

p53 Mutations in Bladder Cancer: Evidence for Exogenous *versus* Endogenous Risk Factors

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ABSTRACT

Bladder cancer is associated with smoking, occupational exposures, and glutathione *S*-transferase (GST) M1 and *N*-acetyltransferase (NAT) 2 polymorphisms that may influence carcinogen metabolism, but somatic *p53* mutations are often CpG dinucleotide G:C-A:T transitions that can occur spontaneously. We conducted a case-control study to determine whether *p53* mutation characteristics might distinguish cases with environmental *versus* endogenous causes. *p53* exons 4–9 were amplified from 146 bladder tumors by PCR, screened by single-strand conformational polymorphism analysis, and sequenced. Thirty-one cases were *p53*-positive, and 112 were *p53*-negative (germ line or silent). G:C-A:T transitions were also subclassified as CpG or non-CpG. Cases and 215 clinic controls were interviewed. *GSTM1*, *NAT1*, and *NAT2* polymorphisms were assayed from peripheral blood. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using logistic and polytomous regression. Case-control ORs for smoking, occupations, and *NAT1**10 genotype were similar for *p53*-positive and *p53*-negative cases. Associations with *GSTM1*-null and *NAT2*-slow genotypes were somewhat stronger for *p53*-positive [OR, 3.3; CI, 1.4–7.8 (*GSTM1* null); OR, 1.8; CI, 0.8–4.0 (*NAT2* slow)] than *p53*-negative cases [OR, 1.5; CI:0.9–2.3 (*GSTM1* null); OR, 0.9; CI, 0.6–1.4 (*NAT2* slow)]. Smoking was strongly associated with CpG G:C-A:T (OR, 15.3; CI:3.6–65) *versus* other G:C-A:T (OR, 1.8; CI, 0.3–9.8). *NAT2* slow genotypes were also associated with CpG G:C-A:T (OR, 6.2; CI:0.7–52), whereas *GSTM1* null was associated with non-CpG G:C-A:T (OR, 7.8; CI, 0.9–65). Associations were not substantially different for case subtypes defined by *p53* mutation status alone. Estimates for *p53* subtypes were imprecise but support *in vitro* evidence that some CpG G:C-A:T transitions may be caused by smoking and other environmental mutagens.

INTRODUCTION

Missense mutations in the *p53* tumor suppressor gene (*TP53*) are the most common somatic mutations identified among cancers (1). Cancer-associated mutations in the highly conserved DNA-binding domain may prevent or inhibit *p53*-mediated cell cycle arrest, DNA repair, programmed cell death, and other protective responses to cell stress and DNA damage (2–4). Some *p53* mutations are associated with specific carcinogens, for example, CC to TT transitions are associated with UV light (5) and G to T transversions are associated with benzo(*a*)pyrene (6, 7). Others may occur spontaneously, most notably G:C to A:T transitions at CpG dinucleotides after 5-methyl cytosine deamination (8). CpG G:C-A:T transitions are particularly common *p53* mutations in breast and colon cancers (9), two cancers for which prominent environmental causes have not been identified. In contrast, CpG G:C-A:T transitions are relatively uncommon in cancers with strong environmental risk factors; namely, cancers of the

lung, skin, and liver. Therefore, it has been proposed that *p53* G:C-A:T transitions at CpG are an attribute of cancers caused by endogenous cellular processes (9–11).

CpG G:C-A:T transitions account for almost 25% of *p53* mutations in bladder cancers (9), but this differs from the “endogenous” pattern associated with colon cancer, where almost half of all *p53* mutations are CpG G:C-A:T (10, 11). The spectrum of bladder cancer *p53* mutations also differs from lung cancer, even though cigarette smoking is probably a contributing cause in over one-third of all bladder cancer cases (12). Specifically, G-T transversions are relatively uncommon (about 8% of bladder cancer *p53* mutations compared with 27% of lung cancer mutations), whereas CpG G:C-A:T transitions are twice as common (22% for bladder *versus* 11% for lung cancer; Ref. 9). This indeterminate pattern of bladder cancer *p53* mutations might be a consequence of etiologic heterogeneity; if so, characteristics of *p53* mutations might distinguish cases caused by smoking or other environmental risk factors from “endogenous” cases with carcinogenic mutations caused by spontaneous cellular processes (13–16). Genetic susceptibility factors for bladder cancer might also vary among *p53* case subtypes, if they increase risks by modifying bladder cell exposures to environmental mutagens. To evaluate these hypotheses, we compared case-control ORs² for bladder cancer case subgroups defined by acquired *p53* mutations to determine whether *p53* subtype-specific associations varied for smoking, occupational exposures, and *GSTM1*, *NAT2*, and *NAT1* polymorphisms that may influence bladder cell exposures to mutagenic byproducts of cigarette smoke.

MATERIALS AND METHODS

Study Participants

Patients with histologically confirmed transitional cell carcinomas of the urinary bladder ($n = 245$) were enrolled from Urology Clinics at the University of North Carolina Hospitals and Duke University Medical Center (17, 18). Controls were 215 patients from the same clinics without a history of cancer (other than nonmelanoma skin cancer), who were frequency-matched to cases based on ethnicity, sex, and 10-year age intervals. Institutional review boards at each participating institution approved all study protocols.

Exposure Data

Demographic and exposure data were obtained from a structured questionnaire administered in person by a trained nurse-interviewer. Years of smoking, pack-years, and current smoking status were based on exposures up to 2 years before initial diagnosis (cases) or interview (controls). Participants provided information on all jobs held at least 2 years, on any work in specific jobs or industries of *a priori* interest, and on occupational or home use of specific products of *a priori* interest that were used on at least 5 occasions. These exposures were counted only if they began more than 2 years before diagnosis

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²The abbreviations used are: OR, odds ratio; CI, confidence interval; GST, glutathione *S*-transferase; NAT, *N*-acetyltransferase; SSCP, single-strand conformational polymorphism; EM, Expectation Maximization; BPDE, benzo(*a*)pyrene diol epoxide; 4-ABP, *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl; TCR, transcription-coupled repair; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

or interview. Exposure data were classified as missing for jobs or products with unknown start dates and years of exposure.

