High Susceptibility of $p53^{+/-}$ Knockout Mice in $N$-Butyl-$N$-(4-hydroxybutyl)nitrosamine Urinary Bladder Carcinogenesis and Lack of Frequent Mutation in Residual Allele


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ABSTRACT

The loss of $p53$ functions is considered to compromise the growth-suppression machinery of the cell and facilitate neoplastic change. In humans, genetic alteration in the $p53$ gene is one of the most frequently observed molecular changes in tumors, including urinary bladder carcinomas. We have investigated the susceptibility of heterozygote $p53$ knockout mice to $N$-butyl-$N$-(4-hydroxybutyl)nitrosamine (BBN) in terms of urinary bladder tumor induction. Both $p53^{+/-}$ knockout mice and C57BL/6 original parent strain were administered 0.002, 0.004, 0.0075 and 0.025% BBN in the drinking water for 20 weeks. As compared with the C57BL/6 strain, greater lesion yields were observed in knockout mice after 20 weeks of treatment. Transitional cell carcinomas were found in 9 (75%) and 12 (100%) of each 12 mice of the 0.0075 and 0.025% BBN treatment groups, respectively, whereas only 1 (11%) and 6 (67%) of each of 9 of the C57BL/6 mice demonstrated tumors. Preneoplastic lesions (dysplasia) were also observed more frequently in the lower dose groups in the knockout mice than C57BL/6 mice. PCR single-strand conformation polymorphism analysis followed by DNA direct sequencing of the $p53$ gene (exons 5–8) extracted from bladder tumors demonstrated mutations in 3 of 11 (27.3%; exon 7) and 8 of 29 (27.6%; exons 5–8) tumors in C57BL/6 and knockout mice, respectively. There was no significant difference in the mutation rates at the residual $p53$ gene between the two cases. All mutations observed in knockout mice were restricted to the normal allele, and none were present in the gene-targeted null allele. In a separate experiment, 5-bromo-2'-deoxyuridine labeling indices after treatment with BBN for 2 or 4 weeks were significantly higher in knockout mice than wild-type mice. Measurement of the urinary concentration of $N$-butyl-$N$-(3-carboxypropyl)nitrosamine, a proximate carcinogenic metabolite, revealed no significant differences between knockout and original parent strain after administration of 0.0075% BBN in the drinking water for 4 weeks. In conclusion, knockout mice are distinctly more sensitive to urinary bladder tumor induction. Both $p53^{+/-}$ knockout mice and C57BL/6 mice. PCR single-strand conformation polymorphism analysis followed by DNA direct sequencing of the $p53$ gene (exons 5–8) extracted from bladder tumors demonstrated mutations in 3 of 11 (27.3%; exon 7) and 8 of 29 (27.6%; exons 5–8) tumors in C57BL/6 and knockout mice, respectively. There was no significant difference in the mutation rates at the residual $p53$ gene between the two cases. All mutations observed in knockout mice were restricted to the normal allele, and none were present in the gene-targeted null allele. In a separate experiment, 5-bromo-2'-deoxyuridine labeling indices after treatment with BBN for 2 or 4 weeks were significantly higher in knockout mice than wild-type mice. Measurement of the urinary concentration of $N$-butyl-$N$-(3-carboxypropyl)nitrosamine, a proximate carcinogenic metabolite, revealed no significant differences between knockout and original parent strain after administration of 0.0075% BBN in the drinking water for 4 weeks. In conclusion, knockout mice are distinctly more sensitive to urinary bladder carcinogenesis induced by BBN than their original parent strain, as evidenced by elevated DNA synthesis during carcinogen administration and an increased tumor yield. The high susceptibility of $p53$ knockout mice appeared to be related to the high level of cell proliferation rather than that of $N$-butyl-$N$-(3-carboxypropyl)nitrosamine in the urine or that of mutations at the $p53$ gene.

