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NAT2 Gene Polymorphism in Bladder Cancer: A Study from North India

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ABSTRACT The relationship between smoking and bladder cancer risk and whether such effect is modified by the variation in NAT2 genotypes is investigated. This case control study was undertaken over a period of 19 months and included 106 bladder cancer patients and 110 controls. The NAT2 genotypes were identified by PCR-RFLP method in peripheral blood DNA samples. Genotype frequencies and the association of the genotypes among patients and controls group were assessed by c2 test and Fisher exact test. The NAT2 phenotypes were not significant in bladder cancer patients (OR=1.18, 95% CI: 0.69-2.03, *P*-value=0.583) alone or in combination with tobacco users (OR=0.84, 95% CI: 0.328-2.125, *P*-value=0.813) when compared with controls. These observations suggest that the NAT2 fast /or slow acetylators genotype is not associated with the risk of developing bladder cancer. Our data further demonstrated that bladder cancer patients who were tobacco users were not susceptible to the risk of developing bladder cancer as no significant association could be established.

INTRODUCTION

N-acetyltransferase 2 (NAT2) is one of the phase II enzyme that participate in the bioconversion of heterocyclic arylamines into electrophilic nitrenium ions, which are important ultimate carcinogens that are directly implicated in tumor initiation process (Hein et al. 1988; Hein et al. 1993). It expresses at high level in liver, and encoded by a polymorphic gene presenting several nucleotide substitutions. Consequently the presence of the different alleles in each individual genome produces a broad range of metabolic phenotypes that vary from fully active rapid metabolizers to the less active alleles of slow metabolizers (Vatsis et al. 1995).

Enzymatic activation and detoxification of carcinogens is a major principle in chemical carcinogenesis (Ames et al. 1973; Miller et al. 1978). Many chemical and dietary carcinogens, such as nitrosoamines and arylamines derived from dietary fat as well as tobacco products, acquire bio-activation and deactivation by NATs enzymes. N-acetyltransferase-2 catalyze the activation and for deactivation of a wide variety of aromatic amines, heterocyclic amines, and hydrazine drugs. This suggests that polymorphism of genes encoding metabolic enzymes may represent potential risk factors (Hein et al.1993; Caporaso et al. 1995; Cascorbi et al. 1999). Recent molecular epidemiological studies have analyzed the relationship between various metabolic enzymes, such as N-acetyltransferases (NATs), cytochrome P450 (CYP) and glutathione S-transferases (GSTs) in bladder cancer to determine as biomarkers (Silverman et al. 1999). In humans, hereditary differences in N-acetylation activity have lead to phenotypic classification of individual as rapid or slow acetylators. It has been reported that genetically variable NATs, CYP P450 and GSTs are involved in the metabolism of drugs, carcinogens and natural products; and therefore act as candidate genes for cancer susceptibility (Caporaso et al. 1995; Guengerich et al. 1995). It is known that human express two forms of N-acetyltransferases: NAT1 and NAT2; both genes are polymorphic. Presently 24 NAT1 and more than 26 NAT2 polymorphism have been identified in humans. A recent review describes the nucleotide and amino-acid changes associated with various alleles and deduced phenotype from genotype. It also summarized results of molecular epidemiologic studies assessing the association of NAT1 and NAT2 genotypes with cancer risk of bladder, colon, breast, lung, head and neck and prostate (Hein et al. 2000; Turesky et al. 1991). Although, some of these studies suggest that NAT1 and NAT2 polymorphisms may or may not

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influence the susceptibility with these cancers. Recent studies have not supported a relationship between NAT1 genotypes and N-acetylation activity (Grant et al. 1997). Furthermore, NAT2 has been reported to exhibit a polymorphism, resulting in the potential expression of four mutant alleles (M1, M2, M3 & M4), which can be identified by RFLP analysis following NAT2 PCR. NAT2 activity is predicted from the detected combination of these NAT2 alleles. Presence of atleast one wild type alleles results in rapid acetylators where as the carriage of two mutant alleles are categorized as a slow acetylator (Inatomi et al.1999). The slow allele is predominant in about 90% Arab population, about 40-60% in Caucasians including Indians, 5-25% in East Asian (Lin et al. 1994; Whoolhouse et al.1997; Xie et al.1997) and 74% in South Indians (Anitha et al.2003). It has been reported that slow acetylators may be at increased risk of bladder and prostate cancer when exposed to environmental arylamines carcinogens, due to their slower inactivation.

The present study was undertaken to examine NAT2 gene polymorphism and to evaluate whether, fast or slow acetylator phenotype is associated with increased risk of bladder cancer when compared with the controls. We also endeavor to examine the association between NAT2 polymorphism with clinical stage and pathological grade of bladder cancer.

