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N-Acetyltransferase 2 genotype, exfoliated urothelial cells and benzidine exposure

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1. ABSTRACT

Most studies report an association of the slow N-acetyltransferase 2 (NAT2) status with elevated bladder cancer risk. In this study, *NAT2* genotypes and the decades-long records of Papanicolaou's grading of exfoliated urothelial cells in a former benzidine-exposed cohort of the Shanghai dyestuff industry (29 bladder cancer patients; 307 non-cancer cohort members, some of them presenting different grades of pre-malignant alterations of exfoliated urothelial cells) were investigated. The cohort members had been enrolled in regular medical surveillance since mid-1980s. No overall increase of slow *NAT2* genotypes in the former benzidine-exposed bladder cancer patients was found, compared with non-diseased members of the same cohort. A lower presentation of the homozygous wild genotype *NAT2 *4/*4* was observed in bladder cancer patients, compared with non-diseased members with averaged Papanicolaou's grading (APG) ≥II (OR=0.31, 95% CI 0.10-0.96, p=0.034) or with APG<II (OR=0.36, 95% CI 0.12-1.10, p=0.063). Nevertheless, neither a protective influence of rapid *NAT2* genotypes on bladder cancer risk nor on pre-malignant cytological alterations could be confirmed by the present data.

2. INTRODUCTION

Arylamine N-acetyltransferases (NATs) (EC 2.3.1.5) are involved in the metabolism of aromatic amines and hydrazines (1). *NAT2* polymorphism affects the individual's acetylating ability (2). *NAT2* polymorphism is based on several point mutations in the coding area (3). A consensus nomenclature for NATs was first published in 1995 (4) and was last updated in 2008 (5). The *NAT2* polymorphism has been reported to be associated with susceptibility for various types of cancer (6-13) or other diseases (14-15) within certain ethnic populations. Many reports documented that NAT2 slow acetylators of Caucasian descent have increased risk of bladder cancer (16-17). The NAT2 slow acetylating genotypes were also suggested to relate with the elevated risk of bladder cancer in the cases without definite occupational exposure to aromatic amines in Eastern Asian populations (18-19), though some inconsistent results were also reported (20-21).

Benzidine, an evident human bladder carcinogen (22) was first introduced for dye synthesis in Shanghai in 1946 and was widely used until it was officially banned for

all industrial purposes in China in 1976 (23). Workers occupationally exposed to benzidine were regarded as a high-risk group for bladder cancer. We have previously investigated the bladder cancer risk in benzidine-exposed workers in the Shanghai dyestuff industry. Our results indicated that the standardized incidence ratio (SIR) for bladder cancer in workers of the Shanghai dyestuff industry reached 35 for the entire cohort and was even higher (up to 75) for those at highly exposed working positions (24). The association of susceptibility to bladder cancer or to pre-malignant cytological alterations of exfoliated urothelial cells with the polymorphic status of some genes involved in xenobiotic metabolism as glutathione S-transferases T1, M1, P1, (GSTT1, GSTM1, GSTP1), N-acetyltransferase 1 *10 and 14*A (NAT1*10, NAT1*14A), and UDP-glucuronosyltransferase 2B7 (UGT2B7) in this cohort have been reported elsewhere (25-27).

Cartwright *et al* (28) first reported a strong association of bladder cancer incidence with the slow acetylating phenotype (96% vs. 57%, OR=16.7; 95% CI 2.16-129.07, P = 0.007) in a group of workers in UK who had been exposed to aromatic amines. In a subgroup of bladder cancer cases, 96% (22/23) of the subjects were slow acetylators, whereas only 57% (54/95) of the local controls were slow acetylators. Lewalter and Miksche (29) reported that 82% of 92 benzidine-exposed chemical workers with bladder cancer were of the slow acetylating phenotype, whereas only 48% of 331 chemical workers who had worked at that plant In Leverkusen, Germany, and had not been diagnosed as bladder cancer patients were of the slow acetylating phenotype. All the subjects enrolled in the above mentioned two studies were of Caucasian origin.

