H-ras Mutations in Rat Urinary Bladder Carcinomas Induced by N-[4-(5-Nitro-2-furyl)-2-thiazolyl]formamide and Sodium Saccharin, Sodium Ascorbate, or Related Salts

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ABSTRACT

Male F344 rats were fed 0.2% N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide for 6 weeks and then fed 3% or 5% sodium saccharin, 5% sodium ascorbate, 3.12% calcium saccharin, 1.34% sodium chloride, or basal diet alone for 72 weeks. Protein and DNA were extracted from 89 bladder tumors [87 transitional cell carcinomas (TCC), 1 papilloma, and 1 sarcoma] from 86 rats. p21 expression was examined by Western blotting using a monoclonal antibody against p21 (NCC-RAS-004). H-ras mutations in exons 1 and 2 were examined by direct sequencing of DNA amplified by polymerase chain reaction. Sequencing results demonstrated mutations at codon 61 (CAA to CGA in 15 TCCs; CAA to CTA in 2 TCCs), at codon 12 (GGA to TGG in 1 TCC), and at codon 13 (GGG to GTG in 1 TCC). Mutations at codon 61 were confirmed by faster mobility of the p21 band in Western blots. The level of p21 expression varied among samples, but many TCCs appeared to express more p21 than controls. The overall incidence of H-ras mutations was 24.4% (21 of 86 rats). The type of chemical used for the promoting phase had essentially no effect on H-ras mutation, suggesting that the effects observed were related to FANFT administration. The frequency of H-ras mutation in each group was negatively related to the incidence of carcinoma (r = -0.85; P < 0.001). Two groups of tumors (with or without the mutated ras gene) were compared for tumor size (reflected by the bladder weight), histological grading, and the presence of invasion. The size of tumors with mutated ras was significantly smaller than those without mutated ras. There was no difference in the histological grading between the two groups. Although not statistically significant, histological invasion was more frequently observed in tumors with mutated ras (14.3%) than in tumors without mutation (3.1%).

INTRODUCTION

Various experimental models have been developed to study the mechanisms of urinary bladder carcinogenesis (1–7). The two-stage, initiation-promotion model has frequently been used in these studies. FANFT is a typical genotoxic bladder carcinogen and is often used as an initiator (1, 2). Sodium saccharin and sodium ascorbate are extensively studied as a class of promoters. Their promoting potential has been shown to be related to changes in the urinary milieu, such as pH or sodium ion concentration (3–6). A bioassay was undertaken to examine the long-term effects of these promoters and related salts, using FANFT as an initiator (7). The results confirmed the promoting activity of sodium saccharin and sodium ascorbate and indicated that calcium saccharin and sodium chloride have weak promoting effects. In the present study, urinary bladder tumors (mostly transitional cell carcinomas) induced in the bioassay were analyzed for the presence of H-ras oncogene activation. The ras protein, p21, was analyzed by Western blotting, and the H-ras gene exons 1 and 2 were sequenced to examine possible mutations at codons 12, 13, or 61.

Protooncogenes are normal cellular genes involved in cellular proliferation and differentiation which have the potential of contributing to the development of a malignancy when their structure or expression is altered (8–13). ras genes are one of the most extensively studied families of protooncogenes (14–29). They have been shown to be activated in various types of tumors, with 5 to 24% of human urinary bladder tumors reported to have activated ras genes (11, 17–23). ras gene activation was also observed in experimentally induced urinary bladder tumors (24–27). We have recently reported the mutational activation of the H-ras gene in rat urinary bladder tumors induced by FANFT (28). No mutation was observed in K- or N-ras genes in the same tumors. The purposes of this study are: (a) to confirm the previous results about the mutational activation of H-ras gene in FANFT-induced tumors; (b) to examine the effect of various promoting agents on H-ras gene activation; and (c) to examine the relationship of several aspects of bladder tumor biology by comparing tumors with or without ras gene activation.

