219-67-6 | | |
| 2,4-Diaminotoluene | 2,4-DAT | 95-80-7 | | |
| 3,3'-Dichlorobenzidine dihydrochloride | DCB | 612-83-9 | | |
| 7,12-Dimethylbenz[a]anthracene | DMBA | 57-97-6 | | |
| 3,3'-Dimethylbenzidine dihydrochloride | DMB | 612-82-8 | | |
| 1,2-Dimethylhydrazine dihydrochloride | DMH | 306-37-6 | | |
| Dimethylnitrosamine | DMN | 62-75-9 | | |
### Table 1. Continued.

| Chemicals | Abbreviation | CAS No. | Reference for carcinogenicity | Ames test | Reference for Ames test | Experimental conditions in this evaluation |
|-----------|--------------|---------|-------------------------------|-----------|-------------------------|------------------------------------------|
| N-Ethyl-N-hydroxyethyl-methylamine | EHEN | 13147-25-6 | 82 | + | 82 | F344 M | 0.1%<sup>a</sup> Water |
| Glycidol | | 556-52-5 | 83 | + | 83 | F344 M | 0.04% Water |
| 4,4'-Methylenebis(2-chloroaniline) | MOCA | 101-14-4 | 84 | + | 118 | F344 M | 0.1% Diet |
| 4,4'-Methyleneedianiline | MDA | 101-77-9 | 81 | + | 118 | F344 M | 0.08% Water |
| N-Methyl-N'-nitro-N-nitrosoguanidine | MNG | 70-25-7 | 81 | + | 118 | F344 M | 10 mg/kg Gavage |
| 2-Nitrofluorenone | 2-NF | 607-57-8 | 85 | + | 85 | F344 M | 0.02% Diet |
| 2-Nitropropane | 2-NP | 79-46-9 | 86 | + | 127 | F344 M | 40 mg/kg Gavage |
| 4-Nitroquinoline 1-oxide | 4NQO | 56-57-5 | 87 | + | 118 | F344 M | 0.005% Water |
| N-Nitrosodiethyiamine | DEN | 55-18-5 | 56 | + | 118 | F344 M | 0.001% Water |
| 5-Nitro-o-toluidine | PNOT | 99-55-8 | 53 | + | 118 | F344 M | 0.01% Diet |
| Potassium bromate | KBrO<sub>3</sub> | 7758-01-2 | 85 | + | 88 | F344 M | 0.05% Water |
| Quercetin hydrate | Quercetin | 849061-97-8 | 89 | + | 118 | SD M | 5% Diet |
| Tris(2,3-dibromopropyl) phosphate | TBPP | 126-72-7 | 90 | + | 90 | F344 M | 0.01% Diet |
| 2,4-Xyline hydrochloride | 2,4-Xyl | 21436-96-4 | 91 | + | 118 | F344 M | 0.4% Diet |
| Acrylamide | | 79-06-1 | 92 | + | 92 | F344 M | 0.005% Water |
| Aniline hydrochloride | Aniline | 142-04-1 | 45 | + | 43 | F344 M | 0.6% Diet |
| Chloroacetic acid | CRA | 115-28-6 | 53 | + | 118 | SD M | 0.125% Diet |
| Chlorobenzene | CB | 108-90-7 | 85 | + | 118 | SD M | 250 mg/kg Gavage |
| Chloroform | CTN | 1897-45-6 | 94 | + | 118 | F344 M | 0.3% Diet |
| Clofibrate | CFB | 637-07-0 | 95 | + | 118 | SD M | 0.5% Diet |
| Decabromodiphenyl ether | DBDE | 1163-19-5 | 96 | + | 118 | SD M | 5% Diet |
| Di-(2-ethylhexyl)phthalate | DEHP | 117-81-7 | 40 | + | 118 | F344 M | 1.2% Diet |
| 1,4-Dioxane | DO | 123-91-1 | 97 | + | 118 | F344 M | 0.5% Water |
| Doxylamine succinate | DOX | 562-10-7 | 75 | + | 118 | SD M | 0.2% Diet |
| DL-Ethionine | DL-En | 67-21-0 | 98 | + | 118 | SD M | 0.1% Diet |
| Furan | | 110-08-9 | 99 | + | 118 | SD M | 0.3 mg/kg Gavage |
| Hexachlorobutadiene | HCB | 87-68-3 | 100 | + | 118 | F344 M | 0.03% Diet |
| d,L-Limonene | LIM | 5899-27-5 | 101 | + | 118 | F344 M | 150 mg/kg Gavage |
| Methimazole | MMI | 60-56-0 | 75 | + | 118 | SD M | 10 mg/kg Gavage |
| Ochratoxin A | OTA | 303-47-9 | 102 | + | 118 | F344 M | 0.21 mg/kg Gavage |
| Poncet 3R | CI16155 | 3564-09-8 | 39 | + | 118 | SD M | 5% Diet |
| 6-Propyl-2-thiouracil | PTU | 51-52-5 | 75 | + | 118 | SD M | 3 mg/kg Gavage |
| Rosiglitazone | RGZ | 122320-73-4 | 87 | + | 67 | F344 M | 50 mg/kg Gavage |
| Thiosacetamide | TAA | 62-55-5 | 103 | + | 118 | F344 M | 0.04% Diet |

