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Genetic polymorphisms of *N*-acetyltransferase 1 and 2 and risk of cigarette smoking-related bladder cancer

F-I Hsieh[1], Y-S Pu[2], H-D Chern[3], L-I Hsu[1], H-Y Chiou[4] and C-J Chen[1]

[1]Graduate Institute of Epidemiology, College of Public Health, 1 Jen-Ai Road Section 1, [2]Department of Oncology, [3]Institute of Pharmaceutical Sciences, College of Medicine, National Taiwan University, [4]Department of Public Health, School of Medicine, Taipei Medical College, Taipei, Taiwan

Summary Aromatic amines from cigarette smoking or occupational exposure, recognized risk factors for bladder cancer, are metabolized by *N*-acetyltransferases (NAT). This study examined the association of (NAT) 1 and 2 genotypes with the risk of smoking-related bladder cancer. A total of 74 pathologically confirmed bladder cancer patients and 184 controls were serially recruited from the National Taiwan University Hospital. History of cigarette smoking and other risk factors for bladder cancer was obtained through standardized questionnaire interview. Peripheral blood lymphocytes were collected from each subject and genotyped for NAT1 and NAT2 by DNA sequencing and polymerase chain reaction-restriction fragment length polymorphism methods. Allele frequency distributions of NAT1 and NAT2 were similar between cases and controls. There was a significant dose–response relationship between the risk of bladder cancer and the quantity and duration of cigarette smoking. The biological gradients were significant among subjects carrying NAT1*10 allele or NAT2 slow acetylators, but not among NAT2 rapid acetylators without NAT1*10 allele. The results are consistent with the hypothesis that NAT1 and NAT2 might modulate the susceptibility to bladder cancer associated with cigarette smoking. © 1999 Cancer Research Campaign

Keywords: *N*-acetyltransferase 1; *N*-acetyltransferase 2; cigarette smoking; bladder cancer

Cigarette smoking is an important risk factor for bladder cancer (Cluade et al, 1986; Jensen et al, 1987; Augustine et al, 1988; Schairer et al, 1988; Burch et al, 1989; Lopez-Abente et al, 1991). Cigarette smoke includes 2-naphthylamine and 4-aminobiphenyl, which are recognized as human and animal carcinogens (International Agency for Research on Cancer, 1986).

The *N*-acetyltransferase (NAT) has two isozymes, NAT1 and NAT2. Each gene is located on chromosome 8 and has an open reading frame of 870-base pair. The enzyme activities of NAT1 and NAT2 are polymorphic (Grant et al, 1992; Weber and Vatsis, 1993; Bell et al, 1995*a*). NAT1 gene has sequence polymorphism in the 3′ untranslated region, the known alleles include NAT1*4 (wild-type), NAT1*10, NAT1*11 and NAT1*3 (Vatsis and Weber, 1993). NAT2 gene has a number of point mutations, which result in decreased NAT activity. The four mutations are NAT2*5 (C at nucleotide 341), NAT2*6 (A at nucleotide 590), NAT2*7 (A at nucleotide 857) and NAT2*14 (A at nucleotide 191) (Vatsis et al, 1995). NAT1*10 allele may increase enzyme activity and two NAT2 mutant alleles may decrease enzyme activity (Hickman and Sim, 1991; Vineis et al, 1994; Bell et al, 1995*a*). Both NAT1 and NAT2 enzymes catalyse the *N*-acetylation of aromatic amine which is considered a detoxifying process. NAT1 also catalyses the *O*-acetylation forming *N*-hydroxyarylamines and converts arylhydroxamic acids into mutagenic acetoxy esters by *N*,*O*-acetyltransferase reaction (Hein et al, 1992; Bartsch et al, 1993; Skipper and Tannenbaum, 1994).

While NAT2 slow acetylators were found to have an increased risk of bladder cancer among cigarette smokers (Risch et al, 1995; Brockmoller et al, 1996), there were inconsistent findings regarding the effect of NAT1 genetic polymorphism on bladder cancer (Okkels et al, 1997; Taylor et al, 1998). The specific aim of this study is to examine the effects of NAT1 and NAT2 on cigarette smoking-related bladder cancer through a case control comparison design.

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Correspondence to: C-J Chen

SUBJECTS AND METHODS

Study subjects

In this case control study, 74 patients affected with urinary bladder cancer were serially recruited from National Taiwan University Hospital. All of them were diagnosed histologically to be affected with transitional cell carcinoma. A total of 184 control subjects within the same age range of cases were recruited from health examination clinic (77.8%) and urology clinic (22.2%) in the same hospital. They were not affected with any cancer. Subjects who had lived in the arseniasis-endemic area were excluded from this study. The proportion of males was 78.4% for cases and 77.7% for controls. Cases and controls had similar frequency for distribution of age. There were 44.6% cases and 54.3% controls with an age below 65 years old. Because there were insufficient male elderly subjects to be selected as controls from the health examination clinic, several male controls were recruited from the urology clinic among patients with benign prostatic hypertrophy. Benign prostatic hypertrophy is a very common condition among elderly males in Taiwan, and no association between NAT and benign prostatic hypertrophy has ever been documented.

