Carcinogenesis vol.29 no.7 pp.1386–1393, 2008
doi:10.1093/carcin/bgn136
Advance Access publication June 9, 2008

Genetic determinants in the metabolism of bladder carcinogens in relation to risk of bladder cancer

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Genetically determined factors that alter the metabolism of tobacco carcinogens can influence an individual's susceptibility to bladder cancer. The associations between the genotypes of glutathione *S*-transferase (*GST*) *M1*, *GSTP1*, *GSTT1* and *N*-acetyltransferase (*NAT*) *1* and the phenotypes of NAT2 and cytochrome P450 (CYP) 1A2 and bladder cancer risk were examined in a case–control study involving 731 bladder cancer patients and 740 control subjects in Los Angeles County, California. Individual null/low-activity genotypes of *GSTM1*, *GSTT1* and *GSTP1* were associated with a 19–48% increase in odds ratio (OR) of bladder cancer. The strongest association was noted for *GSTM1* [OR for the null genotype = 1.48, 95% confidence interval (CI) = 1.19–1.83]. When the three *GST* genes were examined together, there was a monotonic, statistically significant association between increasing number of null/low-activity genotypes and risk (*P* for trend = 0.002). OR (95% CI) for one and two or more null/low-activity *GST* genotypes was 1.42 (1.12–1.81) and 1.71 (1.25–2.34), respectively, relative to the absence of null/low-activity *GST* genotype. NAT2 slow acetylation was associated with doubled risk of bladder cancer among individuals with known high exposures to carcinogenic arylamines (OR = 2.03, 95% CI = 1.12–3.69, *P* = 0.02). The effect of NAT2 slow acetylation was even stronger in the presence of two or more null/low-activity *GST* genotypes. There were no associations between bladder cancer risk and *NAT1* genotype or CYP1A2 phenotype.

Introduction

Cigarette smoking is the most important known risk factor for urinary bladder cancer, accounting for ~50% of all incident cases in the USA (1,2). Selected arylamines [including 2-naphthylamine, benzidine and 4-aminobiphenyl (4-ABP)] present in cigarette smoke and other environmental sources have been identified as human bladder carcinogens (3,4). Metabolic activation is required to biotransform these arylamines into their carcinogenic forms. The first step in this activation pathway is N-oxidation, which is catalyzed by the hepatic cytochrome P450 (CYP) 1A2 isoenzyme (5). Large interindividual variation in CYP1A2 activity is seen in humans and thought to be the basis for genetically determined differences in susceptibility to urinary bladder cancer arising from exposures to arylamines. Phenotypic studies have suggested that CYP1A2 is regulated by at least two mechanisms: one that controls constitutive levels of expression and the other that regulates inducibility (6,7). No polymorphic sequences in the structural *CYP1A2* gene that reflect interindividual differences in enzyme activity have been found. At present, CYP1A2-related activity can be phenotypically measured by assessing caffeine metabolites in urine after ingestion of caffeine (6,8).

Arylamines can be detoxified via N-acetylation catalyzed by *N*-acetyltransferases (NATs) in the liver. Humans' NATs are coded by two distinct genes, *NAT1* and *NAT2* (9). NAT2 is primarily expressed in human liver, whereas NAT1 is detectable in both hepatic and extrahepatic tissues including bladder and colon (9,10). N-acetylation of arylamines, a competing detoxification pathway, is primarily controlled by hepatic NAT2. NAT2, a non-inducible enzyme, has long been known to exhibit polymorphism. In early phenotype-based studies, the slow acetylator phenotype was associated with increased risk of urinary tract cancer (11,12). Genotyping studies demonstrated that slow acetylators, particularly those who were exposed to cigarette smoke or arylamines under occupational settings, were at an increased risk for bladder cancer (13,14). However, studies conducted in USA populations have produced equivocal results (15,16).

Appreciable NAT1 but low NAT2 activities are seen in bladder epithelium (17). O-acetylation of N-hydroxylamines catalyzed by NAT1 in the bladder leads to highly electrophilic N-acetoxy derivatives that can covalently bind to urothelial DNA, a critical step toward malignant cell transformation. Therefore, polymorphic *NAT1* genotypes are thought to modulate an individual's susceptibility to bladder cancer (18). Numerous polymorphisms in the *NAT1* genes in human populations have been reported (19,20). Of particular interest is the *NAT1*10* genotype, which has been associated with higher levels of NAT1 activity and DNA adducts in human bladder tissue (17). Some studies found that *NAT1*10* was overrepresented among bladder cancer patients (10,15). However, epidemiologic results are conflicting (21).

The glutathione *S*-transferases (GSTs) comprise a supergene family of phase 2 detoxifying enzymes that catalyze the conjugation of xenobiotics, including aromatic hydrocarbons, chlorinated compounds and some heterocyclic amines, with glutathione, resulting in enhanced elimination of potential carcinogens (22). Each class consists of one or more isoenzymes with different, but sometimes overlapping, substrate specificity. Genetic polymorphisms have been demonstrated for *GSTM1*, *GSTT1* and *GSTP1* genes. The homozygous deletion polymorphisms (i.e. null genotype) in the *GSTM1* and *GSTT1* genes, respectively, result in total abolishment of their respective enzyme activities in an individual (23). A transition of adenine (A) to guanine (G) at nucleotide 313 in exon 5 of the *GSTP1* gene results in substitution of isoleucine by valine at position 104 in the amino acid sequence (24). The homozygous valine variant (i.e. *GSTP1* BB genotype) exhibits lower specific activity and affinity for the electrophilic substrates of the enzyme (25). Most population-based studies have found that the *GSTM1* null genotype is associated with an increased risk of bladder cancer. Several epidemiologic studies have examined the associations between *GSTT1* or *GSTP1* genotypes and bladder cancer risk. Findings have been equivocal, with the null/low-activity genotypes showing increase, no association or decrease in bladder cancer risk (14,26–30).