### Noncarcinogen (17)

- 2,6-Diaminotoluene | 2,6-DAT | 823-40-5 | 104 | + | 118 | F344 M | 0.1% Diet |
- 8-Hydroxyquinoline | 8-HQ | 148-24-3 | 105 | + | 118 | F344 M | 0.6% Diet |
- 6-Mercaptopurine mono hydrate | 6-MP | 6122-76-1 | 106 | + | 126 | F344 M | 0.01% Diet |
- p-Toluidine hydrochloride | p-Tol | 540-23-8 | 107 | + | 118 | F344 M | 0.2% Diet |
- Amipicillin trihydrate | AMP | 7175-48-2 | 108 | + | 118 | F344 M | 1.35 mg/kg Gavage |
- Anthranilic acid | o-AA | 118-92-3 | 109 | + | 118 | F344 M | 1% Diet |
- Caprolactam | CPL | 105-60-2 | 110 | + | 118 | SD M | 0.75% Diet |
- Carboxin | CBX | 5234-68-4 | 110 | + | 118 | F344 M | 0.2% Diet |
- 4-Chloro-o-toluidine hydrochloride | 4-CT | 3165-93-3 | 60 | + | 118 | F344 M | 1% Diet |
- 5-Chloro-o-toluidine hydrochloride | 5-CT | 6259-42-3 | 83 | + | 118 | F344 M | 0.5% Diet |
- Cyclohexane | CHN | 108-94-1 | 112 | + | 118 | SD M | 0.65% Water |
- Ethionamide | ETH | 536-33-4 | 113 | + | 118 | F344 M | 0.3% Diet |
- 5-Fluouracil | 5-FU | 51-21-8 | 114 | + | 118 | F344 M | 0.0125% Water |
- Hexachlorophene | NAB | 70-30-4 | 115 | + | 118 | SD M | 0.015% Diet |
- 1-Nitropropane | 1-NP | 108-03-2 | 86 | + | 129 | F344 M | 90 mg/kg Gavage |
- Sodium arsenite | NaAsO<sub>2</sub> | 7784-46-5 | 116 | + | 130 | F344 M | 0.0173% Water |
- Sodium L-ascobate | Na-AsA | 134-03-2 | 117 | + | 131 | F344 M | 5% Diet |

<sup>a</sup> +, positive; -, negative.

<sup>b</sup> The administration dose was changed to 0.05% from week 3 due to body weight loss.

<sup>c</sup> The administration dose was changed to 0.0025% from week 2 due to body weight loss.
Characteristics and quantitative analysis of γ-H2AX formation in the rat urinary bladder

