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N-Acetylation phenotype and genotype and risk of bladder cancer in benzidine-exposed workers

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Several studies in subjects occupationally exposed to arylamine carcinogens have shown increased risks for bladder cancer associated with the slow acetylator phenotype. To follow up these reports, a case-control study of *N*-acetylation and bladder cancer risk was carried out among subjects occupationally exposed to benzidine, in benzidine dye production and use facilities in China. Thirty-eight bladder cancer cases and 43 controls from these factories were included for study of acetylation phenotype, by dapsone administration, and for polymorphisms in the *NAT2* gene, by a polymerase chain reaction (PCR)-based test. In contrast to previous studies, no increase in bladder cancer risk was found for the slow *N*-acetylation phenotype (OR = 0.3; 95% CI = 0.1–1.3) or for slow *N*-acetylation-associated double mutations in NAT2 (OR = 0.5; 95% CI = 0.1–1.8). Examination of specific mutations and adjustment for age, weight, city and tobacco use did not alter the results. When examined by level of benzidine exposure in the cases, the bladder cancer risks associated with low (OR= 0.3, 95% CI = 0.0–2.2), medium (OR = 0.7, 95% CI = 0.1–4.5) and high (OR = 0.6, 95% CI = 0.1–3.5) exposure showed no interaction between genotype and benzidine exposure, within the range of exposures experienced by subjects in this study. This study, which is the first to incorporate phenotypic and genotypic analyses, provides evidence that the *NAT2*-related slow *N*-acetylation polymorphism is not associated with an increased risk of bladder cancer in workers exposed to benzidine, and may have a protective effect.

Introduction

In 1979, *N*-acetylation was proposed (1) as a detoxification pathway with respect to arylamine bladder carcinogens. Four subsequent studies in subjects occupationally exposed to arylamine carcinogens (2–5) showed increased risks, ranging from 2- to 17-fold, for bladder cancer associated with the slow acetylator phenotype. The authors concluded that slow acetylation is an important genetic susceptibility risk factor in workers exposed to arylamines, and in particular to benzidine (2–4). Considerations from experimental studies (6) of the metabolism of carcinogenic arylamines indicate that, in humans, acetylation may be an important pathway for detoxification of some arylamines, such as 2-naphthylamine, but for benzidine a protective effect is less likely.

To test the findings of increased risk associated with exposure to benzidine, as suggested from previous epidemiologic studies, we carried out investigations of bladder cancer in workers exposed to benzidine, but not to other carcinogenic arylamines. As we previously reported (7), a retrospective cohort study of 1972 benzidine-exposed people in China identified an overall 25-fold risk for bladder cancer, and showed a clear increase in bladder cancer risk with increasing level of benzidine exposure.

Although benzidine exposure was discontinued in China in 1977, members of this cohort have been followed through bladder cancer screening programs. Here, we report findings from a case-control study of bladder cancer in this population. We assessed phenotypic *N*-acetylation activity in bladder cancer cases and controls by measurement of test drug metabolites. We also studied the underlying genotype. Recently, restriction length polymorphisms (RFLPs*) of the *NAT2* gene have defined at least three mutant alleles associated with the population polymorphism of acetylation (8–11). Moreover, most of the mutations of the *NAT2* gene causing decreased *NAT2* activity have been identified (9,11) and a polymerase chain reaction (PCR)-based test has been developed for the major slow acetylator phenotype-associated mutations (9). This investigation is the first to study both *N*-acetylation phenotype and genotype in relation to bladder cancer risk.

Materials and methods

In 1991, male bladder cancer cases and controls were identified from among surviving members of a 1972–81 retrospective cohort study (7) of workers exposed to benzidine, who were participants in bladder cancer screening programs in Tianjin and Shanghai, China. Exposed subjects had been employed in benzidine production and use facilities for at least 1 year between 1945 and 1977. Review of job histories, materials and products established that the only occupational carcinogenic arylamine exposure in the study factories was to benzidine. Subjects in the screening programs are monitored, on a routine basis, by urine cytology for bladder cancer. The identified cases were pathologically confirmed. The exposed controls, with negative urine cytology, were selected from the cohort of benzidine-exposed workers to be of similar age, in 5 year groups, as the cases, and from the same cities.