INTRODUCTION

Recently, the $p53$ tumor suppressor gene has received much attention because of its propensity for genetic alteration in a wide variety of human neoplasms (1–3). In particular, invasive urinary bladder carcinomas in humans frequently demonstrate mutations of the $p53$ gene (4, 5), suggesting an important role in their generation. Germine $p53$ mutations are associated with an inherited predisposition to early onset of cancer called the Li-Fraumeni syndrome (6–8), and studies of both somatic and germ-line alterations have greatly enhanced our understanding of the pathobiological role of this gene in carcinogenesis, providing useful biological models for studies of the molecular mechanisms leading to cancer development (8).

Numerous experiments involving urinary bladder tumors in rats or mice have been established with the carcinogen BBN given in the drinking water (9–11). Similar to the human cancers, the induced mouse urinary bladder tumors comprise nonpapillary, invasive TCCs and SCCs (11, 12). In contrast, most rat urinary bladder tumors induced by BBN are papillary origin and superficial TCCs (10). Although $p53$ genetic alterations are relatively infrequent in both chemically induced and spontaneous animal tumors (13–19), Masui et al. (20) found BBN-induced rat urinary bladder tumors, demonstrating superficial growth to be an exception. Furthermore, Yamamoto et al. (21) reported frequent $p53$ mutations in BBN-induced invasive urinary bladder carcinomas in NON/Shi mice at similar levels to those observed for human high-grade invasive carcinomas.

A number of reports (22–25) have appeared in the literature of $p53$ knockout mice regarding the spontaneous tumors or susceptibility to chemical carcinogenesis. Donehower et al. (22) revealed that the heterozygous or homozygous $p53$-deficient mice were highly susceptible to some kinds of spontaneous sarcomas (e.g., malignant lymphomas) but not to spontaneous epithelial tumors. Kemp et al. (23) reported that the reduction of $p53$ genes were not related to the increase of initiation or promotion but enhanced malignant progression of chemically induced skin tumors. Harvey et al. (24) showed that a mutant $p53$ transgene accelerated the tumor development in heterozygous but not in nullizygous $p53$-deficient mice, and Tennant et al. (25) reported that $p53^{+/-}$ knockout mice were susceptible to the genotoxic carcinogens but not to the nongenotoxic carcinogens.

In the present study, we examine the role of the $p53$ gene by comparing the susceptibility of knockout mice carrying the heterozygous null allele and the original parent strain (C57BL/6 mice) to BBN. To cast light on mechanistic aspects, comparisons of the frequency of alterations in the $p53$ gene extracted from preneoplastic and neoplastic lesions in the urinary bladder as well as the level of cell proliferation in the urinary bladder epithelium in knockout mice and littermates (wild-type) receiving BBN were made. Furthermore, urinary concentrations of BCPN, a proximate metabolite regarded to have a causal role in bladder carcinogenesis, were measured in the both strains.

MATERIALS AND METHODS

**Animals and Carcinogen.** Heterozygous male $p53$ knockout mice (TSG-$p53^{+/-}$ mice; Ref. 14) and littermates (wild-type) were purchased from GenPharm International Co., Ltd. (Palo Alto, CA). Mice of the C57BL/6 parent strain were purchased from Charles River Japan Inc. (Atsugi, Japan).

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3. The abbreviations used are: BBN, $N$-butyl-$N$-(4-hydroxybutyl)nitrosamine; TCC, transitional cell carcinoma; SCC, squamous cell carcinoma; SSCP, single-strand conformation polymorphism; BrdUrd, 5-bromo-2'-deoxyuridine; BCPN, $N$-butyl-$N$-(3-carboxypropyl)nitrosamine; DEN, diethylnitrosamine.
BBN was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). The animals were housed in a plastic cage (five mice in a cage) with wood chips for bedding in a room kept at 24°C ± 2°C temperature and 40–70% humidity with a 12-h light/dark cycle. They received CDF-1 Laboratory Chow (Charles River Japan, Inc.) in experiments I, II, and III and Oriental MF (Oriental Yeast Co., Ltd., Tokyo, Japan) in experiment IV ad libitum.

