

POLYMORPHISMS OF N-ACETYLTRANSFERASE 2, GLUTATHIONE S-TRANSFERASE MU AND THETA GENES AS RISK FACTORS OF BLADDER CANCER IN RELATION TO ASTHMA AND TUBERCULOSIS

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ABSTRACT

Purpose: We investigated whether the polymorphisms of N-acetyltransferase 2 (NAT2), glutathione S-transferase- μ (GSTM1) and glutathione S-transferase- θ (GSTT1) genes were risk factors of bladder cancer among Korean people in relation to other risk factors.

Materials and Methods: In this case-control investigation of 113 patients with primary bladder cancer and 221 control subjects, we compared the association of bladder cancer with genetic polymorphisms of NAT2, GSTM1 and GSTT1, demographic characteristics, smoking status, and medical histories in a molecular epidemiological way.

Results: The risk of bladder cancer was significantly increased in patients with a medical history of tuberculosis (OR 3.61, 95% CI 1.57 to 8.26) and bronchial asthma (OR 4.15, 95% CI 1.61 to 10.75), while smoking history turned out to be insignificant. GSTM1 null genotype was a significant risk factor of bladder cancer (OR 1.81, 95% CI 1.12 to 2.93). On the other hand, slow acetylator and GSTT1 genotypes were insignificant. Also, we could not find any association between GSTM1, GSTT1, slow acetylator genotypes and bladder cancer risk among smokers. The rapid acetylator, GSTM1 null and GSTT1 null genotypes with a medical history of asthma or tuberculosis combinations were significant risk factors in Korean subjects.

Conclusions: Among Korean subjects, GSTM1 null genotype was a significant risk factor for bladder cancer. The reason why bronchial asthma and tuberculosis are risk factors in Korean subjects is yet unknown, but a variety of factors, including enzyme activities for detoxification, medication for these diseases and immunological background might be involved.

KEY WORDS: bladder neoplasms, polymorphism, genetics, asthma, tuberculosis

Most environmental carcinogens are metabolized via complex enzymatic mechanisms involving activation and inactivation. In some of these metabolisms, genetic differences exist and these individual variations may modulate cancer risk.

N-Acetylation of aromatic amine procarcinogens by N-acetyltransferase 2 (NAT2) is generally considered to be a detoxifying mechanism, because it competes with N-oxidation by cytochrome P450 1A2 which produces reactive hydroxylamines that can be further metabolized to DNA-binding electrophiles.¹ A number of case-control studies have investigated the relationship between acetylator type and bladder cancer risk. In bladder cancer patients, slow acetylators are supposedly more prone to this neoplasm in western countries.¹ However, the risk of bladder cancer in individuals with the slow acetylator genotypes still remains controversial.²

Glutathione (GSH) and GSH-dependent enzymes are involved in cellular metabolisms and detoxification of cytotoxic and carcinogenic products. Accordingly, much interest is concentrated on the significance of polymorphism in the glutathione S-transferase (GST) supergene families, as these enzymes metabolize exogenous and endogenous molecules involved in cell-specific functions, such as proliferation and apoptosis.³ Thus, alleles associated with inappropriate detoxification appear promising candidates for cancer risk.⁴ Mu and theta class GST appear important in the detoxification of

products of oxidative stress, such as lipid hydroperoxides, alkenals and DNA hydroperoxides, as well as potential carcinogens, such as methyl halides and benzo(a)pyrene epoxides.⁵ The absence of GST- μ (GSTM1) activity is due to homozygous deletion of the gene. A similar polymorphism of the GSTT1 encoding the theta class enzymes was recently discovered.⁶ Previous studies have shown a possible link between the GSTM1 null phenotype and susceptibility to cancer.^{4,7} Less is known about the association between GSTT1 and cancer risk, but people with the GSTT1 null genotype show reduced ability to detoxify metabolites of 1,3-butadiene and ethylene oxide.^{6,8} Recently, Deakin et al⁹ and Chen et al¹⁰ showed that the GSTT1 null genotype was a possible risk modifier in the occurrence of cancer.

