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METABOLIC ACTIVATION/INACTIVATION

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Polymorphisms of glutathione *S*-transferase genes (*GSTM1*, *GSTP1* and *GSTT1*) and bladder cancer susceptibility in the Turkish population

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Abstract We investigated the effect of the *GSTM1* and *GSTT1* null genotypes, and *GSTP1* 313 A/G polymorphism on bladder cancer susceptibility in a case control study of 121 bladder cancer patients, and 121 age- and sex-matched controls of the Turkish population. The adjusted odds ratio for age, sex, and smoking status is 1.94 [95% confidence intervals (CI) 1.15–3.26] for the *GSTM1* null genotype, and 1.75 (95% CI 1.03–2.99) for the *GSTP1* 313 A/G or G/G genotypes. *GSTT1* was shown not to be associated with bladder cancer. Combination of the two high-risk genotypes, *GSTM1* null and *GSTP1* 313 A/G or G/G, revealed that the risk increases to 3.91-fold (95% CI 1.88–8.13) compared with the combination of the low-risk genotypes of these loci. In individuals with the combined risk factors of cigarette smoking and the *GSTM1* null genotype, the risk of bladder cancer is 2.81 times (95% CI 1.23–6.35) that of persons who both carry the *GSTM1*-present genotype and do not smoke. Similarly, the risk is 2.38-fold (95% CI 1.12–4.95) for the combined *GSTP1* 313 A/G and G/G genotypes and smoking. These findings support the role for the *GSTM1* null and the *GSTP1* 313 AG or GG genotypes in the development of bladder cancer. Furthermore, gene-gene (*GSTM1-GSTP1*) and gene-environment (*GSTM1*-smoking, *GSTP1*-smoking) interactions increase this risk substantially.

Keywords Bladder cancer · Gene polymorphism · Glutathione transferase

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Introduction

Bladder cancer is the third most common cancer in males, and the eighth most common cancer in females of the Turkish population (Özsarı and Atasever 1997). Environmental risk factors play a substantial role in the development of this malignancy. They include cigarette smoke, chlornaphazine, phenacetin-containing analgesics, cyclophosphamide, arsenic, and occupational exposure to aromatic amines (Johansson and Cohen 1997). The type and amount of exposure to the carcinogen, and its metabolism in the body are important factors for cancer risk. Drug metabolizing enzymes carry out the metabolism of carcinogens in two phases. Phase I enzymes, such as the cytochromes P450, introduce an electrophilic centre, and hence activate the carcinogen. Phase II enzymes detoxify the activated carcinogen by introducing a hydrophilic group such as glutathione in to the metabolite (Lang and Pelkonnen 1999).

Glutathione *S*-transferases (GSTs), which conjugate glutathione, comprise a gene super-family made up of four individual gene families called α, μ, θ and π. Allelic polymorphisms in GST genes have been defined, and disease-association studies were conducted in different populations for various cancers (Strange and Fryer 1999). Among them, the most extensively studied are the *GSTM1* null, the *GSTP1* 313 A/G and the *GSTT1* null polymorphisms. The functional consequences of the *GSTM1* and the *GSTT1* null genotypes are obvious in terms of enzyme activity: no gene, no enzyme, and no activity. The *GSTP1* 313 A/G polymorphism at the nucleotide level leads to an amino acid variation of isoleucine/valine at codon 105 in the protein. The valine

amino acid results in decreased enzyme activity (Ali-Osman et al. 1997). Despite this neat theoretical framework, the results of association studies between GST genotypes and bladder cancer are discordant in different populations (Bell et al. 1993; Zhong et al. 1993; Lin et al. 1994; Anwar et al. 1996; Brockmoller et al. 1996; Kempkes et al. 1996; Okkels et al. 1996; Golka et al. 1997; Harries et al. 1997; Katoh et al. 1999; Salagovic et al. 1999; Georgiou et al. 2000; Mungan et al. 2000; Steinhoff et al. 2000). Although methodological differences might be partially responsible for this discrepancy, the risk might be specific for the studied population because of differences in environmental factors, polymorphism frequencies and gene-gene interactions.