**Experiment I.** At 7–8 weeks of age, 62 male knockout mice were divided into five groups and subjected to BBN treatment in the drinking water as follows: group 1, 10 mice, deionized water (control group); group 2, 13 mice, 0.002% BBN; group 3, 13 mice, 0.004% BBN; group 4, 13 mice, 0.0075% BBN; and group 5, 13 mice, 0.025% BBN. A total of 62 male C57BL/6J mice (7–8 weeks of age) were similarly assigned to five groups and received the same treatments. After 20 weeks of treatment, the mice were killed, and complete autopsies were conducted. The presence of gross lesions was noted, and the urinary bladders were excised. Tumors were partially resected and frozen in liquid nitrogen for gene analysis. Residual urinary bladder tissues were placed in 10% phosphate-buffered formalin and processed for routine H&E staining of wax-embedded sections.

**Isolation of DNA.** Ten-μm frozen sections were cut from tissue embedded in OCT Compound gel (Sakura Finetechnical Co., Ltd., Tokyo, Japan). The first of each series was stained with H&E for histological diagnosis, and the residues were used for isolation of DNA. Areas of lesion were marked with a felt tip pen on the H&E sections, and these were then aligned underneath the corresponding unstained serial sections. Cells from the identified areas were scraped off with a scalpel blade and introduced into sterile disposable tubes, and the cells were scraped off with a scalpel blade and introduced into sterile disposable tubes, and the cell suspension was washed with a 12-h ligh/dark cycle. They received CDF-1 Laboratory Chow (Charles River Japan, Inc.) in experiments I, II, and III and Oriental MF (Oriental Yeast Co., Ltd., Tokyo, Japan) in experiment IV ad libitum.

**PCR amplification.** For screening of samples for p53 mutations, PCR-SSCP analysis and direct sequencing, four pairs of oligonucleotide primers (20mers) for mouse p53 exons 5–8 were designed, based on the published sequences, and used for PCR. The sequences of the primers are given in Table 1. Most primers included both exon and intron portions to avoid amplification of pseudogenes. For screening of samples for p53 mutations, PCR-SSCP analysis was performed according to the methods of Orita et al. (26) with minor modifications. 5′-End labeling of PCR primers was carried out using a Megalabel kit (Takara Shuzo Co., Ltd., Kyoto, Japan). PCR was conducted in a 10-μl reaction volume using a GenAmp DNA amplification kit (Perkin-Elmer Cetus, Norwalk, CT) with 35 cycles of denaturing (94°C) for 35 s, annealing (55°C) for 30 s, and extension (72°C) for 60 s using a Program Control System PC-800 (ASTEC Co., Ltd., Fukuoka, Japan). The pair of oligonucleotide primers used for LA PCR was the same as these for the sense strand in exon 5 and the antisense strand in exon 8 used for PCR-SSCP. After denaturation for 1 min at 94°C, 30 cycles of amplification consisting of 40 s at 68°C (annealing and extension) and 20 s at 98°C (denaturation) were performed. After amplification, the mixture was purified to obtain the target gene by low-melting-temperature agarose gel electrophoresis, phenol/chloroform extraction, and ethanol precipitation. Then the obtained gene was confirmed to be generated from the normal allele of the knockout mice by direct sequencing of the exon 5 region.

**Experiment II.** To confirm differences in susceptibility to BBN carcinogenesis between knockout mice and wild-type littermates, 7–8-week-old animals, 10 mice each, were administered 0.0075% BBN in drinking water. After 20 weeks of treatment, the mice were killed, and their urinary bladders were excised and fixed in 10% phosphate-buffered formalin for subsequent histopathological examination.