MATERIALS AND METHODS

Subjects: The study group consisted of 101 bladder cancer patients (all were transitional cell carcinoma; of which 32% were grade 1, 20 % were grade 2; and 48 % were grade 3 tumors)with mean age (57.3±13.4) and 110 controls with mean age (56.7±13.9. There were no statistical differences in age of patient and control group. Blood sample were obtained between December 2001 to December 2003 from patient and control group. This study was approved by ethical committee of health care and research, of SGPGI under the guidelines of ministry of education, culture and science and technology. The diagnosis of bladder cancer patients was confirmed histopathologically. The control group consisted of age matched 110 normal healthy individuals. Serological (prostate serum antigen), physical (digital rectal examination) and radiological examination were performed in all control individual in order to exclude the possibility of malignancy. The inclusion criteria for the controls were absence of prior history of cancer or pre-cancerous lesions. The consumption of tobacco in any form (cigarette/ bidi (a kind of cigarette used in rural area by the villagers) smoking, chewing tobacco in beetle leaf or gutka etc.) in both groups (cases and controls) was noted through a detailed questionnaire.

PCR-RFLP and Alleles Genotyping: Genomic DNA was isolated from peripheral leucocytes by Proteinase -K digestion and phenol/chloroform method (Sambrook et al.1989) The NAT genotypes were determined using the PCR-RFLP as described previously (Hsieh et al, 1999). 1093 bp PCR product was generated by polymerase chain reaction using the following primer:
Forward 5'-TCTAGCATGAATCACTCTGC-3'
Reverse 5'- GGAACA AATTGG AC TTGG -3'.

Genomic DNA 200ng was added to a PCR mixture, containing 18.5 pmol of each primer, 200mm dNTP (Bangalore Genei, India), 1.5 unit of Taq polymerase (Bangalore Genei, India), and PCR buffer (Bangalore Genei, India) composed of 10mmol/ml Tris HCl Ph=8.4, 50mmol/ml KCl and 2.5 mol/ml $MgCl_{2)}$ in a total volume of 50µl. The thermal cycler PTC-100 for polymerase chain reaction was used. The reaction mixture was subjected to initial denaturation at 94ºC for 5 min, followed by 35 cycles of denaturation;(94ºC, 1 min), annealing (58.5ºC, 1 min) and extension (72ºC, 1 min). The final extension was done at 72ºC for 10 min. Following PCR, 7µl of PCR products were taken in four different tube and digested with four separate enzymes including Kpn1 for NAT2*5 (M1) allele, at 37ºC for 2hrs; Taq1 for NAT2*6(M2) allele, at 56ºC for 4hrs; BamH1 for NAT2*7 (M3) allele at 37ºC for 2hrs; and Msp1/Alu1 for NAT2*14 (M4) allele at 37ºC for 2hrs.Digested products were analyzed on 2% agarose gel for M1, M3, M4 alleles and 3% agarose gels for M2 allele. If the allele could not be identified as M1, M2, M3 or M4 after digestion, then the remaining allele were identified as a NAT2*4 (wild type) allele; since the wild type allele possess the entire restriction site.

Statistical Analysis: Statistical analysis was done using SPSS software programme. Difference in genotype prevalence and association between case and control group were assessed by Chi square and Fisher Exact tests. Odds ratios (OR) and its 95% confidence interval (CI) were obtained by summarizing data over two habit

strata (tobacco users/ non-users). Multivariate analysis, correlation coefficient, odds ratios, *P*-value (two-sided tests) and 95% CI were used to describe the strength of association.

RESULTS

The frequency of NAT2 slow or fast acetylator genotypes was not significant in bladder cancer patients (OR=1.18, 95% CI: 0.69-2.03, *P*-value=0.583) in comparison to controls (Table 1). No significant association between slow phenotypes and tobacco users with bladder cancer was observed in our population (Table 2).

The clinical stage of tumor was categorized into two groups: T1 and T2 (T2+T3+T4). NAT2 slow and fast acetylator genotype was non significant in both the groups as compared to the controls (Table 3).

We also classified pathological findings into three groups: well differentiated (Grade1), moderately differentiated (Grade2) and poorly differentiated (Grade3). We observed that NAT2 slow and fast acetylator genotypes were also insignificant with all grades of tumor (Table 4).

