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p53 gene mutation in *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine-induced urinary bladder tumors and *N*-methyl-*N*-nitrosourea-induced colon tumors of rats

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Abstract

We analyzed *p53* mutations in 17 *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine-induced bladder transitional cell carcinomas (TCCs) with or without areas of squamous cell carcinoma (SCC) of Long–Evans Cinnamon (LEC) and F344 rats, and in 7 *N*-methyl-*N*-nitrosourea-induced colon adenocarcinomas of LEC rats by polymerase chain reaction-single strand conformation polymorphism analysis and DNA sequencing. Of these bladder tumors, one TCC with moderately differentiated SCC had a T to G transversion mutation at codon 141, leading to a Val to Gly amino acid change. No *p53* mutation was found in colon adenocarcinomas. Thus a *p53* gene mutation seems infrequent in these rat bladder and colon carcinogenesis models even in the late stage. © 1997 Elsevier Science Ireland Ltd.

Keywords: *p53* gene; Bladder cancer; Rat; *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; PCR-SSCP

1. Introduction

Carcinogenesis is a multistep process which involves the accumulation of gene alterations [1–3]. Inactivation of the *p53* tumor suppressor gene is one of the most common genetic alterations in many human cancers including lung, breast, urinary bladder and colon cancers [4,5]. It has been reported that the frequency of *p53* mutation is approximately 30–60% in human urinary bladder cancer [6–8], and 50–75% in colon cancer [4,9,10], especially in the late stage.

Hepatocellular carcinomas (HCCs) in China and southern Africa are known to have a specific G to T transversion in codon 249 of the *p53* gene [11–13], and this mutation is thought to be correlated with exposure to aflatoxins. Animal studies have shown that some carcinogens, such as alkylating *N*-nitroso compounds, induce a specific gene alteration. For example, *N*-methyl-*N*-nitrosourea specifically induces a G to A transition at the second nucleotide of codon 12 in the Ha-*ras* gene in various organs of different animals [14], as a result of O^6-methylguanine DNA adduct formation [15].

The LEC rat, a model of human Wilson's disease, develops liver [16] and kidney [17] tumors spontaneously by copper accumulation in these organs

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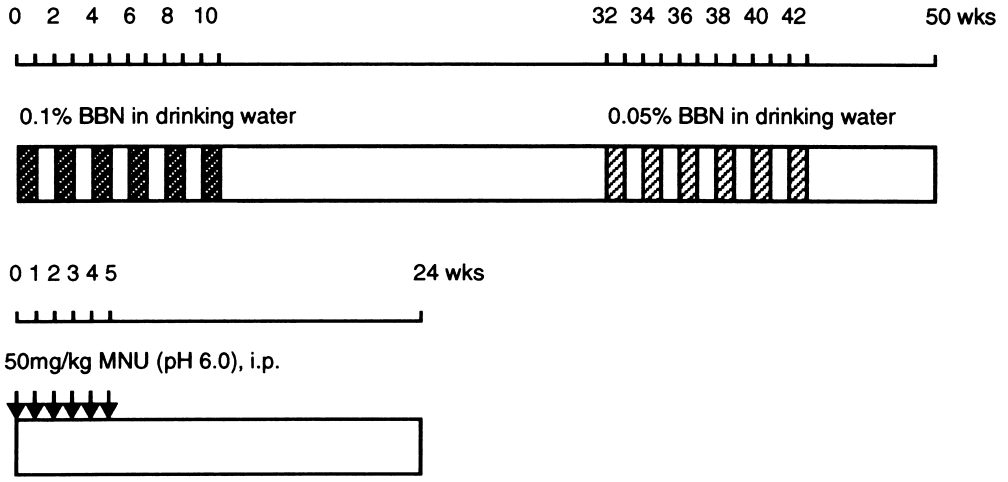

Fig. 1. Experimental design to induce bladder and colon tumors.

[18]. In this study, we screened the *p53* gene mutation in exons 2–9 and a part of exon 10 in carcinogen-induced rat bladder and colon tumors of LEC and F344 rats by the PCR-SSCP method.

2. Materials and methods

2.1. Animal experiments

Seven-week-old male LEC ($n = 3$) and F344 ($n = 7$) rats were given 0.1% BBN in their drinking water every other week from week 0–11, and then 0.05% BBN every other week from week 32–43, and were killed in week 50 to obtain bladder tumors (Fig. 1). Six-week-old male LEC rats ($n = 7$) were injected i.p. with MNU (50 mg/kg body weight) once a week for 6 weeks and killed in week 24 to obtain colon tumors. Seventeen bladder tumors (3–20 mm in size) and 7 colon tumors (4–8 mm in size) were examined. Parts of the tumors were frozen in liquid nitrogen and kept at –80°C until use. The remaining portions of the tumors were fixed in 10% buffered formalin for histological examination.

