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Bladder cancer risk and genetic variation in *AKR1C3* and other metabolizing genes

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Aromatic amines (AAs) and polycyclic aromatic hydrocarbons (PAHs) are carcinogens present in tobacco smoke and functional polymorphisms in *NAT2* and *GSTM1* metabolizing genes are associated with increased bladder cancer risk. We evaluated whether genetic variation in other candidate metabolizing genes are also associated with risk. Candidates included genes that control the transcription of metabolizing genes [aryl hydrocarbon receptor (*AHR*), *AHRR* and aryl hydrocarbon nuclear translocator (*ARNT*)] and genes that activate/detoxify AA or PAH (*AKR1C3*, *CYP1A1*, *CYP1A2*, *CYP1B1*, *CYP3A4*, *EPHX1*, *EPHX2*, *NQO1*, *MPO*, *UGT1A4*, *SULT1A1* and *SULT1A2*). Using genotype data from 1150 cases of urothelial carcinomas and 1149 controls from the Spanish Bladder Cancer Study, we estimated odds ratios (ORs) and 95% confidence intervals (CIs) adjusting for age, gender, region and smoking status. Based on a test for trend, we observed 10 non-redundant single-nucleotide polymorphisms (SNPs) in five genes (*AKR1C3*, *ARNT*, *CYP1A1*, *CYP1B1* and *SULT1A2*) significantly associated with bladder cancer risk. We observed an inverse association with risk for the *AKR1C3* promoter SNP rs1937845 [OR (95% CI) for heterozygote and homozygote variant compared with common homozygote genotype were 0.86 (0.70–1.06) and 0.74 (0.57–0.96), respectively; P for trend = 0.02]. Interestingly, genetic variation in this region has been associated with lung, non-Hodgkin lymphoma and prostate cancer risk. Analysis of additional SNPs to capture most (∼90%) of common genetic variation in *AKR1C3* and haplotype walking analyses based on all *AKR1C3* SNPs (n = 25) suggest two separate regions associated with bladder cancer risk. These results indicate that genetic variation in carcinogen-metabolizing genes, particularly *AKR1C3*, could be associated with bladder cancer risk.

Abbreviations: AA, aromatic amine; AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon nuclear translocator; AKR, aldo–keto reductase; CART, classification trees; CEU, Utah residents with ancestry from northern and western Europe; CI, confidence interval; CYP, cytochrome P450; FDR, false discovery rate; NQO1, NAD(P)H dehydrogenase, quinone 1; OR, odds ratio; PAH, polycyclic aromatic hydrocarbon; SNP, single-nucleotide polymorphism.

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Introduction

Bladder cancer risk is associated with tobacco and occupational exposure to aromatic amines (AAs), and the activation and detoxification of these carcinogens have been implicated in bladder cancer etiology (1–3). Interindividual variations in carcinogen metabolism genes, specifically *NAT2* and *GSTM1*, have been shown to be associated with bladder cancer risk (4–7), and it is thought that variation in other genes that metabolize AA and polycyclic aromatic hydrocarbons (PAHs) may also contribute to genetic susceptibility of bladder cancer (1,8,9).

In bladder carcinogenesis, reactive intermediates of tobacco smoke carcinogens, AA and PAHs can lead to DNA adduct formation and eventually mutation (10–12). PAHs and AA cannot cause mutation directly and must be activated (or detoxified) through a variety of metabolic pathways such as oxidation through cytochrome P450 (CYP)-related enzymes including CYP1A1, CYP1A2, CYP1B1 and CYP3A4 (13–16). In addition to oxidation from CYP enzymes, the two-electron reductase NAD(P)H dehydrogenase, quinone 1 (*NQO1*) can either bioactivate or detoxify quinones from AA or PAH intermediates, depending on the substrate (17). Of more recent interest, the aldo–keto reductase (AKR) gene family has been implicated in carcinogenesis because of their diverse roles in the metabolism of a variety of substrates including PAHs, androgens, estrogens and prostaglandins (18–20). In lung cancer cells, PAHs can be metabolized by members of the AKR family including the family member *AKR1C3*, which can produce PAH metabolites that form DNA adducts or reactive oxygen species leading to oxidative damage (18,19,21). Gene variants in AKR genes have been associated with lung cancer, non-Hodgkin's lymphoma and prostate cancer risk (22–24); however, variants in these genes have not been investigated with respect to bladder cancer risk.

We hypothesized that genetic variation in genes involved in AA and PAH metabolism may be related to bladder cancer risk. To test this hypothesis, we analyzed 65 single-nucleotide polymorphisms (SNPs) in 15 candidate genes that are activated by tobacco carcinogens and control the transcription of metabolizing genes [aryl hydrocarbon receptor (*AHR*), *AHRR* and aryl hydrocarbon nuclear translocator (*ARNT*)] or code for products that activate/detoxify AA or PAH (*AKR1C3*, *CYP1A1*, *CYP1A2*, *CYP1B1*, *CYP3A4*, *EPHX1*, *EPHX2*, *NQO1*, *MPO*, *UGT1A4*, *SULT1A1* and *SULT1A2*) in 1150 cases of urinary bladder transitional cell carcinomas and 1149 controls from the Spanish Bladder Cancer Study.