Standardized personal interview based on a structured questionnaire was carried out to collect information on risk factors, including sociodemographic characteristics, residential and occupational history, habits of cigarette smoking and consumption of

alcohol, tea and coffee, dietary habits, personal and family history of urinary disease and cancers. Duration and quantity of cigarette smoking was inquired in detail. Subjects who had smoked cigarettes more than 3 days a week for more than 6 months were classified as cigarette smokers and the others as non-smokers. A peripheral blood sample was collected from each subject using disposable vacuum syringe containing heparin. DNA was extracted from peripheral lymphocytes by Genomix DNA extraction kit (Talent, Follatoio, Italy) resuspended in deionized distilled water, and stored at –20°C until genotyping.

Genotypes of NAT1 and NAT2

NAT1 was amplified by polymerase chain reaction (PCR) using the primers N1-OA (5′-GCTCACCAGTTATCAACTGAC) and N1-OB (5′-AACCAACATTAAAAGCTTTCT) and resulted in a 233-base pair amplificate. The PCR mixture was composed of 1000–2000 ng DNA, 20 pmole of each NAT1 primers, 1 U *Taq* polymerase (Takara Taq, Takara Shuzo, Japan), 5 µl 10 × PCR buffer (100 mM Tris–HCl (pH 8.3), 500 mM potassium chloride (KCl), 15 mM magnesium chloride ($MgCl_2$)) and 4 µl 2.5 mM deoxynucleoside triphosphates in a final volume of 50 µl. The reaction mixture was placed for 5 min at 94°C, and then subjected to 30 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 45 s. This was followed by a final step at 72°C for 10 min. Negative control was included in each set of PCR analyses. The PCR products were electrophoresed in 1.5% agarose gel (FMC, Rockland, ME, USA) and then extracted the DNA by QIAquick gel extraction kit (QIAGEN, Germany). The product was processed with a Taq-dye terminator cycle sequencing ready reaction kit (Applied Biosystems Inc., Foster City, CA, USA). The fragments were then analysed with an Applied Biosystems 373A automated sequencer with a denaturing 6% polyacrylamide gel.

For NAT2, a PCR was carried out with PCR primers N5 (5′-GGAACAAATTGGACTTGG) and N4 (5′-TCTAGCATGAATCACTCTGC). The PCR mixture was composed of 1000–2000 ng DNA, 50 pmol of each primer, 5 U *Taq* polymerase (Takara Taq, Takara Shuzo, Japan), 5 µl 10 × PCR buffer 100 mM Tris–HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$ and 4 µl 2.5 mM deoxynucleoside triphosphates in a final volume of 50 µl. It was denatured at 94°C for 5 min and subjected to 35 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 90 s. A final 72°C extension for 10 min was performed. The whole intronless NAT2 gene was resulted in a 1093-base pair amplificate. It was then digested with 5 U *Kpn*I (AGS Gmbh, Germany) for NAT2*5 fragment, 10 U *Bam*HI (AGS Gmbh, Germany) for NAT2*7 fragment and 5 U *Alu*I/*Msp*I (Boehringer Mannheim, Germany) for NAT2*14 fragment at 37°C overnight, respectively, and 5 U *Taq*I (Boehringer Mannheim, Germany) for NAT2*6 at 65°C overnight. The NAT2*5, NAT2*7 and NAT2*14 fragments were separated in 2% agarose gel, and NAT2*6 fragments in 3% agarose gel. Individuals with two mutant alleles including NAT2*5, NAT2*6, NAT2*7 or NAT2*14 (Vatsis et al, 1995) were classified as slow acetylators and the others were rapid acetylators.

Statistical analysis

The differences in frequency distributions of bladder cancer risk factors between cases and controls were tested for their statistical significance by χ^2 tests. In the univariate analysis, odds ratios (OR) with a 95% confidence interval (CI) for each risk factor were calculated after the adjustment for age (continuous variable), sex and educational level (categorized variable) through logistic regression analysis. Cases had a slightly older mean age at recruitment, and lower educational levels than controls.

