J Toxicol Pathol 2013; 26: 215–221

Short Communication

Detection of γ-H2AX, a Biomarker for DNA Double-strand Breaks, in Urinary Bladders of *N*-Butyl-*N*-(4-Hydroxybutyl)-Nitrosamine-Treated Rats

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Abstract: To evaluate the potential role of DNA repair in bladder carcinogenesis, we performed an immunohistochemical analysis of expression of various DNA repair enzymes and γ-H2AX, a high-sensitivity marker of DNA double-strand breaks, in the urothelium of male F344 rats treated with *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (BBN), a bladder-specific carcinogen. Our results clearly demonstrated that γ-H2AX aggregation was specifically generated in nuclei of bladder epithelial cells of BBN-treated rats, which was not found in untreated controls or mesenchymal cells. γ-H2AX-positive cells were detected not only in hyperplastic and neoplastic areas but also in the normal-like urothelium after BBN treatment. These data indicate that γ-H2AX has potential as a useful biomarker for early detection of genotoxicity in the rat urinary bladder. To the best of our knowledge, this is the first report demonstrating expression of γ-H2AX during bladder carcinogenesis. (DOI: 10.1293/tox.26.215; J Toxicol Pathol 2013; 26: 215–221)

Key words: urinary bladder, γ-H2AX, DNA repair, double-strand breaks, *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine

Characterization of the genotoxic potential of chemical substances is essential for safety assessment. A battery of complementary tests is generally recommended, including the Ames bacterial mutagenesis test, mouse lymphoma assay (MLA) and chromosomal aberration test. In addition, *in vivo* genotoxicity assays using rodent models have been used to evaluate genetic damage occurring after metabolic processes in living organisms. The micronucleus test of mouse erythrocytes or bone marrow is regarded as one of the standard methods of testing for *in vivo* genotoxicity but has limitations regarding tissue specificity. For instance, diethylnitrosamine (DEN), a well-known hepatic mutagen/carcinogen, has been shown to be negative for the micronucleus test in hematopoietic cells[1]. Thus, the development of *in vivo* evaluation methods to detect organ-specific genotoxicity is a high priority.

Endogenous DNA damage arises from various pathways involving reactive oxygen species, spontaneous hydrolysis and exposure to UV light[2]. Nucleotides affected by sequence errors are usually replaced by repair mechanisms after the removal of a short segment of a damaged strand and copying from the intact complementary strand. Alternatively, broken DNA can be repaired by recombination. The mechanisms of DNA repair are regulated by specific enzymes for each function, and aberrant expression of repair enzymes can cause persistence of damaged DNA and consequent cancer development[2].

Urinary bladder cancer is the seventh most common cancer in males worldwide, with smoking and occupational exposures considered the major risk factors for development of the transitional cell carcinoma[3]. *N*-butyl-*N*-(4-hydroxybutyl)- nitrosamine (BBN) exhibits specific mutagenicity and carcinogenicity for the urinary bladder[4]. Although BBN-treated rodent models have been widely used for investigation of different aspects of bladder cancer[5,6], the mechanisms underlying its tissue-specificity remain unknown. While several previous studies have investigated the association of BBN-induced bladder carcinogenesis and DNA repair[7–9], there are only few published reports focusing on the expression of repair enzymes and phosphorylation of histone proteins. In the present study, to evaluate the potential role of DNA repair in bladder carcinogenesis, we examined the expression pattern of various repair enzymes and phosphorylated histone H2A protein (γ-H2AX) in BBN-treated F344 rats using immunohistochemistry.

BBN was purchased from Tokyo Chemical Industry (Tokyo, Japan). Six-week-old specific pathogen-free male rats (F344/DuCrj; Charles River Laboratories Japan, Yokohama, Japan) were housed in polycarbonate cages with soft chip bedding (Sankyo Labo Service, Tokyo, Japan) in a room with a barrier system controlled for the light/dark cycle (12 hr), ventilation (air exchange rate 18 times per hr), temperature (24 ± 1°C) and relative humidity (55 ± 5%) dur-

Received: 28 October 2012, Accepted: 1 February 2013
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Table 1. Antibodies Used for Immunohistochemistry