Materials and methods

Study population

The Spanish Bladder Cancer Study design has been described elsewhere (4,25,26). In brief, cases were patients with a new diagnosis of histologically confirmed urothelial carcinomas from 1998–2001, aged 21–80 years [mean (standard deviation) = 66 (10) years], of which 87% were males. Controls were selected from patients in the same hospital as the cases, and were admitted for common diseases/conditions that were not known or suspected to have exposures under study. Patients who had a previous diagnosis of cancer of the lower urinary tract (i.e. bladder, renal pelvis, ureters or urethra) were not eligible for the study, as were patients with bladder tumors that were secondary to other malignancies. The distribution of reasons for hospital admission was 37% hernias, 11% other abdominal surgery, 23% fractures, 7% other orthopedic conditions, 12% hydrocele, 4% circulatory conditions, 2% dermatological conditions, 1% ophthalmologic conditions and 3% other diseases. Controls were matched to the cases for age, sex, ethnicity and region. A total of 1219 cases (84% of eligible cases) and 1271 controls (88% of eligible controls) agreed to participate in the study and were interviewed. Of these, 1188 (97%) cases and 1173 (92%) controls provided blood or buccal cell samples for DNA extraction. After excluding individuals for low DNA extraction or quality issues, non-transitional cell histology, individuals not of Caucasian descent or missing information on smoking status, 1150 cases and 1149 controls were available

for analysis. Subjects were categorized as never smokers (29% of controls), if they smoked <100 cigarettes in their lifetime and ever smokers otherwise. Ever smokers were further classified as regular smokers (63% of controls), if they smoked one cigarette per day for 6 months or longer and occasional smokers (8% of controls) otherwise.

Genotyping

Genotyping was performed using three different methods:

(i) TaqMan (Applied Biosystems, Foster City, CA) assays were used to genotype genomic DNA for 16 SNPs (*AHR* R554K rs2066853; *CYP1A1* T461N rs1799814 and I462V rs1048943; *CYP1B1* R48G rs10012, V432L rs1056836 and N453S rs1800440; *EPHX1* Y113H rs1051740 and H139R rs2234922; *NQO1* R139W rs4986998 and P187S rs1800566; *MPO* rs2333227; *SULT1A1* E73Q rs1042011; *SULT1A2* Y62F rs4987024 and *AKR1C3* H5Q rs12529, rs12387 and rs2245191). SNP selection favored variants with expected minor allele frequency ≥0.05 in Caucasians, non-synonymous SNPs, those previously evaluated in relation to bladder cancer risk or those with evidence of functional significance. An additional two TaqMan SNPs were genotyped for *AKR1C3* (rs12242350 and rs4242785) to capture more common genetic variation in this gene according to HapMap (25).

(ii) GoldenGate (Illumina®, San Diego, CA) assay was used to genotype 49 other SNPs (26) (Table I). SNP selection criteria were similar to those used for TaqMan assays, with the exception of *AKR1C3* that covered 60% of the common variation according to HapMap for Utah residents with ancestry from northern and western Europe (CEU) (25). Further, *CYP1A1*, *CYP1B1* and *NQO1* SNPs chosen covered ≥70% of common variation according to HapMap for CEU (25). Sixty-four (5.6%) of the 1150 cases and 116 (10.1%) of the 1149 controls were excluded from the GoldenGate assay due to low DNA amounts available at the time of analysis.

(iii) A SNPlex™ (Applied Biosystems) assay was used to genotype seven SNPs chosen to cover most of the common variation in *AKR1C3*. SNP selection was based on the Carlson methods for capturing common genetic variation (27) ($r^2 \geq 0.8$, minor allele frequency ≥ 0.05) of 97 individuals of European descent from the SNP500Cancer reference population. Of the 1086 cases and 1033 controls used in the GoldenGate assays, we excluded 140 cases and 121 controls with no available DNA at the time of analyses or with poor performance on the iPLEX™ assay. In total, 946 cases (eight with buccal DNA samples) and 912 controls (32 with buccal DNA samples) were used for the SNPlex assays. Table I lists all SNPs determined using TaqMan, GoldenGate or iPLEX assays. TaqMan and iPLEX assays were performed at the Core Genotyping Facility of the Division of Cancer Epidemiology and Genetics, National Cancer Institute, and description and methods for assays can be found at http://snp500cancer.nci.nih.gov (28). All assays were performed using randomly sorted DNA samples from cases and controls, including blinded duplicate samples for quality control. All genotypes studied were in Hardy–Weinberg equilibrium in the control population, except for *CYP1A1* rs2606345 $P = 0.05$; *CYP1B1* rs162556 $P = 0.01$, rs10012 (R48G) $P = 0.009$ and rs1800440 (N453S) $P = 0.0003$; *NQO1* rs689453 $P = 0.04$ and *AKR1C3* rs1937843 and rs7921327, $P = 0.05$. Duplicate quality DNA samples ($n = 93$ pairs) displayed >98% concordance for all assays except for 96% concordance for *CYP1B1* rs10012 (R48G) genotypes and 95% concordance for *AKR1C3* rs1937843 and rs12775701.

Pairwise linkage disequilibrium between SNPs was estimated based on D' and r^2 values using Haploview (http://www.broad.mit.edu/mpg/haploview/index.php) (29).