Later on, a similar study was conducted in 38 bladder cancer cases and 43 non-diseased controls among workers formerly employed in benzidine production facilities in several Chinese cities. Genotyping revealed 5 slow NAT2 acetylators in cases and 10 slow NAT2 acetylators in controls. Phenotyping revealed 3 slow acetylators in cases and 10 slow acetylators in controls. No increase in bladder cancer risk was found for the slow N-acetylating phenotype (OR=0.3; 95% CI 0.1-1.3). The authors concluded that, unlike in Caucasians, the slow NAT2 status is not a risk factor for occupational bladder cancer in Chinese (30). In their expanded study with 68 cases and 107 controls in the same cohort, a protective role of slow *NAT2* genotypes was described after adjustment for cumulative benzidine exposure and lifetime smoking. In the above-mentioned study, six polymorphic loci of the *NAT2* gene ($G_{191}A$, $T_{341}C$, $C_{481}T$, $G_{590}A$, $A_{803}G$, and $G_{857}A$) were genotyped. Only the genotypes without *NAT2*4* allele were classified as slow acetylating (31). Sone (32) reported on NAT2 phenotyping of 20 arylamine-exposed Japanese bladder cancer patients and 82 controls. Ten percent (2/20) of the occupationally exposed bladder cancer cases and 13.4% (11/82) of the controls were of the slow NAT2 phenotype. The data showed no significant association between slow NAT2 phenotype and urothelial cancer in these Japanese workers. In the present work of *NAT2* genotyping in 29 diagnosed bladder cancer cases in a benzidine-exposed cohort, similar frequencies of slow *NAT2* genotype carriers in the benzidine exposed bladder cancer patients and in the healthy individuals were detected (13.8% vs. 12.5%, OR=1.12; 95% CI 0.34-3.70) (21). For further validating the influence of *NAT2* alleles on bladder cancer in occupationally benzidine-exposed Chinese, *NAT2* genotypes and the decades-long record of Papancolaou's grading of exfoliated urothelial cells of an benzidine-exposed cohort in Shanghai were investigated and the association of *NAT2* genotypes with bladder cancer and with averaged Papanicolaou's grading (APG) of exfoliated urothelial cells were evaluated.

3. MATERIALS AND METHODS

3.1. Subjects

This study included two groups of members of a benzidine-exposed cohort of the Shanghai dyestuff industry: All subjects were employees or former employees of dyestuff factories in Shanghai. (1) Twenty-nine patients with histologically diagnosed bladder cancer. Each tumor was classified as papillary transitional cell carcinoma (TCC). The cases had been diagnosed between 1964 and 1998. All were males. Since most members of this cohort are in their advanced age, the numbers of surviving diagnosed bladder cancer patients in the cohort decline every year. (2) Three hundred and seven non-cancer members of the cohort, including 212 males and 95 females. All the subjects in this subgroup have not been diagnosed with bladder cancer or any other malignancies although exfoliated urothelial cells of some subjects revealed different grades of pre-malignant cytological abnormalities by Papanicolaou's grading (33-34). The research cohort of benzidine-exposed workers in the Shanghai dye industry was established in 1984. A follow-up study and regular surveillance have been continuously performed since then. All the subjects included in this study are ethnic Han Chinese (Chinese ethnic majority that represents 93% of the nation's population).

The study was approved by the Ethics Committee of the Shanghai Municipal Center for Disease Prevention and Control (CDC) and met all the legal requirements of Chinese laws and regulations concerned. Each participant provided written informed consent.

3.2. Blood sampling and DNA extraction

Ethylenediaminetetraacetic acid (EDTA) was used as blood anticoagulant. Genomic DNA was prepared from leukocytes after lysis of erythrocytes, incubation with proteinase K, chloroform extraction, and ethanol precipitation as described previously (21).