**Experiment III.** To determine whether early cell proliferation in bladder epithelium of knockout and wild-type mice might vary in response to BBN treatment, groups of five animals were treated with 0.0075% BBN or deionized water for 4 weeks and then injected with 100 mg/kg BrdUrd (Sigma Chemical Co., St. Louis, MO), a thymidine analogue, 1 h before sacrifice. Mice were killed under ethyl ether anesthesia, and urinary bladders were inflated and fixed with 10% phosphate-buffered formalin for 24 h and embedded in paraffin. Tissue sections with 4–5 μm thickness were stained for immunohistochemistry using BrdUrd monoclonal antibody (DAKO A/S, Copenhagen, Denmark) and a Vectastain ABC kit (Burlingame, CA) with 3,3′-diaminobenzidine (Sigma). At least 1500 epithelial nuclei were counted in a urinary bladder tissue section per animal under a light microscope. BrdUrd labeling indices were calculated by dividing the number of labeled nuclei by the total number of nuclei counted, and the results were expressed as percentage values.

**Experiment IV.** Groups of each five C57BL/6J mice, knockout mice, and their littermates were administered 0.0075% BBN in the drinking water. After 4 weeks of treatment, the mice were housed in metabolic cages, and urine samples were collected over 4 h. Determination of BCPN. Determination of BCPN in the urine samples was performed as reported previously (27) with minor modifications. Regardless of the original amount, each urine sample (0.1–1.8 ml) was diluted to 10 ml with distilled water before assay. A 20-μl aliquot of 12 HCl was then added to 3 ml of the diluted sample in a brown tube, followed by extraction with 3 ml of ethyl acetate three times. The organic layers were collected after centrifugation for 10 min at 1500 × g and evaporated to dryness using a speed vacuum concentrator with a cooling trap (Savant SC 100 SpeedVac; Savant Instruments Inc., Farmingdale, NY) below 30°C. The residues, dissolved in ethyl acetate, were spotted on silica gel 70 F254 precoated plates (Wako Pure Chemical Industries, Ltd., Osaka, Japan) that developed with chloroform:methanol:acetic acid (18:1:1) in the dark. Each band corresponding to authentic samples, including BBN and BCPN (RF = 0.68–0.72), was scraped off, and the compounds were eluted from the silica with 4 ml of acetone. The eluates were then concentrated using the speed vacuum concentrator below 30°C, taken up in acetonitrile to make a final volume of 1 ml, and analyzed by HPLC after clarification through a Sartorius MINISART RC4 filter (0.2-μm pore

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**Table 1 p53 primers used for PCR-SSCP analysis and direct sequencing**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer Sequence</th>
</tr>
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<tbody>
<tr>
<td>Exon 5</td>
<td>5′-AGGGCGTGGTTGAGGCTAC-3′ (sense) 5′-GCTCTGACTATGCCTCG-3′ (antisense)</td>
</tr>
<tr>
<td>Exon 6</td>
<td>5′-GCTCTGACTATGCCTCG-3′ (sense) 5′-GCACTCTGGCCTAACGAC-3′ (antisense)</td>
</tr>
<tr>
<td>Exon 7</td>
<td>5′-TACCTGTATGGTCTTCAC-3′ (sense) 5′-CAACTGTCTCTAAGACGCAG-3′ (antisense)</td>
</tr>
<tr>
<td>Exon 8</td>
<td>5′-TGAGCTCAGACGCTTGCT-3′ (sense) 5′-CTCAACAGGCTCCCTAC-3′ (antisense)</td>
</tr>
</tbody>
</table>

*Intron sequences are indicated by underlining.*
URINARY BLADDER CARCINOGENESIS IN p53 KNOCKOUT MICE

C57BL/6 mice Knockout mice

<table>
<thead>
<tr>
<th>BBN(%)</th>
<th>Control</th>
<th>Knockout</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0075</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.025</td>
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</table>

Fig. 1. The incidence of urinary bladder epithelial lesions by BBN treatment for 20 weeks in knockout and C57BL/6 mice.

size; Sartorius Co., Ltd., Gottingen, Germany). HPLC was performed using a SHIMAZU LC9A apparatus (SHIMAZU Co., Ltd., Kyoto, Japan). A 30-μl aliquot of the acetonitrile solution was injected into the HPLC instrument and measured at 239 nm (0.16 absorbance unit full scale). BBN and BCPN were separated using a Jasco Finepak SIL C18 column (4.6 mm inside diameter × 25 cm). N-Nitrosamines were eluted with acetonitrile-20 mM sodium acetate buffer (pH 4.5; 3:7 v/v), with a solvent flow rate of 1.0 ml/min. Under the chromatographic conditions described above, BBN and BCPN had retention times of 10.9 and 7.8 min, respectively. To measure the urinary concentrations of nitrosamine, individual calibration curves were constructed. The recovery of nitrosamine from control urine was 80%.