In this study, we determined the genotypic frequencies of the *GSTM1* null, *GSTP1* 313 A/G and *GSTT1* null polymorphisms in bladder cancer patients, age- and sex-matched controls, and randomly selected individuals to understand whether these polymorphisms are associated with bladder cancer in the Turkish population.

Materials and methods

Peripheral blood samples were collected from 121 patients with bladder cancer (transitional cell carcinoma) diagnosed at Hacettepe University Medical School, and Ankara Numune Hospital. The mean age was 60.15 years, standard deviation 11.10, range 25–87; 72.0% of the patients were smokers, and the male:female ratio was 5:1. Information about sex, age of individual patients, and histopathology of the tumours was obtained from their medical records. Non-smokers are defined as individuals who never smoked, and smokers are individuals who smoked at least one packet of cigarettes daily for 1 year (i.e. one pack-year). The age/sex-matched control group comprised of 121 individuals from Atatürk Chest Disease Research Hospital (non-cancer patients). The mean age was 59.33 years, standard deviation 13.58, range 23–79; 63.8% were smokers and the male:female ratio was 5:1. All three hospitals are in Ankara, and serve patients predominantly from Central Anatolia. Seventy-seven randomly selected Bilkent University students were also included in the study. Informed consent was obtained from all subjects. Genomic DNA was isolated from 700 µl blood by standard phenol-chloroform extraction.

GSTM1 genotyping was performed by simultaneous amplification of GSTM1 primers (Anwar et al. 1996) with CYP2E1 primers (Anwar et al. 1996) in the same polymerase chain reaction (PCR) tube. These primers were:

- G1, 5'-GAA CTC CCT GAA AAG CTA AAG C
- G2, 5'-GTT GGG CTC AAA TAT ACG GTG G
- CYP2E1F, 5'-CCA GTC GAG TCT ACA TTG TCA
- CYP2E1R, 5'-TTC ATT CTG TCT TCT AAC TGG

PCR products were electrophoresed in 2% agarose gels, and visualized by ethidium bromide staining. Null genotype was scored by the presence of a 412-bp *CYP2E1* band in the absence of a 215-bp *GSTM1* fragment.

A313G polymorphism in *GSTP1* was analysed using a previously described PCR-restriction fragment length polymorphism (RFLP) method (Harries et al. 1997). Briefly, amplification was carried out using primers:

- p105F, 5'-ACC CCA GGG CTC TAT GGG AA
- p1051R, 5'-TGA GGG CAC AAG AAG CCC CT

The 176-bp amplified product was digested with *Alw*261 and electrophoresed in 3% NuSieve gel. Presence of the restriction site resulted in two fragments of 91 bp and 85 bp, which was indicative of the G allele.

GSTT1 genotype was determined by using the previously described primers (Bringuier et al. 1998):

- GSTT1F, 5'-AGG CAG CAG TGG GGG AGG ACC
- GSTT1R, 5'-CTC ACC GGA TCA TGG CCA GCA

in combination with the above mentioned GSTP1 primers. A *GSTT1*-specific 138-bp fragment was observed in positive individuals. Null genotype was scored after confirming with at least two independent experiments.

GSTM1 null, *GSTP1* 313 A/G or G/G, and *GSTT1* null are defined as the risk genotypes for statistical analyses. Odds ratio (OR) with 95% confidence intervals (CI), and χ^2 analyses were performed (Daniel 1998). Age-, sex- and smoking-adjusted OR with 95% CI were calculated by a multiple logistic regression model (Hosmer and Lemeshow 1989). Two-gene interactions were calculated by using a previously described model (Yang and Khoury 1997). Analyses were done by using SPSS v.10.0 software.