Antigen	Clonality (clone)	Host species	Dilution	Supplier
γ-H2AX	Poly	Rabbit	1:10	Cell Signaling Technology, Danvers, MA, USA
MRE11	Poly	Rabbit	1:500	Cell Signaling Technology
XRCC1	Mono (33-2-5)	Mouse	1:100	Abcam, Cambridge, MA, USA
MGMT	Mono (SPM287)	Mouse	Ready-to-use	Abcam
MLH1	Mono (EPR3894)	Rabbit	1:250	Abcam
MSH6	Mono (44)	Mouse	1:100	Abcam
APE1	Mono (13B8E5C2)	Mouse	1:5000	Abcam
DDB1	Mono (EPR6089)	Rabbit	1:250	Abcam
ERCC1	Mono (8F1)	Mouse	1:500	Abcam
TREX1	Poly	Rabbit	1:100	Abcam
SMC1	Mono [EPR2879(2)]	Rabbit	1:500	Abcam
RAD18	Mono	Mouse	1:1000	Abcam

Table 2. Immunohistochemical Expression Pattern of DNA Repair Markers

Class	Proteins	Control group		BBN-treated group	
		Urothelium	Mesenchymal cells	Normal-appearing and hyperplastic urothelium	Carcinoma
Double-strand breaks	γ-H2AX	−	−	+	+
	MRE11	+	+	++	++
	XRCC1	+	+	+	+
Direct damage reversal	MGMT	−	−	+	−
Mismatch repair	MLH1	±	±	+	+
	MSH6	+	±	+	+
Base excision repair	APE1	++	++	++	++
Nucleotide excision repair	DDB1	++	++	++	++
	ERCC1	±	−	+	+
Proofreading repair	TREX1	+	++	++	++
Cell cycle checkpoint	SMC1	++	++	++	++
Post-replication repair	RAD18	++	++	++	++

–, Almost negative; ±, scattered positive; +, occasionally positive; ++, extensively positive.

ing the study. The cages and chip bedding were exchanged twice a week. Each animal had free access to basal diet (CRF-1; Oriental Yeast, Tokyo, Japan) and tap water with or without BBN.

Animals were administered 0.05% BBN in their drinking water for 4 weeks and then maintained without any further treatment for 33 weeks. They were sacrificed under deep anesthesia at 43 weeks of age and subjected to laparotomy with excision of the urinary bladder. Untreated rats at 11 weeks of age were also sacrificed as a control group. The experimental design was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences, and the animals were cared for in accordance with institutional guidelines as well as the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, June 1st, 2006).

For histopathological and immunohistochemical examination, the excised urinary bladders were fixed in 10% neutral-buffered formalin for 24 hours, sliced along the longitudinal axis into strips of equal width and embedded in paraffin. Serial sections (4-μm thick) were prepared and stained with hematoxylin and eosin for histological observation and immunohistochemistry for DNA repair-associated proteins: γ-H2AX, MRE11 and XRCC1 (double-strand breaks); MGMT (direct damage reversal); MLH1 and MSH6 (mismatch repair); APE1 (base excision repair); DDB1 and ERCC1 (nucleotide excision repair); TREX1 (proofreading repair); SMC1 (cell cycle checkpoint); and RAD18 (post-replication repair). Table 1 provides details of the sources of the primary antibodies and conditions for the immunohistochemistry. Briefly, the serial sections were deparaffinized and hydrated through a graded series of ethanols and immersed in 3% H_2O_2/methanol solution for inhibition of endogenous peroxidase activity. For antigen retrieval, all sections were autoclaved in 10 mM citrate buffer (pH 6.0) for 15 minutes. After normal rabbit or goat serum treatment, the sections were incubated with primary antibodies for 12 hours at 4°C. Visualization of antibody binding was performed using a Histofine Simple Stain Rat MAX PO kit (Nichirei Corporation, Tokyo, Japan) and 3,3'-diaminobenzidine (DAB). All sections were counterstained with hematoxylin. The results of immunostaining in the epithelial cells, mesenchymal cells (smooth muscle cells and endothelium) and hyperplastic/neoplastic cells of the urinary bladder were classified into four degrees: – (almost negative), ± (scattered positive), + (occasionally positive) and ++