2.2. DNA extraction

High molecular weight DNA was prepared from tumor tissues by proteinase K digestion and phenol/chloroform extraction [19].

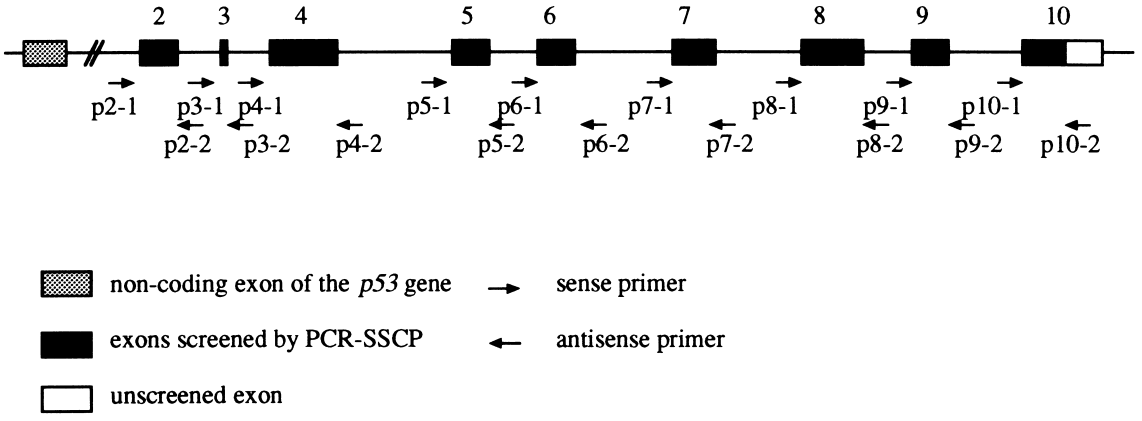

Fig. 2. Schematic presentation of the locations of PCR primers to analyze *p53* mutations.

Table 1

Oligonucleotide primers used for p53 gene analysis

Name	Sequence
P2-1	5′-CAGTACACAGATGTTCTCTC-3′
P2-2	5′-CAGGGGCCCTGTAAGATCCA-3′
P3-1	5′-GCCTGGGGTAAGTGAGATTC-3′
P3-2	5′-TAAGTCCCCTCTTGCCCGGC-3′
P4-1	5′-GTTCTTCTTTGGCCCATCCA-3′
P4-2	5′-AAGCAACTCTTCAGGCCCAC-3′
P5-1	5′-GATTCTTTCTCCTCTCCTAC-3′
P5-2	5′-ACAGGCAGTGCCAGTGCTCA-3′
P6-1	5′-CCCGGCCTCTGACTTATTCT-3′
P6-2	5′-CCCAACCTGGCACACAGCTT-3′
P7-1	5′-CTGTGCCTCCTCTTGTCCCG-3′
P7-2	5′-CTCCACCTTCTTTGTCCTGC-3′
P8-1	5′-AAAGTCACCCCTTGCTCTCT-3′
P8-2	5′-TAATCCAATAATAACCTTGG-3′
P9-1	5′-TGTCCTACTTCATCCTTGCT-3′
P9-2	5′-AGGCGCTGCCCCAGGTCACT-3′
P10-1	5′-CCCTCCCTTTTCTGTATTCC-3′
P10-2	5′-CAGCAGAGACCCAGCAACTA-3′

2.3. Oligonucleotide PCR primers

Nine pairs of primers flanking exons 2–9 and a part of exon 10 of the *p53* gene were synthesized in a Model 392 DNA synthesizer (Perkin-Elmer, Norwalk, CT). All primers except an antisense primer for exon 10 were those of intron sequences to avoid amplification of pseudogenes [20] (Fig. 2). The nucleotide sequences of primers are summarized in Table 1.

