**p53 and H-ras Mutations and Microsatellite Instability in Renal Pelvic Carcinomas of NON/Shi Mice Treated with N-Butyl-N-(4-hydroxybutyl)nitrosamine: Different Genetic Alteration from Urinary Bladder Carcinoma**

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We previously reported p53 mutations to be frequent (greater than 70%), whereas both H-ras mutations and microsatellite instability (MSI) were infrequent (about 10%), in urinary bladder carcinomas (UBCs) and their metastatic foci in the N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN)-induced mouse urothelial carcinogenesis model. In the present study, an analysis of p53 and H-ras mutations as well as MSI was performed on 12 renal pelvic carcinomas (RPCs) and 8 metastatic or invading foci produced by the same experimental procedure. Histologically, 10 of the RPCs were transitional cell carcinomas and the remaining 2 were squamous cell carcinomas. p53 mutations were infrequent and only found in one primary RPC (8%), its metastatic foci and an invading lesion in another animal (in a total 2 of 12; 17%). H-ras mutations were slightly more frequent (found in 3 of 12 animals; 25%), 4 of 5 involving codon 44, GTG to GCG, not a hot-spot reported for human cancers. In two cases, H-ras mutations were confined to lung metastasis and not detectable in their primary RPCs. MSI analysis was available for 6 pairs of primary RPCs and their metastatic foci, and 4 animals (67%) had MSI at one or more microsatellite loci. Overall, the distribution of genetic alterations differed from that in UBCs produced by the same experimental protocol. The results thus suggest that different genetic pathways may participate in carcinogenesis of the upper and lower urinary tract due to BBN.

Key words: Renal pelvic carcinoma — p53 mutation — H-ras mutation — Microsatellite instability — Mouse

The renal pelvis, ureter and urinary bladder are covered with a continuous transitional epithelium (urothelium) which constitutes a large field of homogeneous tissue, although differences in embryology and anatomy exist. The transitional cell carcinoma (TCC) is the most common histological phenotype of human neoplasm arising in the urinary tract.1 Renal pelvic TCCs are relatively rare compared with TCCs of the urinary bladder and account for only 5% of all urothelial tumors.1 Carcinogens excreted in urine target mainly the urinary bladder. However, phenacetin specifically induces renal pelvic carcinomas (RPCs) with prolonged abuse in man as well as in an animal model.2 Therefore, there do exist differences in carcinogen action in the upper and lower urinary tract.

During the process of oncogenesis, alteration of the p53 tumor suppressor gene is one of the most common observations in a variety of human malignant tumors, including colon, lung, breast and urinary bladder cancers.3 In the human urinary bladder, it has been reported that p53 mutations are common in invasive and/or high-grade TCCs and roles in differentiation or tumor progression have therefore been suggested.4–6 The N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN)-induced urothelial carcinogenesis model in male NON/Shi mice, which develop spontaneous hydronephrosis at an incidence of 30% and show high tumor incidence (up to 75%), is very useful for investigating the mechanisms of invasion and/or metastasis.7, 8 Using this mouse model, we recently found p53 alterations to be frequent in invasive urinary bladder carcinomas (UBCs), with or without metastatic foci, whereas ras oncogene alterations were infrequent.9, 10 In addition, microsatellite instability (MSI), suggestive of a defective mismatch repair system, was found to be infrequent (2 out of 28 tumors; 7%) in UBCs produced by the same mouse model.11 In contrast to the concentrated research on the urinary bladder, few reports of molecular analysis of renal pelvic TCCs have been published.12, 13 In the present study, a total of 20 lesions from 12 animals (12 primary RPCs, a single invasive lesion and 7 metastatic foci) were therefore individually evaluated for p53 and H-ras abnormalities. For 6 pairs of primary RPCs and metastatic foci, analysis for MSI was also performed as previously described.11
MATERIALS AND METHODS

Production and isolation of tumors in the renal pelvis
BBN-RPCs were induced by BBN treatment in male NON/Shi mice (Aburahi Lab. of Shionogi Co., Shiga) and pathologically evaluated as previously described.6, 10 Briefly, a total of 240 male mice, aged 6 or 9 weeks old, were treated with BBN in drinking water at as high a concentration as they could tolerate, which ranged between 0.05 and 0.3%. After completion of BBN treatment for 8–12 weeks, mice were maintained without any chemical supplement until killed in a moribund condition under ether anesthesia between weeks 14 and 23 of the experiment. Twelve animals harboring macroscopic tumors in the renal pelvis were found at autopsy and used for further analysis. The largest primary tumors and, if present, representative metastatic foci or aggressively invading lesions were extracted with carefully washed fine scissors and immediately frozen, with special care to avoid mixing of carcinoma cells. A portion of the tumors was processed for histological examination.

Nucleic acid preparation and mutational analysis
RNAs were isolated from frozen tissues by the guanidinium thiocyanate cesium chloride method14 as previously described.9, 10 Polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) analysis and direct sequencing of p53 gene exons 5 to 8 and H-ras gene exons 1 and 2 were performed according to described procedures.9, 10 Analysis of MSI was performed for 6 pairs of primary RPCs and paired metastatic foci as detected earlier,11 using 27 microsatellite primers (D1Mit3, D1Mit7, D1Mit10, D1Mit17, D2Mit13, D3Mit18, D3Mit21, D4Mit12, D4Mit13, D4Mit15, D5Mit11, D6Mit14, D7Nds4, D8Mit13, D9Mit2, D9Mit16, D10Nds1, D10Mit10, D11Nds1, D11Mit14, D13Mit9, D15Mit14, D16Mit4, D17Mit3, D18Mit7, D18Mit12, D19Mit1; obtained from Research Genetics, Huntsville, AL). Reproducibility of mobility-shifts on PCR-SSCP and MSI analysis was confirmed by duplicate analysis.