Based on our validation results, we found that γ-H2AX formation in the urothelium of the urinary bladder of 10-week-old male F344 and SD rats (used as control groups) was rare (mean of all individuals [n=149] in the control group: 1.1‰; range of mean values: 0.4–3.2‰; Fig. 1A). In contrast, various types of γ-H2AX formation, including typical dot-like foci in the nucleus, diffuse positive staining throughout the nucleus, and ring-like patterns consistent with the nuclear membrane (Fig. 1B–D), are observed in bladder carcinogen-treated groups, consistent with results reported in various types of cancer cells and cultured cells exposed to different DNA damaging agents[8]. Although in vitro studies have suggested that pan-nuclear staining and ring-like patterns are associated with clustered DNA damage and apoptosis, respectively, their relevance in vivo has not been clarified[33, 34]. For quantitative analysis, γ-H2AX-positive epithelial cells and whole epithelial cells in the bladder urothelium are counted under a light microscope. We calculated the ratio of γ-H2AX-positive cells by counting more than 1,800 epithelial cells from each animal. Because it can be difficult to detect small foci at low magnification, observation at high magnification is required for accurate counting. The criteria for distinguishing individual cells as positive or negative should be based on a comparison with a concurrent control group owing to interlaboratory differences in the antibodies used and procedures used for immunostaining. As γ-H2AX is also formed in apoptotic cells[35, 36], cells exhibiting morphological features of apoptosis, such as pyknosis, may need to be excluded from the count (Fig. 1B). Nevertheless, in our experience, the number of cells showing γ-H2AX-positivity owing to apoptosis did not increase sufficiently to impact the evaluation.

Statistical analysis

Statistical analysis of the mean values in each group is performed according to the standard protocol for a 28-day repeated-dose toxicity study. Evaluation of carcinogenic potential in the urinary bladder is based on a comparison with the control group, and an increase in the ratio of γ-H2AX-positive cells (p<0.05) is considered positive. In most of our validation experiments, multiple substances were evaluated at a single dose; therefore, we applied Student’s t-test for statistical evaluations. Among the bladder carcinogens evaluated as negative by multiple comparison tests (Dunnett’s test) in our previous study[27], four substances, i.e., phenethyl isothiocyanate, 2,2-bis(bromomethyl)-1,3-propanediol (BMP), uracil, and melamine, showed statistically significant differences when re-evaluated using Student’s t-test[37]. This may be due to the presence of potent genotoxic bladder carcinogens (e.g., BBN and 2-nitroanisole [2-NA]), which induced exceedingly high numbers of positive cells, in groups subjected to multiple comparison tests. Considering

Fig. 1. Representative immunohistochemical findings for γ-H2AX in the urinary bladder of rats orally administered bladder carcinogens for 28 days. A, Untreated control. B, 2-Nitroanisole. C and D, Aristolochic acid I. E, N-Butyl-N-(4-hydroxybutyl)nitrosamine. F, 2,2-Bis(bromomethyl)-1,3-propanediol. γ-H2AX-positive cells show characteristic dot-like intranuclear foci (B, arrowheads and inset). γ-H2AX can be observed in apoptotic cells with pyknosis or nuclear fragmentation (B, arrow). Diffuse positive staining can also be observed throughout the nucleus (C, arrowhead) and in a ring-like pattern consistent with the nuclear membrane (D, arrowhead). γ-H2AX formation can be detected in the proliferative lesions, as well as in the normal-appearing urothelium (E, arrowheads). γ-H2AX formation is mainly induced in the basal layer of the urothelium (B–E), whereas some chemicals induce γ-H2AX specifically in the superficial layer (F, arrowheads). Original magnification: 400×.
the consistently low values in the control group and the fact that γ-H2AX formation induced by bladder carcinogens is clearly dose-dependent, regardless of the genotoxic mechanisms, as discussed below, the increased γ-H2AX ratios in these groups should be detectable in practical toxicity studies using multiple doses of a single chemical.

Results

Results for 100 compounds

Using this method, we and our collaborators have examined a total of 100 substances classified into three categories: bladder carcinogens, non-bladder carcinogens, and noncarcinogens (Table 1). Simultaneously, these compounds were divided into two groups according to their reported mutagenicity in a bacterial reverse mutation test (Ames test): mutagenic and nonmutagenic compounds. The results of the quantitative analysis of γ-H2AX immunostaining in the rat bladder urothelium are shown in Fig. 2. Detailed results for 41 compounds have been previously documented. As expected based on the observation that γ-H2AX is a sensitive marker of DNA damage, the level of γ-H2AX formation tended to be elevated in rats treated with mutagenic (Ames-positive) bladder carcinogens. Significant increases in γ-H2AX-positive cells were also observed for nonmutagenic (Ames-negative) bladder carcinogens, although to a relatively weak extent, probably due to secondary DNA damage associated with replication stress or production of reactive oxygen species caused by sustained proliferative stimulation. γ-H2AX formation in the bladder urothelium of rats treated with non-bladder carcinogens and noncarcinogens, regardless of genotoxicity, remained at the same levels as in the control group, with a few exceptions.