The Los Angeles Bladder Cancer Study was a large-scale, population-based case–control study involving mainly non-Hispanic white subjects (2,31,32). The present study describes our findings on phenotypes/genotypes of CYP1A2, NAT2, *GSTM1*, *GSTP1*, *GSTT1* and *NAT1* in relation to risk of bladder cancer among non-Hispanic white subjects.

Abbreviations: AAMU, 5-acetylamino-6-amino-3-methyluracil; 4-ABP, 4-aminobiphenyl; CI, confidence interval; CYP, cytochrome P450; GST, glutathione *S*-transferase; Hb, hemoglobin; MU, methyluracil; MX, methylxanthine; NAT, *N*-acetyltransferase; OR, odds ratio.

Materials and methods

Study population

The design of the Los Angeles Bladder Cancer Study was described previously (2). Case patients were non-Asians aged 25–64 years, diagnosed with

histologically confirmed bladder cancer between January 1987 and April 1996 and identified through the Los Angeles County Cancer Surveillance Program, one of the Surveillance, Epidemiology and End Results cancer registries in the USA (33). Among 2384 eligible case patients, 210 (9%) died before we could contact them or were too ill to be interviewed; permissions to contact 99 (4%) patients were refused by their physicians and 404 (17%) patients refused to participate in the study. We interviewed the remaining 1671 (70%) bladder cancer patients.

For each case patient, we chose one control subject who was individually matched to the index case patient by gender, date of birth (±5 years), race (non-Hispanic white, Hispanic white or African-American) and neighborhood of residence at the time of cancer diagnosis. The control subjects were identified by a standard procedure defining a sequence of houses on specified neighborhood blocks (2). For the 1671 interviewed case patients, 1586 eligible control subjects were interviewed. Among them, 1090 (69%) were first eligible control subjects, 325 (20%) were second eligible control subjects, 111 (7%) were third eligible control subjects and the remaining 60 (4%) were fourth or higher order eligible control subjects. Permission to conduct the study has been obtained from the Institutional Review Boards at the University of Southern California and the University of Minnesota. Separate informed consent forms for interview and biospecimen collection were obtained from each study participant.

Data collection

A structured questionnaire was used during the in-person interview to request general and exposure information up to 2 years prior to cancer diagnosis for the case patients and 2 years prior to cancer diagnosis of the index case patients for the matched controls. Each subject was asked to report information on demographic characteristics, lifetime use of tobacco products and alcohol, usual adult dietary habits, lifetime occupational history, prior medical conditions and prior use of medications.

Starting from January 1992, all cases and their matched controls were asked additional questions regarding personal use of hair dyes and family history of cancer (34). A supplemental questionnaire soliciting lifetime history of environmental tobacco smoke exposure also was administered to all case patients and control subjects who were non-current users of tobacco (35).

Beginning in January 1992, all case patients ($n = 905$) and control subjects ($n = 867$) of non-Hispanic whites were asked to donate blood and an overnight urine sample. Seventy-three percent (662/905) case patients and 80% (690/867) control subjects consented to blood donation. All blood components (plasma, serum, buffy coat and red cells) were continuously stored at −80°C until analysis. The method for collection of urine samples has been described previously (36). Briefly, all consenting subjects were given two packets of instant coffee (∼70 mg of caffeine per packet) to be drunk between 3 and 6 p.m. The subject collected an overnight urine sample (ending with the first morning void) into a 1 l plastic bottle that was picked up and processed the same day. On the day of collection, the subject was briefly interviewed about caffeine intake (in addition to the prescribed packets of instant coffee) and use of acetaminophen on the previous day because excessive consumption of caffeine (>300 mg or more than four cups of coffee) or use of acetaminophen [an internal standard for the methylxanthin (MX) and urate assays] might affect the validity of the phenotyping assay (37). We also asked subjects whether they used any tobacco products during the past 60 days. The urine specimens were acidified (400 mg of ascorbic acid per 20 mg of urine) prior to storage at −20°C (36).

Laboratory tests

Genotyping. Genomic DNA was isolated from blood lymphocytes. As described in detail previously (38), a standard, multiplex polymerase chain reaction protocol was used to analyze for the presence or absence of the *GSTM1* and *GSTT1* genes. *GSTP1* genotyping was performed according to the method described in Harries *et al.* (26). Among subjects who donated blood samples (662 case patients and 690 control subjects), informative *GSTM1*, *GSTT1* and *GSTP1* genotypes were obtained in 662 (100%), 658 (99.4%) and 657 (99.2%) case patients and 686 (99.4%), 680 (98.6%) and 684 (99.1%) control subjects, respectively.