Statistical Analysis. The two-tailed Student’s t test was performed to compare the total amounts of BBN intake, BrdUrd labeling indices, and urinary concentrations of BCPN using a Yukms statistical computer package (Yukms Co., Ltd., Kawasaki, Japan). Statistical significances were evaluated P of 0.05 and 0.01.

RESULTS

Experiment I. No deaths attributable to the BBN treatment were observed during the experimental period. At the beginning of the experiment, one knockout and one control mouse in the 0.0075% BBN-treated group died of hydrocephalus and thymic lymphoma, respectively. Several C57BL/6 mice died as the result of fighting. There was no significant difference in the body weight gain during the course of the experiment between BBN-treated and control mice except for a decrease in body weight among the 0.025% BBN treated knockout mice during weeks 19–20 and in C57BL/6 mice of the same group at week 20. Total BBN intake was essentially the same for both knockout and C57BL/6 mice (data not shown).

The incidences of urinary bladder lesions by BBN treatment for 20 weeks in knockout and C57BL/6 mice are given in Fig. 1. The TCCs (Fig. 2A) showed atypical morphological features and invasive growth into the submucosa, which is typical of mouse lesions (11, 21); moreover, some cases reached the subserosa. Six of 12 TCCs in animals receiving 0.025% BBN and 2 of 9 TCCs in the 0.0075% BBN group contained SCC elements (50 and 22%, respectively), with proliferation of spindle cell components, and were therefore diagnosed as poorly differentiated SCCs (Fig. 2B). In contrast, no SCCs were present in the urinary bladders of C57BL/6 mice. Dysplasias, considered as a preneoplastic lesion (11), were observed much more frequently in knockout mice than in their C57BL/6 counterparts.

In addition to the epithelial tumors, hemangiomas and hemangiosarcomas (Fig. 2C) were frequently seen in knockout mice treated with BBN at 0.004% or more, whereas they were rarely noted in C57BL/6 mice (Table 2). These lesions were observed in the lamina propria mucosa, and the sarcomas were highly invasive to the muscular layer. No metastasis of either epithelial or nonepithelial tumors to other organs was detected.

PCR-SSCP analysis demonstrated that mobility-shifted bands were restricted to urinary bladder lesions (TCCs, SCCs, and dysplasias) and were not observed for normal epithelium of either knockout or C57BL/6 mice. Mutations in knockout mice were present only in the normal allele and not in the gene-targeted null allele (Table 3A). Interestingly in C57BL/6 mice, mutations were restricted to exon 7 (six of six), and no mutations were found in exons 5, 6, and 8 (Table 3B). One knockout mouse in the 0.025% BBN treatment group contained two different lesions with different mutations, one in exon 5 and the other in exon 6. Almost all of the mutations in exons 5–8 were single-bp substitutions with an amino acid change, except for three silent mutations in knockout mice. In the knockout mice, all

Fig. 2. A, a transitional cell carcinoma induced in p53 knockout mice by 0.0075% BBN treatment for 20 weeks. B, a squamous cell carcinoma induced in p53 knockout mice by 0.0075% BBN treatment for 20 weeks. C, a hemangiosarcoma induced in p53 knockout mice by 0.025% BBN treatment for 20 weeks.
Table 2. Incidences of hemangiomas and hemangiosarcomas in the urinary bladders of knockout and C57BL/6 mice*

<table>
<thead>
<tr>
<th>Mice</th>
<th>Hemangioma</th>
<th>Hemangiosarcoma</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Knockout</td>
<td>0.004 n = 13</td>
<td>0.0075 n = 12</td>
<td>0.025 n = 12</td>
</tr>
<tr>
<td>Hemangioma</td>
<td>3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Hemangiosarcoma</td>
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<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>C57BL/6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemangioma</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hemangiosarcoma</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

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*Nonneoplastic tumors were not observed in either knockout or C57BL/6 mice of the control and 0.002% groups.