Table1: Frequency of NAT2 phenotypes in bladder cancer patients and healthy controls

Patients	*NAT2 phenotype*		*Chi-square*	*P-value*	*OR (95% CI)*
	Slow -acetylators	*Fast- acetylators*			
Controls (N= 110)	59 (53.64%)	51 (46.36%)			1.0 (Reference)
Bladder cancer (N= 101)	51 (50.50%)	50 (49.50%)	0.36	0.583	1.18 (0.69-2.03)

Table 2: Risk of bladder cancer in combination of N-acetyltransferase-2 genotypes (NAT2) genotypes with tobacco users

Tobacco users	*Controls (N=110)*	*Ca- Bladder (N=101)*
Non-users		
Slow -phenotype	45(56.25)	28(48.28)
Fast-phenotype	35(43.75)	30(51.72)
P-value		0.391
OR (95% CI)	1.0(Ref.)	1.378 (0.669-2.715)
Tobacco-users		
Slow -phenotype	14(46.66)	22(51.16)
Fast-phenotype	16(53.34)	21(48.84)
P-value		0.813
OR (95% CI)	1.0(Ref)	0.835 (0.328-2.125)

DISCUSSION

The result presented demonstrates that NAT2 genotype show no relationship to bladder cancer risk when considered alone (OR=1.18, 95% CI: 0.69-2.03, *P*-value=0.583) or in combination with tobacco users (OR=0.84, 95% CI: 0.328-2.125, *P*-value=0.813) as compared to the controls. We also correlated our observation NAT2 genotype in bladder cancer with clinical stage and pathological grade. However no significant

Table 3: Frequency of N-acetyltransferase-2 genotypes (NAT2) of bladder cancer patients categorized by stage of disease

	NAT2 phenotype		*P-value*	*OR (95% CI)*
	Slow -acetylators	*Fast- acetylators*		
Controls (N= 110)	59(53.64%)	51(46.36%)		1.0 (Reference)
Bladder cancer (N= 101)				
T1 stage (n=61)	29(47.55%)	32 (52.45%)	0.523	0.78 (0.42-1.46)
T2+T3+T4 stage (40)	21(52.50%)	19(47.50%)	1.0	0.96 (0.46-1.97)

Table 4: Frequency of N-acetyltransferase-2 genotypes of bladder cancer patients based on grading of tumors

	NAT2 phenotype		*P-value*	*OR (95% CI)*
	Slow -acetylators	*Fast- acetylators*		
Controls (N= 110)	59(53.64%)	51(46.36%)		1.0 (Reference)
Bladder cancer (N= 101)				
G1grade (N= 40)	18 (45%)	22(55%)	0.363	0.71(0.34-1.46)
G2grade (N= 26)	14(53.85%)	12(46.15%)	1.0	1.01(0.43-2.38)
G3grade (N= 35)	18(51.43%)	17(48.57%)	0.848	0.91(0.43-1.96)

association was observed (p>0.59) Our findings concur with previous studies (Taylor et al.1998), who showed no relationship to bladder cancer risk when considered alone or in combination with smoking habit. But some studies have demonstrated that low activity is doubtless a risk factor for bladder cancer, particularly for those individuals who smoke or who are exposed to specific occupational hazards (Marcus et al.2000 a, b). According to the current theory of the role of N-acetyltransferases (NAT1 and, NAT2) in bladder cancer etiology, a decrease in aryl amine N-acetylation rates in the liver enforces N-hydroxylation mediated by CYP-4501A2, which in turn leads to increased concentrations of hydroxylamines in the urinary bladder (Lang et al.1991).

Polymorphisms of NAT and CYP enzymes that activate or detoxify carcinogens and mutagens play a crucial role in carcinogenesis. NAT2 enzyme has been shown to activate carcinogenic aromatic amines, which can arise from tobacco products or dietary intakes or environmental exposures (Caporaso et al. 1995). Human acetylation polymorphism influences both the metabolic activation (O-acetylation) and deactivation (N-acetylation) of aromatic amines via the polymorphic expression of NAT2. It has been hypothesized that the increased susceptibility to urinary bladder cancer for slow acetylators is associated with the decreased deactivation of aromatic amines in the liver, so that excess hydroxylated aromatic amines reach the bladder epithelium where they can induce further activation step (Brockmoller et al. 1996). The deactivation pathway could then be subjected to competetion with activation pathway. Whether NAT serves as an activating or deactivating enzyme depends on the final consequence of the competition of all these pathways, which is related to the polymorphism of CYP enzymes.This suggests that the association of metabolic enzyme gene polymorphisms with bladder cancer may differ according to the metabolic enzymes (NAT, CYP or GST) and ethnic population under study. Due to inadequacy in data on metabolic pathway of various aromatic amines, further studies are mandatory to address this discrepancy.

In conclusion, this study indicates that NAT2 genotype exhibits insignificant association with the risk of bladder cancer, either alone or with tobacco consumpti pathological grade / or clinical stage of disease.

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