Results

The genotype frequencies of the *GSTM1*, *GSTP1* and *GSTT1* polymorphisms in the patients, and the age/sex-matched control groups are summarized in Table 1. A group of randomly selected university students ($n = 77$) was also genotyped to compare with the age/sex-matched control group. In the randomly selected group, the

Table 1 Distribution of the *GSTM1*, *GSTP1* and *GSTT1* genotypes in the age-and sex-matched controls and bladder cancer patients

Locus	Genotype	Group		Odds ratio (95% confidence intervals)		
		Bladder cancer (%) $n = 121$	Control (%) $n = 121$	Crude	Adjusted[a]	*P*
GSTM1	Present	46 (38.02)	66 (54.55)			
	Null	75 (61.98)	55 (45.45)	1.96 (1.18–3.22)	1.94 (1.15–3.26)	0.010
GSTP1	A/A	67 (55.37)	83 (68.60)			
	A/G	42 (34.71)	33 (27.27)			
	G/G	12 (9.92)	5 (4.13)			
	A/G or G/G	54 (44.63)	38 (31.40)	1.76 (1.04–2.94)	1.75 (1.03–2.99)	0.034
GSTT1	Present	97 (80.17)	100 (82.64)			
	Null	24 (19.83)	21 (17.36)	1.17 (0.61–2.22)	1.27 (0.66–2.47)	0.620

[a]Adjusted for age, sex and smoking status

frequency of *GSTM1* null genotype was 46.7% ($P = 0.858$), that of the *GSTT1* null genotype was 17.25% ($P = 0.936$), and the *GSTP1* genotype frequencies were 67.53% (A/A), 31.16% (A/G) and 1.31% (G/G) ($P = 0.820$). These results reveal that the genotype frequencies for the age/sex-matched control group and the randomly selected group are not significantly different, which indicates the absence of bias of ascertainment during the selection of the age/sex-matched control group. The distribution of GST genotypes was in Hardy-Weinberg equilibrium in all three groups. The odds ratio adjusted for age, sex, and smoking status was 1.94 (95% CI 1.15–3.26) for the *GSTM1* null genotype. Since the *GSTP1* 313 G/G genotype frequency was too low in our population, *GSTP1* 313 A/G and G/G genotypes were combined for cancer risk estimation (Katoh et al. 1999). The OR was 1.75 (95% CI 1.03–2.99). Finally, the *GSTT1* null genotype was found not to be a significant risk factor (OR 1.27, 95% CI 0.66–2.47) for bladder cancer.

Combination of the two high-risk genotypes, *GSTM1* null and *GSTP1* 313 A/G or G/G, revealed that the risk increases 3.91-fold (95% CI 1.88–8.13) compared with the combination of the low-risk genotypes of these loci (Table 2). We further investigated the risk associated with the combination of the risk-related genotypes of all three GST loci (Table 3), even though the *GSTT1* null genotype alone does not appear to be a significant risk factor for bladder cancer in the Turkish population. Individuals with all three putative low-risk genotypes, i.e. with the presence of *GSTM1* and *GSTT1* genotypes and the homozygous A/A genotype for *GSTP1*, were designated as the reference group. The calculated odds ratio for the three high-risk genotypes versus no high-risk genotype was 8.00 (95% CI 1.52–287.10).

We evaluated the risk of bladder cancer from GST genotypes by smoking status (Table 4). Among non-smokers we found a slight but statistically non-significant increased risk of bladder cancer associated with the *GSTM1* null (OR 1.95, 95% CI 0.74–5.05), the *GSTP1* A/G or G/G (OR 1.78, 95% CI 0.65–4.80), and the *GSTT1* (OR 1.53, 95% CI 0.51–4.52) genotypes. Among smokers we found a significantly elevated risk of bladder cancer associated with the *GSTM1* null genotype (OR 2.02, 95% CI 1.04–3.93). An association was not observed for either *GSTP1* or *GSTT1*. The effect of the combined contributions of genotype and smoking status to bladder cancer risk is displayed in Table 5. Individuals with the *GSTM1* null genotype who smoke have an increased risk of 2.81 (95% CI 1.23–6.35) compared with the individuals with the *GSTM1* present genotype who do not smoke. Similarly, with respect to the *GSTP1* locus, the risk factor is 2.38 (95% CI 1.12–4.95). An association with the *GSTT1* locus was not found.