NAT1 and *NAT2* polymorphisms were ascertained using RFLP-PCR analysis of DNA extracted from peripheral blood lymphocytes obtained at the time of interview, and results have been reported previously (18). Four *NAT1* alleles were assayed: *NAT1*4* (wild type), *NAT1*3* (C1095A), *NAT1*10* (T1088A, C1095A), and a 9-bp deletion upstream of nucleotide 1088 (previously referred to as *NAT1*11*). Five *NAT2* alleles were assayed: *NAT2*4* (wild type), *NAT2*5D* (C481T), *NAT2*6B* (G590A), *NAT2*7A* (G857A), and *NAT2*14A* (G191A; Ref. 19). Participants were classified as *NAT1*10* if they had at least one *NAT1*10* allele, and as *NAT2* slow if they lacked a *NAT2*4* allele. *GSTM1* alleles were identified using a differential PCR assay, as described previously (17). Participants were classified as *GSTM1* null if both alleles were deleted.

p53 Mutation Status

DNA Extraction. Sections were cut from paraffin-embedded tumor blocks, reviewed by a pathologist, and manually microdissected if they included a substantial amount of nonneoplastic tissue. One or more sections were used to produce 1–3 μ l of tumor lysate DNA from each sample. Samples were digested in 25–200 μ l of lysis buffer containing 1% Triton X-100 and 0.4 μ g/ μ l proteinase K in 1 \times Cetus PCR buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.001% gelatin, and 1.5 mM MgCl₂]. The mixtures were incubated at 56°C for 24 h and 95°C for 10 min to inactivate the proteinase K. The final solution was centrifuged, and the supernatant was stored at 4°C.

PCR Amplification. Tumors were screened for mutations in exons 4–9, which encompass the DNA-binding domain of the encoded protein. Individual exons or exonic fragments were amplified in separate reactions. Due to its size, exon 4 was amplified as two overlapping segments. Exon 5 was amplified as a single 294-bp fragment or as two separate fragments in samples with lower quality DNA. Primer sequences for exons 4–8 were reported elsewhere (20); primers for exon 9 were 5'-GCCTCAGATTCACCTTTTATCAC-3' and 5'-CATTTTCACTGTAGACTGGAAAC-3'. PCR was carried out in a 50- μ l reaction mixture containing 1–3 μ l of tumor lysate DNA, 300 nM of each primer, 1 \times Cetus buffer, 200 μ M of each deoxyribonucleotide, and 1.25–2.5 units of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT). The mixture was overlaid with mineral oil and amplified for 1 cycle at 94°C for 4 min, 55°C for 1 min, and 72°C for 1 min; followed by 33 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and a final extension cycle of 94°C for 1 min and 60°C for 10 min. PCR products were resolved by electrophoresis in nondenaturing 10% polyacrylamide mini-gels and visualized with ethidium bromide staining under UV light.

SSCP. SSCP-PCR was performed using internal nested primers and an aliquot of each PCR product as template. Internal primers for exon 9 were 5'-ATCACCTTTCTTGCTCT-3' and 5'-CATTTTGAGTGTAGACTGG-3'; internal primers for exons 4–8 were reported elsewhere (20). Reactions consisted of a total volume of 20 μ l containing 1 μ l of diluted PCR product; 300 nM of inner primers; 1 \times Cetus buffer; 100 μ M each of dGTP, dTTP and dATP; 15 μ M dCTP; 2.5 units of AmpliTaq polymerase; and 100 μ Ci/ml [α -³²P]dCTP. Cycle conditions were as described for the first PCR. To improve the resolution of exon 4-2 bands, the SSCP-PCR products were digested with 5 units of *AluI* (Boehringer Mannheim) in 25 ml of 1 \times *AluI* buffer. All products were diluted 30–50-fold with 0.1% SDS/10 mM EDTA, mixed with an equal volume (3 μ l) of stop buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF), and denatured at 94°C for 5 min. Electrophoresis in 6% polyacrylamide nondenaturing gels was carried out at room temperature and at 4°C. Each gel included a negative control (wild-type *p53*) and a positive control for the appropriate exon. Samples with abnormal band migration at either temperature were sequenced.

Sequencing. The first PCR product was used as template to generate single-stranded DNA for sequencing. Asymmetric PCR was carried out as described above, but with inner primer concentrations of either 500 nM forward and 10 nM reverse primer or 10 nM forward and 500 nM reverse primer. Forward and reverse PCR products were purified through Centricon-100 concentrators (Millipore Corp., Bedford, MA) and sequenced using a Sequenase version 2.0 sequencing kit (Amersham Biosciences, Piscataway, NJ). If identical mutations were detected in both strands, a second aliquot of the original DNA lysate was amplified and sequenced. Only samples showing the

same mutation on forward and reverse strands in two independent PCR reactions were considered true positives.

Data Analysis

Analyses were run using Stata, Release 6.0 (21). Ninety-nine cases were missing *p53* status because tumor tissue was not available ($n = 88$) or fewer than four *p53* exons were successfully amplified ($n = 11$). *p53*-negative cases had no mutations detected or silent mutations only. *p53*-positive cases had at least one protein altering mutation. For some analyses, *p53*-positive cases were subclassified by type of mutation, including deletions or insertions, G-T and other transversions, G:C to A:T transitions at CpG dinucleotides, and other transitions.

p53 mutations in study cases were compared with *p53* mutational spectra for bladder tumors and other cancers from the IARC *TP53* Mutation Database (Release 7, September 2002; Ref. 9). IARC mutation data were for exons 4–9 only. Splice mutations, intron mutations, and mutations in cell lines or xenographs were not counted (9).

Associations were estimated for potential risk factors when there were at least 15 exposed cases among those with known *p53* status. Unconditional logistic regression models were used to estimate ORs and 95% CIs comparing all bladder cancer cases with controls and *p53*-positive cases with *p53*-negative cases. Unconditional polytomous regression models were used to simultaneously estimate ORs for *p53*-positive cases compared with controls and ORs for *p53*-negative cases compared with controls (22). All models included sex, race (white or other), age at diagnosis or interview (categorized or using upper and lower tail restricted quadratic splines; Ref. 23), and years of smoking (continuous, with a 2-year lag), unless otherwise specified.