MATERIALS AND METHODS

Animal Experiment. The animal experimental design, histopathological criteria, and results were described in a previous report (7). The same group numbers are used in this study. Urinary bladder tumors were induced in male F344 rats initiated by FANFT (from Dr. George T. Bryan, University of Wisconsin) mixed in Prolab 3200 (Groups 1, 2, 4, 7, 10, 13, and 14) or NIH-O7 (Group 20) diets at a level of 0.2% for 6 weeks, followed by Prolab 3200 diet with 5 or 3% sodium saccharin (Groups 1 and 2, respectively), 3.12% calcium saccharin (Group 4), 5% sodium ascorbate (Group 7), 5.2% calcium saccharin with 1.34% sodium chloride (Group 10), or 1.34% sodium chloride (Group 13). Groups 14 and 20 were given Prolab 3200 or NIH-O7 diets alone, respectively, during the promotion stage. Rats were sacrificed when they became moribund, and all surviving rats were killed at the end of the experimental week 78. When fresh and viable bladder tumors larger than 0.7 cm in diameter were available, a part of the tumor was frozen in an ethanol/dry ice bath and kept at −80°C. The urinary bladders were weighed before removing a part of the tumor. The rest of the tumor and the bladder were fixed in Bouin’s solution and embedded in paraffin for histological analysis.

Protein and DNA Extraction. Protein and DNA were extracted from frozen tissues by the method described previously (28) with slight modifications. Protein and DNA were extracted from the same sample.

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3 The abbreviations used are: FANFT, N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide; TCC, transitional cell carcinoma; TTBS, 0.1 M Tris, pH 7.5-0.9% NaCl-0.1% Tween 20.

4 Unpublished data.
Tissues were cut into small pieces in the cell lysis buffer (1% Triton X-0.1% sodium dodecyl sulfate-0.5% sodium deoxycholate-0.1 M NaCl-10 mM sodium phosphate, pH 7.4-1 mM phenylmethanesulfonyl fluoride-aprotinin (100 kallikrein inactivator units/ml)-10 mM sodium phosphate, pH 7.4), homogenized, and centrifuged at 15,000 rpm for 15 min. The supernatant containing protein was saved, and the concentration of the protein was measured by BCA protein assay (Pierce, Rockford, IL) (30). The nuclear pellet was resuspended in the suspension buffer (25 mM EDTA, pH 8.0-75 mM NaCl-1% sodium dodecyl sulfate), and RNase (100 µg/ml; Sigma Chemical Co., St. Louis, MO) and proteinase K (100 µg/ml; Sigma Chemical Co.) were added and then incubated for 2 h at 56°C. DNA was extracted twice with an equal volume of phenol/chloroform (1:1) followed once with chloroform/isoamyl alcohol (24:1). DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes cold ethanol.

**Western Blot Analysis.** Fifty µg of sample proteins were separated in a polyacrylamide gel (14% separating gel with 4% stacking gel) using the Mini-Protein II dual slab cell (Bio-Rad Laboratories, Richmond, CA). Rainbow protein molecular weight markers (Amersham Corp., Arlington Heights, IL) were used as molecular weight markers. Proteins were transferred to Immobilon-P transfer membranes (Millipore Corp., Bedford, MA) using the Trans-Blot SD semidry electrophoretic transfer cell (Bio-Rad Laboratories) with transfer buffer (48 mM Tris-39 mM formamidoamine (24:1). DNA was electrophoresed on 1% agarose gel to check the yield, and the amount of loaded DNA was measured by BCA protein assay (Pierce, Rockford, IL) (30). The nuclear pellet was resuspended in the suspension buffer (25 mM EDTA, pH 8.0-75 mM NaCl-1% sodium dodecyl sulfate), and RNase (100 µg/ml; Sigma Chemical Co., St. Louis, MO) and proteinase K (100 µg/ml; Sigma Chemical Co.) were added and then incubated for 2 h at 56°C. DNA was extracted twice with an equal volume of phenol/chloroform (1:1) followed once with chloroform/isoamyl alcohol (24:1). DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes cold ethanol.