3.3. Genotyping of NAT2 genes

Modified PCR-RFLP procedure (35) was used to determine the mutations on nucleotides 191, 282, 341, 481, 590, 803 and 857 with two sets of primers (for PCR A: Forward: 5'-gtc aca cga gga aat caa atg c-3', and Reverse:5'-acc cag cat cga caa tgt aat tcc tgc cct ca-3' for PCR B: forward: 5'-aat tac att gtc gat gct ggg t-3' and reverse: 5'-aca caa ggg ttt att ttg ttc c-3') (Gibco-BRL Life Technologies, Grand Island, N.Y., U.S.A) were used to amplify two segments in the *NAT2* gene from genomic

DNA. Restriction enzymes *Msp* I, *Fok* I, *Dde* I, *Kpn* I, *Taq* I and *BamH* I (MBI Fermentas, Hannover, MD, U.S.A.) were used to digest PCR products to determine *NAT2* genotype. The *NAT2**13 allele has a nucleotide substitution at position 282 that does not result in an amino acid change. The genotype carriers of **4/*4, *13/*13* or **4/*13* were classified as rapid NAT2 acetylators. Individuals with two variant alleles (other than *NAT2*4, NAT2*13, NAT2*12B*) were classified as slow NAT2 acetylators. Heterozygote genotypes that carry an allele *NAT2 *4, *12B, *13* and one copy of any other *NAT2* allele were taken as intermediate NAT2 acetylators.

3.4. Collection and classification of exfoliated cells

Urine samples were collected from the members of the benzidine cohort at an occupational medical clinic in Shanghai between 9.00 h and 11.00 h a.m. The entire urinates, which were second or later urinates of the day, were immediately spun down, fixed, subsequently stained according to Papanicolaou (33) and classified according to Papanicolaou and Marshall (34) always by the same investigator as described elsewhere in detail (23). The grading classifies exfoliated urothelial cells from grade I (normal findings) to grade V (malignant cells).

The average cytological grading was taken to assess the morphological changes in the exfoliated urothelial cells. An average cytological grading (averaged Papanicolaou's grading score, APG score) was calculated for each subject. The average number of cytological gradings per subject was 10. By the use of an averaged cytological grading over the investigation period, misclassifications due to temporary external effects such as irritation of the urothelium by non-overt diseases or inadequate sampling conditions were minimized. Usually, the cytological gradings of an individual did not change substantially over the period of regular surveillance.

3.5. Statistical analysis

Chi-square test was used to compare the distribution of *NAT2* genotypes in different groups. Cochran-Armitage test was applied to test the trend of percentage of various acetylating genotypes in different subgroups in the exposure cohort. P-values less than 0.05 were regarded as significant. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were also calculated to estimate the risk or the protection due to different genotypes. Hardy-Weinberg equilibrium was used to calculate the expected frequencies of the investigated heterozygous genotypes.

4. RESULTS

In this study, *NAT2* genotypes of 307 non-diseased members of a benzidine-exposed cohort and of 29 occupationally exposed bladder cancer patients were investigated (Table 1). Eighteen different *NAT2* genotypes were observed in this cohort. Some genotypes were found in these groups but not in the general population in the same city (21), i.e. **4/*6B, *13/*6A*, **13/*7B, *12B/*7A, *11/*6A, *5B/*5B, *7A/*7A* and **7B/*14A*. The frequencies for most of these genotypes were lower than 1%, except **12B/*7A* (which represents 1.1% of all investigated groups). In summary, **4/*4* (13.8%), **4/*6A* (31.0%), and **4/*7B* (24.1%) are the most frequent genotypes similar to the general population in the same city.

When taking into account the frequencies of trichotomous distribution of the NAT2 genotypes in these groups, a stepwise decrease of the percentages of the homozygous slow *NAT2* genotypes was observed (from 20.6%, 15.7% to 13.8%) when the averaged Papanicolaou's grading of exfoliated urothelial cells increased (from APG<II to APG≥II, and finally to malignancy). The prevalence of homozygous slow *NAT2* genotypes in the bladder cancer patients group displayed no significant difference, compared with either APG≥II group (13.8% vs. 15.7%, p=0.79) or APG<II group (13.8% vs. 20.6%, p=0.40). A chi-square test on the frequencies of slow, intermediate, rapid acetylating genotypes of subgroups of cancer, APG≥II, APG<II showed no statistically significance (P=0.3404). When a Cochran-Armitage trend test was conducted, no significance was reached in the cases of frequencies of slow, rapid/intermediate between subgroups of cancer, when APG≥II was compared with APG<II (P=0.2225), possibly due to the low frequency of the bladder cancer cases. Among the non-diseased benzidine-exposed workers of the cohort, no significant frequency difference could be detected between APG≥II and APG<II subgroups (15.7% vs. 20.6%, p=0.29). The presentation of homozygous slow *NAT2* genotypes in these bladder cancer patients showed no profound deviation from the general population in the same city, too (13.8% vs. 12.5%, p=0.85) (21).