Complete-data-only models (of data from 215 controls and 146 cases with known *p53* status) were fit using standard maximum likelihood methods. A statistical missing-data technique known as the EM algorithm was used to derive maximum likelihood estimates based on all available data, including exposure data from 99 cases with unknown *p53* status (24). Complete-data-only estimates are unbiased but less precise than EM model estimates when case subtype data are missing completely at random. Complete-data-only estimates may be biased when missing case subtype data are associated with model covariates, but this bias will be reduced when models are fit using EM, as long as missing case subtype data are not also associated with the outcome. Details on the use of EM in case subgroup analyses and results of a simulation study comparing EM with complete-data-only methods are provided elsewhere (25). For this study, EM model ORs were more precise but similar in magnitude to corresponding estimates from models based only on observations with complete *p53* data. However, ORs comparing *p53*-positive and *p53*-negative cases with controls always fell on opposite sides of the corresponding OR for all study cases combined when the EM method was used, whereas ORs based on complete-data-only often did not. This suggests some bias in the complete data estimates because ORs for all study cases combined should correspond to a weighted average of subtype-specific associations (25); therefore, we reported EM model estimates unless otherwise noted.

Joint effects of smoking and gene polymorphisms were modeled using indicator variables for separate or joint exposure to pairs of dichotomous covariates [current smokers up to 2 years prior *versus* former smokers or never-smokers, *GSTM1* null *versus* *GSTM1* functional, any *NAT1*10* allele *versus* none, *NAT2* slow (no *NAT2*4* allele) *versus* other *NAT2* genotypes]. The common referent group for each set of comparisons consisted of former smokers or never-smokers with the referent genotype. Joint ORs were compared with those predicted for average additive effects (26).

Logistic regression models comparing controls and cases with specific types of G:C-A:T transitions were adjusted for age (<60 years, \geq 60 years) and fit using standard maximum likelihood methods. G:C-A:T transitions were subclassified by site (CpG or non-CpG) or by the specific base change (C-T or G-A) to determine whether associations were specific for the location *versus* the type of mutation. Other mutations (insertions/deletions, transversions, or other transitions) were too uncommon to evaluate separately.

RESULTS

p53 mutation status was determined for 146 cases. Of these, 115 (79%) were classified as *p53*-negative (112 cases without detected

mutations and 3 with silent mutations only). Thirty-one (21%) were classified as *p53*-positive, including one with two silent and three missense mutations, one with two missense mutations, and one with a missense mutation and a 17-bp deletion (Table 1). Four *p53*-negative cases had silent codon 36 polymorphisms (27), and two *p53*-positive cases had silent codon 213 polymorphisms (28).

Most mutations (63%) were G:C-A:T transitions, including 10 missense mutations at CpG dinucleotides, and 16 at other sites (9 missense, 2 nonsense, and 5 silent). There were five additional transitions (one T-C and four A-G), four G-T transversions (two missense and two silent), three other transversions, one 17-base deletion, and two single-base insertions. The spectrum of mutations was comparable to bladder tumors in the IARC *TP53* mutation database (Release 7; Ref. 9; Fig. 1).

Forty percent of the original 245 study cases were missing *p53* data, including 88 that were not assayed and 11 with fewer than four exons amplified. These cases were similar to successfully assayed cases with regard to tumor grade but were more likely to have been diagnosed before age 50 years (19% versus 9%) or more than 2 years before interview (71% versus 47%; Table 2).

Women were less likely to have *p53*-positive bladder cancer than

men (*p53*-positive versus *p53*-negative: OR, 0.5; CI, 0.1–1.4; Table 2). *p53*-positive cases were higher grade than *p53*-negative cases (grade IV versus grade I: OR, 6.5; CI, 2.0–22). *p53* mutation status was not clearly related to age at diagnosis or years since first diagnosis.

Only 4 of 36 occupations and 6 of 11 exposures of *a priori* interest met our minimum sample size criteria for evaluation (15 successfully assayed cases in each exposure category). Most estimates were close to the null, and CIs surrounding case:control ORs for *p53*-positive and *p53*-negative cases overlapped (Table 3). There was weak evidence of a subtype-specific association for work as a gasoline station attendant (*p53*-positive: OR, 2.1, CI, 0.8–5.4; *p53*-negative: OR, 0.9, CI, 0.5–1.9) and exposure to soot (*p53*-positive: OR, 1.8, CI, 0.6–5.3; *p53*-negative: OR, 1.1, CI, 0.5–2.4).

Only three *p53*-positive cases never smoked (Table 4). Case-control ORs for *p53*-positive cases were comparable with corresponding ORs for *p53*-negative cases, particularly when current smokers were compared with ex-smokers and never-smokers combined (*p53*-negative OR, 5.3; *p53*-positive OR, 5.6). Case:control ORs for years and pack-years of smoking were also comparable for *p53*-positive and *p53*-negative cases.