NAT1 genotyping was conducted at the National Institute of Environmental Health Science using a mutant primer polymerase chain reaction–restriction fragment length polymorphism method combined with an allele-specific assay as described previously for several published sequence variations in the 3′ region of *NAT1* near the putative polyadenylation signal. Details of this genotyping method have been provided in Bell *et al.* (39). Specifically, we examined *NAT1*4* (presumably the 'wild-type' allele that contains a 'T' at nucleotide 1088 and a 'C' at nucleotide 1095), *NAT1*10* (T1088A, C1095A), *NAT1*3* (C1095A) and *NAT1*11* (a variant that contains a 9 bp deletion in the nucleotides 1065–1090). The rare non-functional *NAT1* alleles (20) and other single-nucleotide polymorphisms in *NAT1* were not analyzed in this study. Positive and negative control samples for each allele were run in each analysis batch. Samples with ambiguous restriction fragment length polymorphism patterns were repeated until resolved. Informative *NAT1* genotypes were obtained in 649 (98.0%) case patients and 677 (98.1%) control subjects. Because the *NAT1*10* allele contains an altered polyadenylation signal and has been associated with elevated levels of DNA adducts (17), genotypes containing at least one *NAT1*10* allele were defined as putative high-risk genotypes and were compared with other non-*NAT1*10* genotypes.

Phenotyping. Urinary caffeine metabolites, namely 5-acetylamino-6-amino-3-methyluracil (AAMU), 1-MX, 1-methyluracil (MU) and 1,7-methylxanthin, were measured by the following methods. Levels of AAMU in urine were determined by a modified procedure of Tang *et al.* (40), using high-performance exclusion chromatography. Quantification of MX, MU and 1,7-methylxanthin in urine was performed according to a modified procedure of Grant *et al.* (41). These analyses were performed with appropriate internal standards. Calibration curves were created during the analysis and used for calculation of concentrations of all analytes. Quality control urine samples spiked with a low, intermediate and high range of the calibration concentrations were analyzed intermittently during the sample runs. Based on the value of a ratio of following urinary caffeine metabolites—AAMU:(AAMU + MX + MU), subjects were classified as either slow (ratio <0.34) or rapid (ratio >0.34) acetylators (42). Similarly, the CYP1A2 index is based on a ratio of urinary metabolites of caffeine—(AAMU + MX + MU):1,7-methylxanthin (37). Higher values of the CYP1A2 index reflect higher CYP1A2 activities. Tobacco is a known inducer of CYP1A2 (43). Thus, as expected, subjects reporting cigarette use during the 60 days prior to urine collection showed significantly higher CYP1A2 values. We used the separate median values of the CYP1A2 index in smokers and non-smokers (4.53 for non-smokers and 8.51 for smokers) to classify subjects into the high (above median) or the low (below median) CYP1A2 activity group. Among subjects who collected overnight urine samples (642 case patients and 609 control subjects), measurements of caffeine metabolites were obtained on 635 (98.9%) case patients and 606 (99.5%) control subjects.

Statistical analysis

To maximize the sample size, we included all subjects (730 cases and 740 controls) with available data on any genotypes or phenotypes of interest for the present analysis. Among 730 cases, 567 (78%) had both genotypes (i.e. any of *GSTM1*, *GSTP1*, *GSTT1* and *NAT1*) and phenotypes (NAT2 and CYP1A2) determined (i.e. provided both blood and urine samples), 95 (13%) had genotypes only and 68 (9%) had phenotypes only. The corresponding figures among controls were 554 (75%), 134 (18%) and 52 (7%). The chi-square test was used to examine differences in the distributions of categorical variables, and the *t*-test was used on continuous variables for comparisons between case patients and control subjects. Unconditional logistic regression models (44) were used to examine the associations between genotypes/phenotypes and risk of bladder cancer. The strength of an exposure–disease association was measured by the odds ratio (OR) and its corresponding 95% confidence interval (CI) and two-sided *P* value. We broke the original matched-pair design in order to maximize the numbers of cases and controls with known genotype values. In all logistic regression models, the matching factors (age, gender) and previously identified risk factors for bladder cancer in this database were included as covariates (2,31,34,45): level of education (high school or below, 1–4 years of college or graduate school or above), currently smoking (no, yes), number of cigarettes smoked per day, number of years of smoking, level of 4-ABP–hemoglobin (Hb) adducts in tertile, use of non-steroidal anti-inflammatory drugs (non-users, <1441 pills or ≥1441 pills used over lifetime), ever use of permanent hair dyes (no, yes) and ever held a high-risk job (truck, bus or taxi driver; hair dresser or barber and aluminum product worker) (no, yes). Data were analyzed for men and women separately and for both sexes combined. The associations between genetically determined factors and bladder cancer risk for women were comparable with those for men. Hence, all results presented were for both sexes combined.

Statistical analyses were performed using the SAS version 9.1 (SAS Institute, Cary, NC) statistical software package. All *P* values are two sided. $P < 0.05$ was considered statistically significant.

Results

The present study included 731 bladder cancer cases with available genotypes and/or phenotypes of interest. Transitional cell carcinoma accounted for 94% ($n = 687$) of total cancer cases. The remaining 6% cases consisted of 12 adenocarcinoma, 6 squamous cell cancer, 15 non-specified papilomas and 11 other or unknown histological type. The distribution of selected characteristics between case patients and control subjects is summarized in Table I. The mean age (standard deviation) of case patients at cancer diagnosis was 55.8 (7.2) years, and the corresponding figure for control subjects at the time of cancer

Table I. Distributions of selected characteristics among non-Hispanic white cases and controls, The Los Angeles Bladder Cancer Study