Statistical analysis

For each individual SNP, we present estimated odds ratios (ORs) and 95% confidence intervals (CIs) using logistic regression models adjusting for gender, age at diagnosis in five categories, region and smoking status (never, occasional, former and current). We also estimated per allele ORs (95% CIs) and performed a test for trend in logistic regression models including each SNP as an ordered categorical variable (genotypes coded as 0 = homozygote common, 1 = heterozygote and 2 = homozygote variant). Exposure to high-risk occupations (including occupations in the textile, laundering, building and construction services) has also been associated with bladder cancer risk. Analyses controlling for occupational exposure did not change interpretation of our results. We evaluated the robustness of our results using the false discovery rate (FDR). FDR is the expected ratio of erroneous rejections of the null hypothesis to the total number of rejected hypothesis among all the SNPs analyzed (30). After excluding SNPs that were highly correlated at $r^2 \geq 0.90$, the FDR method was applied to the P value for trend to results from the remaining 59 SNPs.

Haplotype frequencies for genes with more than one SNP were estimated using HaploStats (version 1.2.1; http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm) using the program language R (http://www.r-project.org/). In order to prioritize regions of interest in the *AKR1C3* gene associated with risk, we used a sliding window method to construct successive haplotypes across *AKR1C3* in windows of two adjacent haplotypes based on all 25 SNPs evaluated. The haplotype sliding window method was performed using HaploStats specifically the haplo.score.slide command (31).

Gene–gene and gene–smoking interactions of SNPs with age, gender, *GSTM1*, *NAT2* and smoking habit were evaluated by introducing interaction terms in logistic regression models, as well as classification trees (CART) using 'rpart' in the program R. CART is an exploratory technique that uses splitting rules to stratify data into groups with homogenous risk (32). Its advantage over logistic regression is the ability to identify subgroups of individuals defined by environmental and/or genetic characteristics that are at high risk, suggesting the presence of gene–gene or gene–environment interactions. Indicator variables for smoking status (ever versus never) and genotypes (homozygous common, heterozygous or homozygous variants) were included in the CART models. A 10-fold cross-validation, two levels of interactions and groups limited to at least 50 individuals were used to reduce overfitted trees to their optimal size. Indicator variables for terminal nodes in the final tree were used in logistic regression models to estimate ORs and 95% CIs.

Unless otherwise specified, statistical analyses were performed with STATA Version 9.1, Special Edition (STATA Corporation, College Station, TX).

Results

The study population was of Caucasian descent, mostly males (87%) with a high proportion of cigarette smokers (63% of controls and 82% of cases were former or current smokers) (4). We evaluated 65 SNPs in 15 carcinogen metabolism genes and their association with bladder cancer risk (Table I and supplementary Table 1, available at *Carcinogenesis* Online).

We observed an inverse association with risk for two highly correlated SNPs *AKR1C3* rs1937845 in the promoter and rs12529 in exon 1 (H5Q) ($r^2 = 0.99$): OR and 95% CI for heterozygote and homozygote variant genotypes compared with the common homozygote was 0.86 (0.70–1.06) and 0.74 (0.57–0.96), respectively; P for trend = 0.02 (Table II). Another non-redundant promoter SNP in *AKR1C3* rs3763676 was associated with increased bladder cancer risk: OR and 95% CI for heterozygote and homozygote variant genotypes compared with the common homozygote was 1.28 (1.05–1.55) and 1.19 (0.89–1.58), respectively; P for trend = 0.05 (Table II). Genotyping of nine additional SNPs (for a total of 25 SNPs) to capture ∼90% of *AKR1C3* common genetic variation according to HapMap (25) identified three additional non-redundant intronic SNP associations (Table II): rs4881400 and rs4641368 were inversely associated with risk (P for trend = 0.03 and 0.01, respectively) and rs12775701 was associated with increased risk (P for trend = 0.04).

AKR1C3 haplotype analysis

We used the haplotype sliding window method to prioritize regions of interest in the *AKR1C3* gene and to identify regions of this gene that might have an association with bladder cancer risk above the effects seen in individual SNP analysis. A graph of transformed P values for successive haplotypes across *AKR1C3* in windows of two and three adjacent haplotypes based on all 25 SNPs evaluated is shown in Figure 1. From the sliding window haplotype analysis based jointly on the P value from all of the tests from these windows and the building of haplotypes of significance in the region, we observe two regions with evidence of an association with bladder cancer risk. The first region is marked by four SNPs we found individually associated with risk between the promoter and intron 1 of the gene (rs1937845, rs3763676, rs12529 and rs1937843) and a second area that was significantly associated with risk between introns 5 and 8 of the gene (rs4881400, rs12775701 and rs4641368). This idea is supported by HapMap data among CEU individuals where a region between intron 4 and intron 5 may represent a break in the gene marked by low linkage disequilibrium (supplementary Figure 1, available at *Carcinogenesis* Online).