Table 1 Characteristics of cases with *p53* mutations detected in exons 4–9

ID	<i>p53</i> mutation				Case characteristics ^a				
	Exon	Codon	Mutation ^b	Result	Grade	Smoking	<i>NAT1</i>	<i>NAT2</i>	<i>GSTM1</i>
G:C to A:T at CpG dinucleotide									
PR363	4	110	CGT-CAT	Arg-His	1	Never	+	+	+
ON119	5	158	CGC-CAC	Arg-His	3	Quit	nd	nd	nd
PR124	5	158	CGC-CAC	Arg-His	3	Current	+	+	+
PR558	5	170	ACG-ATG	Thr-Met	3	Current	+	+	+
PR278	5	181	CGC-CAC	Arg-His	nd	Current	+	–	+
PR282	7	245	GCG-AGC	Gly-Ser	2	Current	–	+	–
PR148	7	248	CGG-CAG	Arg-Gln	3	Current	+	+	–
PR395	8	273	CGT-CAT	Arg-His	4	Current	–	+	+
PR109	8	282	CGG-TGG	Arg-Trp	3	Quit	+	+	–
PR283	8	290	CGC-CAC	Arg-His	3	Current	nd	nd	nd
Other transitions									
PR331	4	88	CCC-CCT	Silent	1	Never	+	+	–
PR171 ^c	5	137	CTG-CTA	Silent	4	Quit	–	+	–
PR171 ^c	5	141	TGC-TGT	Silent	^c	^c	^c	^c	^c
PR171 ^c	5	153	CCC-TCC	Pro-Ser	^c	^c	^c	^c	^c
PR171 ^c	5	161	GCC-GTC	Ala-Val	^c	^c	^c	^c	^c
PR553	5	168	CAC-TAC	His-Tyr	4	Quit	–	+	+
PR167	6	192	CAG-TAG	Gln-Stop	3	Current	–	–	+
PR603	6	192	CAG-TAG	Gln-Stop	3	Quit	+	–	+
PR302	6	205	TAT-TGT	Try-Cys	2	Quit	+	+	–
PR365	6	215	AGT-GGT	Ser-Gly	4	Current	–	+	+
PR267	6	220	TAT-TGT	Tyr-Cys	4	Quit	+	–	+
PR156	7	230	ACC-ATC	Thr-Met	4	Current	+	+	+
PR270	7	234	TAC-TGC	Tyr-Cys	4	Current	–	+	+
PR389	7	239	AAC-AAT	Silent	2	Quit	–	–	–
PR579	7	244	GGC-GAC	Gly-Asp	4	Never	+	–	+
PR122	7	250	CCC-CCT	Silent	4	Never	–	–	+
PR231	8	265	CTG-CCG	Leu-Pro	4	Current	+	+	+
PR228	8	266	GGA-AGA	Gly-Arg	3	Quit	nd	–	+
PR171 ^c	8	285	GAG-AAG	Glu-Lys	^c	^c	^c	^c	^c
PR362 ^c	8	285	GAG-AAG	Glu-Lys	3	Quit	–	–	+
PR362 ^c	8	287	GAG-AAG	Glu-Lys	^c	^c	^c	^c	^c
Transversions									
PR204	4	62	GAA-TAA	Glu-Lys	4	nd	–	–	–
PR227	6	215	AGT-AGA	Ser-Arg	4	Current	–	–	+
ON201	6	216	GTG-GTT	Silent	4	Current	nd	nd	nd
PR402	6	222	CCG-CCT	Silent	2	Current	–	+	–
PR212	7	248	CGG-CTG	Arg-Leu	4	Quit	–	+	–
PR284	8	280	AGA-ACA	Arg-Thr	4	Quit	–	+	+
PR294	8	284	ACA-CCA	Thr-Pro	2	Quit	–	–	+
PR565 ^c	8	286	GAA-GAT	Glu-Asp	2	Current	–	+	–
Insertions and deletions									
PR565 ^c	5	172	–17	Frameshift	^c	^c	^c	^c	^c
ON105	8	267	+G	Frameshift	3	Never	–	+	+
PR145	8	285	+G	Frameshift	3	Quit	+	–	–

^a Grade, clinical grade at initial diagnosis. Smoking, cigarette smoking status 2 years before initial diagnosis. *NAT1*: +, any *NAT1*10* allele; –, no *NAT1*10* allele. *NAT2*: +, slow (no *NAT2*4* allele); –, fast. *GSTM1*: +, homozygous null; –, any wild-type allele. nd, not determined or not available.

^b The specific base involved in each point mutation is underlined. The first 3 bases indicate the wild-type sequence, the second 3 bases are the mutated sequence.

^c Case with multiple mutations.

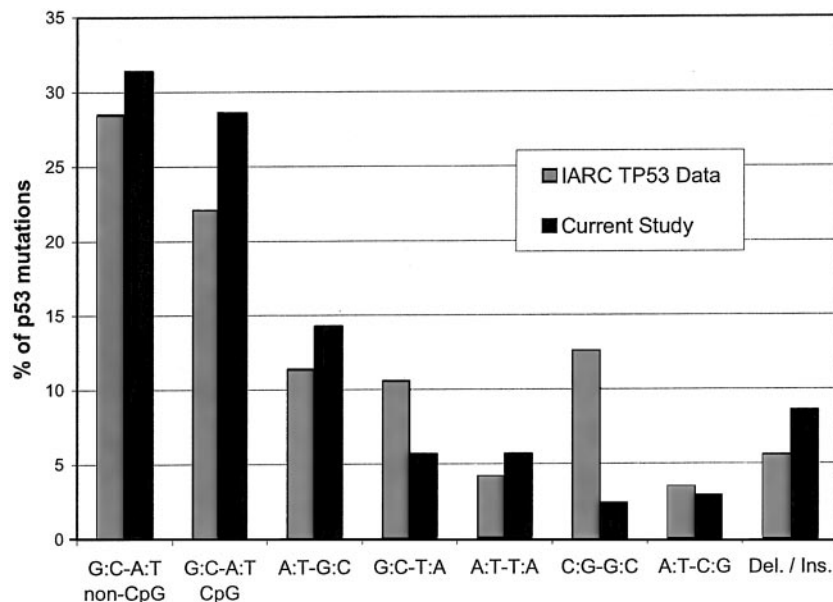


Fig. 1. Relative frequency of *p53* mutation subtypes among bladder tumors in the IARC *TP53* Data Base (Release 7; 9) and bladder cancer cases in the current study. IARC *TP53* data were from 813 bladder tumor mutations in *p53* exons 4–9 only. Splice mutations, intron mutations, and mutations identified from cell lines and xenographs were excluded from this analysis. Current study data included all detected *p53* mutations in exons 4–9 ($n = 32$). G:C-A:T CpG mutations involved CpG dinucleotides, whereas those designated as G:C-A:T non-CpG occurred at other sites. *Del.*, deletions, *Ins.*, insertions.