Polymerase Chain Reaction. Five hundred nineteen base pairs of the H-ras gene including the exons 1 and 2 (32) were amplified by polymerase chain reaction (33) using the GeneAmp DNA amplification kit (Perkin Elmer Cetus Instruments, Norwalk, CT) and the DNA Thermal Cycler (Perkin Elmer Cetus Instruments). Sequences of the primers used for polymerase chain reaction were 5'-AGCTCCTGGTTTGCAACC-3' and 5'-GGTCACCTGTACT- CAGTGG-3'. DNA was amplified by 45 rounds of the step cycle (denaturation, 94°C for 1 min; annealing, 55°C for 1 min; extension, 72°C for 2 min) according to the manufacturer's instructions (33).

Direct Sequencing. After amplification, a fraction of the reaction mix was electrophoresed on 1% agarose gel to check the yield, and the absence of contamination/carryover was confirmed by equally amplified negative controls. The rest of the reaction mix was electrophoresed on 4% polyacrylamide gel using the Mini-Protein II dual slab cell, and a piece of the gel containing amplified ras gene was cut out. DNA was eluted overnight at 37°C in the elution buffer (0.5 mM ammonium acetate-1 mM EDTA, pH 8.0), precipitated with ethanol, and repurified with 2.5 mM ammonium acetate and ethanol. DNA was dissolved in 3.5 µl water, 0.5 µl dimethyl sulfoxide and 1 µl primer (10 pmol) were added, and then the DNA/primer mixture was boiled for 5 min and snap frozen in dry ice/ethanol. To the DNA/primer mixture were added 1 µl reaction buffer (200 mM Tris, pH 7.5-100 mM MgCl2-250 mM NaCl), 0.75 µl dithiothreitol (0.1 M), 1 µl labeling mix (1:10 dilution; 0.75 µM dGTP, dCTP, dTTP), and 2.5 µl α-32P dATP (10 µCi/µl; DuPont, Boston, MA). A 1 µl Sequenase 2.0 (1:8 dilution; United States Biochemical Corporation, Cleveland, OH), and the mixture was incubated for 1 min on ice. The solution (2.2 µl) was added to each 1.25 µl of dideoxynucleotide mix (ddGTP, ddATP, ddTTP, ddCTP, each containing 8 µM of the dideoxynucleotide and 80 µM of the other three dideoxynucleotides, and 50 mM NaCl) and incubated at 47°C for 3-5 min; then the reaction was terminated by the addition of 2 µl stop solution (95% formamide-20 mM EDTA-0.05% bromophenol blue-0.05% xylene cyanol FF). After incubation at 85°C for 5 min, 2 µl of the solution were loaded to the 6% polyacrylamide-8 M urea gel and electrophoresed for about 2 h using a SE1500 sequencer (Hoefer Scientific Instruments, San Francisco, CA). The gel was soaked in 10% acetic acid-12% methanol, dried, and exposed to Kodak XAR-5 film for 2-4 days. The sequence of primers used for sequencing H-ras exons 1 and 2 was 5'-GAGCTCACCCTTATATGGGA-3' and 5'-TTTGGACGACTCTACCAGGA-3', respectively. These primers were purified with Oligo-Pack columns (MilliGen/Biosearch, Burlington, MA).

**RESULTS**

DNA Sequencing. Direct DNA sequencing analysis revealed point mutations in codons 12, 13, and 61 of the H-ras gene. Representative sequencing results are shown in Fig. 1. As summarized in Table 1, the most frequently observed mutation (in 15 TCCs) wasCAA (glutamine) to CGA (arginine) of codon 61. CAA to CTA (leucine) was much less frequent (in 2 TCCs). The same tendency was observed in the previous study (28). Tumor 3 in Group 13 showed G to T and A to G mutations in the first and third nucleotides of codon 12 (normal is GGA, glycine). Since TGA causes termination, this mutation is likely to be TGG (tryptophan). Three TCCs showed the GCC (glycine) to GTC (valine) mutation in codon 13. The total rate of H-ras mutations was 24.4% (21 of 86 rats).