Characteristics	Bladder cancer patients (n = 730)[a]	Control subjects (n = 740)[a]	Two-sided P[b]
Age (years)[c]	55.8 (7.2)	55.7 (8.2)	0.81
Gender			
Males[d]	578 (79.2)	582 (78.7)	0.79
Females[d]	152 (20.8)	158 (21.4)	
Level of education			
High school graduates or below[d]	256 (35.0)	184 (24.9)	<0.0001
College (1–4 years)[d]	337 (46.2)	368 (49.7)	
Graduate school[d]	137 (18.7)	188 (25.4)	
Cigarette smoking			
Never smokers[d]	134 (18.4)	296 (40.0)	<0.0001
Former smokers[d]	279 (38.2)	308 (41.6)	
Current smokers[d]	317 (43.4)	136 (18.4)	
Among all smokers			
Age starting to smoke[c]	17.4 (4.4)	17.9 (4.2)	0.08
Number of cigarettes per day[c]	27.3 (14.0)	22.6 (12.7)	<0.0001
Number of years of smoking[c]	29.6 (12.0)	23.8 (12.9)	<0.0001
Number of pack-years of smoking[c]	42.1 (28.2)	28.8 (23.9)	<0.0001
Alcohol drinking			
Non-drinkers[d]	191 (26.2)	211 (28.5)	0.31
Regular drinkers[d]	539 (73.8)	529 (71.5)	
Among drinkers			
Number of drinks per day[c]	23.7 (33.5)	21.4 (31.2)	0.24
Number of years of drinking[c]	28.2 (13.4)	28.5 (11.7)	0.75
Ever held a high-risk job[e]			
No[d]	620 (84.9)	665 (89.9)	0.004
Yes[d]	110 (15.1)	75 (10.1)	
Ever used permanent hair dyes[f]			
Non-users[d]	81 (53.3)	104 (65.8)	0.02
Regular users[d]	71 (46.7)	54 (34.2)	
4-ABP–Hb adducts (pg/g) in tertile[g]			
Low (<17.9)[e]	137 (20.6)	235 (34.2)	<0.0001
Intermediate (17.9–30.0)[e]	164 (24.6)	230 (33.5)	
High (>30)[e]	365 (54.8)	222 (32.3)	

[a]Including 730 cases and 740 control subjects in all analysis except for those indicated below (see details in the Statistical Analysis section).
[b]*t*-test for continuous variables and chi-square test for categorical variables.
[c]Mean (standard deviation).
[d]Number of subjects (column percentage).
[e]High-risk job includes hair dresser; barber; truck, bus or taxi driver and aluminum product worker.
[f]Among women only (152 cases and 158 controls); regular users of permanent hair dye for 1 year or longer; among regular users, the mean (standard deviation) years of hair dye use for bladder cancer patients and control subjects were 22.6 (9.8) and 19.5 (11.9), respectively.
[g]Including 666 cases and 687 control subjects with available measurements of 4-ABP–Hb adducts.

diagnosis of the index case patient was 55.7 (8.2) years. Approximately 21% of case patients and similar percentage of control subjects were women. Case patients had on average a lower level of education than control subjects but comparable levels of alcohol intake. Cigarette smoking, level of 4-ABP–Hb adducts, occupational history and regular use of permanent hair dyes in women were previously identified risk factors for bladder cancer in this study population (2,34,45).

The main effects of *GST*/NAT/CYP genotypes/phenotypes in relation to risk of bladder cancer in total subjects are summarized in Table II. The strongest association, which was statistically significant, was noted for *GSTM1* (multivariable-adjusted OR = 1.58, 95% CI = 1.25–1.99). Individual null/low-activity genotypes of *GSTM1*, *GSTT1* and *GSTP1* were associated with 58, 24 and 12% increased risk of bladder cancer, respectively, following multivariable adjustment. When the three GST genes were examined together, there was a monotonic, statistically significant association between increasing number of null/low-activity genotypes (range, 0–3) and risk (P for trend = 0.002) (Table II).

Table II. *GSTM1*, *GSTP1*, *GSTT1* and *NAT1* genotypes and NAT2 and CYP1A2 phenotypes in relation to risk of bladder cancer among non-Hispanic whites, The Los Angeles Bladder Cancer Study

Genotypes/phenotypes	Bladder cancer patients[a]	Control subjects[a]	Multivariate-adjusted OR (95% CI)[b]
GSTM1			
Non-null (0)	275	351	1.00
Null (1)	387	335	1.58 (1.25–1.99)
GSTT1			
Non-null (0)	518	556	1.00
Null (1)	140	124	1.24 (0.93–1.66)
GSTP1			
AA	301	284	1.00
AB	274	327	0.83 (0.64–1.06)
BB	82	73	1.02 (0.70–1.49)
AA or *AB* (0)	575	611	1.00
BB (1)	82	73	1.12 (0.78–1.61)
Summed *GST* index			
0	195	262	1.00
1	324	306	1.52 (1.16–1.97)
2–3	136	107	1.80 (1.28–2.53)
P trend			0.002
NAT1			
Non-*NAT1*10*	434	434	1.00
*NAT1*10*	215	243	0.96 (0.75–1.23)
NAT2 phenotype[c]			
Rapid acetylators	271	291	1.00
Slow acetylators	364	315	1.11 (0.87–1.41)
CYP1A2 index[c]			
Low	314	301	1.00
High	321	305	1.00 (0.78–1.27)