GSTM1-null genotype was more strongly associated with *p53*-positive cases (OR, 3.3; CI, 1.4–7.8) than *p53*-negative cases (OR, 1.5; CI, 0.9–2.3; Table 5). *NAT2*-slow genotype was associated with *p53*-positive cases only (*p53*-positive: OR, 1.8; CI, 0.8–4.0; *p53*-negative: OR, 0.9, CI, 0.6–1.4). Case:control ORs for no *NAT1*10* versus any *NAT1*10* allele were similar for *p53*-positive and *p53*-negative cases. Missing polymorphism data may have influenced results, but sensitivity analyses (29) showed that subtype-specific differences in associations with *GSTM1* and *NAT2* would persist under extreme missing data scenarios (for example, if all *p53*-positive

cases with missing genotype had been *NAT2*-fast, and all missing controls had been *NAT2*-slow).

Joint and separate effect estimates for polymorphisms and current smoking were imprecise, and CIs surrounding subtype-specific estimates overlapped. For *p53*-positive bladder cancer, the estimated joint effect of smoking and *GSTM1*-null genotype was more than additive [observed joint OR of 15.4 (CI, 4.7–48) versus predicted joint OR of 6.1, based on OR = 4.6 (CI, 1.0–20) for smokers with active *GSTM1* and OR = 2.5 (CI, 0.9–7.4) for nonsmokers who were *GSTM1* null]. For *p53*-negative cases, joint effect estimates were close to additive

Table 2 Distribution of frequency matching factors and clinical characteristics among study participants, and adjusted case:case ORs and 95% CIs comparing cases with and without protein-altering *p53* mutations

Characteristic	Controls <i>N</i> (%)	All cases <i>N</i> (%)	<i>p53</i> mutation status ^a			OR ^b	CI
			Missing <i>N</i> (%)	Negative <i>N</i> (%)	Positive <i>N</i> (%)		
Sex							
Male	174 (81)	189 (77)	76 (77)	86 (75)	27 (87)	1.0	
Female	41 (19)	56 (23)	23 (23)	29 (25)	4 (13)	0.5	0.1–1.4
Race							
White	200 (93)	223 (91)	95 (96)	100 (87)	28 (90)	1.0	
Black or other	15 (7)	22 (9)	4 (4)	15 (13)	3 (10)	0.8	0.2–2.9
Age (yrs)							
≤50	25 (12)	32 (13)	19 (19)	10 (9)	3 (10)	1.2	0.3–5.6
>50–60	43 (20)	67 (27)	27 (27)	34 (29)	6 (19)	0.8	0.2–2.7
>60–70	90 (42)	92 (38)	35 (35)	42 (36)	15 (48)	1.5	0.6–4.3
>70	57 (27)	55 (22)	18 (18)	29 (25)	7 (23)	1.0	
Years since diagnosis							
≤2		106 (43)	28 (28)	62 (54)	16 (51)	1.0	
>2–5		62 (25)	33 (33)	21 (18)	8 (26)	1.3	0.5–3.8
>5–10		48 (20)	22 (22)	22 (19)	4 (13)	0.7	0.2–2.5
>10		29 (12)	16 (16)	10 (9)	3 (10)	1.5	0.3–6.9
Highest grade							
I or II		93 (38)	39 (39)	49 (43)	5 (16)	1.0	
III		95 (39)	37 (37)	46 (40)	12 (39)	2.7	0.8–8.7
IV		47 (19)	16 (16)	18 (16)	13 (42)	6.5	2.0–22
Missing		10 (4)	7 (7)	2 (2)	1 (3)		

^a *p53*-negative, no mutations detected in exons 4–9 or silent mutations only. *p53*-positive, at least one protein-altering mutation in exons 4–9. Missing, data on *p53* mutation status missing or incomplete.

^b Case:case ORs and 95% CIs based on logistic regression models adjusted for age (categorical), sex, and race.

^c Age at first diagnosis (cases) or interview (controls).

Table 3 Adjusted case:control ORs^a for selected occupations and occupation- or hobby-related exposures^b for all bladder cancer cases, *p53*-mutation positive cases, and *p53*-mutation negative cases^c

		Control		All cases		<i>p53</i> -negative cases			<i>p53</i> -positive cases		
		N	N	OR	CI	N	OR	CI	N	OR	CI
Occupational exposure	No	126	156			79			13		
	Yes	89	84	0.8	0.5–1.2	35	0.7	0.4–1.1	15	1.5	0.6–3.5
Paint thinner or stripper	No	122	160			73			18		
	Yes	93	79	0.7	0.4–1.0	40	0.8	0.5–1.2	10	0.6	0.3–1.5
Organic solvents	No	180	204			97			24		
	Yes	35	36	0.9	0.5–1.5	16	0.9	0.7–1.7	5	1.0	0.3–2.7
Welding or soldering materials	No	162	192			95			19		
	Yes	53	49	0.8	0.5–1.3	19	0.7	0.4–1.2	10	1.5	0.6–3.6
Pesticides or insecticides	No	113	141			71			15		
	Yes	101	97	0.8	0.5–1.1	40	0.7	0.5–1.1	14	1.2	0.5–2.5
Soot	No	198	217			102			24		
	Yes	17	23	1.2	0.6–2.5	11	1.1	0.5–2.4	5	1.8	0.6–5.3
Occupation	No	193	219			103			24		
	Yes	22	22	0.8	0.4–1.6	11	0.7	0.3–1.5	5	1.1	0.4–3.4
Gasoline station attendant	No	190	203			102			22		
	Yes	25	38	1.2	0.6–2.1	12	0.9	0.5–1.9	7	2.1	0.8–5.4
Farming	No	154	174			80			24		
	Yes	60	66	1.1	0.7–1.8	34	1.3	0.8–2.2	5	0.6	0.2–1.7
Truck, bus, or taxi driver	No	188	206			97			22		
	Yes	27	35	1.0	0.6–1.9	17	0.9	0.5–1.8	7	1.5	0.6–3.9

^a ORs and 95% CIs comparing all cases with controls were derived from logistic regression models, whereas estimates comparing *p53*-positive and *p53*-negative cases with controls were derived from polytomous logistic regression models fit using the EM algorithm to account for missing *p53* mutation status data. All models were adjusted for sex, race (white or other), years of smoking (continuous, up to 2 years before diagnosis or interview), age (upper and lower tail-restricted quadratic splines).

^b Occupation- or hobby-related exposures that occurred on at least five occasions, and ever work in specific occupations beginning at least 2 years before diagnosis or interview. Exposures were evaluated only if there were at least 15 cases of known *p53* status in each exposure category.