Benzidine-exposed bladder cancer patients have a lower frequency of the homozygous rapid genotype, compared with non-diseased members of the cohort, although these differences did not reach statistical significance. There was no significant difference of the homozygous rapid genotype in the occupational bladder cancer patients and the non-diseased cohort member when adjusted for gender, smoking status, exposure level and age (data not shown). Furthermore, there were more slow NAT2 acetylators in the group of APG<II, especially in females and non-smokers. But the increase did still not reach statistical significance (Table 3). Benzidine-exposed bladder cancer patients have a significantly lower frequency of the wild *4/*4 genotype than subgroup APG³II whose exfoliated urothelial cells were detected to undergo different degrees of meaningful pre-malignant alterations, but have not been diagnosed with bladder cancer yet (13.8% vs. 33.9%, P=0.03□OR=0.31□95% CI 0.10-0.96). In patients 60 years or older, this significance still exists (14.3% vs. 36.7%, P=0.03□OR=0.29□95% CI 0.09-0.90). Males (13.8% vs. 31.3%, p=0.07, OR=0.35, 95% CI 0.11-1.12) and smoking patients displayed a higher prevalence of **4/*4* genotype than corresponding members in subgroup APG≥II, although only marginal statistical significance was reached. When the **4/*4* frequencies in benzidine-exposed bladder cancer patients and subgroup APG<II were compared, a similar tendency was displayed (Table 3).

Table 1. *NAT2* genotype distribution in the benzidine-exposed cohort, stratified for bladder cancer patients, non-diseased cohort members and two non-diseased cohort member subgroups (S: slow, R: rapid)

***NAT2* genotypes**	**Benzidine-exposed bladder cancer patients (N=29) (n, %)**	**Non-diseased cohort members**		
		APG≥II (N=127) (n, %)	**APG<II (N=180) (n, %)**	**Total of non-diseased members (N=307) (n, %)**
**4/*4*	4 (13.8%)	43 (33.9%)	55 (30.6%)	98 (31.9%)
**4/*13*	0	2 (1.6%)	1 (0.6%)	3 (1.0%)
**13/*13*	2 (6.9%)	0	1 (0.6%)	1 (0.3%)
RR	6 (20.7%)	45 (35.4%)	57 (31.7%)	102 (33.2%)
**4/*5B*	2 (6.9%)	5 (3.9%)	8 (4.4%)	13 (4.2%)
**4/*6A*	9 (31.0%)	35 (27.6%)	43 (23.9%)	78 (25.4%)
**4/*6B*	1 (3.4%)	0	1 (0.6%)	1 (0.3%)
**4/*7B*	7 (24.1%)	20 (15.7%)	28 (15.6%)	48 (15.6%)
**13/*6A*	0	0	2 (1.1%)	2 (0.6%)
**13/*7B*	0	1 (0.8%)	1 (0.6%)	2 (0.6%)
**12B/*7A*	0	1 (0.8%)	3 (1.7%)	4 (1.3%)
RS	19 (65.5%)	62 (48.8%)	86 (47.8%)	148 (48.2%)
**5B/*5B*	1 (3.4%)	0	1 (0.6%)	1 (0.3%)
**5B/*6A*	0	1 (0.8%)	2 (1.1%)	3 (1.0%)
**5B/*7B*	0	1 (0.8%)	2 (1.1%)	3 (1.0%)
**6A/*6A*	2 (6.9%)	4 (3.1%)	8 (4.4%)	12 (3.9%)
**6A/*7B*	1 (3.4%)	12 (9.4%)	19 (10.6%)	31 (10.1%)
**7A/*7A*	0	0	1 (0.6%)	1 (0.3%)
**7B/*14A*	0	0	2 (1.1%)	2 (0.6%)
**7B/*7B*	0	2 (1.6%)	2 (1.1%)	4 (1.3%)
SS	4 (13.8%)	20 (15.7%)	37 (20.6%)	57 (18.6%)