On the day of study, subjects were requested to limit morning food intake to tea and sugar. The purpose of the study was explained and consent received, following Institutional Review Board approved procedures. Beginning at 08.30 h subjects received a 100 mg dose of dapsone (4,4′-diaminodiphenyl sulfone; DDS). Subjects were then interviewed regarding demographics, occupational history, current medications and life-long tobacco use. After 2.5 h, subjects had a light snack. Three hours after DDS administration, 15 ml of blood were drawn in a heparinized tube. The samples were centrifuged and separated into plasma, buffy coat and red blood cell fractions, and stored on dry-ice until transfer to a −70°C freezer. The samples were kept frozen until analysis.

Questionnaire and pathology data were reviewed and edited. For each benzidine-related job held, exposure was assigned, based on job title, as low, medium and high. Our previous investigation (7) indicated that risk of bladder cancer increased dramatically from low to high exposure jobs. Because quantitative levels of benzidine exposure in these jobs are unknown, cumulative benzidine exposure was estimated as:

$$\text{cumulative exposure} = (EXP_i \times DUR_i)$$

*Abbreviations: RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction; DDS, dapsone (4,4′-diaminodiphenyl sulfone); MAD, monoacetyldapsone; OR, odds ration; CI, confidence interval.

where, for each of *i* jobs, EXP is assigned a score of 1 for low, 3 for medium and 9 for high benzidine exposure, and DUR is the duration of exposure in that job.

All samples were analyzed with blinding for study group status. Acetylator phenotype was determined by simultaneous HPLC analysis for plasma DDS and monoacetyldapsone (MAD), using an M6000-A solvent delivery system (water/acetonitrile/acetic acid, 1000:300:25 v/v/v), a U6K injector and a μBondapak C18 column. The designation of acetylator phenotype was based on the ratio of acetylated metabolite (MAD) to parent compound (DDS). Subjects who had MAD/DDS ratios of <0.3 were classified as slow metabolizers (12,13).

For the assessment of *NAT2* genotype, DNA was isolated from buffy coat fractions by phenol and chloroform extractions and isopropanol/sodium acetate precipitation (14). DNA (200–300 ng) was used as a template in six parallel PCR reactions for identification of *NAT2* alleles according to a previously described method (9) with slight modifications, using primers designed to be specific for mutant (M1, M2, M3) or normal wild-type (wt) alleles of the *NAT2* gene. For the genotype comparisons in this study, subjects were classified as having wild-type, one mutation or two mutations in *NAT2*. Based on studies of phenotypic activity, the presence of two mutations is predictive of the slow *N*-acetylator phenotype (15).

Phenotypic analysis was carried out for 80/81 study subjects. All 81 subjects were genotyped. Initial analytic results showed 89% correlation (71/80) between acetylator phenotype (slow versus fast) and genotype (double mutations versus wild-type and single mutations). The nine discordant samples and an additional eight of the 71 concordant samples, representing a range of genotypes, were reanalyzed for both phenotype and genotype, with blinding for case-control status and initial result. None of the eight concordant samples switched categories. Seven of the nine discordances were resolved, with change in either the genotype or phenotype category. The two remaining discordant samples were genotypically double mutations (M2/M3 and M3/M3 respectively), but had the rapid phenotype. Analysis of *N*-acetyltransferase and bladder cancer were carried out for the original and for the corrected data, with nearly identical results. Analyses are presented here for the corrected data.

Odds ratios (OR) and exact (95%) confidence intervals (CI) were calculated as described by Thomas and Gart (16). Logistic regression methods were used for multivariate analyses (17).

Results

For the case-control study, 38 benzidine-exposed bladder cancer cases and 43 benzidine-exposed controls were studied. Cases were diagnosed, on average, 8.5 years (SD 5.2 years) prior to study. Thirty-two cases were transitional cell carcinomas, 11 with papillary elements. One adenocarcinoma, one mixed transitional cell and adenocarcinoma, and four unspecified bladder cancer cases completed the series.