^c *p53*-negative cases, no *p53* mutations detected in exons 4–9 or silent mutations only. *p53*-positive cases, at least one protein-altering mutation in exons 4–9.

[observed joint OR = 6.6 (CI, 3.3–13) versus predicted OR = 6.3, based on OR = 5.7 (CI, 2.8–12) for smokers with active *GSTM1* and OR = 1.6 (CI, 0.9–2.6 for nonsmokers who were *GSTM1* null)]. Other joint effect estimates (for *NAT1*10* with smoking, *NAT2*-slow genotypes with smoking, and *NAT1*10* with *NAT2*-slow genotypes) were comparable for *p53*-positive and *p53*-negative cases.

ORs for smoking and *GSTM1*, *NAT1*, and *NAT2* polymorphisms were comparable for cases with any G-A versus any C-T transition, but ORs differed between cases with CpG G:C-A:T transitions (10 cases: 8 G-A and 2 C-T) and cases with non-CpG G:C-A:T (8 cases: 4 G-A and 4 C-T; Table 6). Current smoking was strongly associated with CpG G:C-A:T (OR, 15.3; CI, 3.6–65), but not G:C-A:T transitions at other sites (OR, 1.8; CI, 0.3–9.8; Table 6). *NAT2*-slow genotype was positively associated with CpG G:C-A:T (OR, 6.2; CI, 0.7–52) and inversely associated with other G:C-A:T transitions (OR,

0.5; CI, 0.1–2.3). Missing *NAT2* data may have influenced estimates because ORs for *NAT2* and cases with CpG G:C-A:T could have ranged from 1.8 to 8.5, depending on the genotype of two CpG-positive cases and nine controls with unknown *NAT2*. *GSTM1*-null genotype was more strongly associated with non-CpG G:C-A:T transitions (OR, 7.8; CI, 0.9–65) than with CpG G:C-A:T (OR, 1.9; CI, 0.4–8.0).

DISCUSSION

The association between smoking and bladder cancer did not differ substantially between cases with and without *p53* mutations, consistent with the results of other studies (30–32). However, current smoking was more strongly associated with cases that had CpG G:C-A:T transitions than cases with other types of *p53* mutations. Associations

Table 4 Adjusted case:control ORs^a for cigarette smoking (up to 2 years before initial diagnosis or interview)^b for all bladder cancer cases, *p53*-mutation positive cases, and *p53*-mutation negative cases^c

Exposure	Control		All cases		<i>p53</i> -negative cases			<i>p53</i> -positive cases		
	N	N	OR	CI	N	OR	CI	N	OR	CI
Cigarette smoking	79	40	1.0		21	1.0		3	1.0	
	Ex-smoker	104	87	2.1	1.2–3.5	44	1.9	1.1–3.4	13	3.3
Current smoker	32	117	8.7	4.8–16	50	8.1	4.3–15	14	13.0	3.4–49
	Never or ex-smoker	183	127	1.0		65	1.0		16	1.0
Current smoker	32	117	5.3	3.4–8.5	50	5.3	3.2–8.7	14	5.6	2.5–13
	Years of smoking									
0–20	129	80	1.0		40	1.0		8	1.0	
>20–40	59	88	2.7	1.7–4.4	39	2.5	1.5–4.3	13	3.7	1.4–9.6
>40	27	75	6.8	3.9–12	35	6.8	3.7–13	9	7.2	2.4–22
Pack-years	125	86	1.0		41	1.0		9	1.0	
	>20–40	42	59	2.5	1.5–4.1	24	2.4	1.4–4.3	7	2.7
>40	46	97	4.2	2.6–6.8	48	4.1	2.4–7.1	14	4.3	1.7–11

^a ORs and 95% CIs comparing all cases with controls were derived from logistic regression models, whereas estimates comparing *p53*-positive and *p53*-negative cases with controls were derived from polytomous logistic regression models fit using the EM algorithm to account for missing *p53* mutation status data. All models were adjusted for sex, race (white or other), and age (upper and lower tail-restricted quadratic splines).

^b Years and pack-years of smoking were counted up to 2 years before initial diagnosis or interview (for controls). Ex-smokers quit more than 2 years before diagnosis or interview, current smokers smoked 2 years before diagnosis or interview.

^c *p53*-negative cases, no *p53* mutations detected in exons 4–9 or silent mutations only. *p53*-positive cases, at least one protein-altering mutation in exons 4–9.

Table 5 Adjusted case:control ORs^a for *NAT1*, *NAT2*, and *GSTM1* polymorphisms and all bladder cancer cases, *p53*-mutation positive cases, and *p53*-mutation negative cases^b

Genotype ^c	Control		All cases		<i>p53</i> -negative cases			<i>p53</i> -positive cases		
	N	N	OR	CI	N	OR	CI	N	OR	CI
<i>NAT1</i>										
Any *10 allele	134	132			56			16		
No *10 allele	73	102	1.5	1.0–2.3	54	1.5	1.0–2.4	12	1.4	0.6–3.1
Missing	8	11			5			3		
<i>NAT2</i>										
Fast	95	111			57			11		
Slow	111	123	1.0	0.7–1.5	53	0.9	0.6–1.4	18	1.8	0.8–4.0
Missing	9	11			5			2		
<i>GSTM1</i>										
Active	112	93			50			9		
Homozygous null	101	137	1.7	1.1–2.6	59	1.5	0.9–2.3	20	3.3	1.4–7.8
Missing	2	15			6			2		

^a ORs and 95% CIs comparing all cases with controls from logistic regression models. Estimates comparing *p53* mutation subtypes with controls from polytomous logistic regression models fit using the EM algorithm to account for missing *p53* mutation status data. All models adjusted for sex, race (white or other), years of smoking (continuous, up to 2 years before diagnosis or interview), age (upper and lower tail-restricted quadratic splines).

^b *p53*-negative cases, no *p53* mutations detected in exons 4–9 or silent mutations only. *p53*-positive cases, at least one protein-altering mutation in exons 4–9.