[a]The following subjects with missing information on genotypes or phenotypes were excluded from the relevant analysis: *GSTM1* on 4 control subjects, *GSTT1* on 4 case patients and 10 control subjects, *GSTP1* on 5 case patients and 6 control subjects, summed *GST* index on 7 case patients and 15 controls, *NAT1* on 18 case patients and 22 controls and NAT2 and CYP1A2 on 8 case patients and 3 controls.
[b]ORs were derived from unconditional logistic regression models that included the following covariates: age (years), gender, level of education (high school or below, 1–4 years of college, graduate school or above), currently smoking (no, yes), number of cigarettes smoked per day, number of years of smoking, total number of non-steroidal anti-inflammatory drug pills used over lifetime (non-user, <1441 and 1441+), ever use of permanent hair dye (no, yes), ever held a high-risk job (no, yes) and 4-ABP–Hb adducts in tertile (a separate category was created for 65 cases and 53 controls with missing values who were included the multivariate analysis).
[c]See the Materials and Methods section for the definitions for the rapid/slow acetylators as well as for the low/high CYP1A2 index.

The NAT2 slow acetylators were associated with a statistically significant 26% increase in risk of bladder cancer (OR = 1.26, 95% CI = 1.00–1.57, P = 0.046) after adjustment for age and sex. The multivariate-adjusted OR became statistically non-significant (OR = 1.11, 95% CI = 0.87–1.41). Neither *NAT1*10* genotype nor CYP1A2 phenotype was associated with increased risk of bladder cancer in the present study (Table II).

We examined the *GST*/NAT/CYP genotypes/phenotypes and risk associations according to subjects' smoking history and his/her levels of 4-ABP–Hb adducts (Table III). The associations between individual and the composite *GST* genotypes and the NAT2 phenotype and risk of bladder cancer were uniformly stronger among individuals with known high exposure to carcinogenic arylamines, namely heavy smokers (≥30 pack-years) and/or those possessing high levels of 4-ABP–Hb adducts (>30 pg/g Hb). In this subgroup of subjects, the OR (95% CI) for one and two to three versus zero null/low-activity GST genotypes was 2.33 (1.29–4.22) and 3.35 (1.49–7.56), respectively

(P for trend = 0.002). The comparable figure for NAT2 slow versus rapid acetylation was 2.06 (1.21–3.49).

The joint effect of NAT2 phenotype and *GST* genotypes in relation to bladder cancer risk is presented in Table IV. Among subjects with known high exposure to carcinogenic arylamines, NAT2 slow acetylation was associated with an increase in bladder cancer risk regardless of *GST* genotype profile. In contrast, among subjects with lower levels of exposure to carcinogenic arylamines, the association between NAT2 slow acetylation and increased bladder cancer risk was apparent only among those possessing the high-risk *GST* genotype profile (two or more null/low-activity genotypes of *GSTM1*, *GSTT1* and *GSTP1*). This possible interaction effect was statistically borderline significant (P for interaction = 0.09). The overall joint effect of NAT2 phenotype and *GST* genotypes on bladder cancer risk was most pronounced among subjects with known high exposure to carcinogenic arylamines. Compared with NAT2 rapid acetylators without null/low-activity *GST* genotype, individuals possessing the NAT2 slow acetylation phenotype and two or more null/low-activity *GST* genotype had an OR of 9.37 (95% CI = 2.15–40.86) for bladder cancer based on a relatively small sample size (22 cases and three controls) (Table IV).

We also examined the pair-wise effects of *NAT1* × *GST*, CYP1A2 × *GST*, NAT2 × *NAT1*, *NAT1* × CYP1A2 and NAT2 × CYP1A2 genotypes/phenotypes on bladder cancer risk. There was no obvious synergism between these gene pairs and risk.

Discussion

Our study demonstrates that null/low-activity genotypes of the *GSTM1*, *GSTT1* and *GSTP1* loci independently contributed toward risk of bladder cancer in the host. The strongest association was noted for *GSTM1*. The magnitude of the *GSTM1*–bladder cancer association in the present study (OR = 1.53) was comparable with earlier reports (14,46,47). The *GST* genotypes–bladder cancer association was strongest among subjects with known high exposure to carcinogenic arylamines (namely heavy smoking and/or high level of 4-ABP–Hb adducts). These findings are biologically plausible. Cigarette smoking is recognized to be the most important risk factor for bladder cancer, point sources of bladder carcinogens—polycyclic aromatic hydrocarbons and arylamines in the USA population. *GSTM1*, and probably *GSTT1* and *GSTP1*, participates in the detoxification of polycyclic aromatic hydrocarbon in promoting the conjugation of carcinogenic electrophiles with glutathione, thus enhancing excretion in the urine. We analyzed our data under the assumption that the effects of *GSTM1*, *GSTT1* and *GSTP1* on risk are additive in bladder cancer. It is important to note that separate univariate analysis of individual genes would misclassify individuals with null/low-activity genotypes of one gene into the group of non-null/high-activity genotype of the other. For example, among 518 bladder cancer patients with at least one *GSTT1* allele in the present study, 290 had homozygous deletion of *GSTM1* and 69 had homozygous low-activity allele of *GSTP1*. Prior epidemiologic studies performing univariate analyses of *GSTT1* and *GSTP1* yielded similar, null findings as in the present study. Studies that examined the combined effects of *GSTM1* null, *GSTT1* null and/or *GSTP1* BB genotypes on risk generally noted stronger effects than *GSTM1* null alone (28,29,48). Thus, prior observations are supportive of our novel contention that *GSTM1*, *GSTT1* and *GSTP1* jointly contribute to the host's risk level of bladder cancer in an additive manner.