A summary of the bladder carcinogenicity of various compounds, as evaluated by γ-H2AX immunostaining, is shown in Table 2. The most important result was that for 39 bladder carcinogens showed positive results (sensitivity = 84.6%), leaving only six false negatives. Among the 26 mutagenic and 13 nonmutagenic bladder carcinogens, 24 (92.3%) and 9 (69.2%) were positive, respectively, indicating that mutagenic substances could be detected with higher sensitivity even as a single marker, combining multiple biomarkers may further improve sensitivity. Differentiation and stem cell-related markers of the bladder mucosa are promising candidates, given that abnormalities in differentiation processes, such as disordered expression of uroplakin, a specific marker of superficial cells, reportedly occur during bladder carcinogenesis. We performed immunohistochemical analyses of bladder tissue and cancer stem cell markers, including cytokeratin 14 (KRT14), aldehyde dehydrogenase 1A1 (ALDH1A1), and CD44, using urinary bladder samples derived from rats orally administered 14 bladder carcinogens and five non-bladder carcinogens for 28 days. In rats treated with bladder carcinogens, increased expression of KRT14, ALDH1A1, and CD44 was observed in 9, 10, and 10 of the 14 groups, respectively, whereas the five non-bladder carcinogens did not induce upregulation of these markers. Importantly, two of the three bladder carcinogens with negative results on γ-H2AX immunostaining (4-amino-2-nitrophenol [ANP] and dimethylarsinic acid [DMA]) could increase the expression of stem cell markers. We recently confirmed that these stem cell markers are indeed expressed in focal proliferative lesions, including tumors, and that ALDH1A1 and CD44 expression can persist of 0 and 77% for malignant bladder tumors in rats treated with 0.0001 and 0.001% BBN for 2 years, respectively. In addition, the ratios of γ-H2AX-positive cells were also clearly different in rats treated with 1 and 3% melamine for 28 days, with bladder cancer incidence rates of 5 and 79% after 36 weeks of treatment, respectively, reflecting the strength of carcinogenicity. Moreover, a dose-dependent increase in γ-H2AX formation was also observed in the 2-day treatment group for both BBN and melamine, suggesting that this method could be applied to short-term studies.

To examine changes in γ-H2AX formation in the bladder carcinogenesis in different species, 6-week-old male B6C3F1 mice were orally administered 12 chemicals for 28 days. All substances that were carcinogenic to the mouse bladder caused significant increases in γ-H2AX-positive cells, as was observed in rats, whereas the levels of γ-H2AX-positive cells in mice treated with non-bladder carcinogens remained the same as those in the control group. Importantly, 2-NA, a potent bladder carcinogen in rats but does not cause bladder tumors in mice, significantly enhanced γ-H2AX formation in rats only, indicating that this method could reflect species differences between rats and mice.

Stem cell markers as complementary factors for γ-H2AX

As described above, although immunohistochemistry for γ-H2AX could detect bladder carcinogens with high sensitivity even as a single marker, combining multiple bio-markers may further improve sensitivity. Differentiation and stem cell-related markers of the bladder mucosa are promising candidates, given that abnormalities in differentiation processes, such as disordered expression of uroplakin, a specific marker of superficial cells, reportedly occur during bladder carcinogenesis. We performed immunohistochemical analyses of bladder tissue and cancer stem cell markers, including cytokeratin 14 (KRT14), aldehyde dehydrogenase 1A1 (ALDH1A1), and CD44, using urinary bladder samples derived from rats orally administered 14 bladder carcinogens and five non-bladder carcinogens for 28 days. In rats treated with bladder carcinogens, increased expression of KRT14, ALDH1A1, and CD44 was observed in 9, 10, and 10 of the 14 groups, respectively, whereas the five non-bladder carcinogens did not induce upregulation of these markers. Importantly, two of the three bladder carcinogens with negative results on γ-H2AX immunostaining (4-amino-2-nitrophenol [ANP] and dimethylarsinic acid [DMA]) could increase the expression of stem cell markers. We recently confirmed that these stem cell markers are indeed expressed in focal proliferative lesions, including tumors, and that ALDH1A1 and CD44 expression can persist
Fig. 2. Quantitative analysis of γ-H2AX formation in the urothelium of rats orally administered chemicals for 28 days. γ-H2AX staining was evaluated by determining the average number of γ-H2AX-positive epithelial cells per 1000 cells. Each group contained 4–6 animals. Values represent the means ± standard deviations. * and **: significantly different from the corresponding control at p<0.05 and 0.01, respectively (Student’s t-test). NS, not significant. A, Control and bladder carcinogen-treated groups. B, Non-bladder carcinogen- and noncarcinogen-treated groups.
Detection of Bladder Carcinogens by γ-H2AX Immunostaining