Cases and controls were similar with regard to city of residence, age and weight (Table I). There was a moderate excess of smokers in the bladder cancer case group, with a risk of bladder cancer of 1.4 (95% CI = 0.3–6.1) associated with <20 pack-years of cigarette use, and 1.6 (95% CI = 0.4–5.8) associated with 20+ pack-years of cigarette use. Cases and controls were similar with regard to years of exposure to benzidine, but cases had substantially higher cumulative exposure ($P < 0.001$). When benzidine exposure was categorized into low (<30 cumulative level-years), medium (30–59 cumulative level-years) and high (60+ cumulative level-years) exposure, the risks associated with medium and high exposure, relative to low exposure, were OR= 2.6 (95% CI = 0.8–8.9) and OR = 6.7 (95% CI = 1.7–33.6) respectively.

Among these benzidine-exposed subjects, the risk for bladder cancer associated with the slow acetylator phenotype (MAD/DDS < 0.03) is OR = 0.3 (95% CI = 0.1–1.3) and the risk associated with the *NAT2* genotype predictive for slow acetylation (two mutations) is OR = 0.50 (95% CI = 0.1–1.8) (Table II). Although two subjects were discordant for phenotype and genotype, by either measure, risks of 2-fold or greater are excluded by the upper bounds of the 95% CI. Rather, the point estimates of risk are <1.0, for both phenotype (OR = 0.3) and genotype (OR = 0.5), suggesting a possible protective effect associated with slow *N*-acetylation. Adjustment for age (50–59, 60–69, 70+), weight, city or tobacco use (none, 1–20 pack-years, 20+ pack-years) did not alter this finding. Additional analyses, with respect to specific mutations (M1, M2, M3, or their combinations), did not reveal any further associations.

The risk for bladder cancer associated with the slow acetylator predictive genotype (two mutations) was OR = 0.3 (95% CI = 0.0–2.2) for low exposure, OR = 0.7 (95% CI = 0.1–4.5) for medium exposure and OR = 0.6 (95% CI = 0.1–3.5) for high exposure to benzidine, showing no interaction for bladder cancer between genotype and benzidine exposure, within the range of exposures experienced by subjects in this study.

Table I. Selected variables by study group for benzidine-exposed bladder cancer cases and controls

	Cases		Controls	
	n	(%)	*n*	(%)
City				
Tianjin	31	(82)	36	(84)
Shanghai	7	(18)	7	(16)
Tobacco pack-years				
0	7	(18)	11	(26)
<20	11	(30)	12	(28)
20+	20	(57)	20	(46)
	Mean	(SD)	Mean	(SD)
Age	63.3	(6.8)	63.2	(7.0)
Weight (kg)	70.2	(10.5)	68.4	(9.6)
Benzidine exposure				
Years exposed	11.8	(6.9)	10.0	(8.2)
Cumulative level	53.2	(44.7)	24.1	(21.5)[a]

[a]$P < 0.001$ for difference between the means by Student's *t*-test.

Table II. *N*-Acetylator phenotype (dapsone) and genotype (*NAT2*) associated relative risk for bladder in benzidine-exposed cases and controls

	Phenotype (MAD/DDS)		Genotype (no. of mutations)	
	Fast (>0.3), *n*	Slow (<0.3), *n*	Fast (0–1), *n*	Slow (2), *n*
Cases[a]	34	3	33	5
Controls	33	10	33	10
	Risk of bladder cancer		Risk of bladder cancer	
Odds ratio	1.0	0.3	1.0	0.5
95% CI		(0.1–1.3)		(0.1–1.8)

[a]For cases, phenotype ($n = 37$, one subject not phenotyped), genotype ($n = 38$). Of five cases with two mutations in *NAT2*, only three had the slow acetylator phenotype.

Discussion

A previous study (7) demonstrated an increased risk for bladder cancer in a cohort of Chinese workers employed in the manufacture and use of benzidine. There was no known exposure, in the study factories, to other carcinogenic arylamines, and specifically, there was no exposure to 2-naphthylamine or 4-aminobiphenyl. In the present investigation in bladder cancer cases and controls from this population, we show that the slow *N*-acetylation phenotype and the *NAT2* genotype, predictive for slow acetylation (two mutations), do not contribute to the benzidine-associated increased risk for bladder cancer, and may be associated with a protective effect.