2.4. PCR-SSCP analysis

PCR-SSCP analysis was performed by the method of Orita et al. [21] with a minor modification. Samples of 5 μl of the PCR mixture contained 50 ng of genomic DNA as a template, 5 pmol each of primers, 0.125 nmol each of dNTPs, 0.25 μl of [α-^{32}P]dCTP (110 TBq/mmol; 370 MBq/ml) and 0.25 units of Taq DNA polymerase (Takara, Ohtsu, Japan) in the buffer supplied by Takara. Thirty cycles of the reaction at 94, 55 and 72°C each for 1 min were carried out in a thermal cycler (GeneAmp™ PCR System 9600, Perkin-Elmer). The resultant PCR product was mixed with 245 μl of a formamide dye mixture (95% for-mamide, 20 mM EDTA, 0.05% xylene cyanol and 0.05% bromophenol blue) and heated at 95°C for 3 min for denaturation. Then 1 μl of the mixture was applied to 6% polyacrylamide gel containing 45 mM Tris–borate (pH 8.3) and 1 mM EDTA. All PCR products were analyzed by PCR-SSCP at glycerol concentrations of 0, 5 or 10%, respectively. Electrophoresis was performed at 15 W for 3–5 h with cooling by a fan at room temperature. The gel was dried on filter paper and exposed to an X-ray film with an intensifying screen overnight at –80°C. In each case, the mobility shift on PCR-SSCP analysis was reproducible.

2.5. DNA sequencing

The portion of the dried polyacrylamide gel containing DNA with abnormal mobility found on SSCP analysis was cut out and DNA was extracted overnight from the gel with 50 μl of water at 37°C. DNA fragments re-amplified by PCR with the extracted DNAs as templates and the same primers as used in the original PCR were subcloned into TA vectors with the original TA cloning™ Kit (Invitrogen, San Diego, CA). Eight individual clones of each sample with a mobility shift were sequenced by the fluorescence-based dideoxy sequencing method with a DNA sequencer (Model 377, Perkin-Elmer).

Table 2

A *p53* mutation in BBN-induced bladder tumors and MNU-induced colon tumors

	Histology of the tumors	No. of samples	*p53* mutation		
			Incidence	Codon	Nucleotide and amino acid change
Bladder	TCC	10	0	–	
	TCC with SCC	7	1	141	GTG (Val) → GGG (Gly)
Colon	Adenocarcinoma	7	0	–	

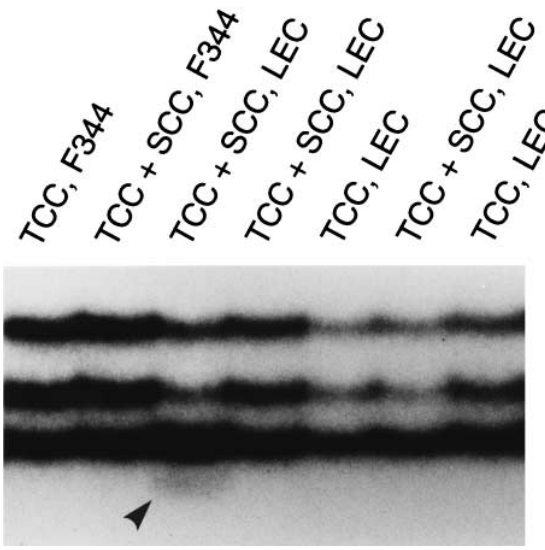

Fig. 3. Results of PCR-SSCP analysis of the *p53* gene exon 5 in BBN-induced rat bladder tumors. A shifted band was observed in a TCC with moderately differentiated SCC in an LEC rat (arrowhead).

3. Results

Data are summarized in Table 2. Bladder tumors from 3 LEC and 7 F344 rats included 10 pure TCCs and 7 TCCs with areas of well to moderately differentiated SCC histologically. On PCR-SSCP analysis of DNA samples extracted from these bladder tumors, a band shift of exon 5 was detected in one tumor of an LEC rat (Fig. 3). Sequence analysis showed a T to G transversion mutation in codon 141, leading to a Val (GTG) to Gly (GGG) amino acid change (Fig. 4). This bladder tumor was identified as a TCC with areas of moderately differentiated SCC, and its histological grade of malignancy was the highest among the bladder tumors examined (Fig. 5).

All colon tumors of LEC rats were adenocarcinomas invading the submucosa and subserosa. We did not detect any *p53* mutations in these colon tumors.