Discussion

GSTM1, *GSTP1*, and *GSTT1* polymorphisms were analysed in 121 bladder cancer patients, and 121 age/sex-matched controls. When the two groups were compared, the odds ratio for the *GSTM1* null genotype in bladder cancer patients is 1.94, and for the *GSTP1* 313 A/G or G/G genotypes is 1.75. The *GSTT1* null geno-

Table 2 Combination of the *GSTM1* null with *GSTP1* 313 AG or GG genotypes and bladder cancer risk

Genotype of risk	*GSTM1*	*GSTP1*	Group		Odds ratio (95% confidence intervals)	
			Bladder cancer ($n = 121$)	Control ($n = 121$)	Crude	Adjusted[a]
None[b]	Present	A/A	24	41	1.00 (reference)	1.00 (reference)
One	Null	A/A	43	42	1.75 (0.94–3.25)	2.07 (1.00–4.30)
	Present	A/G, G/G	22	25	1.50 (0.69–3.74)	1.89 (0.91–3.93)
Two	Null	A/G, G/G	32	13	4.20 (1.85–9.58)	3.91 (1.88–8.13)

[a]Adjusted for age, sex and smoking status
[b]Group that includes the combination of no-risk genotypes and is used as the reference for odds ratio analysis

Table 3 GST genotype distribution and risk associated with genotype combinations

Combination	Locus			Group		Odds ratio (95% confidence interval)
	GSTM1	*GSTP1*	*GSTT1*	Bladder cancer ($n = 121$)	Controls ($n = 121$)	
Three high-risk genotypes	Null	A/G or G/G	Null	8	2	8.00 (1.52–287.10)
Two high-risk genotypes	Null	A/G or G/G	Present	24	11	4.36 (1.75–10.80)
	Null	A/A	Null	7	8	1.75 (0.54–5.52)
	Present	A/G or G/G	Null	2	4	1.00 (0.16–5.58)
One high-risk genotype	Null	A/A	Present	36	34	2.11 (1.06–4.41)
	Present	A/G or G/G	Present	20	21	1.90 (0.84–1.69)
	Present	A/A	Null	7	7	2.00 (0.60–6.61)
No high-risk genotype	Present	A/A	Present	17	34	1.00 (reference)

Table 4 Distribution of GST genotypes stratified according to smoking status in cancer cases and controls. For bladder cancer patients and controls, the number of cases of each genotype is shown together with percentage in parentheses

Locus	Genotype	Non smokers			Smokers		
		Bladder cancer	Control	Odds ratio (95%CI)	Bladder cancer	Control	Odds ratio (95%CI)
GSTM1	Present	12 (38.70)	21 (55.20)		27 (33.75)	34 (50.70)	
	Null	19 (61.30)	17 (44.80)	1.95 (0.74–5.05)	53 (66.25)	33 (49.30)	2.02 (1.04–3.93)
GSTP1	AA	18 (58.06)	27 (71.05)		45 (56.25)	45 (67.10)	
	AG/GG	13 (41.94)	11 (28.95)	1.78 (0.65–4.80)	35 (43.75)	22 (32.90)	1.59 (0.83–3.03)
GSTT1	Present	22 (70.90)	30 (78.90)		66 (82.50)	56 (83.50)	
	Null	9 (29.10)	8 (21.10)	1.53 (0.51–4.52)	14 (17.50)	11 (16.50)	1.08 (0.42–2.51)

Table 5 Combined risk of bladder cancer associated with smoking and GST genotypes

Smoking status	Odds raio (95% confidence intervals)					
	GSTM1		*GSTP1*		*GSTT1*	
	Present	Null	A/A	A/G or G/G	Present	Null
No	1	1.95 (0.74–5.06)	1	1.77 (0.65–4.75)	1	1.53 (0.53–4.34)
Yes	1.38 (0.73–2.58)	2.81 (1.23–6.35)	1.50 (0.72–3.06)	2.38 (1.12–4.95)	1.60 (0.83–3.06)	1.73 (0.77–3.74)

