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The Role of Human Glutathione S-Transferases M1 and T1 in Individual Susceptibility to Bladder Cancer

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Summary

Several genes involved in the metabolism of carcinogens have been found to be polymorphic in the human population, and specific alleles are associated with increased risk of cancer at various sites. This study is focused on the polymorphic enzymes glutathione S-transferase M1 (GST M1) and T1 (GST T1) that are involved in the detoxification of many xenobiotics involved in the etiology of bladder cancer. To investigate the role of GST M1 and GST T1 in bladder carcinogenesis, the polymerase chain reaction was used to determine GSTM1 and GSTT1 genotypes of cancer patients (n = 76) and controls (n = 248). The proportion of putative risk GSTM1 null genotype in the case group was 52.6 %, compared to 49.6% in the control group, but the GSTT1 0/0 frequency in the bladder cancer group was significantly higher (P = 0.04) in comparison with the control group (27.6 vs 16.9 %). Individuals lacking the GSTT1 gene are at an approximately 1.9-fold higher risk (OR = 1.87, C.I. 95 % = 1.03-3.42) of developing bladder cancer in comparison with individuals with at least one active allele in the GSTT1 locus. A significantly higher incidence of GSTM1 deletion genotype (P = 0.02) was found in smokers with bladder cancer compared to the controls (70.6 vs 49.6 %). Smokers lacking the GSTM1 gene are at an approximately 2.4-fold higher risk of bladder cancer (OR = 2.44, C.I. 95 % = 1.10-5.30). The effect of smoking associated with the GSTT1 0/0 genotype was not found to affect the risk of bladder cancer.

Key words

Xenobiotic metabolism • Bladder cancer • GSTM1 • GSTT1 • Genetic polymorphism

Introduction

Bladder cancer belongs to human neoplastic diseases most strongly linked to occupational and environmental exposures to chemical carcinogens. According to epidemiological estimations, occupational exposure to chemicals may explain 10-30 % of bladder cancers (Brockmöller *et al.* 1996). Bladder cancer was first related to occupational exposure to aniline-derived dyes at the end of the 19th century (Rehn 1895) and was experimentally induced with β-naphtylamine in dogs (Hueper 1938). The most potent carcinogens associated with the development of occupational urothelial cancer are 2-naphtylamine, 4-aminobiphenyl and benzidine (British Association of Urological Surgeons 1988). It is important to consider that arylamines and polycyclic aromatic hydrocarbons are present in the environment, notably from cigarette smoke. Smoking has been

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identified as an important risk factor in bladder cancer (Bell *et al.* 1993, Vineis 1994). Some drugs, alcohol, saccharin, coffee (Viscoli *et al.* 1993) and other factors have also been proposed as exogenous chemical risk factors of bladder cancer (Kunze *et al.* 1992). Many of these naturally occurring or synthetic compounds require enzymatic activation to become ultimate carcinogens that may finally react with cellular macromolecules such as DNA. Therefore, more common genetic traits that substantially control the metabolic activation or detoxification of carcinogenic chemicals to their DNA-damaging intermediates appear to be important risk factor. The superfamily of cytochrome P450 phase I enzymes catalyses the oxidative metabolism of most endogenous and exogenous chemicals. While this housekeeping process converts them to water-soluble, readily excretable forms, it also creates electrophilic intermediates (Roots *et al.* 1992). Activated intermediates of this metabolism are thought to be the ultimate initiating carcinogens, electrophiles that react with DNA bases. In contrast, phase II enzymes generally detoxify carcinogenic metabolites by conjugating them with glucuronide, glutathione or sulfate to produce hydrophilic products excretable in urine. Genetic differences in these pathways are likely to be a major source of interindividual variation in the susceptibility to cancer (Nebert 1991). Genetic polymorphism exists in a number of phase I enzymes and phase II enzymes. It is conceivable that individuals with genotypes associated with a more efficient activating enzyme and a less efficient inactivating enzyme might be at a particularly high risk of cancer, if exposed to genotoxicants.