The cause of bladder cancer is not fully explained by single risk factor and many kinds of enzymes would be involved in the metabolism of carcinogens excreted in urine. In this study, we investigated whether the polymorphisms of NAT2, GSTM1 and GSTT1 genes were risk factors of bladder cancer among Korean people in relation to other risk factors in a molecular epidemiological way.

MATERIALS AND METHODS

A hospital-based, age and sex matched case-control investigation was carried out in 113 patients with primary bladder cancer hospitalized in the Department of Urology of Chungbuk National University Hospital, Cheongju, Korea, and 221 control subjects from 1997 thru 1998. Controls were recruited in the same department and included benign disease, mainly BPH and stress incontinence. Cases and controls were interviewed by a trained interviewer before therapy,

Accepted for publication January 19, 2000.

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with a standard questionnaire including a detailed history of cigarette smoking, dietary habits, drug use, medical histories and occupational status. Informed consent was given by each subject and 5 ml. of blood from each patient and control individual was sampled with 0.1 ml. EDTA (15%). DNA extraction was performed according to Lahiri and Nurnberger.¹¹

For the detection of 4 NAT2 alleles, described by Abe et al,¹² we synthesized 3 sets of allele-specific primers (table 1) on a DNA synthesizer (Applied Biosystems, CA). Primers were designed according to the known NAT2 sequence. The PCR mixture of sets 1 and 2 contained 100 ng. genomic DNA, 10 pmol. of each oligonucleotide primer, dNTPs (each at 20 mM), MgCl₂ (25 mM) and 0.25 unit *Taq* DNA polymerase in a total volume of 25 μ L. The PCR conditions of sets 1 and 2 were denaturation at 94C for 1 1/2 minutes, annealing at 57C 1 1/2 minutes and extension at 74C 1 minute. In set 3, 200 ng. genomic DNA, 0.25 unit *Taq* DNA polymerase, dNTPs (each at 10 mM) and 20 pmol. of each primer were applied to a total volume of 25 μ L. The PCR conditions were denaturation at 94C 15 seconds, annealing at 55C 30 seconds and extension at 74C 1 minute. After incubation of the mixture for 5 minutes at 94C for denaturation, 30 cycles of amplification were performed with final extension period of 5 minutes at 74C, using a DNA Thermal Cycler (Perkin-Elmer Cetus). Ten μ L. aliquots of PCR products were digested with restriction enzymes *Bam*H1, *Taq*I and *Asp*718 using the method described by Abe et al¹². The digested PCR products (5 μ L.) were electrophoresed on a 2% agarose gel or 9% polyacrylamide gel and stained with ethidium bromide.

A multiplex PCR method was used to detect the presence or absence of the GSTM1 and GSTT1 genes in genomic DNA samples, as described previously.^{4,6,10} In brief, the primers used in the PCR were sense (5'-GAACTCCCTGAAAAGCT-AAAGC-3') and anti-sense (5'-GTTGGGCTCAAATATACGG-TGG-3') for GSTM1; sense (5'-TTCCTTACTGGTCCTCACATC-TC-3') and anti-sense (5'-TCACCGGAT CAT- GGCCAGCA-3') for GSTT1; sense (5'-ACCCCCACTGAAAAGATGA-3') and anti-sense (5'-ATCT- TCAAACCT- CCATGATG-3') for beta-2-microglobulin (β_2 m). DNA (100 ng.) was amplified in a total volume of 25 μ L. containing 10 pmol. of each primer, 1 unit of *Taq* polymerase, 1.5 mM. MgCl₂ and PCR buffer. Following an initial denaturation step at 94C for 3 minutes, 35 cycles of amplification were carried out at 94C for 30 seconds, 62C for 30 seconds, and 74C for 30 seconds. The amplified products were electrophoresed on a 9% polyacrylamide gel. Both positive and negative samples were analyzed in each experiment, and GSTM1 and GSTT1 genotypes were not scored unless the PCR product from the internal reference gene (β_2 m) was evident.

