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p53 mutations in phenacetin-associated human urothelial carcinomas

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Chronic abuse of the analgesic drug phenacetin is associated with an increased risk of development of transitional cell carcinomas of the urinary tract. It is unclear whether phenacetin acts through chronic tissue damage (phenacetin nephropathy) or via a genotoxic metabolite causing promutagenic DNA lesions. In the present study, we investigated 15 urothelial carcinomas from 13 patients with evidence of phenacetin abuse. Tumors were screened for *p53* mutations in exons 5–8 by single-strand conformation polymorphism (SSCP) analysis, followed by direct sequencing of PCR-amplified DNA. *p53* Mutations were detected in 8/14 primary tumors (57%). All except one were missense mutations located in exon 5 (three mutations), exon 6 (one), exon 7 (two) and exon 8 (one). The type of mutation varied, with a preference for CpG sites. A frameshift mutation resulting from the insertion of a single cytosine at codons 151/152 was detected in a bladder tumor and its lung metastasis. Urothelial carcinomas located in the renal pelvis and in the ureter of the same patient exhibited two different mutations, strongly suggesting that they developed independently. Another patient had tumors in the renal pelvis and bladder, both of which contained the same *p53* mutation, indicating intracavitary metastatic spread. This demonstrates that screening of *p53* mutations allows the clonal origin of tumors in patients with multiple primary and metastatic lesions to be determined. None of the tumors investigated contained mutations in codons 12, 13 or 61 of H-*ras* or K-*ras* protooncogenes.

Introduction

Habitual use of phenacetin or phenacetin-containing analgesics is known to cause renal papillary necrosis, chronic interstitial nephritis and capillary sclerosis of the lower urinary tract (1). In addition, some patients with long-term addiction develop urothelial carcinomas (2–5). Moreover, patients with a history of phenacetin abuse have a significantly higher risk of developing multiple tumors of the urinary tract than non-abusers (4). There is increasing evidence that the type and genomic distribution of mutations in oncogenes and tumor suppressor genes may be typical or even specific for the carcinogen which initiated the multi-step process of malignant transformation. Examples of this type of genetic tumor epidemiology include aflatoxin B_1-induced hepatocellular carcinomas (6), tobacco versus uranium-associated lung cancer (7) and squamous cell carcinomas of the skin following UV exposure (8). The objective of the present study was, therefore, to identify possible specific *p53* mutations in urothelial carcinomas resulting from phenacetin intake.

*Abbreviations: SSCP, single-strand conformation polymorphism; PCR, polymerase chain reaction.

Materials and methods

Tumors from 13 patients were investigated (Table I). Surgical biopsies were obtained from seven patients (cases 1–4, 7, 8 and 10). Samples from five additional patients (cases 5, 6, 9, 11–13) were collected at post mortem examination. Two separate urinary tract tumors were analyzed in each of two patients (cases 10 and 13), and in one patient (case 11) a bladder tumor and a lung metastasis were investigated. Twelve out of 13 patients had a clinically documented history of long-term phenacetin abuse and kidney lesions typical for phenacetin nephropathy, i.e. renal capillary sclerosis, renal papillary necrosis and chronic interstitial nephritis (Table I). In one patient (case 9), there was no proven history of phenacetin abuse but the presence of capillary sclerosis and papillary necrosis was considered sufficient evidence of phenacetin nephropathy (1,4). Tumors were graded according to WHO guidelines (9) into well-differentiated (grade I), moderately differentiated (grade II) and poorly differentiated carcinomas (grade III).

DNA was extracted from formalin-fixed, paraffin-embedded tissue according to the protocol described by Wright and Manos (10). Briefly, tumor tissue was scraped off serial histological sections after comparison with a representative section stained with H&E. Samples were deparaffinized with xylene and washed with absolute ethanol. Dried samples were treated with 200 μg/ml of proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) in 100 μl digestion buffer (50 mM Tris, pH 8.5; 1 mM EDTA; 0.5% Tween 20) for 3 h at 55°C. After inactivation of proteinase K by heating at 95°C for 10 min, samples were kept at −20°C until PCR amplification. DNA from frozen tumor tissue was extracted by phenol and chloroform (11).