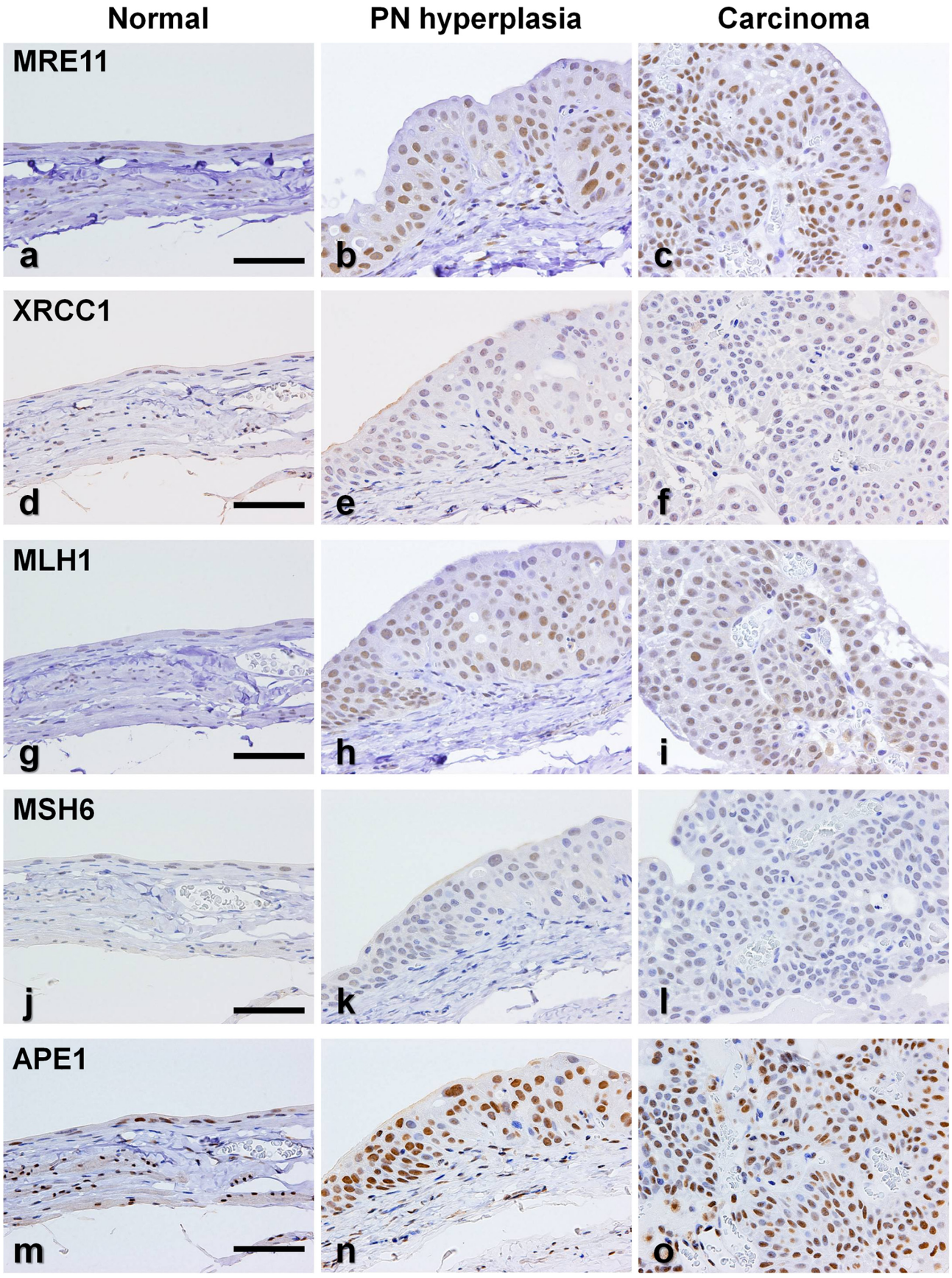

Fig. 1. Immunohistochemistry of DNA repair enzymes for double-strand breaks, mismatch repair and base excision repair in urinary bladder tissue of F344 rats. Left column, untreated control; center column, BBN-induced PN hyperplasia; right column, BBN-induced carcinoma. Bars = 50 µm. (a–c) MRE11. (d–f) XRCC1. (g–i) MLH1. (j–l) MSH6. (m–o) APE1.