**Table 2. Detection of Bladder Carcinogens by Immunohistochemistry for γ-H2AX**

| Increased γ-H2AX formation | Bladder carcinogen | Non-bladder carcinogen and noncarcinogen |
|----------------------------|--------------------|-----------------------------------------|
|                            | Ames-positive      | Ames-negative                           | Ames-positive | Ames-negative |
| Positive                   | 24                 | 9                                       | 1<sup>a</sup> | 2<sup>b</sup> |
| Negative                   | 2<sup>c</sup>      | 4<sup>d</sup>                           | 27           | 31           |
| Sensitivity or specificity | 92.3%              | 69.2%                                   | 96.4%        | 93.9%        |

|                | 84.6% (33/39)     | 95.1% (58/61)                          |

<sup>a</sup> 4-Aminoazobenzene hydrochloride.

<sup>b</sup> 4-Chloro-o-toluidine hydrochloride and sodium arsenite.

<sup>c</sup> 4-Amino-2-nitrophenol and N-nitrosomorpholine.

<sup>d</sup> m-Cresidine, dimethylarsinic acid, pioglitazone hydrochloride, and sulfasalazine.

**Fig. 3.** Dose-dependency of γ-H2AX formation in the bladder urothelium of rats orally administered Ames-positive (N-butyl-N-(4-hydroxybutyl)nitrosamine [BBN]) and Ames-negative (melamine) bladder carcinogens for 28 days. γ-H2AX staining was evaluated by determining the average number of γ-H2AX-positive epithelial cells per 1,000 cells. Each group contained 5 animals. * and **: significantly different from the control at p<0.05 and 0.01, respectively (Dunnett’s multiple comparison test). †: significantly different from the control at p<0.01 (Jonckheere’s trend test).

**Fig. 4.** Reproducibility of γ-H2AX formation in the bladder urothelium of male F344 rats administered 0.05% N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) in drinking water for 28 days in three separate experiments. γ-H2AX staining was evaluated by determining the average number of γ-H2AX-positive epithelial cells per 1,000 cells. Each group contained 5 animals. Values represent the means ± standard deviations.
long-term in the normal-appearing urothelium after BBN withdrawal, as determined in a time-course analysis using a BBN-induced rat bladder cancer model. Thus, these bladder stem cell markers can be useful as complementary markers for γ-H2AX in the early detection of bladder carcinogens.

KRT14 and CD44 expression in the bladder urothelium is an indicator of stem cell potential, and KRT14 may possess more substantial primitive stem cell properties than CD44. In addition, subpopulations of CD44-positive cells in bladder tumors co-expressing ALDH1A1 exhibit more potent clonogenic and tumorigenic properties, suggesting that these co-expressing cells are primitive cancer stem cells. ALDH1A is an essential enzyme that participates in the metabolism of retinol (vitamin A) to retinoic acid (RA) and plays an important role in the RA signaling pathway, which mediates the morphogenesis of many organs, including the kidney and urinary bladder. In the urinary bladder, RA is required for urothelial differentiation and regeneration, specifically for the formation of intermediate cells and superficial cells. Thus, these three stem cell markers may reflect different stages and mechanisms of urothelial differentiation and could be employed to elucidate the mechanisms of bladder carcinogenesis.