Interaction with AKR1C3 SNPs and bladder cancer risk factors

We evaluated interactions between the *AKR1C3* SNPs significantly associated with risk and other risk factors (gender, age, smoking

Table I. Sixty-five SNPs in carcinogen-metabolizing genes and nine additional *AKR1C3* SNPs evaluated in the Spanish Bladder Cancer Study

Gene	Gene name	Chromosomal location	Nucleotide change	Amino acid change	dbSNP ID	Minor allele frequency in control population	Assay (Oligopool, GoldenGate, Taqman or iPLEX)
AHR	Aryl hydrocarbon receptor	7p15	Ex1+185G>A		rs7796976	0.25	GoldenGate
			IVS7+33C>A		rs2074113	0.11	GoldenGate
			Ex10+501G>A	R554K	rs2066853	0.13	Taqman
ARNT	Aryl hydrocarbon receptor nuclear translocating protein	1q21	−991G>A		rs7517566	0.10	GoldenGate
			IVS5+726T>C		rs2864873	0.40	GoldenGate
			IVS6+123A>G		rs2256355	0.39	GoldenGate
			IVS6+205A>G		rs1027699	0.39	GoldenGate
			Ex7+81G>C		rs2228099	0.40	GoldenGate
			IVS12−662G>A		rs1889740	0.40	GoldenGate
AHRR	Aryl hydrocarbon receptor repressor (competes with ARNT for AHR binding)	5p15.3	2912 bp 3′ of STP G>C		rs34847072	0.34	GoldenGate
			3152 bp 3′ of STP T>G		rs10078	0.27	GoldenGate
CYP1A1	Cytochrome p450, family 1, subfamily A, polypeptide 1	15q22–q24	−17961C>T		rs2472299	0.35	GoldenGate
			−9893G>A		rs17861115	0.04	GoldenGate
			IVS1+606T>G		rs2606345	0.36	GoldenGate
			IVS1−728C>T		rs4646421	0.11	GoldenGate
			Ex7+129C>A	T461N	rs1799814	0.05	Taqman
			Ex7+131A>G	I462V	rs1048943	0.15	Taqman
			11599 bp 3′ of STP G>C		rs2198843	0.16	GoldenGate
CYP1A2	Cytochrome p450, family 1, subfamily A, polypeptide 2	15q22-qter	IVS1−154A>C		rs762551	0.36	GoldenGate
CYP1B1	Cytochrome p450, family 1, subfamily B, polypeptide 1	2p21	−5329G>A		rs10175368	0.28	Ilumina
			−4977A>G		rs162555	0.23	Ilumina
			−3922T>C		rs162556	0.45	Ilumina
			Ex2+143C>G	R48G	rs10012	0.25	Taqman
			Ex3+251C>G	V432L	rs1056836	0.44	Taqman
			Ex3+315A>G	N453S	rs1800440	0.20	Taqman
			Ex3+939A>C		rs162562	0.18	Ilumina
			Ex3+1284T>G		rs10916	0.17	Ilumina
EPHX1	Epoxide hydrolase 1, microsomal	1q42.1	−4786C>A		rs2854461	0.31	GoldenGate
			IVS1−1310G>A		rs2671272	0.24	GoldenGate
			IVS1−1127A>G		rs3738043	0.10	GoldenGate
			IVS1−1067C>T		rs2854456	0.22	GoldenGate
			IVS3+114C>G		rs2260863	0.31	GoldenGate
			Ex3−28T>C	Y113H	rs1051740	0.26	Taqman
			Ex4+52A>G	H139R	rs2234922	0.19	Taqman
			Ex8+31C>T	N357N	rs1051741	0.10	GoldenGate
EPHX2	Epoxide hydrolase 2, microsomal	8p21–p12	Ex19+4A>C	P531P	rs1126452	0.22	GoldenGate
NQO1	NAD(P)H dehydrogenase, quinone 1	16q22.1	IVS1−27C>G		rs689452	0.12	GoldenGate
			Ex2+65G>A	E24E	rs689453	0.08	GoldenGate
			Ex4−3C>T	R139W	rs4986998	0.03	Taqman
			Ex6+40C>T	P187S	rs1800566	0.23	Taqman
			Ex6−455C>T		rs10517	0.13	GoldenGate
MPO	Myeloperoxidase	17q23.1	−642G>A		rs2333227	0.26	Taqman
			IVS11−6A>C		rs2071409	0.16	GoldenGate
UGT1A4	UDP glucuronosyltransferase 1, family, polypeptide A4	q37	Ex5−402C>G		rs1042640	0.22	GoldenGate
SULT1A1	Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	16p12.1	Ex7−49G>C	E73Q	rs1042011	No variation	Taqman
SULT1A2	Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 2	16p12.1	Ex4+37A>T	Y62F	rs4987024	0.01	Taqman
			336 bp 3′ of STP T>C		rs3194168	0.16	GoldenGate
CYP3A4	Cytochrome p450, family 3, subfamily A, polypeptide 4	7q21.1	−8890G>A		rs??	0.05	GoldenGate
			−391A>G		rs2740574	0.04	Taqman
AKR1C3	Aldo–keto reductase, family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)	10p15–p14	−32346T>C		rs10795241	0.28	GoldenGate
			−23066G>C		rs28943575	0.10	GoldenGate
			−18314C>A		rs6601899	0.14	GoldenGate
			−4048C>T		rs17134288	0.33	GoldenGate
			−1632C>T		rs28942669	0.07	GoldenGate
			−1423T>C		rs11252937	0.33	GoldenGate
			−488A>G		rs1937845	0.46	GoldenGate
			−137A>G		rs3763676	0.33	GoldenGate
			Ex1−70C>G	H5Q	rs12529	0.46	Taqman
			IVS1+195A>G		rs1937843	0.29	iPLEX[a]
			IVS1−756A>G		rs17396032	0.04	iPLEX[a]