The relative associations between cases and controls were assessed by calculating odds ratio (OR) from contingency tables. Adjusted OR and 95% confidence intervals (95% CI) were calculated by using multiple logistic analysis model controlling the effects of other factors including age and sex.

RESULTS

Table 2 outlines the risk of bladder cancer according to the

TABLE 1. Sequences of the amplification primers in the 5' to 3' orientation

Set	Primer	PCR Product (bp)*	Restriction Site
1	S ACAATACAGATCTGGTCGAG	393	BamH1
	A AGCATGAATCACTCTGCTTC		
2	S CTTAATTCTCATCTCCTGCC	117	TaqI.
	A TGTTGGAGACGCTGCAGGT		
3	S TGACGGCAGGAATTACATTGTC	559	Asp718
	A ACACAAGGGTTTATTTTGTTC		

S and A respectively indicate the sense and anti-sense primers.

* Size of the amplified fragments obtained with each pair of primers.

TABLE 2. Comparison of various factors and 95% CI between patient and control groups

	Patient	Control	OR	95% CI
Sex				
Male	93	184	1.00	—
Female	20	37	0.94	0.51–1.70
Drinking Hx				
No drinking	37	83	1.00	—
Stop drinking	25	32	1.75	0.91–3.36
Drinking	44	100	0.99	0.58–1.67
Smoking Hx				
No smoking	24	59	1.00	—
Smoking	88	161	1.34	0.78–2.31
Vasectomy Hx				
No	96	209	1.00	—
Yes	9	10	1.96	0.77–4.98
Thyroid disease Hx				
No	105	217	1.00	—
Yes	2	0	—	—
GI disease Hx				
No	89	171	1.00	—
Yes	19	31	1.18	0.63–2.20
Liver disease Hx				
No	105	215	1.00	—
Yes	1	4	0.51	0.06–4.63
GB disease Hx				
No	103	212	1.00	—
Yes	4	4	2.06	0.50–8.40
HT Hx				
No	92	190	1.00	—
Yes	15	19	1.63	0.79–3.34
CHD Hx				
No	101	205	1.00	—
Yes	5	8	1.27	0.40–3.98
DM Hx				
No	101	206	1.00	—
Yes	6	12	1.02	0.37–2.79
Intestinal polyp Hx				
No	105	218	1.00	—
Yes	0	0	—	—
Tbc Hx				
No	91	205	1.00	—
Yes	16	10	3.61	1.57–8.26
Bronchial asthma Hx				
No	93	208	1.00	—
Yes	13	7	4.15	1.61–10.75

Hx: history, GI: gastrointestinal GB: gall bladder, HT: hypertension, DM: diabetes mellitus, CHD: coronary heart disease, Tbc: tuberculosis. OR was adjusted for age and sex.

gender, smoking and medical history. Of the cases and controls, 78.6% and 73.2%, respectively, had a history of smoking over 400 cigarettes in their lifetime. Smoking history turned out to be insignificant as a risk factor of bladder cancer (OR 1.34, 95% CI 0.78 to 2.31) in Korean subjects, and occupation could not be tested because of the extremely small number of occupational histories related to the risk of bladder cancer. The risk of bladder cancer was significantly increased in patients with a medical history of tuberculosis and bronchial asthma, and the OR (95% CI) was 3.61 (1.57 to 8.26), and 4.15 (1.61 to 10.75), respectively.

The distribution of NAT2 genotypes in Koreans is shown in table 3. The electrophoretic pattern of PCR-based restriction fragment length polymorphism (RFLP) for NAT2 is shown in fig. 1. The NAT2 genotypes were categorized as homozygous mutant (slow), and heterozygous wild-type mutant and ho-

TABLE 3. NAT2 genotype between cases and controls

Genotype	No. of Cases	No. of Controls	Phenotype
1,1	58	95	Rapid
1,2	20	47	Rapid
1,3	22	48	Rapid
1,4	4	5	Rapid
2,2	0	1	Slow
2,3	5	12	Slow
3,3	3	11	Slow
	112	219	