Summary of mutation frequencies in the p53 gene at exons 5 to 8 from preneoplastic dysplasias, TCCs, and SCCs are shown in Table 4A. In knockout mice, the frequencies of the mutation were 3 of 16 dysplasias, 4 of 21 TCCs, and 4 of 8 SCCs. In C57BL/6 mice, 3 of 12 dysplasias and 3 of 11 TCCs demonstrated mutations. The distribution of the mutation frequency for urinary bladder carcinomas in the BBN treatment group is shown in Table 4B. There was no significant difference in the mutation frequency of p53 gene between knockout and C57BL/6 mice for either preneoplastic or neoplastic lesions. Furthermore, no influence of staging of the tumors was apparent (Table 4A). DNAs extracted from hemangiomas and hemangiosarcomas in knockout and C57BL/6 mice had no mutations.

Experiment II. In agreement with the results of the experiment, the knockout mice were much more sensitive to BBN carcinogenesis than their littermates (wild-type). After treatment with 0.0075% BBN, the incidences of urinary bladder carcinomas as well as preneoplastic lesions were significantly higher in knockout mice compared to C57BL/6 mice.

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Table 3A. p53 mutations in knockout mice urinary bladder lesions

<table>
<thead>
<tr>
<th>Histopathology (treatment)</th>
<th>Exon</th>
<th>Kind of mutation</th>
<th>Character change in amino acid</th>
<th>Transition/Transversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dysplasia (0.004% BBN)</td>
<td>5</td>
<td>164 CTC→TAG</td>
<td>Hydrophilic, noncharge</td>
<td>Transition</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gln→stop codon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dysplasia (0.004% BBN)</td>
<td>7</td>
<td>234 ATG→AGG</td>
<td>Hydrophobic</td>
<td>Transversion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Met→Arg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dysplasia (0.004% BBN)</td>
<td>7</td>
<td>252 ATC→ACC</td>
<td>Hydrophobic</td>
<td>Transversion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ile→Thr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCC Transversion (0.0075% BBN)</td>
<td>7</td>
<td>148 CCT→TCT</td>
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<td>Transition</td>
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<tr>
<td></td>
<td></td>
<td>Pro→Ser</td>
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<tr>
<td>TCC (0.025% BBN)</td>
<td>6</td>
<td>199 TAT→TAA</td>
<td>Hydrophilic, noncharge</td>
<td>Transversion</td>
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<tr>
<td></td>
<td></td>
<td>Thr→stop codon</td>
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<td></td>
</tr>
<tr>
<td>SCC (0.0075% BBN)</td>
<td>8</td>
<td>206 CGG→TGG</td>
<td>Plus charge</td>
<td>Transition</td>
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<tr>
<td></td>
<td></td>
<td>Arg→Trp</td>
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<tr>
<td>SCC (0.025% BBN)</td>
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<td>170 GTG→GCG</td>
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<tr>
<td></td>
<td></td>
<td>Val→Ala</td>
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</table>