Hereditary difference in N-acetylation activity among individuals has been recognized for >50 years (49). Our study began in early 1990s, which was before the complete identification and characterization of genetic variants of the NAT2 gene (50). Thus, a phenotypic assay was chosen for our study to determine subject's acetylator status, a common approach used before the genome era. An additional benefit for use of this caffeine-based assay was the simultaneous determination of both NAT2 and CYP1A2 phenotypes from a single assay on the same urine sample (42,51).

Numerous epidemiological studies across different populations have examined the role of NAT2 phenotype/genotype in relation to bladder cancer risk. A recent meta-analysis including >5000 bladder cancer cases showed a statistically significant 40% increase in risk of bladder cancer in individuals possessing the *NAT2* slow-acetylation genotypes (14). A case–control study of Caucasians in the USA reported an ~30% increase in risk of bladder cancer for *NAT2* slow acetylation genotypes overall and 2-fold increase in risk among heavy smokers (16). The present study demonstrated that NAT2 slow acetylation phenotype plays a significant role in the risk of bladder cancer, especially among subjects with known high exposure to carcinogenic arylamines (i.e. heavy smoking and high levels of 4-ABP–Hb adducts).

Our study represents that, for the first time, NAT2 acetylation status is examined in combination with a composite index of *GST* genotypes that presumably capture greater impact of the three *GST* genes on bladder cancer development. Our results suggest a potential modifying effect between *NAT2* and the *GST*s among subjects with no or low exposure to carcinogenic arylamines (i.e. light smokers and/or low levels of 4-ABP–Hb adducts), although the test for multiplicative effect was not statistically significant (P = 0.09) given relatively small sample size. Among individuals possessing an unfavorable *GST* genotype profile, NAT2 slow acetylation status shows an ~60% (i.e. the ratio of 1.77 over 1.12 = 1.58, 95% CI = 0.80–1.35) increase in risk for bladder cancer, whereas no risk increase for NAT2 slow acetylation status was seen for subjects with more favorable *GST* genotypes.

The role of *NAT1* gene in the etiology of bladder cancer is controversial. Taylor *et al.* (15) reported a statistically significant increase in risk of bladder cancer associated with the *NAT1*10* genotype among heavy smokers in the USA. However, a later study in Germany reported a reduced risk of bladder cancer associated with the *NAT1*10* genotype, especially among non-smokers or those who never held high-risk occupations (21). A recent meta-analysis including >2500 bladder cancer cases and similar number of controls reported a null association between putative rapid *NAT1* genotypes and risk of bladder cancer (52). Our findings of a null association between the *NAT1*10* genotype and bladder cancer risk was consistent with most epidemiologic studies across different populations (14,16,53,54). Earlier, we had reported that levels of 4-ABP–Hb adducts were comparable between individuals with and without the putative high-risk *NAT1*10* genotype (55). While NAT1 appears to play a role in the metabolism of aromatic amines, the relationship between NAT phenotype and *NAT1*10* genotype remains uncertain and it is not surprising that the overall effect of *NAT1* genotype on risk is negligible.

High CYP1A2 activity has been linked to elevated urinary mutagenicity in smokers (56,57) and high 4-ABP–Hb adduct levels (58), a marker associated with increased risk of bladder cancer (45). CYP1A2 activity in humans appears to be determined by both heritable and environmental factors. So far, all genetic polymorphisms identified in the CYP1A2 gene are located either in the 5′ flanking regulatory region or the introns (59–63). Neither individual single-nucleotide polymorphisms nor haplotypes could unequivocally predict the CYP1A2 metabolic phenotype in humans (62–65). Several environmental factors have been found to influence hepatic CYP1A2 expression and activity. Cigarette smoking (43,66,67), consumption of high-temperature cooked meat (7) and cruciferous vegetables (68,69) can enhance, whereas use of oral contraceptives (43,67,70) reduces CYP1A2 metabolic capacity.

Two epidemiologic studies have examined the association between the CYP1A2 phenotype and risk of bladder cancer. A case–control study involving 100 bladder cancer patients and 84 control subjects from an Asian population reported a statistically significantly higher capacity for 3-demethylation of theophylline, a marker for CYP1A2 activity, than control subjects (71), whereas a case–control study of 53 bladder cancer patients and 96 control subjects in a Canadian population (>90% of subjects were Caucasians) did not find any difference in plasma or urinary indices of CYP1A2 activity between bladder cancer patients and control subjects (72). The present study with a larger sample size (635 case patients and 606 control subjects) did not detect any effect of the CYP1A2 index on risk of bladder cancer.

Table III. *GSTM1*, *GSTP1*, *GSTT1* and *NAT1* genotypes and NAT2 and CYP1A2 phenotypes in relation to risk of bladder cancer among non-Hispanic whites by levels of smoking and 4-ABP–Hb adducts, The Los Angeles Bladder Cancer Study