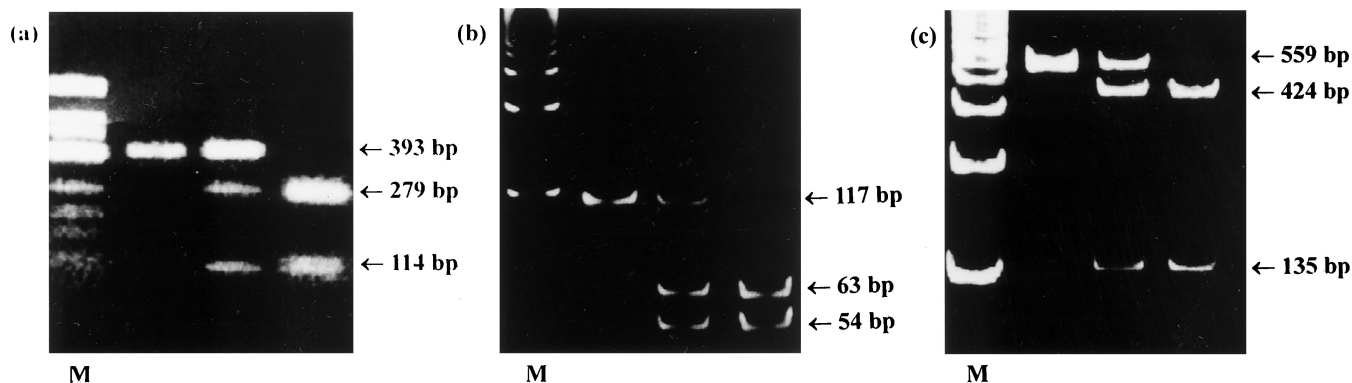


FIG. 1. Electrophoretic patterns of genotyping of NAT2 by PCR-based RFLP. PCR products were digested with *Bam*H1, *Taq*1 and *Asp*718. A, DNA lacking *Bam*H1 site gives 393 bp band and DNA possessing *Bam*H1 site gives 279 and 114 bp bands. M is size marker (ϕ x174/*Hinf*I). B, DNA lacking *Taq*1 site gives 117 bp band and DNA possessing *Taq*1 site gives 63 and 54 bp bands. M is size marker (123 bp DNA ladder). C, DNA lacking *Asp*718 site gives 559 bp band and DNA possessing *Asp*718 site gives 424 and 135 bp bands. M is size marker (123 bp DNA ladder).

mozygous of the case and the control groups (rapid). The NAT2 genotype frequencies of the case group (7.1% slow and 92.9% rapid) were not significantly different from the control group (11.0% slow and 89.0% rapid) in this analysis ($X^2 = 3.12$, $p > 0.05$). Also, the NAT2 allele frequencies did not significantly differ between the case and the control groups (table 4). Figure 2 shows 9% polyacrylamide gel demonstrating multiplex PCR genotyping of genomic DNA samples for the detection of GSTM1 and GSTT1 gene deletion. The GSTM1 null frequencies were 69.6% for the cases and 55.9% for the controls (OR 1.81, 95% CI 1.12 to 2.93), showing a significant risk of bladder cancer. The GSTT1 null type was 42.0% and 45.9% in the bladder cancer patients and control subjects, respectively. The OR (95% CI) of GSTT1 null type was 0.85 (0.54 to 1.35) with no statistical significance (table 5).