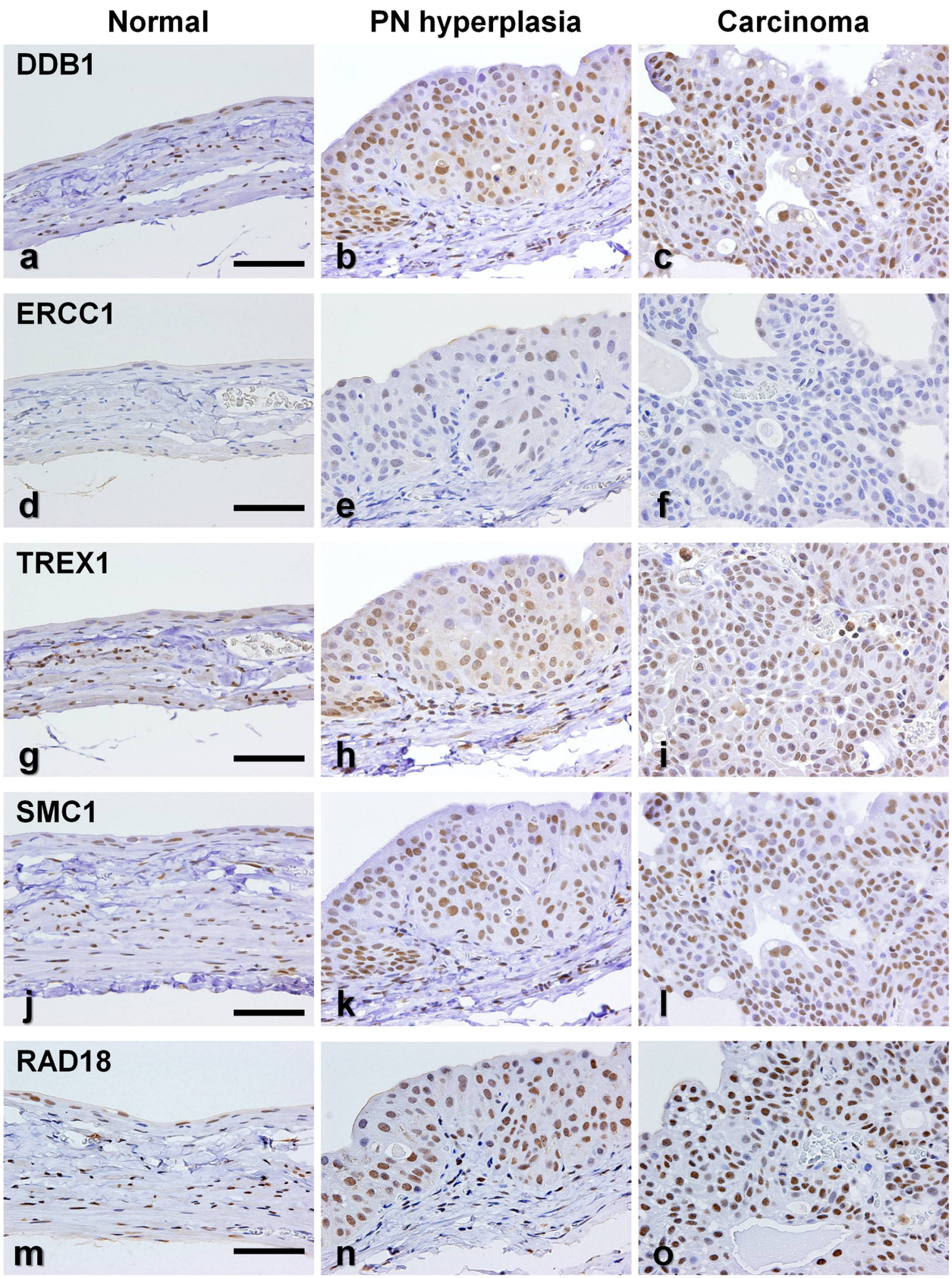

Fig. 2. Immunohistochemistry of DNA repair enzymes for nucleotide excision repair, proofreading repair, cell cycle checkpoint and post-replication repair in urinary bladder tissue of F344 rats. Left column, untreated control; center column, BBN-induced PN hyperplasia; right column, BBN-induced carcinoma. Bars = 50 µm. (a–c) DDB1. (d–f) ERCC1. (g–i) TREX1. (j–l) SMC1. (m–o) RAD18.