Table I. *Continued*

Gene	Gene name	Chromosomal location	Nucleotide change	Amino acid change	dbSNP ID	Minor allele frequency in control population	Assay (Oligopool, GoldenGate, Taqman or iPLEX)
			Ex3−58A>G	K104K	rs12387	0.15	Taqman
			IVS3+73C>A		rs2245191	0.29	Taqman
			IVS4+218G>A		rs1937841	0.06	iPLEX[a]
			IVS5+1214A>C		rs4559587	0.07	iPLEX[a]
			IVS5−256T>G		rs4881400	0.23	iPLEX[a]
			IVS5−230C>G		rs12242350	0.30	Taqman[a]
			IVS7+336G>A		rs12775701	0.37	iPLEX[a]
			IVS8+40A>G		rs2275928	0.37	GoldenGate
			IVS8+584C>T		rs4641368	0.14	iPLEX[a]
			IVS8-509G>A		rs4242785	0.21	Taqman[a]
			1532 bp 3′ of STP G>C		rs10904422	0.16	GoldenGate
			1875 bp 3′ of STP A>G		rs7070041	0.29	GoldenGate
			9757 bp 3′ of STP A>G		rs7921327	0.30	GoldenGate
			12259 bp 3′ of STP A>G		rs1937920	0.29	GoldenGate

TaqMan genotyping assays were performed on 1150 cases and 1149 controls. GoldenGate genotyping assays were performed among 1086 cases and 1033 controls. iPLEX genotyping assays were performed on 946 cases and 912 controls. See Materials and Methods for details.
[a]The nine additional SNPs genotyped to capture ∼90% of common genetic variation in *AKR1C3*.

Table II. SNPs in the *AKR1C3* gene associated with bladder cancer risk, adjusted for gender, age, region and smoking status (1150 cases and 1149 controls)

AKR1C3 SNP	Genotype	Cases		Controls		OR	95% CI	*P* value
		n	%	*n*	%			
rs1937845 (GoldenGate assay)	AA	355	33	300	29	1.00	Reference	
−488A>G	AG	541	50	518	50	0.86	0.70–1.06	0.15
Highly correlated with rs12529	GG	189	17	215	21	0.74	0.57–0.96	0.02
Per allele risk						0.86	0.75–0.98	0.02
rs3763676 (GoldenGate assay)	AA	443	41	471	46	1.00	Reference	
−137A>G	AG	498	46	433	42	1.28	1.05–1.55	0.01
Highly correlated with rs1937843	GG	145	13	128	12	1.19	0.89–1.58	0.23
Per allele risk						1.14	1.00–1.30	0.05
rs4881400 (iPLEX assay)	TT	558	63	523	60	1.00	Reference	
IVS5-256T>G	GT	294	33	294	34	0.92	0.75–1.14	0.45
	GG	32	4	56	6	0.53	0.33–0.85	0.01
Per allele risk						0.83	0.70–0.98	0.03
rs12775701 (iPLEX assay)	GG	230	26	263	30	1.00	Reference	
IVS7+336A>G	GA	437	49	413	47	1.22	0.96–1.54	0.10
	AA	221	25	198	23	1.34	1.02–1.76	0.04
Per allele risk						1.16	1.01–1.33	0.04
rs4641368 (iPLEX assay)	CC	663	75	610	70	1.00	Reference	
IVS8+584C>T	CT	212	24	239	27	0.82	0.66–1.03	0.09
	TT	11	1	24	3	0.42	0.20–0.91	0.03
Per allele risk						0.78	0.64–0.95	0.01

This table shows findings for a subset of the 24 SNPs evaluated in the *AKR1C3* gene significantly associated with bladder cancer risk. See supplementary Table 1 (available at *Carcinogenesis* Online) for findings on all SNPs evaluated; GoldenGate genotyping assays were performed among 1086 cases and 1033 controls. iPLEX genotyping assays were performed on 946 cases and 912 controls. See Materials and Methods and Table I for details.
Note: Differences between cell counts in table and total number of cases and controls are due to missing genotype data.

status, *NAT2* and *GSTM1* genotypes). Analyses among ever smokers suggested that the increased risk associated with rs3763676 was limited to ever smokers: OR (95% CI) for heterozygote and homozygotes compared with common homozygotes, respectively; for never smokers 0.92 (0.60–1.42) and 0.68 (0.32–1.43) and for ever smokers 1.37 (1.11–1.69) and 1.33 (0.98–1.80), *P* for interaction = 0.06 (Table III). The increased risk associated with rs3763676 and smoking was not substantially different for former or current smokers (supplementary Table 2, available at *Carcinogenesis* Online). The four other non-redundant SNPs associated with risk did not show significant effect modification by smoking status (Table III). Analyses stratified by *GSTM1* present and null genotypes suggested stronger associations among subjects with *GSTM1* present for the *AKR1C3* rs4881400 SNP: per allele OR (95% CI) = 0.65 (0.49–0.85) for *GSTM1* present and 0.95 (0.76–1.18) for *GSTM1* null, *P* for interaction = 0.03 (Table IV).

