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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)]

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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.[9, 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 agressively 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 method[14] as previously described.[9] 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.

Statistical analysis All 2×2 tables were analyzed by the χ^2 test or Fisher's exact probability test (Stat View-J 4.02). *P* values less than 0.05 were considered statistically significant.

RESULTS

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

Table I. Genetic Alterations in Mouse Renal Pelvic Carcinomas and Metastases

Animal no.	Tumor type	Histology	Grade /stage	Genetic alterations		
				p53 mutation	H-*ras* mutation	MSI[a]
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			NE
	invasion to aorta	TCC+AD	G3	codon 155 CGC to TGC		NE
7	RPC	TCC	G3/T4			D16Mit4
	lung meta.	TCC	G3			
8	RPC	SCC	Mod./T3		codon 44 GTG to GCG	
	lung meta.	SCC	Mod.		codon 12 GGA to GTA codon 44 GTG to GCG	D11Mit14
9	RPC	TCC	G3/T3			
	lung meta.	TCC	G3		codon 44 GTG to GCG	
10	RPC	TCC+AD	G3/T3			
	lung meta.	TCC+AD	G3		codon 44 GTG to GCG	
11	RPC	SCC	Mod./T4	codon 227 ACC to ATC		D11Mit14
	splenic LN	SCC	Mod.	codon 227 ACC to ATC		
12	RPC	TCC+SA	G3/T4			D11Mit14, D15Mit14, D18Mit7
	diaphragm meta.	SA	G3			D18Mit7

a) 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.

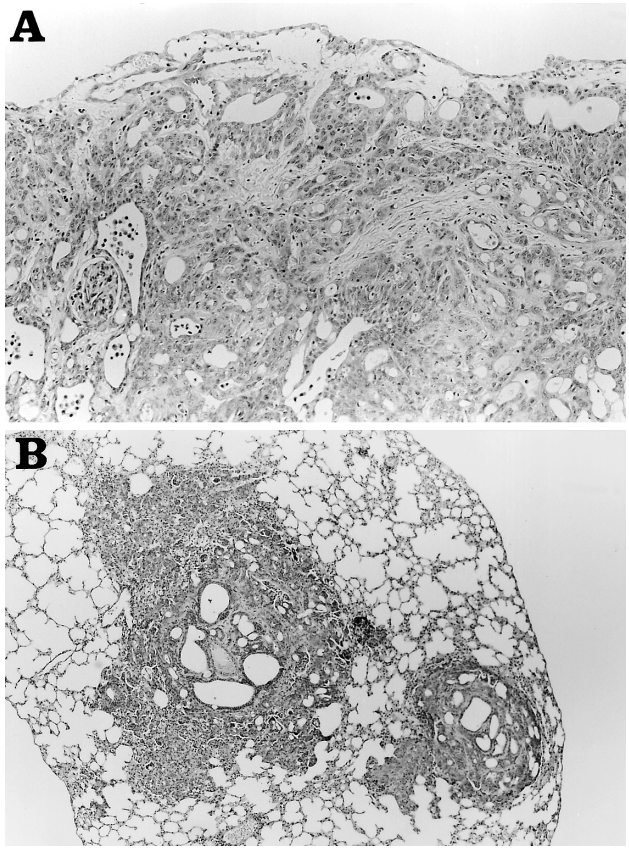

Fig. 1. Histological appearance of a renal pelvic TCC with tubular formation (adenocarcinoma) induced by BBN (animal #10). (A) Invasive growth in the renal parenchyma is evident. H&E: ×50. (B) Lung metastasis in the same animal showing the same histological features. H&E: ×50.

metastatic foci in some animals. Incidences of UBCs and RPCs were 180/240 (75%) and 17/240 (7%), respectively, and 13 of the 17 RPCs (76%) were metastatic. Metastatic foci selected for the present mutational analysis were located in the lung in 5, diaphragm in one and splenic lymph nodes in one animal (Table I). The RPC in animal #6 had directly invaded along the abdominal aorta and an invasive lesion distant from the primary was taken for analysis. Of the 12 primary RPCs, 10 were TCCs and the remaining 2 were squamous cell carcinomas (SCCs). Eight out of 10 primary renal pelvic TCCs were grade 3 and two were grade 2 (Fig. 1).[15] Both SCCs were moderately differentiated. All RPCs invaded beyond the muscularis mucosa into peripelvic fat or renal parenchyma. Two TCCs (animals #1 and #12) exhibited proliferation of sarcomatous components with spindle cells. In two animals (#6 and #10), TCCs were accompanied by subpopulations of tubular elements (adenocarcinoma). All pairs of primary and advanced lesions (animals #5 to #12) showed consistent pathological features (Fig. 1).

Mutational analysis of the *p53* and H-*ras* genes Table I shows the results of the mutation analysis of *p53* and H-*ras* genes, as well as MSI analysis. *p53* mutations were found in 2 of 12 (17%) cases and in animal #11, and the ACC-to-ATC (Thr to Ile) mutation at codon 227 was common in primary RPC and its metastatic foci (Fig. 2a). On the other hand, the CGC-to-TGC (Arg to Cys) mutation at codon 155 of *p53* was confined to the invasive lesion in animal #6 and not found in the primary RPC. The result was a C-to-T transition in all cases.