Table 2. Homozygous slow *NAT2* (SS) genotypes in the total cohort, stratified for cytological findings

		***NAT2* genotype**		**Risk (genotype of corresponding APG≥II were taken as reference)**		**Risk (genotype of corresponding APG<II were taken as reference)**	
All the cohort members		*SS* genotypes	Other	P	OR (95% CI)	P	OR (95% CI)
	Benzidine-exposed occupational bladder cancer patients	4 (13.8%)	25 (86.2%)	0.792	0.86 (0.27-2.73)	0.395	0.62 (0.20-1.89)
	APG≥II	20 (15.7%)	107 (84.3%)	Ref.	Ref.	0.286	0.72 (0.40-1.31)
	APG<II	37 (20.6%)	143 (79.4%)	0.286	1.38 (0.76-2.52)	Ref.	Ref.

APG: Averaged Papanicolaou's grading

Table 3. *NAT2* wildtype (*NAT2 *4/*4*) related risk compared to all other *NAT2* genotypes in the total cohort and in different subgroups

Population		***NAT2* genotypes**		**Risk (genotype of corresponding APG≥II were taken as reference)**		**Risk (genotype of corresponding APG<II were taken as reference)**	
Subgroups		**4/*4*	Other	P	OR (95% CI)	P	OR (95% CI)
All the cohort members	Benzidine-exposed occupational bladder cancer patients	4 (13.8%)	25 (86.2%)	0.034	0.31 (0.10-0.96)	0.063	0.36 (0.12-1.10)
	APG≥II	43 (33.9%)	84 (66.1%)	Ref.	Ref.	0.541	1.16 (0.72-1.89)
	APG<II	55 (30.6%)	125 (69.4%)	0.541	0.86 (0.53-1.40)	Ref.	Ref.
60 and older	Benzidine-exposed occupational bladder cancer patients	4 (14.3%)	24 (85.7%)	0.026	0.29 (0.09-0.90)	0.076	0.36 (0.11-1.15)
	APG≥II	33 (36.7%)	57 (63.3%)	Ref.	Ref.	0.493	1.25 (0.66-2.37)
	APG<II	25 (31.6%)	54 (68.4%)	0.493	0.80 (0.42-1.51)	Ref.	Ref.
Male	Benzidine-exposed occupational bladder cancer patients	4 (13.8%)	25 (86.2%)	0.068	0.35 (0.11-1.12)	0.061	0.36 (0.12-1.09)
	APG≥II	25 (31.3%)	55 (68.8%)	Ref.	Ref.	0.977	1.01 (0.55-1.84)
	APG<II	41 (31.1%)	91 (68.9%)	0.977	0.99 (0.55-1.81)	Ref.	Ref.
Smoker	Benzidine-exposed occupational bladder cancer patients	1 (5.6%)	17 (94.4%)	—	—	0.034	0.13 (0.02-1.11)
	APG<II	16 (30.2%)	37 (66.8%)	—	—	Ref.	Ref.

APG: Averaged Papanicolaou's grading

Table 4. *NAT2* genotypes in the highly exposed subgroup of the benzidine-exposed cohort and the subgroups "smokers" and "60 years and older" (S: slow, R: rapid)