^c *NAT2*: fast, any *NAT2**4 allele; slow, no *NAT2**4 allele; *GSTM1*: active, any active allele; homozygous null, no active allele.

between smoking and bladder cancers with CpG G:C-A:T transitions have not been specifically evaluated by others, but smokers were overrepresented among cases with CpG G:C-A:T in three (15, 30, 31) of five (14, 33) published studies where smoking status was linked to specific mutations. In the largest of these, 7 of 8 CpG G:C-A:T cases were current smokers, while only 40 of 84 (48%) of all cases were current smokers (30). Similarly, 7 of 10 CpG G:C-A:T cases were current smokers in our study, compared with only 117/244 (48%) of all cases combined. These findings contradict expectations, if the assumption that CpG G:C-A:T mutations are simply a byproduct of endogenous processes is correct (10).

Several biological mechanisms could account for associations with bladder cancer subtypes defined by somatic *p53* mutations, with the most obvious involving an exposure that is a direct cause of *p53* DNA damage (34). Smoking is associated with increased mutagenic BPDE and aromatic amine 4-ABP DNA adducts in urothelial cells (32, 35–38). BPDE adducts are thought to be the primary cause of *p53* G-T transversions in lung tumors, which occur more often than expected at CpG sites (39). Cytosine residues in *p53* CpG dinucleotides appear to be consistently methylated (8), and guanines adjacent to 5-methylcytosines may be particularly susceptible to BPDE-mediated mutations because BPDE adducts formed at higher than expected rates at methylated CpG in experimental systems (40, 41). Repair of BPDE adducts

also may be inhibited at methylated CpG (39). BPDE guanine adducts have been strongly associated with G-T transversions *in vitro* (6, 7, 42), but mutations that occur *in vivo* may be influenced by the local environment (43–45), with G-A transitions occurring as the predominant mutation in some contexts (46). Like BPDE, 4-ABP forms bulky adducts at guanine bases that may cause G-A transitions as well as transversions (44). 4-ABP may also cause oxidative damage (47), and 5-methylcytosine residues may be more susceptible to oxidative damage than unmethylated cytosines (48). Taken as a whole, these findings suggest that smoking-associated BPDE, 4-ABP, or related carcinogens might cause or enhance the formation of G:C-A:T transitions at CpG sites (45).

Associations between *p53*-positive cases and gene polymorphisms that might influence the activation and detoxification of BPDE and 4-ABP may clarify the role of these compounds in bladder cancer etiology (31). Glutathione conjugation by *GSTM1* may detoxify BPDE, and the inactive *GSTM1*-null genotype has been associated with increased BPDE adducts in lung tissue (49) and increased urine mutagenicity (50). In our study, *p53*-positive cases were more likely to be *GSTM1* null than *p53*-negative cases, in agreement with two previous case:case analyses (31, 51). We also found evidence of a greater than additive effect of *GSTM1*-null genotype and current smoking among *p53*-positive cases, but not *p53*-negative cases. How-

Table 6 Case:control ORs^a for current smoking and *NAT1*, *NAT2*, and *GSTM1* polymorphisms among bladder cancer cases with *p53* G:C-A:T mutations, by type of mutation

Exposure ^c	Controls		<i>p53</i> G:C-A:T mutation subtype ^b										
	N	G:C-A:T (CpG)			G:C-A:T (non-CpG)			Any G-A			Any C-T		
		N	OR	CI	N	OR	CI	N	OR	CI	N	OR	CI
Smoker													
No	183	3			6			6			4		
Yes	32	7	15.3	3.6–65	2	1.8	0.3–9.8	6	5.6	1.9–19	4	6.7	1.5–29
Missing	0	0			0			0			0		
<i>NAT1</i>													
Any *10	134	3			4			4			4		
No *10	73	5	3.0	0.7–13	3	1.4	0.3–6.3	5	2.3	0.6–8.7	4	1.9	0.4–7.6
Missing	8	2			1			3			0		
<i>NAT2</i>													
Fast	95	1			5			4			2		
Slow	111	7	6.2	0.7–52	3	0.5	0.1–2.3	6	1.4	0.4–5.2	6	2.5	0.5–13
Missing	9	2			0			2			0		
<i>GSTM1</i>													
Active	112	3			1			3			2		
Null	101	5	1.9	0.4–8.0	7	7.8	0.9–65	7	2.6	0.7–11	6	3.3	0.7–17
Missing	2	2			0			2			0		

^a ORs and 95% CIs from logistic regression models adjusted for age (60 years or less, older than 60 at diagnosis or interview).

^b Nonsynonymous G:C-A:T mutations only. Categories are not mutually exclusive.

^c Smoker, current smoker 2 years before diagnosis or interview. *NAT2*: fast, any *NAT2**4 allele; slow, no *NAT2**4 allele; *GSTM1*: active, any active allele; null, homozygous null, no active allele.

ever, the association with *GSTM1* null was stronger for cases with G:C-A:T transitions at non-CpG versus CpG sites; therefore, the association between *GSTM1* null and *p53*-positive bladder cancers could not have been driven by an excess of CpG mutations. We had inadequate numbers of transversion-positive cases to evaluate them as a distinct outcome, but three of six were *GSTM1* null. In the study by Brockmoller *et al.* (31), bladder cancer cases with *p53* transversions were more likely to be *GSTM1* null (6 of 7) than cases with *p53* transitions (8 of 18).

4-ABP metabolism begins with *N*-hydroxylation by cytochrome P450 enzymes, which generates an active intermediate that may be *N*-acetylated in a detoxification reaction or *O*-acetylated to a more reactive product. *NAT1* and *NAT2* may catalyze both reactions, but hepatic *NAT2* is thought to be primarily responsible for detoxification, whereas *NAT1* activity in bladder cells may increase local *O*-acetylation. *NAT2*-slow (52) and *NAT1*10* (53) genotypes have been associated with increased 4-ABP DNA adducts in bladder cells, although not all studies have confirmed these results (32, 54). We did not find an association between *NAT1*10* genotype and *p53* mutations, but we did find a positive association between *NAT2*-slow genotypes and *p53*-positive bladder cancer that was stronger for cases with CpG G:C-A:T than other case subtypes. In contrast, previous case:case analyses reported no association between *NAT2* and *p53* (31) or a stronger association with *p53*-negative cases (51). Brockmoller *et al.* (31) reported that the *NAT2*-slow genotype was more strongly associated with cases that had *p53* transversion mutations than with other cases; in our study, three of six transversion-positive cases were *NAT2* slow.