Western Blotting. Fig. 2 shows the p21 bands obtained from Western blotting, where N stands for normal bladder controls and the number indicates the tumor number in each group. Group 1, Tumors 1 and 2, were from one rat. Similarly, Group 1, Tumors 15 and 16, and Group 7, Tumors 17 and 18, respectively, were from one rat. Normal p21 is observed as two bands. The more slowly migrating band is unmodified cystolic p21, and the more quickly migrating band represents the processed p21 attached to the plasma membrane (34-36). Some samples, as shown in Fig. 2, showed a third band migrating faster than normal p21 bands. This extra band indicated the presence of a mutation of p21 at codon 61 (37). The extra bands were observed in Group 1, Tumors 10 and 15; Group 2, Tumor 4; Group 4, Tumors 2, 4, and 6; Group 7, Tumors 4, 5, and 10; Group 10, Tumor 9; Group 14, Tumors 2 and 3; and Group 20, Tumors 1, 2, and 4. These were all confirmed by DNA sequencing to have a mutation at the 61st codon of the H-ras gene. The tumors which had a mutation in codon 61 but showed less obvious change in the p21 bands were Group 1, Tumor 20, and Group 13, Tumor 6. Group 7, Tumor 14 had the more quickly migrating extra band, but it appeared slightly different from other quickly migrating bands of codon 61 mutants. DNA sequencing analysis showed no mutation in codon 61 of the H-ras gene. Codon 12 mutant (Group 4, Tumor 3) and codon 13 mutants (Group 1, Tumor 8; Group 10, Tumor 14; and Group 13, Tumor 7) revealed by DNA sequencing analysis did not show obvious changes in migration of the p21 bands. As for the expression level of p21, many tumor samples appeared to express more p21 than controls. Although subjective, more than one-half of the tumors showed higher levels of p21 than the controls in Fig. 2.

**H-ras Mutation and Histology of Tumors.** The tumors were divided into two groups (with or without H-ras mutation), and the bladder weights and the histology were compared (Table 2). The bladder weight was a general measure of the size of the bladder weight was a general measure of the size of the...
H-ras MUTATIONS IN RAT BLADDER TUMORS

Fig. 1. Direct sequencing of the H-ras gene exon 1 (A and B) and exon 2 (C and D). Sequences around codons 12 and 13 (A and B) and codons 59 to 61 (C and D) are shown. A, Group 13. Tumor 3. G to T and A to G mutations in the first and third portion of codon 12; B, Group 13. Tumor 7. G to T transversion in the second portion of codon 13; C, Group 14. Tumor 3. A to G transition in the second portion of codon 61; D, Group 20. Tumor 1. A to T transversion in the second portion of codon 61.