H-*ras* mutations were found in 3 of 12 cases (25%). Of the total of 5 detected, 4 were at codon 44, GTG to GCG (Val to Ala). In 2 cases (animals #9 and #10), H-*ras* mutations were confined to lung metastasis and not found in the primary RPCs. In addition, although the mutations at codon 44 were common in primary RPC and lung metastasis of animal #8, an additional GGA-to-GTA (Gly to Val) mutation at H-*ras* codon 12 was found in the lung metastasis (Fig. 2b).

MSI analysis was available for 6 pairs of primary RPCs and their metastatic foci, and 4 animals (67%) had MS alterations rather than MSI at one or more microsatellite loci (Fig. 2c). In animals #7 and #11, MS alterations were positive in primary RPC but not in metastatic foci. In contrast, only the metastatic focus was positive in animal #8. Interestingly, MS alterations were positive for 3 microsatellite loci (D11Mit14, D15Mit14 and D18Mit7) in the primary RPC of animal #12 and only one locus (D18Mit7) was positive in metastatic foci. Since the abnormal mobility-shifts at the D18Mit7 locus were identical in both primary and metastatic foci (Fig. 2c), a subpopulation with this alteration might have selectively metastasized.

DISCUSSION

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

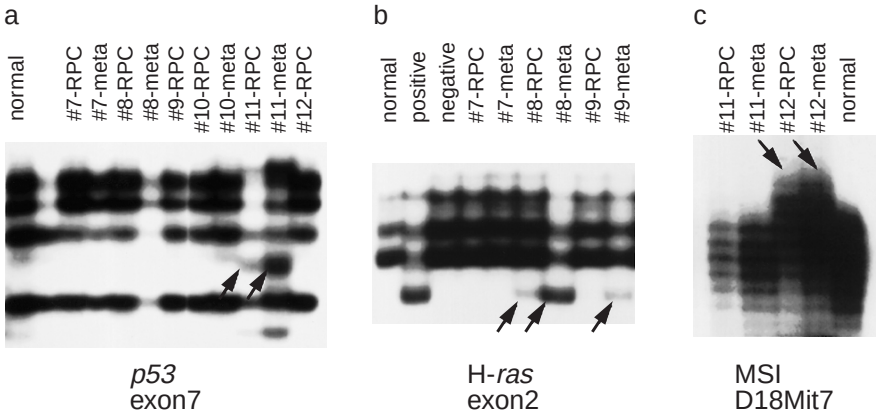

Fig. 2. Results of PCR-SSCP analysis of *p53* gene exon 7 (a) and H-*ras* gene exon 2 (b) and for MSI (c) in mouse RPCs. In (a), mobility-shifted bands for animal #11 are in the same position for both the primary RPC and its metastasis, an identical *p53* mutation being found (codon 227). (b) Although the intensity of the mobility-shifted band of the metastatic focus of animal #8 is strong, that in the primary RPC is relatively weak, suggesting that a small subpopulation with this H-*ras* mutation (codon 44) selectively metastasized. In (c), MSI at the D18Mit7 locus is apparent in both the primary RPC and its metastatic focus. Normal, control DNA from an animal not receiving chemical supplement. Positive and negative, positive and negative controls for this locus, respectively, as detailed in our previous report (ref. 11).

Table II. Summary of Findings for *p53* and H-*ras* Mutations and Microsatellite Instability (MSI) in Mouse Urothelial Carcinomas and Metastases

	p53 mutation (%)		H-*ras* mutation (%)		MSI (%)	
Renal pelvic carcinomas (RPCs)						
Primary RPCs	1/12[a)] (8)		1/12 (8)		3/6 (50)	
Advanced lesions (meta./inv.)[b)]	2/8 (25)		3/8 (38)		2/6 (33)	
Total (/animal)	2/12 (17)		3/12 (25)		4/6 (67)	
Urinary bladder carcinomas (UBCs)[c)]						
Primary UBCs		$P=0.0002$[d)]		NS		$P=0.0043$
without metastasis	14/18 (78)		3/28 (11)		2/18 (11)	
with metastasis	9/10 (90)		1/10 (10)		0/10 (0)	
Metastatic foci	7/10 (70)		1/10 (10)		0/10 (0)	
Total (/animal)	23/28 (82)		4/28 (14)		2/28 (7)	

a) No. of animals with mutations/no. of animals examined.
b) Abbreviations used are: meta., metastasis; inv., invasive.
c) Based on data from our previous publications (refs. 9–11).
d) The significance of differences in mutation frequencies was assessed using Fisher's exact probability test; NS, not 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,[16, 17)] but no information is available concerning differences in generation of the ultimate carcinogen between the upper and lower urinary tract. The spectrum of base-pair substitutions as a "genetic footprint" of carcinogen exposure may provide clues.[18)] 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 in 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.

In conclusion, the distribution of genetic alterations may be different between RPCs and UBCs produced by the same experimental protocol in mice, so that the results suggest distinct site-specificity in carcinogenic actions of BBN or different genetic pathways participating in carcinogenic processes in the upper and lower urinary tract. We recently found *p53* mutations to be frequent in human UBCs, but infrequent in RPCs as assessed by p53 yeast functional assay.[20] In addition, cyclin D1 gene amplification was found in human renal pelvic TCCs, as determined by fluorescence *in situ* hybridization.[21] Further detailed analysis of the molecular events occurring in urothelial neoplasia is necessary using both human and rodent materials.

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