**Chemicals with false-negative results**

In total, six bladder carcinogens exhibited false-negative results on γ-H2AX immunostaining: two with mutagenicity (ANP and N-nitrosomorpholine [NMOR]) and four without mutagenicity (m-cresidine [m-Cre], DMA, pioglitazone hydrochloride [PGZ], and sulfasalazine [SSZ]). In a 2-year carcinogenicity study, ANP, an aromatic amine, induced bladder tumors (33%, 13/39) and hyperplasia in male rats in the high-dose (0.25% in the diet) group, whereas no bladder tumors were detected in the low-dose (0.125%) group. DMA is an arsenic-containing compound, and the incidence of bladder tumors in male F344 rats treated with 0.005 and 0.02% DMA in drinking water was 26 (8/31) and 39% (12/31), respectively. Among the six substances, these two compounds, associated with relatively high incidences of bladder cancer, can be detected by combined evaluation with bladder stem cell markers.

NMOR is a nitrosamine that induces liver tumors in rats at high frequencies. In contrast, its carcinogenicity in the bladder is weak, with a reported bladder tumor incidence of up to 4.2% (benign papillomas only) and unclear dose-dependency. Although m-Cre is a structural isomer of p-cresidine (p-Cre), its potential for bladder carcinogenicity is weaker than that of p-Cre, and the incidence of bladder cancer with orally administered m-Cre (160 mg/kg/day) was 11.4% (5/44). PGZ, a peroxisome proliferator-activated receptor-γ agonist, is an anti-diabetic drug known to induce benign and malignant bladder tumors in male rats; however, the frequency remains low (11.7%, 7/60). SSZ, a sulfonamide used as an anti-inflammatory agent, induces benign papillomas in rats with suspected urolithiasis at a low frequency (12%, 6/50). Thus, all substances that exhibited false-negative results for γ-H2AX and negative results for bladder stem cell markers elicited benign tumors only or low tumor incidence.

**Chemicals with false-positive results**

The three non-bladder carcinogens that showed false-positive results on γ-H2AX immunostaining were mutagenic (4-aminoazobenzene hydrochloride [AAB]) and two nonmutagenic substances (4-chloro-α-toluidine hydrochloride [4-CT] and sodium arsenite [NaAsO2]). 4-CT is an aromatic amine with a structure similar to that of α-toluidine, and epidemiological studies have revealed a possible association between occupational exposure and bladder cancer in humans. In contrast, carcinogenicity studies in rats (highest dose: 0.4 or 0.5% in the diet) have shown no evidence of carcinogenicity in any organ, including the urinary bladder. Thus, these three stem cell markers were observed at a dose of 0.5%, whereas levels similar to those in the control group were observed at a dose of 0.5%, which was used in carcinogenicity studies (manuscript in preparation). This result was similar to that observed in our previous study using o-toluidine, which induced severe mucosal damage and a significant increase in γ-H2AX-positive cells in the 0.8% group, but not in the 0.4% group. These results suggest that there may be a threshold for the appearance of specific metabolites (or unchanged forms) capable of causing damage to the bladder urothelium and inducing DNA damage or cell proliferation in the urine. Although additional studies are needed, the possibility that 4-CT is carcinogenic to the rat urinary bladder cannot be excluded when long-term administration at high doses is possible.

NaAsO2 is a type of inorganic arsenic compound that, combined with other arsenic compounds, has been shown by several epidemiological studies to be a cause of human bladder cancer. Although only one case of bladder cancer was observed in female rats in the middle-dose group after 2 years of drinking water administration, the authors concluded that the relevance of this result should not be underestimated, given that no cases were found in background data from over 2,000 female rats. In addition, the short-term dietary administration of NaAsO2 caused hyperplasia of the rat bladder urothelium in a dose-dependent manner. Although AAB is an aromatic amine, information regarding its potential carcinogenicity remains limited to older studies. To the best of our knowledge, there are no reports of carcinogenicity tests conducted using standard methods. Therefore, additional studies on the bladder carcinogenicity of NaAsO2 and AAB are recommended.
Table 3. Detection of Bladder Carcinogens by Immunohistochemistry for Ki67

| Increased Ki67 expression | Bladder carcinogen | Non-bladder carcinogen and noncarcinogen |
|---------------------------|--------------------|----------------------------------------|
|                           | Ames-positive | Ames-negative | Ames-positive | Ames-negative |
| Positive                  | 19            | 7             | 2           | 3           |
| Negative                  | 7            | 6             | 13          | 9           |
| Sensitivity or specificity| 73.1%         | 53.8%         | 86.7%       | 75.0%       |

* 4-Aminoazobenzene hydrochloride and 8-hydroxyquinoline.
* 4-Chloro-o-toluidine hydrochloride, sodium L-ascorbate, and sodium arsenite.
* 4-Amino-2-nitrophenol, disperse blue 1, N-nitrosobis(2-oxopropyl)amine, N-nitroso-N-methylurea, N-nitrosomorpholine, 2-nitrosotoluene, and o-toluidine hydrochloride.
* m-Cresidine, dimethylylarsinic acid, N-nitrosodiphenylamine, pioglitazone hydrochloride, sulfasalazine, and trisodium nitritolriacetate monohydrate.