Table 3B. p53 mutations in C57BL/6 mice lesions

<table>
<thead>
<tr>
<th>Histopathology (treatment)</th>
<th>Exon</th>
<th>Kind of mutation</th>
<th>Character change in amino acid</th>
<th>Transition/Transversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dysplasia (0.0075% BBN)</td>
<td>7</td>
<td>251 ATC→AGC</td>
<td>Hydrophobic</td>
<td>Transversion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ile→Ser</td>
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<tr>
<td>Dysplasia (0.0075% BBN)</td>
<td>7</td>
<td>252 ATC→ACC</td>
<td>Hydrophobic</td>
<td>Transversion</td>
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<tr>
<td></td>
<td></td>
<td>Ile→Thr</td>
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<tr>
<td>Dysplasia (0.0075% BBN)</td>
<td>7</td>
<td>258 AGT→AAT</td>
<td>Hydrophilic, noncharge</td>
<td>Transversion</td>
</tr>
<tr>
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<td></td>
<td>Ser→Asn</td>
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<tr>
<td>TCC (0.025% BBN)</td>
<td>7</td>
<td>253 ACA→AAA</td>
<td>Hydrophilic, noncharge</td>
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<td>Thr→Lys</td>
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<tr>
<td></td>
<td></td>
<td>Tyr→Asp</td>
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<tr>
<td>TCC (0.025% BBN)</td>
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<td>251 ATC→AGC</td>
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<td></td>
<td></td>
<td>Ile→Ser</td>
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The present study demonstrated that heterozygous p53 knockout mice are very much more sensitive to the urinary bladder carcinogenesis of BBN than their parent strain. Comparison of the yield of TCCs with the treatment dose of 0.075% BBN indicated at least a 3-fold increase in the knockout case. Considering the fact that SCC was observed only in knockout mice (50% in 0.025% BBN and 22% in 0.0075% BBN), knockout mice appear to be more sensitive to carcinogenesis and abnormal differentiation than C57BL/6 mice. Although Yamamoto et al. (21) demonstrated that the most frequently observed carcinomas in NON-Shi mice treated with 0.05-0.2% BBN for 28 weeks were SCCs, SCCs were found in some knockout mice in the present study. Tannan et al. (11) described the incidence of SCC to be about two times higher than that of TCC after treatment of B6C3F1 mice with N-ethyl-N-(4-carboxybutyl)nitrosamine with substitution of an N-ethyl for the N-butyl base of BBN. Their experimental data indicated that the longer the N-ethyl-N-(4-carboxybutyl)nitrosamine treatment duration, the higher the incidence of SCC.

In addition to the epithelial tumors, non-epithelial tumors (hemangiomas and hemangiosarcomas) were frequently observed in knockout mice. Kemp (28) reported that hepatic hemangiosarcomas rapidly appeared in p53 null mice treated with DEN and noted that the hepatic endothelial cell may be more sensitive to DEN-induced transformation in the absence of p53 than is the hepatic parenchymal cell. Their assumption was supported by the results of Harvey et al. (29), who showed an increase in hepatic hemangiosarcomas but not in hepatocellular adenomas or carcinomas in p53-deficient mice chronically exposed to dimethylnitrosamine, a carcinogen with a similar mechanism of action to DEN. The present results are consistent with the previous results that p53 knockout mice are more sensitive to the induction of hemangiomas and hemangiosarcomas by carcinogen treatments than C57BL/6 mice.

If the urinary concentration of BCPN, a proximate carcinogenic metabolite of BBN, was higher in knockout mice than in normal mice, increased sensitivity of urinary bladder carcinomas in knockout mice would be a matter of course. However, there was no significant difference in urinary concentrations of BCPN among the three types of mice.

Despite the difference in tumor incidence, PCR-SSCP analysis and direct sequencing of DNA extracted from preneoplastic and neoplastic lesions revealed similar p53 mutation frequencies (exons 5–8) in knockout and C57BL/6 mice. In this connection, point mutations, deletions, rearrangements, and allelic loss of the p53 gene have been reported in a variety of human tumors. Thus, we believe that the mutation of the p53 gene occurs in a dominant-negative manner and not as a definitive event for induction of bladder tumor. The present results are in line with the earlier conclusion that mutations of the p53 gene may not confer any growth advantage in two-stage urinary bladder carcinogenesis in the rat (30). We found that the cell proliferation in the urinary bladder epithelium after BBN treatment was significantly greater in knockout mice than in wild-type mice. Activation of cell proliferation may be related to the loss of cell cycle arrest and would be expected to play an important role in greater tumor yield in knockout mice.

Our speculation regarding the high susceptibility of knockout mice to urinary bladder carcinogenesis is as follows. DNA damage occurred at some key genes associated with BBN treatment in urinary

| Experiment IV. Values for urinary excretion of BCPN, a proximate carcinogen of BBN, after administration of 0.0075% BBN to knockout, wild-type, and C57BL/6 mice for 4 weeks are presented in Table 7. No significant differences in the concentration of BCPN in urine between the less susceptible wild-type, C57BL/6, and the more susceptible knockout mice were evident.