Single-strand conformation polymorphism (SSCP*) analysis was performed using a modification of the method of Orita *et al.* (12). Polymerase chain reaction (PCR) was performed with 2 μl of DNA solution extracted from formalin-fixed tissue or 200 ng of genomic DNA from frozen tissue, 10 pmol of each primer, 125 μM of dNTPs, 1 μCi of [α-^{32}P]dCTP (Amersham, sp. act. 3000 Ci/mmol), 10 mM Tris (pH 8.3), 50 mM KCl, 1.25 mM $MgCl_2$, and 0.5 U Taq polymerase (Perkin-Elmer Cetus) in a final volume of 20 μl. After adding 10 μl of mineral oil (Sigma), 35 cycles of denaturation (95°C) for 50 s, annealing (58°C) for 50 s and extension (72°C) for 70 s were carried out using an automated DNA Thermal Cycler (Perkin-Elmer Cetus). The primer sequences for the *p53* gene were reported previously (13–15). Aliquots (5 μl) of the amplification mixture were mixed with 5 μl of sequencing stop solution (United States Biochemical, Cleveland, OH), heated at 95°C for 5 min and immediately loaded onto a 6% polyacrylamide non-denaturing gel containing 10% glycerol. Gels were run at 8 W for 13–15 h at room temperature. Gels were dried and autoradiography was performed with an intensifying screen for 12–72 h.

For the samples scored positive with SSCP analysis, PCR was performed with 10 μl of DNA extracted from formalin-fixed tissue or 1 μg of genomic DNA extracted from frozen tissue, 20 pmol each primer, 125 μM dNTPs, 10 mM Tris (pH 8.3), 50 mM KCl, 1.25 mM $MgCl_2$, and 2.5 U Taq polymerase (Perkin-Elmer Cetus) in a final volume of 100 μl. Exons 1 and 2 of H-, K-*ras* genes were also amplified and directly sequenced as described above. Primer sequences for *ras* genes were described previously (16). After amplification, 70 μl of the PCR reaction were electrophoresed on a 4% agarose gel (3% low melting and 1% regular agarose). The specific bands were excised, electroeluted in 0.5× TBE buffer, purified by Elutip-D columns (Schleicher and Schuell GmbH, Dassel, Germany) and precipitated with ethanol. Dried DNA was resuspended in 12 μl of distilled water.

Direct sequencing was performed using amplification primers for *p53* exons. The following internal sequencing primers were used for *ras* genes: 5′-TCCAC-AAAATGGTTCTGGAT for H-*ras* codon 12/13; 5′-AGACGTGCCTGTTGG-ACATC for H-*ras* codon 61; 5′-CGTCCACAAAATGATTCTGA for K-*ras* codon 12/13; 5′-GTAATTGATGGAGAAACCTG for K-*ras* codon 61. A modified Sanger dideoxynucleotide sequencing method was used that allows efficient extension labeling of the sequencing primer. Briefly, template DNA (4 μl) and primer (10 pmol) in 10 μl buffer (20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 25 mM NaCl; United States Biochemical) containing 10% (v/v) DMSO,

Table I. *p53* mutations in human phenacetin-induced urothelial carcinomas

Case	Sex	Age	History of abuse	Capillary sclerosis	Nephritis[a]	Papillary necrosis	Tumor localization	WHO grade	*p53* mutation
1	m	47	yes	+	+	+	ureter	I	
2	m	60	yes	+	+	+	ureter	II	
3	m	66	yes	+	+	+	renal pelvis	II	
4	f	52	yes	+	+	+	renal pelvis	III	
5	f	52	yes	+	+	+	renal pelvis	III	
6	m	56	yes	+	+	+	ureter	III	
7	f	58	yes	+	+	+	renal pelvis	III	exon 8, codon 282: CGG → TGG (Arg → Trp)
8	f	61	yes	−	+	+	renal pelvis	III	exon 5, codon 158: CGC → GGC (Arg → Gly)
9	m	64	uncertain	+	−	+	bladder	III	exon 5, codon 179: CAT → CAC (His → Arg)
10	f	65	yes	+	+	+	renal pelvis	III	exon 7, codon 249: AGG → ATG (Arg → Met)
							ureter	III	exon 7, codon 232: ATC → TTC (Ile → Phe)
11	f	67	yes	+	+	+	bladder	III	exon 5, codon 151/152: insertion of 1 C (frameshift)
							lung metastasis	III	exon 5, codon 151/152: insertion of 1 C (frameshift)
12	f	68	yes	+	+	+	bladder	III	exon 6, codon 193: CAT → CGT (His → Arg)
13	f	69	yes	+	+	+	renal pelvis	III	exon 5, codon 175: CGC → CAC (Arg → His)
							bladder	III	exon 5, codon 175: CGC → CAC (Arg → His)