### Features of γ-H2AX Immunostaining and Future Perspectives in Carcinogenicity Assessment

**Comparison with other predictive factors for bladder carcinogenicity**

Several bladder carcinogens used in this validation could induce diffuse simple hyperplasia of the bladder urothelium after 28 days of administration. Hyperplasia of the urothelium has been suggested as a useful predictive factor of bladder carcinogenicity when combined with 28-day and 13-week repeated-dose toxicity studies. Our proposed method using γ-H2AX immunostaining allows evaluation within a shorter experimental period and with fewer animals (five rats per group). More importantly, γ-H2AX formation was observed not only in proliferative lesions but also in the surrounding normal-appearing urothelium (Fig. 1C–E) and in rats treated with several bladder carcinogens such as 2-acetylaminofluorene (2-AAF) and cyclophosphamide monohydrate, which do not induce pathological findings after short-term administration; these findings indicate that γ-H2AX formation may be a biomarker that precedes the formation of histological lesions. Indeed, in an experiment with multiple doses of BBN, a significant increase in γ-H2AX formation was observed after 28 days of administration of a lower dose (0.01%) than at the dose (0.01%) at which histopathological findings, such as mononuclear cell infiltration and simple hyperplasia, were observed. In addition, significant increases in γ-H2AX-positive cells were observed on day 2, when no these bladder lesions were detected. These results suggest that assessing bladder carcinogenicity using γ-H2AX formation as an indicator is more sensitive than assessments based on histopathological findings. The increase in γ-H2AX-positive cells at two days after the initiation of BBN administration suggests that an evaluation period shorter than 28 days may be possible, warranting further investigations.

Ki67 is a well-known cell proliferation marker widely used as a predictive biomarker for malignancy and cancer prognosis. To evaluate the ability of these markers to detect bladder carcinogens, we examined the ratio of Ki67- and γ-H2AX-positive cells in the bladder urothelium (Table 3). The sensitivity and specificity for detecting bladder carcinogens by Ki67 were 66.7% (26/39) and 81.5% (22/27), respectively, both of which were lower than those of γ-H2AX. Bladder carcinogens with relatively low ratios of γ-H2AX-positive cells tended to be negative for Ki67, indicating the superior sensitivity of γ-H2AX formation. In the experiment with multiple BBN doses described above, a significant increase in Ki67 expression was detected at high doses compared with γ-H2AX on days 2 and 28.

In contrast, in the experiment with multiple doses of melamine, γ-H2AX formation and Ki67 expression increased at the same dose on day 28, whereas Ki67 expression increased after administration of a lower dose on day 2. These results suggest that γ-H2AX formation induced by melamine, a nongenotoxic substance, is associated with indirect DNA damage secondary to increased cell proliferative activity rather than direct DNA damage. Interestingly, double immunofluorescence staining for γ-H2AX and Ki67 revealed that most γ-H2AX-positive cells in the melamine-treated group simultaneously displayed Ki67 expression, whereas there were many γ-H2AX single-positive cells in the BBN-treated group. Taken together, these findings showed that, although Ki67 immunostaining alone is insufficient for the early detection of bladder carcinogens, double staining with γ-H2AX may discriminate the involvement of genotoxic mechanisms in bladder carcinogenesis.