RESULTS

While fewer cases (10%) than controls (20%) were habitual coffee drinkers, there was no significant association between coffee drinking and bladder cancer after adjustment for age, sex and educational level (OR = 0.6, 95% CI = 0.2–1.4). The proportions of habitual alcohol drinking were 30% for cases and 24% for controls, a multivariate-adjusted OR of 1.1 (95% CI 0.6–2.2). Forty-five per cent cases and 48% controls were tea drinkers with a multivariate-adjusted OR of 0.9 (95% CI 0.5–1.7). Few study subjects had well documented occupational exposures related to bladder cancer. Only one case and one control were engaged in printing and painting industry, and three cases and two controls were professional drivers. The consumption of coffee, alcohol and tea, as well as the above occupational exposures, were not considered as confounding factors.

Table 1 compares the cigarette smoking habit between bladder cancer cases and healthy controls. Cases had a higher percentage of cigarette smokers than controls ($0.05 < P < 0.10$) showing an OR of developing bladder cancer of 1.90 (95% CI 0.99–3.64) after

Table 1 Comparison of cigarette smoking habit between bladder cancer cases and healthy controls

		Cases		Controls		Adjusted odds ratio[a]
Cigarette smoking	**Group**	**No.**	**(%)**	**No.**	**(%)**	**(95% CI)**
Habit	No	35	(47.3)	119	(64.7)	1.00 (referent)
	Yes	39	(52.7)	65	(35.3)	1.91 (0.99–3.64)[d]
Duration (year)[b]	0	35	(49.3)	119	(66.5)	1.00 (referent)
	<33	12	(16.9)	27	(15.1)	1.41 (0.58–3.42)
	≥33	24	(33.8)	33	(18.4)	2.52 (1.16–5.50)[c]
Quantity (packs per day)[b]	0	35	(47.9)	119	(65.7)	1.00 (referent)
	<1	18	(24.7)	36	(19.9)	1.76 (0.82–3.78)
	≥1	20	(27.4)	26	(14.4)	2.35 (1.05–5.24)[c]

[a]Adjustment for age, sex and educational level through logistic regression analysis. [b]Duration and/or quantity of cigarette consumption were not available for three cases and six controls [c]$P < 0.05$ bases on the significance test of the odds ratio. [d]$0.05 < P < 0.10$ bases on the significance test of the odds ratio. [e]$P < 0.05$ bases on the trend test. CI: confidence interval.

Table 2 Comparison of NAT 1 and NAT 2 allele and genotype frequency between cases of bladder cancer and controls

		Cases		Controls		
Gene	**Allele/Genotype**	**No.**	**(%)**	**No.**	**(%)**	***P*-value[a]**
NAT1[b]	*3	4	(3.1)	11	(3.2)	0.92
	*4	75	(57.7)	186	(54.4)	
	*10	50	(38.5)	143	(41.8)	
	*11	1	(0.7)	2	(0.6)	
NAT2[b]	NAT2*4 (wild)	85	(58.2)	192	(52.6)	0.70
	NAT2*5	6	(4.1)	22	(6.0)	
	NAT2*6	33	(22.6)	99	(27.0)	
	NAT2*7	21	(14.4)	51	(13.9)	
	NAT2*14	1	(0.7)	2	(0.5)	
NAT1	Without *10	25	(38.5)	60	(35.1)	0.63
	With *10	40	(61.5)	111	(64.9)	
NAT2	Slow acetylator	15	(20.6)	44	(24.0)	0.55
	Rapid acetylator	58	(79.4)	139	(76.0)	
NAT1/NAT2	Without *10/slow	6	(9.4)	18	(10.6)	0.53
	Without *10/rapid	18	(28.1)	42	(24.7)	
	With *10/slow	5	(7.8)	25	(14.7)	
	With *10/rapid	35	(54.7)	85	(50.0)	

[a]Based on χ^2 test. [b]NAT1 genotypes were not available for nine cases and 13 controls, while NAT2 genotypes were not available for one case and one control.

Table 3 Association between cigarette smoking and bladder cancer risk stratified by genotypes of NAT1 and NAT2

		Adjusted odds ratio (95% confidence interval)[a]	
Cigarette smoking	**Group**	**NAT2 rapid acetylator Without NAT1 *10**	**With NAT1 *10 or NAT2 slow acetylator**
Habit	No	1.00 (referent)	1.00 (referent)
	Yes	2.07 (0.32–13.30)	2.34 (1.03–5.31)[b]
Duration (year)	0	1.00 (referent)	1.00 (referent)
	<33	0.55 (0.04–7.83)	1.78 (0.58–5.45)[b]
	≥33	4.33 (0.56–33.41)	3.08 (1.14–8.32)[b,c]
Quantity (pack/day)	0	1.00 (referent)	1.00 (referent)
	<1	2.65 (0.34–20.70)	2.13 (0.82–5.53)
	≥1	1.49 (0.16–13.93)	3.22 (1.17–8.86)[b,c]

[a]Adjustment for age, sex and educational level through logistic regression analysis. [b]$P < 0.05$ based on the significance test of the odds ratio. [c]$P < 0.05$ based on the trend test.

adjustment for age, sex and educational level. There were significant dose–response relationships between the risk of bladder cancer and the duration and quantity of cigarette smoking.