4. Discussion

p53 mutations generally occur within exons 5–8 in human cancers [4,5]. To test whether different hotspots of the *p53* gene exist in rodents, we screened all coding regions of the rat *p53* gene except a part of exon 10. In the present study, we found a mutation in exon 5, but its frequency was lower than that reported in human bladder or colon cancer. Possible reasons for the low incidence of the *p53* mutation in rats are (a) in spite of the large size of their tumors and the presence of areas of SCC the stage of tumors in rats is earlier than that in humans because usually no metastases are observed in animals with carcinogen-induced bladder

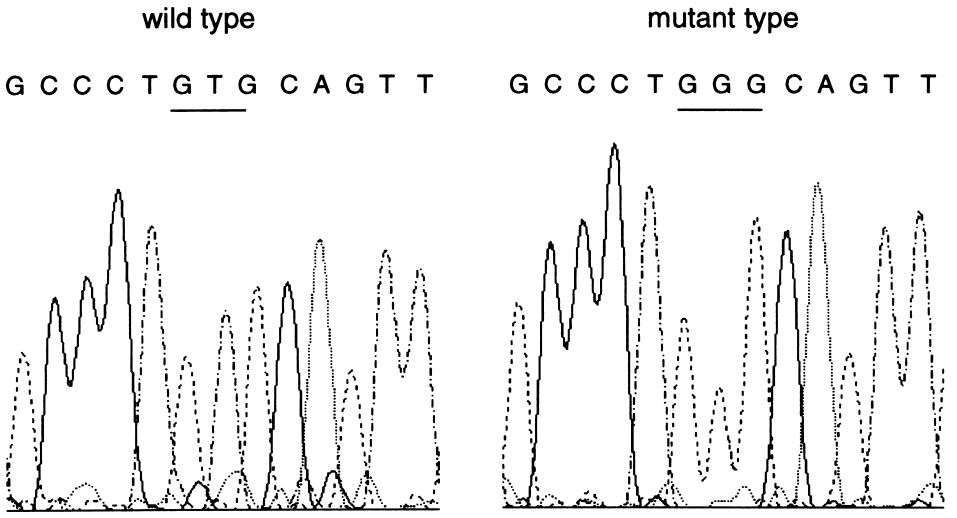

Fig. 4. DNA sequences in samples of bladder tumors. GTG (Val) to GGG (Gly) transversion at codon 141 of the *p53* gene is shown.

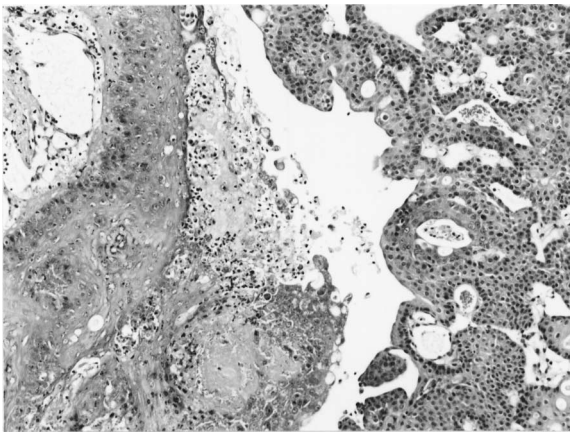

Fig. 5. Histology of a bladder tumor with a *p53* mutation. TCC with moderately differentiated SCC (HE × 99).

tumors and (b) there may be a species difference in the incidence of *p53* mutations.

It is reported that the incidence of *p53* gene mutations in BBN-induced bladder tumors in F344 rats is 30–67% [22,23], and that in *N*-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide-induced tumors in F344 rats is 0–7% [24,25]. In our study, we detected a *p53* mutation in 6% (1/17) of BBN-induced bladder tumors. One factor for this discrepancy in incidence might be a difference in the period of administration of carcinogens, the period of administration of BBN in our study being shorter than that in the study of Masui et al. [22,23]. The GTG (Val) to GGG (Gly) mutation in codon 141 detected in the present study has been observed in BBN-induced small TCC [22] in addition to T → G mutations in other codons [5,7,22]. Although G → A and G → T mutations are common in the *p53* gene, a T → G mutation is rare in human cancers [5].

In a recent study, we did not find a p53 mutation in spontaneously developed HCCs in LEC rats, but found it in diethylnitrosamine or *N*-ethyl-*N*-hydroxyethyl-nitrosamine-induced HCCs (unpublished data). Nagao et al. [26] reported that all *Apc* gene mutations in rat colon tumors induced by 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine, a heterocyclic amine, were specific GGGA to GGA frameshift mutations in various codons due to deletion of G, but they found no *p53* mutations [27]. Animal models provide opportunities for studies on carcinogen-specific or organ-specific mutations. As the database on mutations in laboratory animals is limited, more studies on these mutations induced by various carcinogens are necessary.

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