type was not found to be associated with a significantly increased bladder cancer risk (Table 1). Our observation for the *GSTM1* null genotype parallels that of a recent meta-analysis study, although our figure of 1.94 is higher than the reported OR of 1.5 in the meta-analysis (Johns and Houlston 2000). Association of the *GSTP1* 313 A/G or G/G genotypes with bladder cancer in the Turkish population is in accord with findings in the British population (Harries et al. 1997), but not with those in the Japanese (Katoh et al. 1999) or German (Steinhoff et al. 2000) populations. The lack of association between bladder cancer and the *GSTT1* locus is in agreement with studies in the Greek (Georgiou et al. 2000) and German (Kempkes et al. 1996; Steinhoff et al. 2000) populations, but not with results for Slovaks (Salagovic et al. 1999).

Bladder cancer is a malignancy in which, in addition to the genetic status of the individual, gene-environment interactions are thought to play an important role. Smoking is one of the important environmental risk factors. Since GSTs are involved in the metabolism of smoking-related carcinogens such as epoxides and polycylic aromatic hydrocarbons (Lang and Pelkonnen 1999; Autrup 2000), we analysed the risk of bladder cancer from GST genotypes by smoking status (Table 4), and the combined risk of bladder cancer associated with smoking and GST genotypes (Table 5). In order to examine the genetic risk for bladder cancer independently by eliminating the contribution of smoking, we stratified the subjects by smoking status. An association was observed only in individuals who smoke and carry the *GSTM1* null genotype (OR 2.02, 95% CI 1.04–3.93). However, we would like to note that the stratification process, which reduced the number of samples analysed, might have resulted in statistically non-significant confidence intervals in the remaining groups. Combined analyses of smoking status and GST genotypes indicate an interaction between smoking and the *GSTM1* null genotype as well as the *GSTP1* A/G or G/G genotypes. The risk figures are 2.81 and 2.38, respectively. This observation is in accordance with the results from studies in the U.S.A. (Bell et al. 1993) but not with those from Dutch (Mungan et al. 2000) or Korean (Kim et al. 2000) studies. No data were available in the literature for the association of *GSTP1* locus and bladder cancer.

We observed that combination of the *GSTM1* null and the *GSTP1* 313 A/G or G/G genotypes leads to approximately a four-fold increased risk of cancer compared with the combination of the low-risk genotypes of these loci. This observation suggests that gene-gene interactions may contribute to genetic susceptibility for bladder cancer. Simultaneous analysis of the *GSTM1* and *GSTP1* loci was conducted for bladder cancer in only one study from Germany (Steinhoff et al. 2000) in which an increased risk was not observed. On the other hand, in a Japanese lung cancer study (Kihara et al. 1999) and a U.S. breast cancer study (Helzlsouer et al. 1998) where the high-risk genotypes of the *GSTM1* and *GSTP1* loci were analysed simultaneously, an increased risk on combination of high-risk genotypes was detected. Interestingly, when we combined the three risk genotypes, the odds ratio figure increases to eight. However, this observation should be interpreted with caution and validated by further studies using a larger sample size.

The data from the age/sex-matched controls and the randomly selected individuals were combined since they did not differ significantly. These combined data were then compared with the previously reported data from

Caucasian populations by employing a test of homogeneity (Daniel 1998). Neither the *GSTM1* nor the *GSTT1* loci differed significantly (Vineis et. al. 1999) among the studies including a previously reported Turkish study (Oke et al. 1998). However, the frequency of the *GSTP1* 313 genotypes in the Turkish study differs from the previously reported British (Harries et al. 1997) ($P = 0.001$), German (Steinhoff et.al. 2000) ($P = 0.008$), Norwegian (Ryberg et al. 1997) ($P = 0.001$), and European American (Watson et al. 1997) ($P = 0.015$) population frequencies.

To our knowledge, this is the first genetic study on the association of GSTs with bladder cancer in the Turkish population. We have demonstrated a risk of bladder cancer associated with the *GSTM1* null and the *GSTP1* 313 A/G or G/G genotypes. Furthermore, gene-gene (*GSTM1-GSTP1*) and gene-environment (*GSTM1*-smoking, *GSTP1*-smoking) interactions increase this risk substantially.

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