	Pack-years of smoking			Level of 4-ABP–Hb adducts (pg/g Hb)			Level of exposure to bladder carcinogens	
	Never smokers (0)	<30	≥30	Low (<17.9)	Intermediate (17.9–30.0)	High (>30.0)	Low[a]	High[a]
GSTM1								
Non-null (0)	54/141[b]	83/123	138/87	49/106	71/124	151/119	179/299	92/50
Null (1)	70/134	115/119	202/82	87/128	89/105	208/100	262/298	122/35
OR for null versus non-null[c]	1.48	1.54	1.72	1.42	1.45	1.82	1.51	2.06
95% CI	0.95–2.30	1.03–2.30	1.16–2.55	0.88–2.28	0.94–2.24	1.27–2.60	1.16–1.96	1.21–3.49
GSTT1								
Non-null (0)	96/221	159/199	263/136	99/186	134/188	280/179	348/483	165/70
Null (1)	27/50	37/43	76/31	36/46	24/40	78/37	89/110	49/13
OR for null versus non-null[c]	1.36	1.17	1.28	1.54	0.89	1.40	1.19	1.54
95% CI	0.79–2.34	0.70–1.93	0.79–2.09	0.90–2.63	0.50–1.60	0.89–2.21	0.86–1.65	0.77–3.06
GSTP1								
AA or *AB* (0)	113/245	171/214	291/152	122/202	144/206	303/200	390/531	179/77
BB (1)	11/29	25/29	46/15	12/31	15/22	54/19	48/65	33/7
OR for *BB* versus *AA* or *AB*[c]	0.82	1.04	1.60	0.55	0.94	1.80	0.95	2.05
95% CI	0.39–1.75	0.57–1.89	0.84–3.04	0.26–1.17	0.45–1.95	1.02–3.19	0.63–1.45	0.85–4.93
Summed *GST* index[d]								
0	41/102	62/92	92/68	34/75	55/94	103/91	132/220	60/40
1	58/126	94/110	172/170	68/113	79/102	175/91	218/274	104/32
2–3	24/31	39/39	73/27	32/43	24/31	78/31	86/95	48/10
OR for 1 versus 0[c]	1.25	1.36	1.99	1.24	1.40	1.83	1.37	2.33
95% CI	0.76–2.06	0.86–2.14	1.21–2.91	0.72–2.14	0.87–2.26	1.22–2.72	1.02–1.84	1.29–4.22
OR for 2–3 versus 0[c]	1.66	1.64	2.21	1.54	1.32	2.47	1.58	3.35
95% CI	0.87–3.17	0.92–2.92	1.25–3.91	0.80–2.96	0.67–2.58	1.46–4.19	1.08–2.32	1.49–7.56
P for trend	0.434	0.185	0.001	0.162	0.478	<0.001	0.041	0.002
NAT1								
Non-*NAT1*10*	75/117	129/149	230/108	86/142	115/142	228/148	293/376	136/56
*NAT1*10*	48/93	66/91	101/59	44/90	44/84	125/68	140/214	73/28
OR for *10 versus non*10[c]	1.32	0.98	0.75	1.01	0.65	1.25	0.93	1.10
95% CI	0.84–2.08	0.64–1.49	0.50–1.14	0.62–1.64	0.41–1.03	0.85–1.81	0.70–1.22	0.63–1.92
NAT2 phenotype[e]								
Rapid acetylators	61/127	94/97	116/67	52/91	61/85	133/89	177/231	69/34
Slow acetylators	56/118	98/114	210/83	58/93	81/105	188/91	210/256	117/33
OR for slow versus rapid[c]	0.99	0.92	1.51[f]	0.96	0.87	1.42	0.99	2.03
95% CI	0.63–1.56	0.61–1.39	0.99–2.29	0.57–1.61	0.54–1.41	0.96–2.08	0.74–1.32	1.12–3.69
CYP1A2 index[d]								
Low	51/123	97/105	166/73	59/107	71/90	156/80	194/253	92/24
High	66/122	95/106	160/77	51/77	71/100	165/100	193/234	94/43
OR for high versus low[c]	1.26	0.92	0.91	1.35	1.00	0.82	1.13	0.57
95% CI	0.80–1.99	0.60–1.40	0.60–1.37	0.81–2.27	0.63–1.60	0.56–1.21	0.85–1.50	0.31–1.04

[a]The high-exposure group consisted of smokers of ≥30 pack-years with high level of 4-ABP–Hb adducts (>30 pg/g Hb), whereas the low exposure group included all other subjects who never smoked or smoked <30 pack-years of cigarettes or had a relatively low level of 4-ABP–Hb adducts (≤30 pg/g Hb).
[b]The number of bladder cancer patients/number of control subjects; subjects with missing information on genotypes or phenotypes listed in the footnote of Table II were excluded from the relevant analysis. In addition, 65 bladder cancer patients and 53 control subjects with unknown 4-ABP–Hb adducts were excluded from the subgroup analyses stratified by 4-ABP–Hb adducts alone or in combination of cigarette smoking.
[c]ORs were derived from unconditional logistic regression models that included the following covariates: age (years), gender, level of education (high school or below, 1–4 years of college, graduate school or above), number of cigarettes smoked per day, number of years of smoking, total number of non-steroidal anti-inflammatory drug pills used over lifetime (non-user, <1441 and 1441+), ever use of permanent hair dyes (no, yes), ever held a high-risk job (no, yes) and 4-ABP–Hb adducts in tertile (a separate category was created for 65 cases and 53 controls with unknown 4-ABP–Hb adducts who were included in the subgroup analyses stratified by pack-years of smoking alone).
[d]One was assigned to the GSTM1 null, GSTP1 BB or GSTT1 null genotypes, respectively, whereas zero was assigned to the GSTM1 non-null, GSTP1 AA or AB or GSTT1 non-null genotypes, respectively.
[e]See the Materials and Methods for the definitions for the rapid/slow acetylators as well as for the low/high CYP1A2 index.
[f]Two-sided $P = 0.053$ for test for OR = 1.0.