	NAT2 genotypes	Benzidine-exposed patients (n, %)	Non-diseased cohort members		
			APG≥II (n, %)	APG<II (n, %)	Total of non-diseased members (n, %)
All the most highly exposed cohort members	**4/*4*	4 (16.7%)	20 (32.8%)	32 (32 %)	52 (32.3%)
	RR	6 (25.0%)	21 (34.4%)	33 (33%)	54 (33.6%)
	RS	15 (62.5%)	31 (50.8%)	46 (46%)	77 (47.8%)
	SS	3 (12.5%)	9 (14.8%)	21 (21%)	30 (18.6%)
Highly exposed smokers in benzidine-exposed cohort	**4/*4*	1 (6.7%)	9 (34.6%)	12 (36.4%)	21 (35.6%)
	RR	2 (13.3 %)	9 (34.6%)	12 (36.4%)	21 (35.6%)
	RS	10 (66.7%)	12 (46.2%)	11 (33.3%)	23 (39.0%)
	SS	3 (20.0%)	5 (19.2%)	10 (30.3%)	15 (25.4%)
Highly exposed workers aged 60 and older in benzidine-exposed cohort	**4/*4*	4 (17.4%)	16 (35.6%)	15 (35.7%)	31 (35.6%)
	RR	6 (26.1 %)	16 (35.6%)	15 (35.7%)	31 (35.6%)
	RS	15 (65.2%)	23 (51.1%)	18 (42.9%)	41 (47.1%)
	SS	2 (8.7%)	6 (13.3%)	9 (21.4 %)	15 (17.2%)

APG: Averaged Papanicolaou's grading, R: Rapid,S: Slow

Table 5. Homozygous slow *NAT2* genotypes in highly exposed cohort and the subgroups "smokers" and "60 years and older", stratified for cytological findings (SS: Homozygous slow *NAT2* genotype)

		***NAT2* genotype**		**Risk (genotype of corresponding APG≥II were taken as reference)**		**Risk (genotype of corresponding APG<II were taken as reference)**	
		SS genotypes	Other genotypes	*P*	OR (95% CI)	*P*	OR (95% CI)
Most highly exposed cohort members	Benzidine-exposed occupational bladder cancer patients	3 (12.5%)	21 (87.5%)	0.788	0.83 (0.21-3.35) (0.27-2.73)	0.344	0.54 (0.15-2.00)
	APG≥II	9 (14.8%)	52 (85.2%)	Ref.	Ref.	0.323	0.65 (0.28-1.54)
	APG<II	21 (21.0%)	79 (79.0%)	0.323	1.54 (0.65-3.61)	Ref.	Ref.
Highly exposed smokers of benzidine-exposed cohort	Benzidine-exposed occupational bladder cancer patients	3 (20.0%)	12 (80.0%)	0.952	1.05 (0.21-5.19)	0.457	0.58 (0.13-2.49)
	APG≥II	5 (19.2%)	21 (80.8%)	Ref.	Ref.	0.332	0.55 (0.16-1.87)
	APG<II	10 (30.3%)	23 (69.7%)	0.332	1.83 (0.54-6.22)	Ref.	Ref.
Highly exposed workers aged 60 and older of benzidine-exposed cohort	Benzidine-exposed occupational bladder cancer patients	2 (8.7%)	21 (91.3%)	0.574	0.62 (0.12-3.34)	0.190	0.35 (0.07-1.78)
	APG≥II	6 (13.3%)	39 (86.7%)	Ref.	Ref.	0.318	0.56 (0.18-1.75)
	APG<II	9 (21.4 %)	33 (78.8%)	0.318	1.77 (0.12-3.34)	Ref.	Ref.

APG: Averaged Papanicolaou's grading, SS: Homozygous slow

The individuals' age, high exposure, smoking history (smoking during the benzidine exposure years) were among the non-genetically determined risk factors. The cohort members who were taking the most exposed working positions in the production factories of benzidine-based dyes and who were smokers then were considered as the "highest risk subjects" in this "risk cohort". Since there is no reliable data of environmental pollution in the factories in the 1940s-1960s available, the cohort members were classified into three subgroups according to their job in the production process with decreasing exposure levels. The subgroup with working posts for managing materials (benzidine was the most important one) and dye synthesis were classified as the highest exposed one among the three. There were 185 members from high exposure subgroups (including 145 males and 40 females; age at sampling time: 42-87 years, median 63 years; mean: 62.8 ±10.3) included in the present study.