Other SNP associations with bladder cancer risk

Five SNPs in four other genes were also found to be significantly associated with bladder cancer risk (supplementary Table 1, available at *Carcinogenesis* Online)—rs7517566 in the *ARNT* promoter was associated with increased risk: per allele OR (95% CI) = 1.24 (1.01–1.52), *P* for trend = 0.04. Two non-redundant SNPs in the *CYP1A1* gene in the promoter and 3′ of the STP codon were associated with bladder cancer risk: *CYP1A1* rs2472299 per allele OR (95% CI) = 0.87 (0.76–1.00), *P*-trend = 0.04 and *CYP1A1* rs2198843 per

allele OR (95% CI) = 1.20 (1.02–1.41), *P*-trend = 0.03. One SNP in the promoter of the *CYP1B1* gene rs162555 had an inverse association with risk: per allele OR (95% CI) = 0.86 (0.73–1.00), *P*-trend = 0.05. Lastly, a rare *SULT1A2* SNP rs4987024 (Y62F) was inversely associated with risk, OR and 95% CI for heterozygote variant compared with common homozygote genotype was 0.43 (0.20–0.95), *P* = 0.04. Analysis for potential interactions between these SNPs significantly associated with risk and other risk factors (gender, age, smoking status, *NAT2* and *GSTM1* genotypes) did not reveal any significant interactions (data not shown). Haplotype analysis in the rest of the genes did not reveal any common haplotype significantly associated with risk (data not shown).

Classification tree analysis

To further explore potential gene–gene and gene–smoking interactions, we performed classification tree (CART) analysis of all SNPs evaluated in this report as well as *NAT2* and *GSTM1* present/null genotypes. Smoking status was the most important predictor of case–control status in this analysis, followed by *GSTM1* genotype that was important among both ever and never smokers (Figure 2). Consistent with previous reports (4), analyses suggested that the *NAT2* genotype is most important in determining risk among smokers with the *GSTM1*-null genotype. Analyses also suggested that associations for SNPs in *AKR1C3* and *ARNT* might be modified by combinations of smoking status and *GSTM1* genotype.

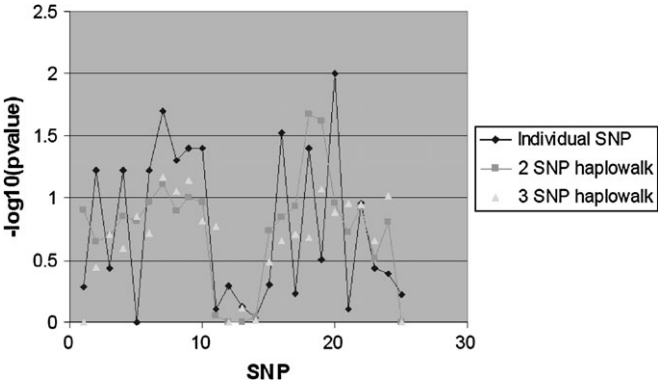

Fig. 1. *AKR1C3* graph of *P* values and sliding haplotypes and bladder cancer risk in 870 cases and 853 controls. Graph of *P* values from individual SNP and haplotype walking analyses. Haplotype walking analysis was done using two and three adjacent SNPs from the 25 SNPs analyzed. SNPs 1–25 are in 5′–3′order and SNPs in bold were significantly associated with risk: rs10795241, rs28943575, rs6601899, rs17134288, rs28942669, rs11252937, **rs1937845**, **rs3763676**, **rs12529**, **rs1937843**, rs17396032, rs12387, rs2245191, rs1937841, rs4559587, **rs4881400**, rs12242350, **rs12775701**, rs2275928, **rs4641368**, rs4242785, rs10904422, rs7070041, rs7921327 and rs1937920.

Discussion

Our evaluation of 65 SNPs in 15 metabolizing candidate genes revealed 10 non-redundant associations in five genes *AKR1C3*, *ARNT*, *CYP1A1*, *CYP1B1* and *SULT1A2*. Additional follow-up analysis of *AKR1C3* using nine additional SNPs that captured ∼90% of common genetic variation identified three additional non-redundant associations, making a total of five non-redundant associations with the *AKR1C3* gene.

AKR1C3 genetic variants associated with bladder cancer risk

AKR1C3 is a member of the AKR superfamily that can act on a variety of substrates including hormones, prostaglandins and PAHs (18). AKR family members could be important for smoking-related cancers as AKR1C1–AKR1C4 have been implicated in PAH activation (33). Furthermore, *AKR1C1*, *AKR1C3* and *AKR1B10* have been found to be induced by cigarette smoke condensate, diesel exhaust and PAH exposure *in vitro* (21,34) and it is thought that their induced expression is a function of their involvement in the metabolism of PAH exposures. To our knowledge, this is the first report to extensively evaluate genetic variation in *AKR1C3* with bladder cancer risk. We observed two regions of the *AKR1C3* gene associated with risk: one area was marked by the promoter and intron 1 of the gene (rs1937845, rs3763676, rs12529 and rs1937843) and a second area that was significantly associated with risk was marked between introns 5 and 8 of the gene (rs4881400, rs12775701 and rs4641368). Recently, another *AKR1C3* SNP in the 5′ region of rs7741 (P30P) has been associated with increased familial prostate cancer risk (24) and according to HapMap (25), this SNP is highly correlated (r^2 = 1.0) with the promoter SNP rs3763676 associated with increased bladder cancer risk in our study. Furthermore, rs3763676 has been shown to have altered transcriptional activity in reporter assays using human cells *in vitro* (35), suggesting that lower expression of *AKR1C3* may increase risk for bladder cancer. The non-synonymous SNP rs12529 (H5Q) has been associated with increased lung cancer risk in a Chinese population, with the strongest association observed among individuals exposed to smoky coals (which have high levels of PAHs) (22).