Epidemiological studies can be limited by biased selection of study subjects and inaccurate measurement of risk factors. As in previous studies of *N*-acetylation in arylamine dye workers (2–5), prevalent bladder cancer cases were studied here. One concern with this approach is that disease status and its treatment may affect the measure of phenotype. Genotype, however, provides an independent assessment, and is not affected by disease status. Second, if *N*-acetylation is associated with aggressiveness of bladder cancer and consequently with survival, study of surviving cases could give biased results. We saw no association between *N*-acetylation and year of diagnosis, suggesting no relationship between acetylation activity and survivorship. Others (2,18–20) also have not found a consistent relationship between *N*-acetylation and tumor aggressiveness. Third, this was the only study of the *N*-acetylation polymorphism carried out in a non-Western population occupationally exposed to arylamines. We cannot rule out ethnic differences in metabolic pathways not studied. Further, as others have shown in Asians (8) and Caucasians (9), discordances were observed between genotype and phenotype results for some subjects. The findings, however, with respect to the association with bladder cancer, were essentially the same for all analyses. Finally, any protective effect of *N*-acetylation could have been overwhelmed by high benzidine exposure; however, we found no evidence of an *N*-acetylation-associated excess risk in cases with relatively low or relatively high exposure to benzidine. Adjustment for age, weight, geographic location and tobacco use did not alter the conclusions.

In 1979, *N*-acetylation was proposed (1) as a detoxification pathway with respect to arylamine bladder carcinogens. Studies of arylamine–hemoglobin adducts support this theory. Slow acetylators have increased smoking-related 4-aminobiphenyl adducts (21), and increased occupational aniline-associated adducts (22). In epidemiologic studies of bladder cancer, however, non-occupationally exposed cases and smoking-related cases have shown only a moderate association with the slow acetylator phenotype (23).

Only in studies of workers heavily exposed to arylamines has a strong association been shown between acetylator phenotype and bladder cancer risk. Cartwright *et al.* (2) identified a 17-fold relative risk for bladder cancer for slow acetylators among workers employed in the dye-manufacturing trade in Huddersfield, UK. Studies of bladder cancer cases occupationally exposed to arylamines in Poland (3), Germany (4) and the United States (5) show 9-, 4- and 2-fold risks respectively, associated with the slow acetylator phenotype. The wide variability in estimates of the risk associated with the slow acetylator phenotype suggests heterogeneity of exposure or of other important factors. Although benzidine was reported as the major exposure in these investigations (2–4), at least in the Huddersfield study (24) 2-naphthylamine was also used. The studies provided little detail on historical occupational exposure, making it difficult to assess the specific types of arylamine exposures that actually took place. Given the greater potency of 2-naphthylamine, compared to benzidine, as a human bladder carcinogen (25–27) and the suggested importance of *N*-acetylation in detoxification of 2-naphthylamine as a bladder carcinogen (1), the increased risk for bladder cancer observed in association with the slow acetylation phenotype in previous occupational studies may have been due to 2-naphthylamine, or related exposures.

The occupational exposure to arylamines in the present study was exclusively to benzidine. Experimental evidence indicates that the model proposed by Lower (1) for the deactivation of aromatic amines as bladder carcinogens by *N*-acetylation is not appropriate for benzidine. Benzidine is a substrate for *N*-acetylation in humans (28), yielding monoacetyl- and diacetylbenzidine in the urine of exposed workers (29,30). Monoacetylbenzidine is subject to *N*-hydroxylation via cytochrome P450, yielding experimental reactive metabolites (31), and *N*-hydroxylated monoacetylbenzidine has been shown to be a potent bladder carcinogen in heterotopic rat bladder (32). Additionally, extrahepatic tissue, including human bladder cytosol, exhibits polymorphic *N*-acetylation activity (33). This work suggests that fast *N*-acetylation activity may be associated with increased risk for benzidine-induced bladder cancer. However, another mechanism has been proposed. Benzidine is readily metabolized by prostaglandin-H-synthase to an activated diimine (34) in human bladder (35), while mono- and diacetylated benzidine are not substrates for this enzyme (36). This would suggest that slow *N*-acetylation activity may be associated with increased risk for benzidine-induced bladder cancer.

Although the relative importance of the putative metabolic pathways for benzidine activation to a proximate bladder carcinogen in humans has not been directly described, this epidemiologic analysis shows that slow *N*-acetylation is not a risk factor for benzidine-induced bladder cancer, and may have a protective effect.

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