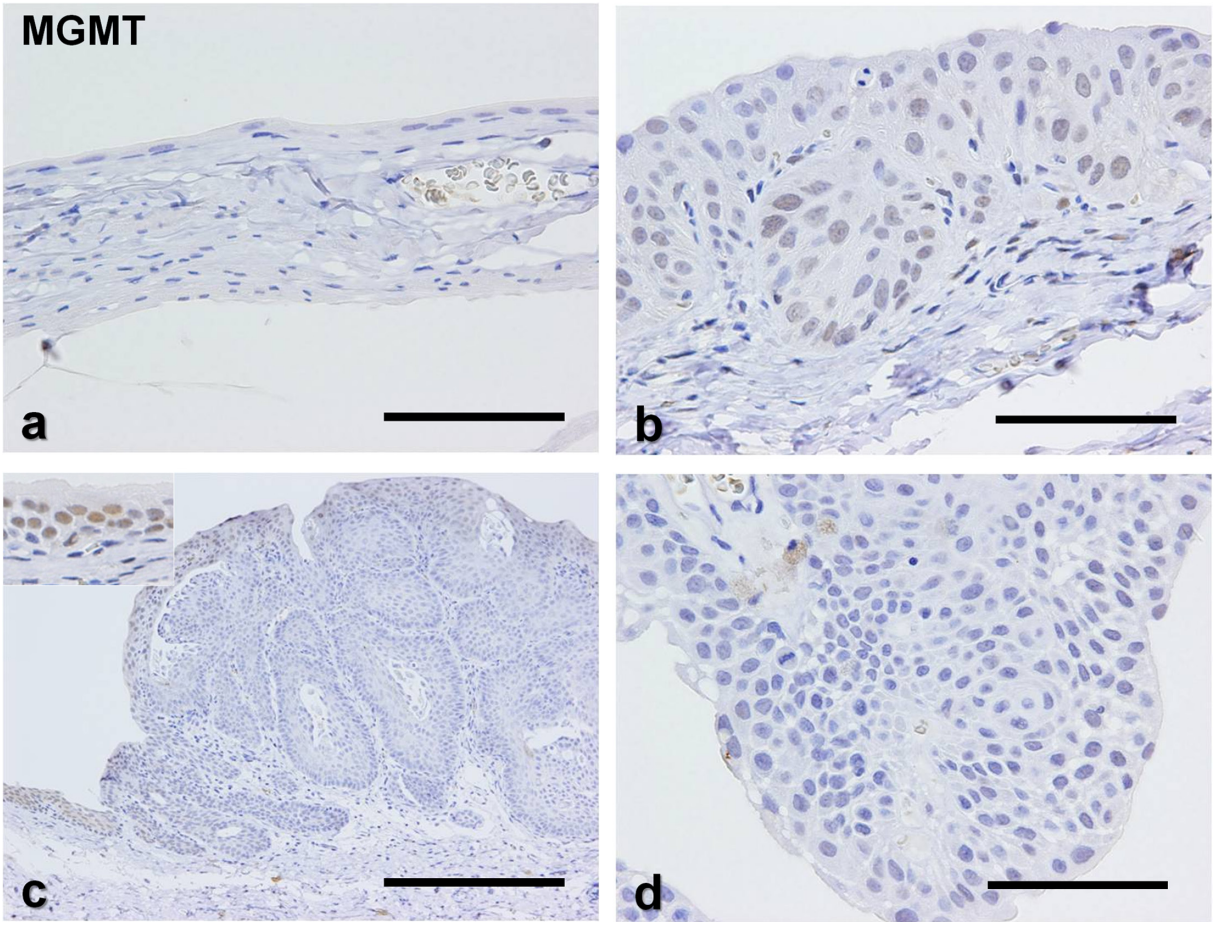

Fig. 3. Immunohistochemical findings for MGMT in urinary bladder tissue of F344 rats. Bars = 50 (a, b and d) or 200 (c) μm. (a) Untreated control. (b) BBN-induced PN hyperplasia. (c and d) BBN-induced bladder carcinoma. Surface epithelial cells (c) and the adjacent normal-looking urothelium (c, insert) are positive, while the neoplastic cells are negative.

(extensively positive).