[a]Chronic interstitial nephritis.

were denatured at 95°C for 5 min and immediately frozen in dry ice/ethanol. Six microliters containing an appropriate α-^{32}P-labeled dideoxynucleotide (Amersham, sp. act. 3000 Ci/mmol) and 4 U of Sequenase Version 2.0 (United States Biochemical) in 17 mM dithiothreitol were added. The radioactive nucleotide was chosen to be the one immediately incorporated adjacent to the primer. After preincubation for 2 min at room temperature, 3.6 μl aliquots were mixed with 2.5 μl termination mix (80 μM dNTPs, 8 μM of one ddNTP) and incubated for 10 min at 37°C. Samples were loaded onto 6% denaturing polyacrylamide gels containing 7 M urea. Autoradiography was carried out as above.

Results

SSCP analysis and direct sequencing revealed *p53* point mutations in 10/16 tumors (Table I). All tumors with a mutation of the *p53* gene were poorly differentiated (WHO grade III). All except one consisted of missense mutations resulting in single amino acid changes. Three of these were GC → AT transitions at CpG dinucleotide sites. In addition, a frameshift mutation resulting from the insertion of a single cytosine residue in a run of five cytosines was detected at codons 150/151 of exon 5. One bladder tumor (case 9) and one renal pelvis tumor (case 10) appeared to have undergone loss of heterozygosity as evidenced by the absence of the wild-type base in the sequencing autoradiographs. In the other tumors, both the mutated and the wild-type base were detected, suggesting that these tumors had retained one normal allele. Two typical sequencing autoradiographs are shown in Figure 1. Two patients had multiple tumors of the urinary tract. In one case (case 10) one tumor was located in the renal pelvis and a second tumor in the ureter. The two tumors contained different mutations in exon 7, located in codons 249 and 232 respectively. In case 13, two tumors localized in the renal pelvis and the bladder both carried the same mutation, a GC → AT transition at codon 175 in exon 5. In one patient (case 11) we found the same mutation in both a primary urothelial carcinoma of the bladder and a lung metastasis.

Mutations at codons 12, 13 or 61 of the H-*ras* and K-*ras* protooncogenes were not detected in any of the tumors.

Discussion

Chronic abuse of phenacetin causes tumor formation in the human urinary tract (3−5). Often, multiple tumors are observed, with preferential location in the bladder (>50%), the renal pelvis (30−40%) and in the ureter (5−15%). In contrast, >90% of

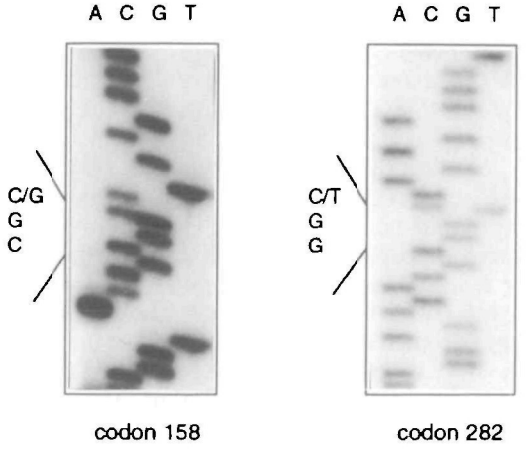

Fig. 1. DNA sequence analysis of *p53* mutations in phenacetin-induced urothelial carcinomas. **Left**, tumor of the renal pelvis (Table I, case 8) with a CG → GC transversion at codon 158 in exon 5. **Right**, tumor of the renal pelvis (Table I, case 7) with a CG → TA transition at a CpG pair at codon 282 in exon 8. Mutated with wild-type sequences are visible in both tumors.