Table 1 H-ras mutations of urinary bladder tumors

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rats</th>
<th>No. of tumors</th>
<th>12TGG</th>
<th>13GTC</th>
<th>61CGA</th>
<th>61CTA</th>
<th>Total</th>
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<tr>
<td>1</td>
<td>FANFT → 5% NaS</td>
<td>18</td>
<td>20</td>
<td>0</td>
<td>1 (5.6)</td>
<td>3 (16.7)</td>
<td>0</td>
<td>4 (22.2)</td>
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<tr>
<td>2</td>
<td>FANFT → 3% NaS</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>1 (10.0)</td>
<td>0</td>
<td>1 (10.0)</td>
</tr>
<tr>
<td>4</td>
<td>FANFT → 3.12% CaS</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>1 (16.7)</td>
<td>3 (50.0)</td>
<td>0</td>
<td>4 (66.7)</td>
</tr>
<tr>
<td>7</td>
<td>FANFT → 5% NaAsc</td>
<td>17</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>3 (17.6)</td>
<td>0</td>
<td>3 (17.6)</td>
</tr>
<tr>
<td>10</td>
<td>FANFT → 5.2% CaS + 1.34% NaCl</td>
<td>17</td>
<td>17</td>
<td>0</td>
<td>1 (5.9)</td>
<td>1 (5.9)</td>
<td>0</td>
<td>2 (11.8)</td>
</tr>
<tr>
<td>13</td>
<td>FANFT → 1.34% NaCl</td>
<td>9</td>
<td>9</td>
<td>0</td>
<td>1 (11.1)</td>
<td>0</td>
<td>1 (11.1)</td>
<td>2 (22.2)</td>
</tr>
<tr>
<td>14</td>
<td>FANFT → control</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>2 (66.7)</td>
<td>0</td>
<td>2 (66.7)</td>
</tr>
<tr>
<td>20</td>
<td>FANFT → control</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>2 (33.3)</td>
<td>1 (16.7)</td>
<td>3 (50.0)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>86</td>
<td>89</td>
<td>1 (1.2)</td>
<td>3 (3.5)</td>
<td>15 (17.4)</td>
<td>2 (2.3)</td>
<td>21 (24.4)</td>
</tr>
</tbody>
</table>

* Groups are numbered according to the system in Ref. 7.
* Chemicals for Groups 1 to 14 were mixed with Prolab 3200 diet. NIH-07 was used as a basal diet in Group 20.
* NaS, sodium saccharin; CaS, calcium saccharin; NaAsc, sodium ascorbate.

Fig. 2. Western blot analysis of p21. p21 bands were detected with NCC-RAS-004 mouse monoclonal antibody and visualized using Vectastain ABC kit. Only p21 bands are shown. N, normal bladder control; numbers, tumor number in each group. Faster migrating p21 bands (codon 61 mutants) are obvious in the following samples: Group 1, Tumors 10 and 15; Group 2, Tumor 4; Group 4, Tumors 2, 4, and 6; Group 7, Tumors 4, 5, and 10; Group 10, Tumor 9; Group 14, Tumors 2 and 3; Group 20, Tumors 1, 2, and 4. The codon 12 mutant (Group 4, Tumor 3) and codon 13 mutants (Group 1, Tumor 8; Group 10, Tumor 14; Group 13, Tumor 7) do not show an obvious change in the migration of p21 bands. Many tumor samples are showing higher expression of p21 than controls.
tumors present. The average urinary bladder weight of rats bearing tumors with H-ras mutation was significantly smaller than that of rats bearing tumors without H-ras mutation (P < 0.01 by Student's t test). There was no difference in the histological grading of transitional cell carcinomas between the two groups. Although not significant by the χ² test, histological invasion was more frequently observed in tumors with H-ras mutation (14.3%) than in tumors without mutation (3.1%).

DISCUSSION

In this study, we demonstrated H-ras mutations in urinary bladder carcinomas induced by FANFT and various tumor promoters. CAA (glutamine) to CGA (arginine) at codon 61 was most frequently observed (17.4%); CAA to CTA (leucine) was much less frequent (2.3%). The same tendency was seen previously (28). The CAA to AAA (lysine) mutation, which is common in chemically induced tumors (10, 12), was not observed in this or previous studies (28). As shown in Fig. 1, one TCC showed two mutations in codon 12 (G to T in the first nucleotide and A to G in the third nucleotide). Since TGA causes termination, this mutation is likely to be TGG (tryptophan). In any event, this is a rare mutation. Three TCCs showed GCC (glycine) to GTC (valine) mutation at codon 13. Although not common, a point mutation at codon 13 of the H-ras gene in human bladder cancer, causing substitution of glycine to cysteine (20) or to arginine (23), has been reported.