<table>
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<th>DISCUSSION</th>
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<table>
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<tr>
<th><strong>Table 4A. Mutation frequencies of p53 gene (exons 5–8) in dysplasias, TCCs, and SCCs</strong></th>
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<td><strong>Mice</strong></td>
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<tr>
<td>Knockout</td>
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<td>C57BL/6</td>
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<sup>a</sup> DNA samples were extracted from TCCs and SCCs.

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<th><strong>Table 4B. Number of carcinomas with a p53 gene mutation in exons 5–8&lt;sup&gt;a&lt;/sup&gt;</strong></th>
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<tr>
<td><strong>BBN (%)</strong></td>
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<tr>
<td>Total</td>
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<tr>
<td>No. of tumors examined</td>
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<td>Tumors with mutation</td>
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<th><strong>Table 5 Incidences of urinary bladder lesions in knockout and wild-type mice after treatment with 0.0075% BBN for 20 weeks</strong></th>
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<tr>
<td><strong>Histopathology findings</strong></td>
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<td></td>
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<tr>
<td>No. of animals examined</td>
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<tr>
<td>Dysplasia</td>
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<tr>
<td>TCC</td>
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<td>SCC</td>
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<th><strong>Table 6 BrdUrd labeling indices in the urinary bladder epithelium of knockout and wild-type mice after treatment with 0.0075% BBN for 2 or 4 weeks</strong></th>
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<tr>
<td><strong>No. of animals with lesions</strong></td>
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<tr>
<td>Knockout</td>
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<tr>
<td>Wild-type</td>
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<sup>a</sup> Control animals were treated with deionized water for 4 weeks.

<sup>b</sup> BrdUrd labeling index (%; mean ± SD).

<sup>c</sup> P < 0.01 versus control (knockout mice; Student’s t test).

<sup>d</sup> P < 0.01 versus wild-type (2-week treatment; Student’s t test).

<sup>e</sup> P < 0.01 versus wild-type (4-week treatment; Student’s t test).

<sup>f</sup> P < 0.01 versus control (wild-type mice; Student’s t test).

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<th><strong>Table 7 Total urinary concentration of BCPN in knockout, wild-type, and C57BL/6 mice after treatment with 0.0075% BBN for 2 weeks</strong></th>
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<tr>
<td><strong>Mice</strong></td>
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<sup>a</sup> Mean ± SD. Statistical significance was not observed (Student’s t test).
bladders of both knockout and C57BL/6 mice in the same manner. It is unlikely that p53 is a prime target but contributes to the growth advantage by genetic alteration. After DNA damage, the p53 genes in normal mice are presumably leading to arrest of the cell cycle in the G1 phase until genetic alterations are repaired. On the other hand, knockout mice might not induce a sufficient amount of p53 protein after DNA damage, so that knockout mice might produce less p53-dependent p21, which is considered to be cyclin-dependent kinase inhibitor, than wild-type mice. In fact, we recently found decreased p21 mRNA expression in the urinary bladder of the knockout mice treated with BBN. This may have been abrogated to a certain extent in the knockout mice containing one germ-line null allele, as evidenced by the greater cell proliferation.

In conclusion, knockout mice are distinctly more sensitive to urinary bladder carcinogenesis induced by BBN than the parent strain. This is presumably related to the elevated cell proliferation rate already observed in the early stage after treatment of BBN. The cell proliferation rate appears to be more important than the mutation rate at the p53 gene.

REFERENCES


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High Susceptibility of $p53(+/\cdot)$ Knockout Mice in $N$-Butyl-$N$-(4-hydroxybutyl)nitrosamine Urinary Bladder Carcinogenesis and Lack of Frequent Mutation in Residual Allele

Keisuke Ozaki, Tokuo Sukata, Shinji Yamamoto, et al.