The subgroups were further stratified for smoking status and age. Seventy-four (all male; age: 48-87 years; median 66; mean 66.0±10.6) of 185 highly exposed cohort members were ever smokers (i.e., smokers at the time of exposure). There was a total of 110 members belonging to the subgroup of age 60 or older (including 97 males and 13 females; age 60-87 with median at 70; mean±SD: 70.1±6.4). Regarding the frequencies of homozygous slow *NAT2* genotypes and *NAT2*4/*4* genotypes between diagnosed bladder cancer cases and non-diseased members with APG≥II and APG<II within each stratification, no significant deviation of the homozygous slow *NAT2* genotype percentage could be found in any case (see Table 5). Only in the case of highly occupational exposed ever smokers a statistical significant decreased presentation of *NAT2*4/*4* was observed in diagnosed bladder cancer patients, compared either with subjects of APG≥II (6.7% vs. 34.6%, OR=0.14; 95% CI 0.02-1.20, p=0.045) or with the individuals of APG<II (6.7% vs. 36.4%, OR=0.13; 95% CI 0.02-1.07, p=0.032) (see Table 6).

Table 6. *NAT2* wild type (*NAT2 *4/*4*) in the highly exposed cohort members and the subgroups" 60 years and older" and "smokers", stratified for cytological findings

Population		***NAT2* genotype**		**Risk (genotype of corresponding APG≥II were taken as reference)**		**Risk (genotype of corresponding APG<II were taken as reference)**	
Subgroups		**4/*4*	Others genotypes	P	OR (95% CI)	P	OR (95% CI)
All the highly exposed cohort members	Benzidine-exposed occupational bladder cancer patients	4 (16.7%)	20 (83.3%)	0.137	0.41 (0.12-1.36)	0.137	0.43 (0.13-1.35)
	APG≥II	20 (32.8%)	41 (67.2%)	Ref.	Ref.	0.918	1.04 (0.53-2.05)
	APG<II	32 (32%)	68 (68%)	0.918	0.97 (0.41-1.90)	Ref.	Ref.
60 and older	Benzidine-exposed occupational bladder cancer patients	4 (17.4%)	19 (82.6%)	0.120	0.38 (0.11-1.32)	0.120	0.38 (0.11-1.32)
	APG≥II	16 (35.6%)	29 (64.4%)	Ref.	Ref.	0.998	0.99 (0.41-2.39)
	APG<II	15 (35.7%)	27 (64.3%)	0.998	1.01 (0.42-2.42)	Ref.	Ref.
Smoker	Benzidine-exposed occupational bladder cancer patients	1 (6.7%)	14 (93.3%)	0.045	0.14 (0.02-1.20)	0.032	0.13 (0.02-1.07)
	APG≥II	9 (34.6%)	17 (65.4%)	Ref.	Ref.	0.889	0.93 (0.32-2.72)
	APG<II	12 (36.4%)	21 (63.6%)	0.889	1.08 (0.37-3.16)	Ref.	Ref.

APG: Averaged Papanicolaou's grading

5. DISCUSSION

It has been suggested that bladder cancer risk is not only related to the exposure to carcinogens but is also modulated by genetically based susceptibility factors. It was widely believed that the slow acetylating genotypes of N-acetyltransferase 2 (NAT2) were associated with an elevated risk of bladder cancer, especially for those who had been occupationally exposed to aromatic amines (28-29). However, Hayes *et al.* (30) and Carreon *et al.* (31) found that this was not the case in benzidine-exposed Chinese dyestuff workers. The percentage of the less metabolic active 'slow' NAT2 acetylating status depends on the ethnic origin. In Chinese and in Japanese populations, the percentage of slow NAT2 acetylators is between 10 and 20%, while in Caucasian populations it is between 50 and 65%. Slow acetylation status was suggested to play a protective role, at least in occupationally exposed Chinese people. At the time of the present study, there were only 29 surviving cancer patients in the investigated benzidine-exposed cohort. A relationship of bladder cancer risk and 17 years average results of Papanicolaou's grading was analyzed.