Bladder cancer risk was estimated for Korean GSTM1 and GSTT1 null subjects with their smoking history. The OR (95% CI) with smoking history was 2.31 (0.83 to 6.45) in GSTM1 null individuals, and 1.87 (0.78 to 4.49) in GSTT1 null subjects. The OR (95% CI) in the case-control pairs without smoking history were 0.85 (0.30 to 2.44) for GSTM1 null type and 0.91 (0.32 to 2.58) for GSTT1 null type. GSTM1 and GSTT1 genotypes were unrelated to smoking status in bladder cancer. Also, we could not find a significant association between the slow acetylators genotypes and bladder cancer risk among smokers (OR 6.79, 95% CI 0.67 to 68.82). Compared with slow acetylators genotypes without asthma history, the rapid acetylators genotypes and asthma history combinations were significant risk factors of bladder cancer (OR 6.75, 95% CI 1.80 to 25.41). Compared with the combinations of slow acetylators genotypes and no tuberculosis history, the OR of carrying the combined rapid acetylators genotypes with tuberculosis history was 6.46 and the 95% CI was 1.88 to 22.22. Analysis of GSTM1 and GSTT1 was extended to include medical history. When the cases and controls with GSTM1- or GSTT1-positive genotype without asthma or tuberculosis history were used as a reference group, the GSTM1 null genotype and asthma or tuberculosis history combinations were significant risk factors for bladder cancer, in which the respective OR (95% CI) was 9.25 (2.26 to 37.81) and 7.65 (2.15 to 27.21). Also, a significantly increased risk was associated with the GSTT1 null genotype in patients with asthma history (OR 19.19, 95% CI 2.30 to 160.07) and with tuberculosis history (OR 4.00, 95% CI 1.20 to 13.31). On the

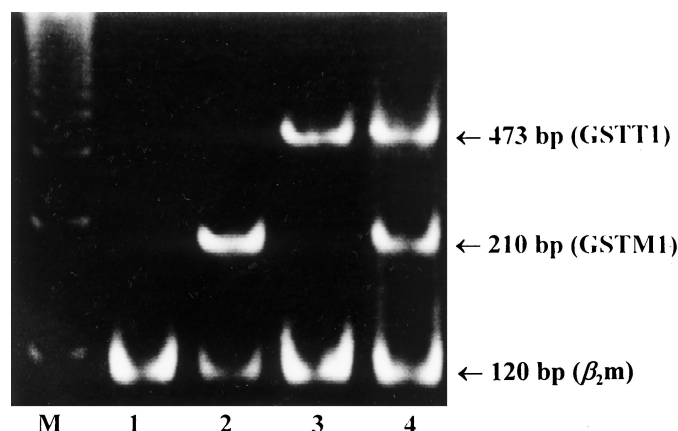


FIG. 2. 9% polyacrylamide gel demonstrating multiplex PCR genotyping of genomic DNA samples for detection of GSTM1 and GSTT1 gene deletion. Absence of 473 bp band indicates GSTT1 null genotype; absence of 210 bp band indicates GSTM1 null genotype; β_m was coamplified in all samples. Lane M, size marker (123 bp DNA ladder); lane 1, GST1(-)/GST(-); lane 2, GSTT1(-)/GSTM1(+); lane 3, GSTT1(+)/GSTM1(-); lane 4, GSTT1(+)/GSTM1(+).

TABLE 5. OR for association of GSTM1 and GSTT1 genotypes to bladder cancer

Genotype	Cases	Controls	OR	95% CI
GSTM1				
Positive	34	97	1.00	
Null	78	123	1.81	1.12-2.93
GSTT1				
Positive	65	119	1.00	
Null	47	101	0.85	0.54-1.35

OR was adjusted for age and sex.

other hand, we did not find an increased risk of the GSTM1 and GSTT1 null genotypes in bladder cancer patients in relation to other medical history.

In multiple logistic analysis controlling the effects of other risk factors, GSTM1 null type (OR 1.80, 95% CI 1.07 to 3.05), and history of tuberculosis (OR 2.99, 95% CI 1.22 to 7.32) and of bronchial asthma (OR 3.38, 95% CI 1.24 to 9.22) were significant risk factors of bladder cancer, while slow acetylators and GSTT1 null type were not.

DISCUSSION

Investigators of chemical carcinogenesis have long known that polymorphism exists in the ability of humans to acetylate drugs or chemicals. Slow and rapid acetylators were shown to have variable susceptibility to bladder cancer. Cartwright et al¹