This study focuses on the glutahione S-transferases (GSTs) that, among phase II enzymes, have attracted most of recent interest. The glutathione S-transferases play a central role in the detoxification and consist of a superfamily of enzymes involved in the conjugation of a wide range of electrophilic substrates with glutathione. The GSTs may be expected to protect individuals from cancer by deactivating reactive chemical species, such as polycyclic aromatic hydrocarbon epoxides and arylamines. The mammalian GSTs can be differentiated into four classes (alpha, mu, pi and theta) of cytosolic enzymes and two membrane-bound enzymes. The enzymes are expressed in almost all tissues, including bladder urothelial tissue (Brockmöller *et al.* 1994). Two human cytosolic GST enzymes – μ-class enzyme GST M1 and the θ-class enzyme GST T1, have been shown to be polymorphic in humans (Pemble *et al.* 1994, Seidegard *et al.* 1986). In both cases, gene deletion is responsible for the existence of a null allele. Carriers of homozygous deletions in the GSTM1 and GSTT1 genes lack GST M1 and GST T1 enzyme activity, respectively. Deficiencies of these enzymes do not permit effective metabolism of compounds involved in carcinogenesis and may result in an increased risk of somatic mutations, leading to tumor formation. Therefore, individuals with inherited GSTM1 0/0 or GSTT1 0/0 genotypes may be at increased cancer risk.

In the present study, we investigated the GSTM1 0/0 and GSTT1 0/0 genotypes as a risk factor in bladder cancer, using the polymerase chain reaction in a case-control study.

Material and Methods

Subjects

Bladder cancer case patients (76) were enrolled from Urology Clinics at the University Hospital in Košice. All patients were either outpatients for follow-up cystoscopy, or were inpatients for surgical removal of a bladder tumor. In order to explore racial differences in GSTM1 and GSTT1 genes frequencies, we determined GSTM1 and GSTT1 genotypes in a community-based sample of 248 healthy, unrelated individuals.

All individuals were of Caucasian origin and were asked to complete a questionnaire regarding any chemical exposure they might have either as a result of their occupation or smoking habits. Only individuals who smoked cigarettes were included as smokers. Non-smokers were only those who had never smoked. The questionnaire also included information on age, gender, alcohol consumption and family history of chronic diseases. Mean age was 67.1 years for patients and 58.7 years for the controls. The studied population is described in Table 1.

Identification of GSTM1 and GSTT1 genotypes

Enzyme GST M1 is encoded by *GSTM1* gene on chromosome 1p13.3 and isoenzyme GST T1 by *GSTT1* gene on chromosome 22q11.2. Genotype analysis of large populations by PCR is both cost- and time-consuming. We have therefore used the simultaneous amplification of GSTM1 and GSTT1 genomic fragments in the same reaction (Arand *et al.* 1996). In a single PCR reaction, we combined two sets of primer pairs previously used for the separate amplification of GSTM1 and GSTT1 genomic

fragments and a third pair of compatible primers for the additional amplification of an albumin gene fragment that was used as an internal positive control for the success of the amplification reaction.

Blood (3 ml) was withdrawn into EDTA by routine venepuncture and stored at –20 °C until DNA extraction was carried out by phenol-chloroform. Genomic DNA was prepared from separated leukocytes by proteinase K-phenol-chloroform extraction. DNA (50-100 ng) was amplified in a final volume of 50 µl containing 3 µg/ml of each GSTM1 primer, 1 µg/ml of each GSTT1 primer, 600 ng/ml of albumin primers, 200 µmol deoxynucleoside triphosphates, 2 U Taq polymerase (Promega), PCR buffer and 2.5 mM $MgCl_2$, overlaid with one drop of mineral oil. PCR was carried out in a Techne Progene thermal cycler. After 2-min pretreatment at 95 °C, the reaction mixture was subjected to 30 cycles of 94 °C for 1 min, 64 °C for 1 min, and 72 °C for 1 min. This was followed by a final step at 72 °C for 5 min. The resulting DNA fragments were separated by electrophoresis on 2 % agarose gel containing 0.4 µg ethidium bromide/ml, and subsequently visualized by UV detection. The involvement of GSTM1 deficiency was concluded from the absence of the specific 215 base pair (bp) fragment and GSTT1 deficiency from the absence of 480 bp fragment. Albumin used as internal positive control resulted in a constant 350 bp band in all samples.

Three alleles at the GSTM1 locus have been described: GSTM1*A, GSTM1*B and GSTM1*0 alleles. GSTM1*A and GSTM1*B code for enzymes of similar catalytic activity (they differ only by a Lys/Asn substitution at amino acid 172 that has no apparent effect on function), but the GSTM1*0 (null allele) produces no catalytically active enzyme, apparently because of partial or total gene depletion (Rebbeck 1997, Strange 1993). The "+" allele designation, which was used for an "active" allele, does not differentiate between the GSTM1*A and GSTM1*B alleles. Because of the small percentage of individuals with GSTM1 +/+ genotype and because GSTM1 +/0 and +/+ genotypes have similar expression levels (Bell *et al.* 1993), individuals with GSTM1 +/0 and GSTM1 +/+ genotypes were grouped in our analysis together as GSTM1 +.