The data displayed no overall increase of homozygous slow *NAT2* genotypes in the former benzidine-exposed bladder cancer patients, compared with non-diseased exposed cohort members. This is in line with the reports of Hayes *et al.* (30) and of Carreon *et al.* (31) that *NAT2* slow acetylating genotypes is not a risk factor for bladder cancer related with benzidine-only exposure as well as no influence on the higher grade of pre-malignant cytological alterations of exfoliated urothelial cells in the case of occupational benzidine exposure was observed. However, present data fail to provide a convincing proof for the hypothesis of Carreon *et al.* (31) that *NAT2* slow acetylating genotypes would serve as a protective factor in the case of benzidine exposure.

A significantly decreased frequency of the homozygous wild genotype *NAT2*4/*4* (corresponding to rapid acetylating phenotype) in benzidine- exposed bladder cancer patients was observed in the present investigation. This finding is in line with a protective role of NAT2 rapid acetylators in formerly benzidine-exposed Chinese individuals. However, one should be cautious to postulate that *NAT2*4/*4* genotype might play a protective role in the development of bladder cancer at least in the case of occupational exposure of Chinese individuals to benzidine, since some *in vitro* data indicated that it is unlikely that NAT2 is involved in the biotransformation of benzidine (36-37).

To understand the results of this study, several aspects should be taken into account:

(1) The different exposure scenario: benzidine is an aromatic diamine. The metabolic pathways of benzidine are different from that of aromatic monoamines (38). The subjects' exposure description in the fundamental study of Cartwright *et al.* (28) included benzidine and some other aromatic amines, such as 2-naphthylamine. The study on workers at a Leverkusen-based plant also presents a high percentage of slow NAT2 acetylators, which comes close to the percentage reported by Cartwright *et al.* In contrast to the workers investigated by Cartwright *et al.*, the Leverkusen workers were exposed to benzidine (and possibly some other aromatic amines at the same time), but not to 2-naphthylamine (29). In the present study, all cohort members were definitely exposed to benzidine.

(2) The preferential substrate spectrum of NAT2: Human liver slices incubated with 3H-benzidine revealed acetyl-benzidine (8.8 % ± 3.6 %), diacetyl-benzidine (73 % ± 2.5 %), but no unmetabolized benzidine (36). Nevertheless, other findings of Zenser *et al.* (36) indicate benzidine is not a preferential substrate for NAT2 in the

human liver. When a liver slice was simultaneously incubated with sulfamethazine (SMZ) (specific substrate of NAT2) and benzidine, benzidine metabolism was not influenced. Data including *NAT2* and *NAT1* genotypes remained inconclusive, possible due to the limited number of 8 investigated liver samples. Nevertheless, these findings indicate that, at least regarding benzidine, NAT2 does not represent the acetylation capacity of an individual alone. More work is required to elucidate the physiological and biochemical mechanisms both for elevated bladder cancer risk, as observed in many studies in Caucasians, and for a suggested protective role of slow NAT2 genotypes, as observed in studies in Chinese.

(3) The complexity of benzidine metabolic pathways in the human body: Benzidine has complicated metabolic pathways in humans. Besides N-acetylation, benzidine and its metabolites can be glucuronidated, catalysed by UDP-glucuronyltransferases (39-40), and hydroxylated by CYP450 1A family members in the liver (41). In the bladder, benzidine can also be transformed by prostaglandin H synthase (42). Polymorphisms of different enzyme coding genes may be also involved in bladder carcinogenesis of benzidine. Further work is required to elucidate the relationship between polymorphisms of other metabolic enzymes which are involved in the metabolism of benzidine and its metabolites.

(4) Different ethnic background: The studies of Cartwright *et al.* (28), Lewalter and Miksche (29) and of Golka *et al.* (43-44) were all conducted in Caucasians. In the Shanghai population, the most frequent genotypes are **4/*4* (31%), **4/*6A* (27%), and **4/*7B* (24%) (21). In contrast, common *NAT2* genotypes found in Caucasians are **5B/*6A* (25%), **5B/*5B* (16%), **4/*6A* (13%), and **4/*5B* (18%) (45), which may vary between different Caucasian study groups (46). Thus, the trichotomous spectrum of *NAT2* genotype in general Shanghai population is the following: rapid NAT2 acetylators 33.0%; intermediate rapid NAT2 acetylators 54.5%; slow NAT2 acetylators 12.5%, which is clearly different from that in Caucasians.

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