Histopathologically, the incidences of papillary-nodular (PN) hyperplasia and transitional cell carcinoma in the urinary bladder were 73.3% and 46.7%, respectively. The results of immunohistochemistry are summarized in Table 2. The nuclei of the BBN-stimulated normal-looking urothelium, PN hyperplasia and carcinoma showed positive staining for XRCC1 (Fig. 1d–f), MSH6 (Fig. 1j–l), APE1 (Fig. 1m–o), DDB1 (Fig. 2a–c), SMC1 (Fig. 2j–l) and RAD18 (Fig. 2m–o) similar to that of untreated control tissue. MRE11 (Fig. 1a–c), MLH1 (Fig. 1g–i), ERCC1 (Fig. 2d–f) and TREX1 (Fig. 2g–i) were expressed in the BBN-treated normal-appearing urothelium, PN hyperplasia and carcinoma more extensively than in the control group.

Most epithelial cells of the normal bladder in the control rats were negative for MGMT (Fig. 3a) and γ-H2AX (Fig. 4a). Contrary to the occasional positive staining in the BBN-stimulated normal urothelium and PN hyperplasia, carcinomas were entirely negative for MGMT (Fig. 3b–d). In the BBN-treated group, γ-H2AX-positive granules were observed not only in the nuclei of hyperplastic and neoplastic cells but also in the normal-appearing epithelium (Fig. 4b–d). It was noteworthy that some mitotic chromosomes of neoplastic cells were strongly positive for γ-H2AX (Fig. 4d).

H2AX is a variant of the histone 2A family, which plays important roles not only in DNA packing but also in DNA repair. The rapid phosphorylation of H2AX at Serine 139, to become γ-H2AX, occurs in response to DNA double-strand breaks[10]. The phosphorylation of H2AX happens over a large region of chromatin surrounding double-strand breaks, leading to the accumulation of repair proteins[11]. The repair of double-strand breaks is extremely important to an individual, because this damage can lead to genome instability and cancer development[12]. Therefore, since its discovery in 1998[13], γ-H2AX has been used as a tool in multiple scientific fields, such as the *in vitro* assessment of preclinical drugs[14–16]. In addition, γ-H2AX has been applied to evaluation of DNA damage and genotoxicity screening for chemical agents[17–19]. In the present study, we demonstrated that γ-H2AX foci developed in the bladder epithelium of BBN-treated F344 rats using immunohistochemistry. The γ-H2AX-positive cells were observed not only in the proliferative lesions including PN hyperplasia and transitional cell carcinoma but also in the normal-like urothelium thirty-