The total incidence of H-ras mutations in the present study was 24.4% (21 of 86 rats), while that in our previous study (28) was 42.9% (6 of 14 rats). As discussed below, the difference in the incidence of H-ras mutation might be negatively related to the strength of the carcinogenic stress applied to the rats, which was reflected by the incidence of carcinomas induced in the given treatment protocols. In the previous experiment (28, 38), the carcinoma incidence (6.7 to 30%) of groups used for the study of H-ras mutation was lower than in the present one (30.8 to 97.4%), mostly because of the lower dose or shorter period of FANFT administration (0.1 or 0.2% FANFT for 1 to 4 weeks in the previous experiment; 0.2% FANFT for 6 weeks in the present experiment; Ref. 7).

The type of chemical used for the promoting phase had essentially no effect on H-ras mutation, suggesting that the effects observed were related to FANFT administration. There was, however, a tendency for H-ras mutations to occur more frequently in the groups in which sampling numbers were small. The small sampling number reflected a small number of reasonably sized (0.7 x 0.7 x 0.7 cm) tumors in a given group, which was related to the potency of the promoting chemical. Fig. 3 shows the relationship between the incidence of carcinoma (data from Ref. 7) and H-ras mutation (present data). The incidence of H-ras mutation is negatively related to the incidence of carcinoma. The Pearson product-moment correlation coefficient (r) is −0.845 and is significant at P < 0.01 (39). This result, together with the above discussion about the differences in H-ras mutation incidences between the present and previous studies, suggests that H-ras mutation is more frequently observed in groups in which the incidence of bladder carcinoma is low. In other words, cells with H-ras mutation might have a selective advantage over others with a weak carcinogenic stimulus. Furthermore, this also strongly suggests the existence of mechanisms other than H-ras activation for the development of carcinomas in the presence of a stronger carcinogenic environment. The present data are in accordance with the study by Zhang et al. (40), in which reduction in the frequency of activated ras genes was observed in rat mammary carcinomas with increasing doses of N-methyl-N-nitrosourea. Brown et al. (29) have provided evidence that different mutations in Ha-ras are produced with different carcinogens, and the various mutations affect the probability of progression to malignancy. Theodorescu et al. (41) have similarly found that expression of Ha-ras may be related to progression of human bladder transitional cell carcinomas.

Comparing tumors with or without H-ras mutations (Table 2) revealed significant differences in the urinary bladder weight, which reflected the size of the tumor(s) present. The size of tumors with an H-ras mutation is significantly less than that of tumors without an H-ras mutation. This might indicate that an H-ras mutation is not essential for tumor growth. Since large tumors observed in the present experiment are often accompanied by marked mesenchymal proliferation or sarcomatous growth, some other factors might contribute to the growth of these interstitial elements.

| Table 2. Comparison of tumor groups with or without H-ras mutation |
|------------------------|------------------------|------------------------|------------------------|------------------------|
| **Histology (%)**      | **Transitional cell carcinoma** | **Sarcoma/papilloma** | **Invasion** |
|                       | G1 | G2   | G3   | 0 | 3 (14.3) |
| Tumors with H-ras mutation | 21 | 1.51 ± 1.39 | 5 (23.8) | 14 (66.7) | 2 (9.5) | 0 | 3 (14.3) |
| Tumors without H-ras mutation | 65 | 2.92 ± 3.14 | 10 (15.4) | 47 (72.3) | 6 (9.2) | 2 (3.1) | 2 (3.1) |

*Mean ± SD. Two groups are significantly different at P < 0.01.*
there was no apparent difference in the histological grading of TCC between tumors with or without H-ras mutations. Although not statistically significant, histological invasion was observed more frequently in tumors with H-ras mutation (14.3%) than in those without mutation (3.1%). There was no distant metastasis in either group.

The results from Western blotting (Fig. 2) showed that many tumors expressed more p21 than control urothelium. Since the expression of p21 was not measured quantitatively, this observation is rather subjective. However, some tumors are obviously expressing more p21 than others, and this overexpression is observed with normal p21 as well as mutant p21; furthermore, the incidence of p21 overexpression appeared more frequently than H-ras mutation.

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REFERENCES


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