Table III. Stratified results by smoking status for seven SNPs in the *AKR1C3* gene associated with bladder cancer risk in the Spanish Bladder Cancer Study, controlling for region, age and gender

SNP	Smoking status	Homozygous common		Heterozygous		Homozygous variant		Heterozygous			Homozygous variant			*P* interaction
		Cases	Controls	Cases	Controls	Cases	Controls	OR	95% CI	*P* value	OR	95% CI	*P* value	
rs1937845[a]	Never	45	87	79	152	24	58	0.96	0.60–1.52	0.85	0.86	0.46–1.58	0.62	
−488A>G	Ever	310	213	462	366	165	157	0.86	0.69–1.07	0.18	0.71	0.54–0.95	0.02	0.58
rs3763676[b]	Never	63	119	73	143	12	34	0.92	0.60–1.42	0.72	0.68	0.32–1.43	0.31	
−137A>G	Ever	380	352	425	290	133	94	1.37	1.11–1.69	0.003	1.33	0.98–1.80	0.07	0.06
rs4881400	Never	75	154	45	83	5	14	1.10	0.68–1.77	0.69	0.94	0.31–2.81	0.91	
IVS5−256T>G	Ever	483	369	249	211	27	42	0.88	0.70–1.11	0.29	0.48	0.29–0.80	0.005	0.21
rs12775701	Never	26	70	70	121	27	65	1.37	0.78–2.40	0.27	1.05	0.54–2.03	0.89	
IVS7+336A>G	Ever	204	193	367	292	194	133	1.18	0.92–1.52	0.19	1.41	1.05–1.90	0.02	0.40
rs4641368	Never	90	172	34	72	2	7	0.92	0.55–1.51	0.73	0.70	0.14–3.61	0.67	
IVS8+584C>T	Ever	573	438	178	167	9	17	0.81	0.63–1.03	0.09	0.40	0.17–0.91	0.03	0.50

This table shows findings for a subset of the 24 SNPs evaluated in the *AKR1C3* gene significantly associated with bladder cancer risk. See supplementary Table 2 (available at *Carcinogenesis* Online) for stratified analysis on all SNPs evaluated.
[a]*AKR1C3* SNPs rs1937845 and rs12529 are highly correlated (r^2 = 0.99).
[b]*AKR1C3* SNPs rs3763676 and rs1937843 are highly correlated (r^2 = 0.93).

Table IV. Stratified results by GSTM1 present/null status for seven SNPs in the *AKR1C3* gene associated with bladder cancer risk in the Spanish Bladder Cancer Study, controlling for smoking status, region, age and gender

SNP	GSTM1 null/present	Homozygous common		Heterozygous		Homozygous variant		Heterozygous			Homozygous variant			*P* interaction
		Cases	Controls	Cases	Controls	Cases	Controls	OR	95% CI	*P* value	OR	95% CI	*P* value	
rs1937845[a]	Present	135	137	186	267	72	94	0.67	0.48–0.92	0.01	0.72	0.48–1.09	0.12	
−488A>G	Null	216	161	352	245	115	118	1.07	0.81–1.40	0.64	0.75	0.53–1.06	0.10	0.58
rs3763676[b]	Present	157	228	177	209	60	60	1.32	0.98–1.78	0.07	1.55	1.00–2.39	0.05	
−137A>G	Null	283	238	317	220	83	66	1.24	0.96–1.60	0.10	0.98	0.67–1.43	0.90	0.19
rs12529[a]	Present	136	135	185	254	73	88	0.71	0.52–0.98	0.04	0.78	0.52–1.18	0.24	
Ex1−70C>G (H5Q)	Null	215	155	352	241	115	117	1.05	0.79–1.38	0.75	0.73	0.52–1.03	0.07	0.91
rs1937843[b]	Present	126	190	146	172	47	52	1.38	0.99–1.92	0.06	1.53	0.95–2.47	0.08	
IVS1+195A>G	Null	235	209	247	179	70	54	1.23	0.93–1.63	0.14	1.08	0.71–1.64	0.72	0.30
rs4881400	Present	470	386	80	58	2	2	0.67	0.48–0.93	0.02	0.38	0.17–0.86	0.02	
IVS5−256T>G	Null	224	243	89	147	9	25	1.13	0.85–1.49	0.41	0.60	0.33–1.10	0.10	0.03
rs12775701	Present	82	121	153	213	87	83	1.10	0.77–1.59	0.60	1.76	1.14–2.71	0.01	
IVS7+336A>G	Null	145	140	281	196	131	112	1.35	0.99–1.85	0.06	1.12	0.78–1.60	0.54	0.15
rs4641368	Present	264	301	60	104	1	12	0.68	0.47–0.99	0.04	0.08	0.01–0.71	0.02	
IVS8+584C>T	Null	392	302	150	133	10	12	0.87	0.65–1.17	0.35	0.65	0.26–1.59	0.34	0.10

This table shows findings for a subset of the 24 SNPs evaluated in the *AKR1C3* gene significantly associated with bladder cancer risk.
[a]*AKR1C3* SNPs rs1937845 and rs12529 are highly correlated ($r^2 = 0.99$).
[b]*AKR1C3* SNPs rs3763676 and rs1937843 are highly correlated ($r^2 \geq 0.93$).
Note: Differences between cell counts in table and total number of cases and controls are due to missing genotype data.