The present study had several strengths including population-based study design, relatively large sample size and homogenous study population (non-Hispanic whites only). The comprehensive assessment of environmental exposures including smoking, occupation and personal use of permanent hair dyes allows for controlling for their potential confounding effect on the associations between genotypes and phenotypes of interest and risk of bladder cancer. More importantly, the measurement of 4-ABP–Hb adducts, a validated

Table IV. Joint effect of NAT2 phenotype and *GST* genotypes on risk of bladder cancer among non-Hispanic whites by level of exposure to bladder carcinogens, The Los Angeles Bladder Cancer Study

NAT2 phenotype status by level of exposure to bladder carcinogens[a]	Summed *GST* genotype index[b]		
	0	1	2–3
All subjects			
Rapid acetylators			
Cases/controls[c]	72/99	120/114	51/44
OR (95% CI)[d]	1.00 (referent)	1.44 (0.93–2.21)	1.43 (0.83–2.48)
Slow acetylators[c]			
Cases/controls[c]	91/113	155/134	66/37
OR (95% CI)[d]	0.88 (0.56–1.38)	1.50 (0.99–2.27)	2.27 (1.32–3.91)
Low exposure			
Rapid acetylators			
Cases/controls[c]	55/85	91/100	29/39
OR (95% CI)[d]	1.00 (referent)	1.34 (0.83–2.14)	1.12 (0.60–2.08)
Slow acetylators			
Cases/controls[c]	54/96	103/123	44/34
OR (95% CI)[d]	0.73 (0.44–1.21)	1.21 (0.77–1.92)	1.77 (0.97–3.22)
High exposure			
Rapid acetylators			
Cases/controls[c]	17/14	29/14	22/5
OR (95% CI)[d]	1.00 (referent)	2.00 (0.72–5.53)	3.96 (1.12–14.03)
Slow acetylators			
Cases/controls[c]	37/17	52/11	22/3
OR (95% CI)[d]	2.24 (0.84–5.94)	5.12 (1.81–14.46)	9.37 (2.15–40.86)

[a]See the Materials and Methods for the definition for the rapid/slow acetyplators. The high-exposure group consisted of smokers of ≥30 pack-years with high level of 4-ABP–Hb adducts (>30 pg/g Hb), whereas the low exposure group included all other subjects who never smoked or smoked <30 pack-years of cigarettes or had a relatively low level of 4-ABP–Hb adducts (≤30 pg/g Hb).

[b]One was assigned to the GSTM1 null, GSTP1 BB or GSTT1 null genotypes, respectively, whereas zero was assigned to the GSTM1 non-null, GSTP1 AA or AB or GSTT1 non-null genotypes, respectively.

[c]The number of bladder cancer patients/number of control subjects; a total of 555 bladder cancer patients and 541 control subjects who had complete data on all three GST genotypes, NAT2 phenotype and 4-ABP–Hb adducts were included in the analysis.

[d]ORs were derived from unconditional logistic regression models that included the following covariates: age (years), gender, level of education (high school or below, 1–4 years of college, graduate school or above), currently smoking (no, yes), number of cigarettes smoked per day, number of years of smoking, total number of non-steroidal anti-inflammatory drug pills used over lifetime (non-user, <1441 and 1441+), ever use of permanent hair dyes (no, yes), ever held a high-risk job (no, yes) and 4-ABP–Hb adducts in tertile (for all subjects only).

biomarker for exposure to bladder carcinogens—arylamines, enhanced our ability for controlling the potential confounding effect of 4-ABP from various, as-yet-unidentified sources on the genotype/phenotype–bladder cancer association. Another strength of the present study was the determination of NAT2 and CYP1A2 status based on phenotype-based assays, which presumably represent their 'true' enzyme activity determined by both endogenous (i.e. genetic) and exogenous (i.e. environmental) factors. On the other hand, this phenotype-based approach has inherent limitation to distinguish the genetically determined effect from environmental influence on the respective enzymes, especially in the case of a large number of environmental factors that potentially affect the enzyme phenotype. For example, paraxanthine, isoniazid and sulfamethzine are substrates of NAT2. Several environmental factors have been found to influence hepatic CYP1A2 expression and activity. Cigarette smoking (43,66,67), consumption of high-temperature cooked meat (7) and cruciferous vegetables (68,69) can enhance, whereas use of oral contraceptives (43,67,70) reduces CYP1A2 metabolic capacity. Therefore, incomplete assessment and controlling for these environmental factors could lead to the misclassification of NAT2 and CYP1A2 phenotype status.

In summary, this study confirms the role of *GSTM1* in bladder cancer and is the first study suggesting that the null/low-activity genotypes of the *GSTM1*, *GSTT1* and *GSTP1* may additively contribute to risk of bladder cancer in the host. NAT2 slow acetylation status is related to increased risk of bladder cancer among subjects with known high exposure to carcinogenic arylamines. The present study also suggests a potential modifying effect between NAT2 and GST genes on bladder cancer, especially among subjects with exposure to carcinogenic arylamines.

Funding

United States Public Health Service (R01 CA65726, R01 CA114665, P01 CA17054, P01 ES05622 and P30 ES07048); National Institute of Environmental Health Sciences, National Institutes of Health.

Acknowledgements

We thank Ms Susan Roberts and Ms Kazuko Arakawa of the University of Southern California for data collection and management.

Conflict of Interest Statement: None declared.

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Received February 25, 2008; revised April 28, 2008; accepted May 30, 2008