TABLE 4. NAT2 allele frequency of cases and controls

Allele	Cases (%)	Controls (%)
1	162 (72.3%)	290 (66.2%)
2	25 (11.2%)	61 (13.9%)
3	33 (14.7%)	82 (18.7%)
4	4 (1.8%)	5 (1.1%)

showed a 17-fold increase in the risk of bladder cancer among slow acetylators, who made up to 96% of patients exposed to aromatic amines. Several comparative studies among different ethnic groups observed less than 20% and more than 55% of slow acetylators among Asians and whites. Hein¹³ combined data from 12 studies and noted a statistically significant relative risk for bladder cancer of 1.5 in slow versus rapid acetylators (95% CI 1.2 to 1.7). Among Korean subjects, we determined in the present study that 7.1% of bladder cancer patients and 11.0% of controls had slow acetylator genotypes. Yu et al¹⁴ observed that a difference in the prevalence of slow acetylators (the high-risk phenotype) among the three ethnic groups (whites, blacks, and Asians) closely paralleled their varying incidence of bladder cancer. It might be inferred that the lower frequencies of slow acetylator genotypes in Koreans relative to the westerners could result in less incidence of bladder cancer. However, the present analysis revealed no significant relationship between acetylator genotypes and bladder cancer risk in Koreans.

GSTM1 null genotype has attracted much attention due to its possible association with increased susceptibility to certain malignancies such as lung cancer⁷ and bladder cancer,^{4,15} although the high risk of bladder cancer in individuals with the GSTM1 null genotype is still controversial. Our data demonstrated an association between the GSTM1 null genotype and an increased risk of bladder cancer (OR 1.81). On the other hand, few studies showed GSTT1 being a possible risk factor in bladder cancer. Kempkes et al¹⁶ reported that the frequency of GSTT1 null genotype was similar in patients with urothelial cancer and controls. Brockmöller et al¹⁷ found no significant difference in the GSTT1 genotype between urothelial cancer cases and controls in all subjects including smokers and non-smokers, although a significantly high frequency of GSTT1 null genotype in the cancer cases was observed in the non-smoker group. Our previous data showed no increased risk of urothelial cancer in individuals with the GSTT1 null genotype among the Japanese.¹⁵ Our results confirm that GSTT1 null genotype is not a significant risk factor for bladder cancer.

We also investigated the effects of concurrent GSTM1, GSTT1 and NAT2 genotypes on bladder cancer risk. Okkels et al¹⁸ demonstrated that the NAT2 and GSTM1 genotypes were not associated with increased risk of bladder cancer among Danish people. Brockmöller et al¹⁷ reported that a combination of GSTM1- and GSTT1-positive genotypes was overexpressed in bladder cancer patients; however, this increase was not significant. In our Japanese data, the frequency of the GSTM1 null/GSTT1-positive genotype was significantly higher in the urothelial cancer patients than in controls.¹⁵ Although there might be an increased risk of urothelial cancer in Japanese individuals with the GSTT1-positive/GSTM1 null genotype, analysis of genetic combinations of GSTM1/GSTT1/NAT2 did not reveal any combination of NAT2, GSTM1 and GSTT1 genotypes associated with increased bladder cancer risk among Korean people. The OR of GSTT1-positive/GSTM1 null to GSTT1-positive/GSTM1-positive genotype was greater than the null value, although not statistically significant. Since risk of bladder cancer in GSTM1 null subjects depends on their smoking status,¹⁵ a difference in the proportion of smoking subjects could cause the discrepancy in statistical significance between the Japanese and the present data.

Cigarette smoking has been strongly associated with the development of bladder cancer for at least 20 years in more than 30 case-control studies in western populations. Nevertheless our data demonstrated that smoking was not a significant risk factor for bladder cancer. The prevalence of smoking in Korean adult males is very high compared with western people. In the present study, over 70% of the subjects were smokers. Since the extent of cumulative smoking has been considered to have a closer correlation with cancer

incidence, we analyzed the statistical significance of cumulative smoking (pack-year) instead of smoking history. However, no statistical significance was resulted (data not shown).

Arylamines are smoke constituents (for example, 2-naphthylamine and 4-aminobiphenyl) and it has been demonstrated that smokers have higher arylamine DNA adducts than non-smokers.¹⁹ The carcinogenic forms of arylamines are formed by bioactivation in the liver in a cytochrome P450 1A2-catalyzed reaction. In a competing hepatic detoxifying reaction (N-acetylation), NAT enzymes (predominantly NAT2) inactivate the proximate carcinogens. Therefore, although no strong epidemiological data support a link between NAT2 status and smoking-induced bladder cancer, arylamines found in cigarette smoke may play an important role in smoking-induced bladder carcinogenesis.^{13,19} However, we failed to find a significant association between acetylator genotypes and bladder cancer among smokers in Korean population.