urothelial carcinomas not associated with phenacetin abuse are located in the bladder (4). It has been calculated that after intake of a total dose of ~5 kg of phenacetin, the mean latency period for tumor formation in the renal pelvis and ureter is 25 years (5). Phenacetin also induces benign and malignant tumors in the urinary tract of rats (17,18) and mice (19), but the mechanism of malignant transformation has remained enigmatic. Tumorigenicity studies in rodents suggested that phenacetin may act as a complete genotoxic carcinogen when fed chronically at doses of 0.6−2.5% in the diet (17,19). It is weakly mutagenic in bacteria but only after metabolic activation by hamster liver microsomes (20−22). Following phenacetin administration to rats, a marginal though statistically significant increase in DNA fragmentation was observed in the kidney (21). *In vitro* DNA binding of the phenacetin metabolite *N*-hydroxyphenacetin has been reported (23), but to date no DNA adducts of phenacetin or its metabolites have been identified. On the other hand, there is considerable evidence that phenacetin may act as a potent co-carcinogen. It enhances the induction of urothelial carcinomas in rats exposed to *N*-(4-(5-nitro-2-furyl)-2-thiazolyl)formamide (FANFT) (24) or *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine (25).

It is assumed that this effect is due to the phenacetin-induced stimulation of urothelial cell proliferation (26,27).

The present study revealed that phenacetin-associated urothelial carcinomas contain a high frequency (57%) of *p53* mutations, which thus may significantly contribute to the multi-step process of malignant transformation. However, there were neither specific mutations nor mutational hotspots, in contrast to various other tumors induced by chemical carcinogens and UV irradiation (6–8,28). Three of eight mutations were GC → AT transitions at CpG pairs, a type of mutation thought to result from spontaneous deamination of 5-methylcytosine (29). In addition, two were AT → GC transitions which may arise through deamination of adenine to hypoxanthine: Chronic inflammation is associated with activation of macrophages which in turn produce high levels of nitric oxide, a known deaminating agent (30). Chronic inflammation of the urothel as a cause of malignant transformation is illustrated by schistosomiasis, a chronic parasitic disease known to be associated with an increased risk for bladder cancer in humans (31). The results of this study do not clarify the mechnism by which phenacetin induces urothelial carcinomas in man. The absence of specific mutations and of mutational hotspots may be suggestive of a mechanism involving chronic tissue damage and stimulation of urothelial proliferation; however, our results do not exclude tumor induction via specific promutagenic DNA lesions generated by phenacetin metabolites. Our data are compatible with previous molecular genetic studies of human bladder tumors showing that *p53* mutations occur at an incidence of 50–60% and prevail in invasive transitional cell carcinomas rather than in superficial bladder cancers (32,33). In a recent study on bladder carcinomas in patients with long-term exposure to cigarette smoke, GC → CG transversions were most frequent. In contrast to our data on phenacetin-associated carcinomas, one third of neoplasms with *p53* mutations had double mutations, usually tandem mutations on the same allele which were not observed in similar tumors from non-smokers (34).

Mutations at codons 12, 13 or 61 of the H-*ras* and K-*ras* protooncogenes were not detected in any of the tumors. This is consistent with previous reports showing a low incidence or even absence of H-*ras* and K-*ras* mutations in urothelial carcinomas not associated with exposure to phenacetin (35–42).

p53 mutations have been reported to persist and are usually identical in primary tumors and their metastases (43). This may serve to distinguish between the occurrence of multiple primary tumors and metastatic spread, particularly when the tumors are localized in the same tissue. In the present series, we identified an identical frameshift mutation in a bladder tumor and its lung metastasis (case 11). In another patient (case 13), two urothelial tumors in the renal pelvis and the bladder were diagnosed as multiple primary tumors but contained the same *p53* point mutation. This suggests that the renal tumor was the primary neoplasm which had spread via the urine to the bladder. This contrasts with a third case (case 10) in which urothelial carcinomas were located in the renal pelvis and ureter. They carried different *p53* point mutations, located in codons 249 and 232 respectively, indicating that the two tumors arose independently. Multifocal neoplasms that differed in *p53* mutations have also been reported for human esophageal cancer (44). These examples demonstrate that screening for *p53* point mutations may prove to be very useful in determining the clonal origin of tumors in patients with multiple primary and metastatic lesions, in addition to identifying or excluding putative causative agents or their carcinogenic metabolites.

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