Similarly, two functionally different genotypes in GSTT1 have been identified (Pemble *et al.* 1994) that are denoted here as GSTT1 0/0 (homozygous deletion genotype) and GSTT1 + (genotypes with one or two undeleted alleles).

Statistics

χ^2 tests were used for examining homogeneity between patients and the controls. Since some genotype frequencies were small, the StatXact-Turbo statistical package was used to obtain exact P values. The results are reported as odds ratios (ORs) and 95 % confidence intervals (CI). The odds ratio (OR) is defined as the odds of a case patient having the GSTM1 and GSTT1 0/0 genotypes divided by the odds of a control subject having the GSTM1 and GSTT1 null genotypes, respectively. The OR can be used as an estimate of the risk of cancer for those with the GSTM1 and GSTT1 null genotype relative to those with the GSTM1 and GSTT1 +/+ or +/0 genotypes, respectively.

Table 1. Summary of the bladder cancer patients and control groups

	Bladder cancer n =76	Controls n = 248
Gender		
Male	70 (92.1 %)	142 (57.3 %)
Female	6 (7.9 %)	106 (42.7 %)
Age		
<55 years	9 (11.8 %)	169 (68.2 %)
>55 years	67 (88.2 %)	79 (31.8 %)
Smoking status		
Non-smokers	42 (55.3 %)	147 (59.3 %)
Smokers	34 (44.7 %)	101 (40.7 %)

Results and Discussion

In contrast to most of earlier studies, which were based on phenotypic measurements of enzyme activity, our investigation has relied on molecular genetic testing (using PCR technique). Genotyping is advantageous because it can unequivocally distinguish between GSTM1, GSTT1 and other glutathione transferase gene family members and can reveal the inherited DNA sequence polymorphism that forms the basis for a specific lifetime phenotypic trait. The GSTM1 and GSTT1 deletion variants have been useful for molecular epidemiological studies of cancer, because they separate the studied subjects into two well-defined susceptibility classes: those who are and those who are not able to

detoxify potential carcinogens by metabolic pathways regulated by GSTM1 or GSTT1.

Table 2 presents the data on the overall proportions of the GSTM1 and GSTT1 genotypes in a given the studied case and control groups (including the respective ORs). In the control population from our geographic region (Slovakia), the frequencies of GSTM1 and GSTT1 null genotypes were 49.6 and 16.9 %, respectively. In the whole group, there was only a marginal difference in the incidence of GSTM1 0/0 individuals between patients and the controls (52.6 vs 49.6 %). However, the GSTT1 0/0 frequency in the group with bladder cancer was significantly higher (P = 0.04) in comparison with the control group (27.6 vs 16.9 %). Individuals lacking the GSTT1 gene are at an approximately 1.9-fold higher risk of developing bladder cancer (OR = 1.87, C.I. 95 % = 1.03-3.42) in comparison with individuals having at least one active allele in the GSTT1 locus.

Table 2. Percentage distribution of the GSTM1 and GSTT1 genotypes in the examined groups

	GSTM1–	GSTM1+	OR	C.I. 95 %	P
Control group	49.6 % (123)	50.4 % (125)	1.00	–	
Bladder cancer group	52.6 % (40)	47.4 % (36)	1.13	(0.68-1.89)	0.69
	GSTT1–	GSTT1+	OR	C.I. 95 %	P
Control group	16.9 % (42)	83.1 % (206)	1.00	–	
Bladder cancer group	27.6 % (21)	72.4 % (55)	1.87	(1.03-3.42)	0.04

GSTM1– and GSTT1– indicate genotypes GSTM1 0/0 and GSTT1 0/0, respectively. GSTM1+, GSTT1+ indicate genotypes GSTM1 +/+ or GSTM1 +/0, GSTT1 +/0 or GSTT1 +/+, respectively. OR indicates odds ratio of GST M1- or GST T1-deficient over GST M1- or GST T1-active individuals in cases versus controls; 95 % C.I. indicates 95 % confidence interval.

If the glutathione S-transferase M1 and T1 enzymes play an important role in the detoxification of tobacco smoke-derived carcinogens, we might expect to find a different risk factor associated with the GSTM1 and GSTT1 null genotypes depending on smoking status. After grouping the patients according to their smoking status (Table 3), the frequency of GSTM1 deletion genotype compared to the control group (70.6 vs 49.6 %) increased significantly (P = 0.02) in the bladder cancer group of smokers. Smokers lacking the GSTM1 gene are at an approximately 2.4-fold higher risk for bladder cancer (OR = 2.44, C.I. 95 % = 1.10-5.30). Among non-smokers, we did not find any significant association between the GSTM1 0/0 genotype and cancer risk.