GSTM1 catalyzes the conjugation of reduced glutathione to several polycyclic aromatic hydrocarbon epoxides generated from cigarette smoke.⁵ Cigarette smokers who were GSTM1 null genotype had a 1.8-fold greater risk of developing bladder cancer than the smokers with GSTM1-positive genotype. Non-smokers who lacked this gene, however, had a similar risk of developing bladder cancer to non-smokers who had it, thus supporting the notion that this gene might play a role in developing smoking-induced bladder cancer.⁴ The GSTT1 polymorphism is of interest as well because monohalomethanes and others, which are metabolized by GSTT1, are present in cigarette smoke.²⁰ As opposed to Katoh et al's¹⁵ report that the GSTM1 null genotype as a risk factor for urothelial cancer was dependent on smoking status, our data indicated that GSTM1 genotype was unrelated to smoking status in bladder cancer. Although the GSTM1 null genotype in smokers was not a significant risk factor in this study, the OR, 2.31, was greater than that of the Japanese study, 1.98. Similar studies on the influence of the GSTT1 null genotype as a risk factor have been published by Kempkes et al¹⁶ and Brockmöller et al¹⁷. They observed a significantly increased risk of bladder cancer associated with the GSTT1 null genotype in non-smokers. In our study, the GSTT1 null genotype for the risk of bladder cancer did not differ significantly between the cases and the controls. The reason for this discrepancy is yet unclear, but a variety of factors including race might have been introduced into the tests for genetic susceptibility.

Medical histories of tuberculosis and bronchial asthma were significant risk factors for bladder cancer (OR 3.16 and 4.15, respectively). Prior to this study, only one epidemiological report was published from Finland finding that the incidence of bladder cancer in men was slightly increased among patients with bronchial asthma (SIR 1.25%),²¹ which is consistent with our results. With regard to tuberculosis, no data have been published for tuberculosis associated with bladder cancer. The proportions of the GSTT1 null²² and the rapid acetylator genotypes¹⁴ are relatively high in Korean population compared with other ethnic groups, and Korea has been a peculiar country with high tuberculosis prevalence. Therefore, more bladder cancer patients could be related to tuberculosis in Koreans with the GSTT1 null or the rapid acetylator genotypes. Our data showed relatively high ORs of carrying the combined tuberculosis with GSTT1 null and rapid acetylator genotypes. In the population of fewer tuberculosis patients and also with lower proportions of GSTT1 null or rapid acetylator genotypes, significant relationship may not be found between bladder cancer and tuberculosis in a statistical sense.

The risk of bladder cancer in tuberculosis and bronchial asthma with GSTM1 and GSTT1 null genotypes was signif-

icantly high in Korean subjects. We suggest that GSTM1 and GSTT1 null genotypes, with consequent absence of these enzymatic function, could result in decreased detoxification properties, including decreased conjugation inactivation of unknown reactive intermediates in relation to drugs for tuberculosis and bronchial asthma, making them capable of interaction with DNA, which might enhance bladder cancer development, although the mechanisms are unknown.

CONCLUSIONS

The present study demonstrated for the first time an association between genotypes of detoxification enzymes, asthma, tuberculosis and susceptibility to bladder cancer. Our data confirm that the GSTM1 null genotype is a significant risk factor for bladder cancer among Korean subjects. The reason why bronchial asthma and tuberculosis are risk factors of bladder cancer in Korean subjects is yet unknown, but a variety of factors, including enzyme activities for detoxification, medication for these diseases and immunological background might be involved. To predict bladder cancer risk, several urinary metabolites of drugs for tuberculosis and bronchial asthma and enzymatic activities of the above mentioned enzymes should be taken into consideration. Studies are now in progress with respect to this.

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