**Significance of γ-H2AX formation in bladder carcinogenesis**

The bladder urothelium consists of three layers: basal, intermediate, and superficial layers. Among these, increased γ-H2AX formation was mainly observed in the basal layer (Fig. 1B–E). Notably, stem cells located in the basal layer are closely associated with the regeneration and carcinogenesis of the bladder epithelium. The high prevalence of γ-H2AX formation in the basal layer may indicate that direct and indirect DNA damage caused by bladder carcinogens occurs in stem cells. In contrast, some bladder carcinogens (e.g., BMP and DMA) induce γ-H2AX formation mainly in the epithelial cells of the superficial...
layer (Fig. 1F)\(^{27}\). DMA is known to cause acute cytotoxicity in superficial cells\(^{152}\), inducing regenerative proliferation and secondary DNA damage. Additionally, 2-AAF induced γ-H2AX formation in the basal layer of rats, whereas 2-AAF-treated mice showed more frequent γ-H2AX-positive cells in the superficial layer, with decreased and disorganized expression of uroplakin III, a superficial cell marker\(^{27, 136}\). In a previous study, electron microscopy revealed mouse-specific cytotoxicity in superficial cells following 2-AAF treatment, reflecting species-specific differences in carcinogenic mechanisms\(^{153}\).

For several bladder carcinogens, we set a 2-week recovery period after 28 days of treatment and examined changes in the number of γ-H2AX- and Ki67-positive cells. The results showed that Ki67 expression rapidly decreased, similar to those observed in the control group, whereas γ-H2AX formation also decreased but remained at a significantly high level\(^{27-29}\). These results suggest that γ-H2AX formation in the urothelium is not a transient response to chemical exposure but may be closely associated with the long-term process of bladder carcinogenesis. Furthermore, we recently demonstrated that γ-H2AX formation in the urothelium persists for a prolonged period after BBN withdrawal using a time-course analysis of changes in γ-H2AX formation and stem cell marker expression in a BBN-induced rat bladder cancer model\(^{139}\). Both external factors, including genotoxic agents, and endogenous factors, such as aging, are known to cause persistent γ-H2AX formation, which is not completely repaired\(^{154}\). This persistent γ-H2AX formation may facilitate carcinogenesis via induction of genomic instability\(^{155}\).

Extrapolation to humans and possible applications in other organs

As discussed above, γ-H2AX immunostaining is useful for the early detection of bladder carcinogens; however, this method is predictive of the results of 2-year carcinogenicity studies in rats. In particular, although bladder cancer induced by physical stimulation via calculi or crystalluria is often observed in rodents, in many cases, the findings cannot be extrapolated to humans because of species differences in urine pH and ion concentrations\(^{156}\). Thus, the ability to detect carcinogens regardless of their genotoxicity is an advantage afforded by this method, and additional information is required to determine whether a threshold value can be established. Determination of the carcinogenicity to humans of a substance that significantly increases γ-H2AX-positive cells should be made using the same approach as for conventional carcinogenicity testing.

The development clinically or histopathologically detectable tumors from normal tissue takes a long time. The concept of the “field effect” (also referred to as “field change” or “field carcinization”) has been proposed for developing various human tumors, such as bladder, esophageal, and mammary gland tumors, in which a population of cells with shared gene mutations gradually expands their area and replaces the normal mucosa\(^{157-159}\). Urothelial carcinoma of the bladder is generally classified into two types, papillary/luminal and non-papillary/basal, and it has been suggested that both types may develop through the field effect via their inherent specific pathways\(^{160}\). The field effect is thought to arise from the histologically normal-appearing urothelium at the very beginning of bladder carcinogenesis, and the γ-H2AX formation observed in our study may be a relevant indicator. In addition, the application of γ-H2AX to other organs, such as the lungs and stomach, has recently been investigated in rodents\(^{161, 162}\). Further studies are needed to examine whether these results can be applied to the main target organs typically examined in carcinogenicity tests, including the liver and kidneys. It could be possible to use markers other than γ-H2AX, such as stem cell markers for the urinary bladder, in a combined evaluation method for targeting organ-specific stem cell characteristics and carcinogenic mechanisms.

Conclusions

The γ-H2AX immunostaining approach described herein is a rapid, reliable, and practical method for the early detection of bladder carcinogens with high sensitivity and specificity. This method does not require additional animal experiments and can be performed by conventional immunohistochemical analysis using formalin-fixed paraffin-embedded tissues. Therefore, this approach can be readily incorporated into existing 28-day repeated-dose toxicity studies as designated in the OECD guidelines. This method can also be applied as a screening evaluation method for assessing the carcinogenicity of a wide range of chemical substances, ranging from industrial chemicals to pharmaceuticals.

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