These data suggest that the presence of the GSTM1 gene product, glutathione S-transferase M1, exerts a protective effect against smoking-induced cancer. It is presumed that glutathione S-transferase M1 would act to conjugate smoking-associated carcinogens, rendering them inactive in the urine and noncarcinogenic to the bladder (Bell *et al.* 1993). Four recent case-control independent studies all suggest that GSTM1 deficiency is associated with an increased bladder cancer risk in smokers (Bell *et al.* 1993, Daly *et al.* 1993, Lafuente *et al.* 1993, Brockmöller *et al.* 1994). In support of this observation, the mutagenicity in the urine of smokers with the GSTM1 0/0 genotype was increased (Hirvonen *et al.* 1994). There are many potential carcinogenic and mutagenic substrates for GST M1 in cigarette smoke (Ketterer *et al.* 1992). Several polycyclic aromatic hydrocarbons epoxides generated from cigarette smoke, including the potent carcinogen benzo[a]pyrene-7,8-diol-9,10-oxide, are known substrates for glutathione S-transferase M1, but GST M1 may also be protective

against other classes of carcinogens present in cigarette smoke, such as nitrosamines or aromatic amines. Our results have demonstrate that the genetic risk from the GSTM1 0/0 genotype depends on the exposure to the carcinogens. Absence of the GSTM1 gene significantly increases the risk to persons with exposure to the carcinogens in tobacco smoke, but does not represent the increased risk for persons without such exposure.

Table 3. Risk of bladder cancer from GSTM1 and GSTT1 null genotypes by smoking status

		GSTM1–	GSTM1+	OR (C.I. 95 %)	P
Controls		49.6 % (123)	50.4 % (125)	1.00 –	
Bladder	Non-smokers	33.3 % (14)	66.7 % (28)	0.51 (0.30-1.00)	0.07
cancer	Smokers	70.6 % (24)	29.4 % (10)	2.44 (1.10-5.30)	0.02
		GSTT1–	GSTT1+	OR (C.I. 95 %)	P
Controls		16.9 % (42)	83.1 % (206)	1.00 –	
Bladder	Non-smokers	28.6 % (12)	71.4 % (30)	1.96 (0.93-4.10)	0.09
cancer	Smokers	26.4 % (9)	73.6 % (25)	1.77 (0.77-4.10)	0.21

GSTM1– and GSTT1– indicate genotypes GSTM1 0/0 and GSTT1 0/0, respectively. GSTM1+ and GSTT1+ indicate genotypes GSTM1 +/+ or GSTM1 +/0, GSTT1 +/0 or GSTT1 +/+, respectively. OR indicates odds ratio of GST M1- or GST T1-deficient over GST M1- or GST T1-active individuals in cases versus controls; 95 % C.I. indicates 95 % confidence interval.

Our hypothesis that the GSTT1 null genotype effect would be greater in smokers due to exposure to chemical carcinogens in cigarette smoke has not been confirmed. We did not find any effect of smoking associated with the GSTT1 0/0 genotype on bladder cancer risk. However, a higher (but non-significant) risk of bladder cancer for GSTT1 null genotype was surprisingly detected among non-smokers (OR = 1.96, C.I. 95 % = 0.93-4.10, p = 0.09). The same results were obtained by Kempkes *et al.* (1996) who reported an even 3.8-fold increase of bladder cancer risk among non-smokers with the GSTT1 0/0 genotype. These trends suggest that GST T1 is involved in the deactivation of carcinogens acting in urinary bladder carcinogenesis among non-smokers and that also other carcinogens than those from cigarette smoking have an important tumor-inducing or tumor-promoting role in bladder cancer. Thus a differential role of GST T1 with these substances might be postulated. Further studies should identify, whether the exposure to carcinogens caused increased bladder cancer risk in GST T1-deficient individuals.

In conclusion, bladder cancer is a common multifactorial disease which is known to be associated with exposure to chemical carcinogens. The development of bladder cancer has been associated with occupational exposure to aromatic amines, e.g. dye workers, and non-occupational exposure to carcinogens, e.g. smoking. Because of the association between carcinogen exposure and bladder cancer, we investigated whether genetic factors that modulate the internal dose of carcinogen can affect the risk of developing disease. We have used a molecular epidemiological approach that incorporates the detection of a common defect in the carcinogen-metabolism gene into a case-control study. This genotypic analysis should permit a clearer understanding of the relationship between polymorphism of genes involved in carcinogen metabolism and individual susceptibility to the mutagenic and carcinogenic actions of specific chemical exposures.

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