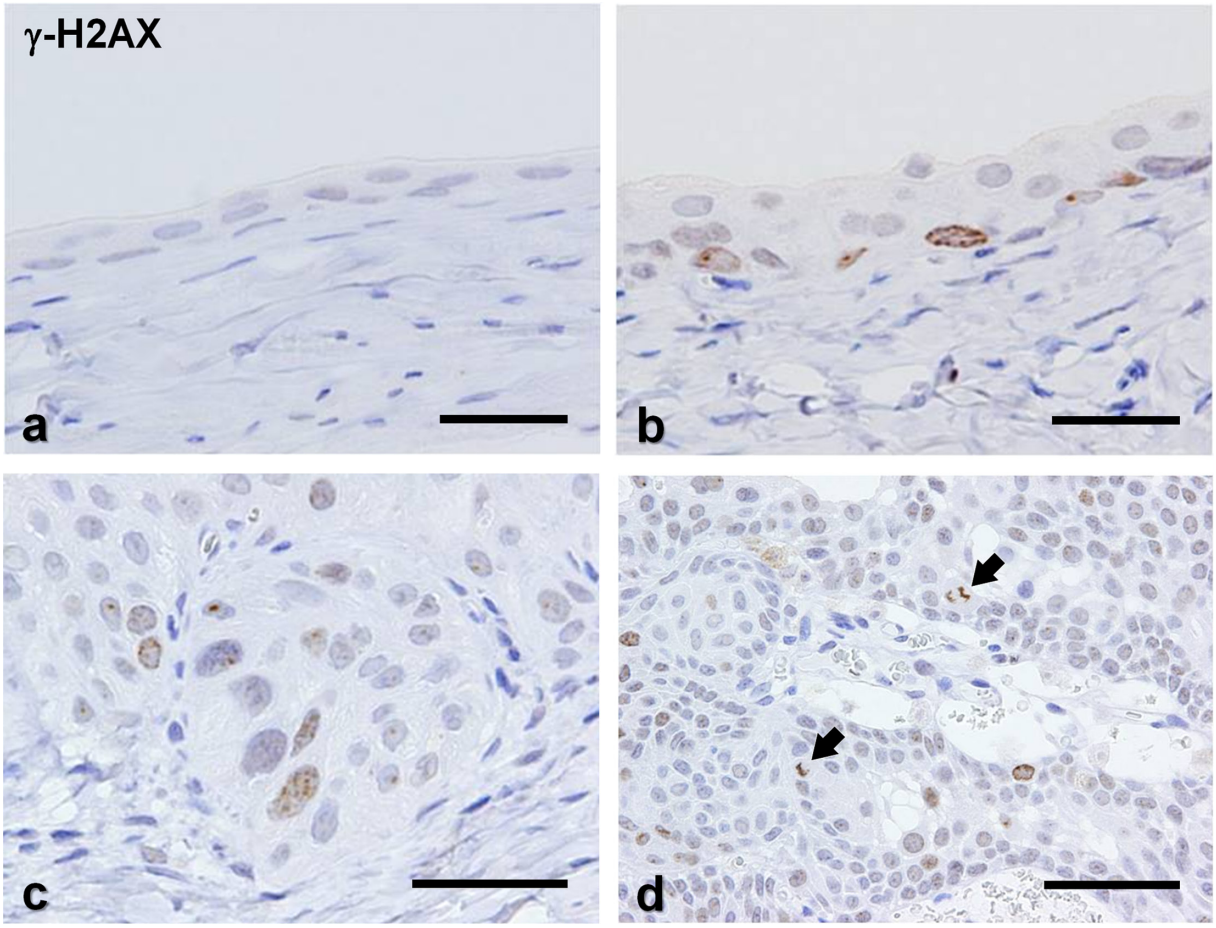

Fig. 4. Immunohistochemical findings for γ-H2AX in urinary bladder tissue of F344 rats. Bars = 25 (a, b and c) or 50 (d) μm. (a) Untreated control. (b) BBN-stimulated bladder epithelium. (c) BBN-induced PN hyperplasia. (d) BBN-induced carcinoma. Note mitotic neoplastic cells (arrows) are positive.

three weeks after BBN treatment. The results indicate that BBN-induced DNA damage in bladder epithelial cells persists for a long period beyond the duration of administration. Our results also suggest the possibility that γ-H2AX is suitable for the early detection of *in vivo* genotoxicity in the urinary bladder and that the tissue-specific carcinogenicity of BBN is associated with double-strand breaks in the bladder epithelial cells.

MGMT is known as a major enzyme for direct damage reversal such as oxidization of guanine residues or alkylation of DNA bases[20]. In this study, MGMT expression was occasionally observed in BBN-treated hyperplastic cells, while the non-treated urothelium was negative. Interestingly, the neoplastic cells were almost negative for MGMT, suggesting that MGMT acts only in the initial stage of bladder carcinogenesis. Yoshimi *et al.* previously reported that the DNA repair response to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) is reduced in the hyperplastic bladder epithelium of BBN-treated rats[21]. These results indicate that suppression of DNA repair enzymes in specific stages of cancer development could have key roles in BBN-induced bladder carcinogenesis.

MRE11, MLH1, ERCC1 and TREX1 here showed increased expression in BBN-treated rats as compared with control rats. These enzymes may be associated with BBN-induced DNA damage and cancer development, at least to some extent. As shown in control rats, other DNA repair enzymes examined in this study (XRCC1, MSH6, APE1, DDB1, SMC1 and RAD18) are constitutively expressed in epithelial or mesenchymal cells of the urinary bladder. Since the expression pattern was similar in BBN-treated rats, these enzymes appear unlikely to be directly associated with BBN-induced bladder carcinogenesis.

In summary, the present study demonstrated that BBN treatment increased expression of several DNA repair enzymes and phosphorylation of histone H2AX in the urinary bladder. These results suggest that γ-H2AX could have potential as a useful biomarker in the early detection of genotoxicity in the rat urinary bladder. To the best of our knowledge, this is the first report demonstrating expression of γ-H2AX during bladder carcinogenesis. Although further investigations are needed, including comparison of results with non-genotoxic bladder carcinogens, our present study indicated potential application of γ-H2AX for rapid evaluation of tissue-specific genotoxicity.

Acknowledgments: We thank Ayako Saikawa and Yoshimi Komatsu for expert technical assistance in processing histological materials. This work was supported by a Grant-in-Aid from the Ministry of Health, Labour and Welfare, Japan.

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