Eight of ten CpG G:C-A:T transitions among our cases were G-A, which suggests the possibility of strand bias in CpG G:C-A:T mutations. Strand bias is associated with preferential repair of damage on the transcribed DNA strand during TCR, a process triggered by bulky DNA adducts, including BPDE and 4-ABP guanine adducts. Strand bias would not be expected as a consequence of spontaneous deamination of methylated cytosine residues to thymine because this would normally be corrected with equal efficiency on both DNA strands (10) via base excision repair (55). Therefore, evidence of strand bias supports the hypothesis that some CpG transitions might be caused by preferential adduction of guanine residues in CpG dinucleotides. Evidence of G-A strand bias in CpG G:C-A:T transitions has not been specifically noted by others (15, 30–33), but G-A account for 59% of CpG G:C-A:T among primary bladder cancers in the IARC *TP53* mutation database (Release 7; Ref. 9). Several other cancers in the database also appear to have more G-A than C-T transitions at CpG sites, including breast cancer (62% G-A), ovarian cancer (59% G-A), hematopoietic cancers (69% G-A), and colon and colorectal cancers (59% G-A). A notable exception is lung cancer, where only 47% of CpG G:C-A:T transitions are G-A. The extent of this apparent strand bias in CpG G:C-A:T is far less pronounced than strand bias seen in G:C-T:A transversions (about 89% G-T in lung cancer), and IARC *TP53* data must be interpreted with some caution because they are derived from peer-reviewed reports that cannot be independently verified (9). Nonetheless, these data suggest that at least some CpG G:C-A:T transitions may be a consequence of lesions that induce TCR, such as BPDE and 4-ABP adducts (56), and some forms of oxidative damage (57, 58).

NNK and other tobacco-associated nitrosamines are carcinogens that also might be preferentially associated with G:C-A:T at CpG sites. Nitrosamine-mediated guanine alkylation (for example, to *O*⁶-methylguanine) typically results in G:C-A:T mutations (59, 60), and there is experimental evidence that direct repair of alkylated guanines by *O*⁶-methylguanine DNA methyltransferase enzymes is inhibited at methylated CpG (61). We would not expect NNK-mediated *p53*

mutations to be associated with the *NAT1*, *NAT2*, and *GSTM1* polymorphisms we evaluated because nitrosamine activation occurs via CYP450-mediated α -hydroxylation, and detoxification is primarily via glucuronide conjugation (62). Strand bias also would not be expected because non-bulky adducts caused by NNK and other alkylating agents do not trigger TCR (56).

Our study included more *p53*-positive bladder cancer cases than previous studies of bladder cancer and environmental exposures (14, 15, 30–33, 51, 63, 64), but numbers of cases with specific types of *p53* mutations were small, and associations may have occurred by chance. For example, random error might explain discordant associations for *GSTM1*-null and *NAT2*-slow genotypes with G:C-A:T at non-CpG versus CpG sites. On the other hand, the spectrum of *p53* mutations among our cases was similar to bladder tumors in the IARC *TP53* database (Fig. 1; 9), and we confirmed previous findings that acquired *p53* mutations are associated with higher grade bladder cancers (13–15, 30–33, 51, 65–67). False positive results due to PCR contamination or Taq polymerase errors were unlikely, given the variability of the mutations that were detected and the stringency of our laboratory protocol, which required verification of all abnormal *p53* sequences in an independently amplified sample of DNA. Some mutations may not have been detected in exons that were not successfully amplified, but samples missing data for three or more exons were excluded from analyses ($n = 11$). False negative results might have occurred if mutant sequences were substantially diluted by wild-type DNA from normal cells, but nonneoplastic cells were removed from tissue sections before DNA extraction when possible, and SSCP electrophoresis was run at both room temperature and 4°C to increase sensitivity. Our use of clinic-based controls could have biased estimates if bladder cancer risk factors were associated with conditions leading to clinic participation (68), but most study controls were being treated for impotence and benign prostatic hyperplasia, conditions not known to have risk factors in common with bladder cancer. Our study included incident and prevalent bladder cancer cases, with about one-third having been diagnosed >5 years before interview. This may have distorted relations with factors associated with survival, but years since diagnosis was not related to *p53* status among assayed cases.

In conclusion, our results did not indicate a pronounced difference in risk factors for case subtypes defined by *p53* mutation status alone, although several occupations and occupational exposures, as well as *NAT2*-slow and *GSTM1*-null genotypes, were more common among *p53*-positive cases. However, we did find evidence that risk factors varied when cases were classified by type of *p53* mutation: smoking and *NAT2*-slow genotypes were associated with G:C-A:T transitions at CpG dinucleotides, whereas *GSTM1*-null genotype was associated with G:C-A:T transitions at other sites. Although common smoking-related adducts are associated with G-T transversions in experimental systems, they can also cause G-A transitions and other mutations (43, 44), and there is increasing evidence that CpG bases may be more susceptible than other sites to attack by environmental mutagens (8, 45, 48, 69, 70). In addition, DNA repair may be inhibited at CpG, including TCR of bulky adducts (39) and direct repair of *O*⁶-methylguanine adducts by *O*⁶-methylguanine DNA methyltransferase (61). Finally, tissue-specific selective pressures related to the specific functional effects of individual mutations might have a substantial influence on *p53* mutational spectra associated with environmental exposures among different cancers because the effects of specific *p53* mutations vary, with some causing only partial loss of function, whereas others may cause functional gains (71, 72). Our findings require confirmation in a larger study but are compatible with hypotheses suggesting that G:C-A:T transitions at CpG may be caused by smoking and other environmental exposures, as well as by endoge-

nous processes (41, 43, 45, 70). If this is true, a larger proportion of carcinogenic *p53* mutations in bladder cancers (and possibly other "endogenous" cancers) might be caused by modifiable environmental exposures than has previously been assumed (11).

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