RESULTS

Histological findings
At autopsy, tumor masses caused kidney enlargement with invasion toward the renal hilus or

| Animal no. | Tumor type | Histology | Grade/stage | p53 mutation | H-ras mutation | MSIa |
|------------|------------|-----------|-------------|--------------|----------------|------|
| 1          | RPC        | TCC+SA    | G3/T4       | -            | -              | NE   |
| 2          | RPC        | TCC       | G3/T3       | -            | -              | NE   |
| 3          | RPC        | TCC       | G2/T3       | -            | -              | NE   |
| 4          | RPC        | TCC       | G3/T4       | -            | -              | NE   |
| 5          | RPC        | TCC       | G2/T3       | -            | -              | NE   |
| Lung meta. | TCC        | G2        | -           | -            | -              | NE   |
| 6          | RPC        | TCC+AD    | G3/T4       | codon 155 CGC to TGC | - | NE |
| Invasion to aorta | TCC+AD | G3 | codon 155 CGC to TGC | D16Mit4 | |
| Lung meta. | TCC        | G3        | -           | -            | -              | NE   |
| 7          | RPC        | SCC       | Mod./T3     | codon 44 GTG to GCG | - | |
| Lung meta. | SCC        | Mod.      | -           | codon 12 GGA to GTA | D11Mit14 | |
| 8          | RPC        | TCC       | G3/T3       | -            | codon 44 GTG to GCG | |
| Lung meta. | TCC        | G3        | -           | codon 44 GTG to GCG | |
| 9          | RPC        | TCC       | G3/T3       | -            | -              | D11Mit14 |
| Lung meta. | TCC+AD     | G3        | -           | codon 44 GTG to GCG | |
| Lung meta. | TCC+AD     | G3        | -           | codon 44 GTG to GCG | |
| 10         | RPC        | SCC       | Mod./T4     | codon 227 ACC to ATC | D11Mit14 | |
| Splenic LN | SCC        | Mod.      | -           | codon 227 ACC to ATC | |
| 11         | RPC        | TCC+SA    | G3/T4       | D11Mit14, D15Mit14, D18Mit7 | |
| Diaphragm meta. | SA   | G3        | -           | D18Mit7 | |

Abbreviations used are: MSI, microsatellite instability; RPC, renal pelvic carcinoma; TCC, transitional cell carcinoma; SA, carcinoma with sarcomatous components; NE, not examined; meta., metastasis; AD, adenocarcinoma; SCC, squamous cell carcinoma; Mod., moderately differentiated; LN, lymph node.

1279 Microsatellite Instability in Mice RPC
In the present study, we detected infrequent incidences of p53 and H-ras genes mutations, and frequent MS alterations. We also statistically examined the differences of the mutational incidences of these genes between RPCs in this paper and UBCs based on the previous published data from the same animal experiment (Table II). All the data of the UBCs in Table II are from the same animal experiment reported in our previous reports. The frequency of p53 mutation was significantly lower than that observed for mouse UBCs (Table II, \( P = 0.0002 \), Fisher’s probability test), but that for MS alteration was significantly higher (Table II, \( P = 0.0043 \)).\(^{11} \) In addition, the predominating histological types of RPCs and UBCs also varied; the proportions of TCCs and SCCs were 11/28 (39%) and 17/28 (61%) for UBCs, as opposed to 10/12 (83%) and 2/12 (17%) for RPCs, although no significant
relationship was found between the existence of genetic alterations and histological features.

The findings imply two possibilities. Firstly, distinct carcinogenic actions of BBN might occur in the upper and lower urinary tract. Secondly, different genetic pathways may participate in the underlying carcinogenic processes. Metabolism of BBN in vivo has been investigated using animal models, but no information is available concerning differences in generation of the ultimate carcino-

gen between the upper and lower urinary tract. The spectrum of base-pair substitutions as a “genetic footprint” of carcinogen exposure may provide clues. However, in our experiment, while the small numbers of mutations in RPCs do not allow a more precise assessment, there does not appear to be any mutational cell-type specificity between SCCs and TCCs. Further analysis, for example, with examination of the levels of DNA-adducts is necessary for clarification.
Since no p53, or H-ras mutations or MS alteration were found in the 4 RPCs without detectable metastatic foci (animals #1 to #4, Table I), and all except one (animal #5) case with advanced lesions (aggressive invasion or metastasis) harbored one or more changes, the genetic alteration presumably played a role in progression. We previously reported that mutational inactivation of the p53 gene might be a significant step in progression of mouse urinary bladder carcinomas.9, 10, 19) In the present study, the p53 mutation at codon 227 was common in the primary RPC and its metastatic foci of animal #11, suggesting their clonal relationship. As for animal #6, p53 mutation at codon 155 was confined to the invasive lesion and a link with progression was suggested. Therefore, although the frequency of the p53 mutation was low, inactivation of the p53 tumor suppressor gene might have contributed to the development of advanced-stage mouse RPCs.

The types of H-ras mutations found here have not been reported in human cancers as far as we know, but were previously demonstrated in other experiments with BBN-induced mouse UBCs.9, 10) The data may thus point to a new hot spot for this gene in BBN-induced mouse UBCs, though the reason why this mutation should only occur in mouse urinary tract carcinomas is unclear. Since the mutational events, however, were limited to a small number of cases, further studies are necessary to clarify their significance.

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