The frequency distributions of NAT1 and NAT2 alleles and genotypes in cases and controls are compared in Table 2. Cases and controls had similar allele frequency distributions of NAT1 and NAT2. A total of 61.5% cases and 64.9% controls had one or two alleles of NAT1*10; 20.6% cases and 24.0% controls were NAT2 slow acetylators. The genotype frequency distributions of NAT1 and NAT2 were alike in cases and controls. The combined frequency distribution of NAT1 and NAT2 was also similar in cases and controls.

As shown in Table 3, the OR of developing bladder cancer for cigarette smoking was further analysed by stratifying study subjects according to NAT1 and NAT2 genotypes. Among cases, those subjects carrying NAT1*10 allele and NAT2 slow acetylators had an increased bladder cancer risk associated with cigarette smoking, they were classified as one group to be compared with cases with NAT2 rapid acetylators and without NAT1*10 allele as another group. A significant association between bladder cancer and cigarette smoking was observed among subjects carrying NAT1*10 allele or NAT2 slow acetylators showing an OR of 2.34 (95% CI 1.03–5.31), but not among NAT2 rapid acetylators without NAT1*10 allele (OR 2.07, 95% CI 0.32–13.30). The dose–response relationships between the risk of bladder cancer and the quantity and duration of cigarette smoking were also statistically significant among those who had NAT1*10 allele or NAT2 slow acetylators, but not for cases with NAT2 rapid acetylators but without NAT1*10 allele.

DISCUSSION

As in previous studies of cigarette smoking and bladder cancer, we also observed an increased risk of bladder cancer among cigarette smokers in this study. The metabolism of carcinogenic arylamines in tobacco smoke is mediated by enzymes including NAT1 and NAT2. Both NAT1 and NAT2 are genotypically and phenotypically polymorphic with variable genotype frequencies in

different ethnic groups. The allele frequency of NAT1*10 in controls of this study was 41.8%, which is significantly higher ($\chi^2 = 4.00$, $P < 0.05$) than the 30.0% observed in Caucasians (Bell et al, 1995*b*). The frequency of NAT2 slow acetylator in controls of this study was 24.0%, which is similar to that observed among Chinese in Hong Kong (27.0%) (Lin et al, 1993) and lower than that among American Caucasians (55.0%) (Bell et al, 1993). The percentage of NAT2 slow acetylator is significantly lower in Taiwanese than in American Caucasians ($\chi^2 = 20.14$, $P < 0.001$).

NAT2 slow acetylators were found to have an increased risk of bladder cancer among cigarette smokers (Risch et al, 1995; Brockmoller et al, 1996). The NAT1*10 allele alone was reported to be a risk factor in one study (Taylor et al, 1998), but not in another (Okkels et al, 1997). In this study, the genetic polymorphism of neither NAT1 nor NAT2 was significantly associated with the development of bladder cancer. However, the significant dose–response relationships between cigarette smoking and bladder cancer were observed in this study among those with NAT1*10 or NAT2 slow acetylators, but not among NAT2 rapid acetylators without NAT1*10 allele.

Some aromatic amines are metabolically inactivated through acetylation in the liver mainly by NAT2 (Kadlubar and Badawi, 1995). Rapid NAT2 acetylators are considered to have a higher level of non-toxic metabolites and a lower risk of bladder cancer than slow NAT2 acetylators. Some other arylamines were catalysed by cytochrome P450 1A2 in the liver, and the *N*-hydroxy arylamine metabolites can then enter the blood stream. The *N*-hydroxy arylamine metabolites are further metabolized into highly electrophilic *N*-acetoxy derivatives by NAT1, which is expressed mainly in urinary bladder epithelium (Kirlin et al, 1989; Frederickson et al, 1994). This will lead to an elevated level of arylamine-DNA adducts and an increased risk of bladder cancer. The dose–response relationship between cigarette smoking and bladder cancer is thus significant among cigarette smokers who have NAT1*10 allele and/or cigarette-smoking NAT2 slow acetylators.

In addition to NAT1 and NAT2, the metabolism of arylamines also involves a number of other enzymes including sulphotransferases (Kato and Yamazoe, 1994), prostaglandin H synthase (Smith et al, 1992), cytochrome P450 1A2 (Butler et al, 1989; Fleming et al, 1994) and glucuronyltransferases. These enzymes may also contribute to the metabolic steps of arylamines relevant to the development of bladder cancer. Further examination of effects of these enzymes on the cigarette smoking-related bladder cancer in humans is noteworthy.

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