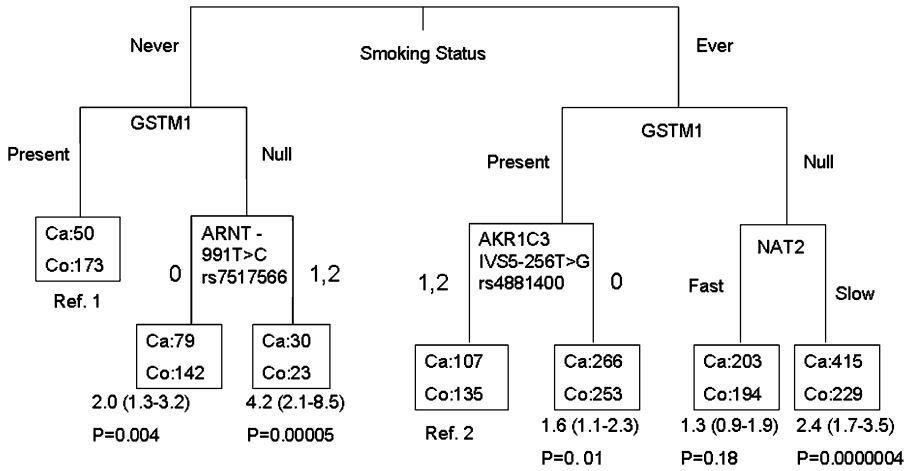

Fig. 2. Classification tree model for cigarette smoking and carcinogen metabolism polymorphisms and bladder cancer risk. Ca = cases and Co = controls. ORs and *P*s under the terminal nodes are for genotype–bladder cancer associations within the smoking categories estimated from a logistic regression model. Codes for genotypes: 0 = homozygote common variant and 1,2 = heterozygote and homozygote variant, respectively.

In contrast, this SNP was inversely associated with bladder cancer risk in our study population of Caucasian origin, with no evidence for modification by smoking. These differences could reflect environmental, metabolic or allelic frequency differences among ethnic groups. It is interesting to note that the allele frequency data from the dbSNP database for *AKR1C3* rs12529 and rs4881400 from HapMap show that the most common genotype in Caucasians is the minor allele in Chinese Han, Japanese from Tokyo (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=rs12529 and http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=4881400). However, replication of these findings is needed before the differences in risk estimates between different cancers in various populations can be explained.

NQO1 genetic variants

NQO1's activity has been found to have both detoxifying properties by reducing the presence of hydroquinones that can be excreted as well as activating nitroaromatic amines present in tobacco smoke (17,36,37,38). The variant allele of *NQO1* rs1800566 (P187S) has been shown to have reduced quinone reductase activity from *in vitro* studies (39–41). A recent meta-analysis of six bladder cancer studies of 1410 cases and 1485 controls in Caucasian populations suggest an increased risk for the rs1800566 (P187S) CT/TT genotype of 1.20 (1.00–1.43) (36). We did not confirm this association and found estimates to be inversely related to risk.

There have been limited studies of other polymorphisms in other metabolizing genes and bladder cancer risk. In this report, we found that in addition to *AKR1C3*, we observe five other SNP associations in four other genes namely: the *CYP1A1* and *CYP1B1* genes that are mostly extrahepatic microsomal enzymes, which can activate PAHs and AAs (13), the *ARNT* gene that interacts with the *AHR* to activate transcription in target promoters of metabolizing genes (42) and lastly, the sulfotransferase *SULT1A2*, which can activate or detoxify PAHs and AAs through conjugation of sulfonation (43).

CART analysis suggested the presence of gene–gene and gene–smoking interactions. As expected, smoking was the main predictor of risk followed by GSTM1, which influenced risk for both smokers and non-smokers. Analyses suggested an interaction between smoking, GSTM1 and NAT2 with the highest risk among individuals that were *NAT2* slow acetylators and *GSTM1* null. Finally, SNPs in the *AKR1C3* and *ARNT* genes further contributed to risk stratification. CART is an exploratory technique that may overfit data; therefore, OR estimates for specific genotype combinations in these models need to be interpreted with caution and confirmed in other studies.

Analyzed SNPs in *CYP1A1*, *CYP1B1*, *NQO1* and *AKR1C3* captured >80% of common genetic variation [according to HapMap (25)]; however, coverage for other genes was limited. The most highly significant SNPs had FDR values of 0.27, indicating that our findings need to be interpreted with caution until replicated and evaluated in meta- and pooled analyses. Strengths of our study include a large sample size with high participation rates and a extensive analysis of genetic variation in four carcinogen metabolism genes. Our paper provides additional evidence that the *AKR1C3* locus may be important in the pathogenesis of multiple cancer sites. If findings are confirmed, they could lead to further understanding of bladder carcinogenesis as well as to the identification of subsets of individuals susceptible to carcinogens. However, the public health implications of such findings are unclear.

Supplementary material

Supplementary Figure 1 and Tables 1–3 can be found at http://carcin.oxfordjournals.org/

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