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GENETIC POLYMORPHISMS OF GENES GSTM1 AND CYP2D6 AND BLADDER CANCER

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ABSTRACT

Objective: To study the relationship between GSTM1 and CYP2D6 polymorphisms and exposure to risk factors, and the occurrence of bladder cancer.

Patients and Methods: The study included 77 patients with fully characterized transitional cell carcinoma of the bladder, from whom a complete history was taken, and 191 healthy individuals, who served as controls for the genetic assessment. Using a polymerase chain reaction technique, GSTM1 polymorphisms were studied in all patients and controls, and CYP2D6 polymorphisms were studied in 53 patients and 99 controls. Chi-squared test was used for statistical analysis.

Results: GSTM1 null genotype was detected in 75.3% of patients and in 57.1% of controls, and the difference was statistically significant ($\chi^2 = 7.79$; $p = 0.005$). This difference was due exclusively to individuals with tumors Ta/T1 (81.5% were GSTM1 deficient, $\chi^2 = 10.7$; $p = 0.001$). More smokers (85.7%) than non-smokers (62.8%) demonstrated the GSTM1 null phenotype ($\chi^2 = 5.36$; $p = 0.02$), and 95.8% of heavy smokers (> 40 pack-year) were GSTM1 null. Familial history of tumors was associated with GSTM1 null: 91.7% of patients with familial history versus 67.9% without such history showed the null phenotype ($\chi^2 = 5.01$; $p = 0.02$). Patients and controls were not significantly different in respect to frequency of CYP2D6 polymorphisms. Among patients, no significant association between CYP2D6 polymorphisms and tumor characteristics was found.

Conclusions: GSTM1 null genotype seems to be associated with bladder tumor occurrence, particularly "superficial" tumors (Ta/T1). This association is stronger in individuals with exposure to tobacco smoke. CYP2D6 gene does not seem to play any significant role in bladder tumor development.

Key words: bladder, bladder cancer, malignancy susceptibility, genetic polymorphisms, GSTM1, CYP2D6
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INTRODUCTION

Susceptibility to cancer is thought to depend on interplay between genetic factors and environmental chemical carcinogens. The xenobiotic-metabolising machinery includes oxidative enzymes (phase-I), which may inactivate carcinogens or, eventually, activate compounds to become carcinogenic, and phase-II conjugating enzymes, considered mainly protective since they detoxify a number of reactive chemical carcinogens (1).

Some enzymes involved in the metabolism of xenobiotics are polymorphically expressed on a Mendelian hereditary basis. Thus, different individuals (and different populations) may be differently affected by the exposure to risk factors. Recent works have addressed the issue that susceptibility to many cancers (including bladder cancer) may vary according to polymorphisms of the genes CYP2D6 and GSTM1. The gene CYP2D6 encodes for a phase I enzyme of the cytochrome P450 superfamily, the debrisoquine hydroxilase, whose substracts include aromatic amines and tobacco nitrosamines (2). Five to 10% of Caucasians are recessive homozygous, and are termed poor metabolizers (in opposition to extensive metabolizers, whom can be homozygous or heterozygous) as they are unable to metabolize various substances. The issue on the implications of

CYP2D6 polymorphisms, in bladder cancer is not settled. Some researchers did not find any relation between CYP2D6 polymorphisms and bladder cancer (3,4,5), others concluded for an increased risk of aggressive bladder cancer in extensive metabolizers (6), and others for an increased risk in extensive metabolizers who are simultaneously GSTM1 deficient (7). Glutathione-S-transferases (GSTs) are phase-II enzymes with many functions, including detoxification of polycyclic aromatic hydrocarbons from tobacco smoke and aromatic amines (8,9). Glutathione-S-transferase M1 (GSTM1), a member of the GSTs superfamily, is polymorphic in humans, and about 50% of Caucasians have the null genotype, and are devoid of GSTM1 enzymatic activity. Several studies have pointed out that individuals with the null phenotype are in greater risk of developing bladder cancer (3,7,10,11). However, this matter is still under debate, and other workers did not find such a relation (12,13).

In this study, GSTM1 and CYP2D6 polymorphisms in bladder cancer patients were studied, and results were compared with those found in a community-based sample of healthy volunteers.

PATIENTS AND METHODS

The study population comprised 77 patients (60 males, 17 females) with transitional cell cancer (TCC) of the bladder and 191 healthy volunteers, who served as controls for the genetic characterization. Patients and healthy volunteers were Caucasians from the center of Portugal. After giving informed consent, all patients answered a standardized questionnaire pertaining smoking habits (non-smoker / smoker / ex-smoker; number of pack-years smoked [one pack-year meaning 7300 cigarettes smoked]), alcohol consumption (considered excessive if superior to 100g per day), medications, exposition to known risk factors for bladder cancer (chemicals, motor exhaust, etc.), and history of tumors in first and second degree relatives. All relevant data about the disease, like age at diagnosis, T-category and grade of the tumor [UICC], occurrence of relapses and their characteristics, were transcribed into a data sheet. Peripheral blood samples were collected from all patients and healthy volunteers into tubes with Na_2EDTA at pH8, and genotyping of the GSTM1 locus (77 patients and 104 healthy volunteers) and of the CYP2D6 locus (53 patients and 99 healthy volunteers) was done using a polymerase chain reaction (PCR) technique in a Omnigene® equipment. DNA was extracted by standard manual methods (14-16). Three primers were used for GSTM1 (P1: 5´-CGCCATCTTG TGCTACATTGCCCG-3´; P2: 5´-ATCTTCTCCTC TTCTGTCTC-3´; P3: 5´-TTCTGGATTGTAGCAGA TCA-3´) (9). P1 and P3 amplify a 230 base pairs (bp) product specific for the GSTM1 gene. P1 and P2 can anneal to either GSTM1 or GSTM4 genes and originate a 157bp product used as internal control. Each PCR reaction mixture comprised approximately 50 ng of isolated DNA, 2.5 µl of 10x PCR buffer (final concentration: 50 mM KCl, 10 mM Tris-HCl pH 8.4, 1.5 mM $MgCl_2$), 0.2 mM of each nucleotide (dATP, dTTP, dGTP, dCTP), 1.25 ml of 5% DMSO, 25 ng of primers P1 and P2 and 50 ng of P3 and 0.5 U of Taq DNA polymerase, to a final volume of 25 ml. A total of 30 PCR cycles with denaturation at 94ºC for 60 sec, annealing at 52ºC for 60 sec and extension at 72ºC for 60 sec was performed. An initial DNA denaturation stage at 95ºC and a final stage with annealing at 52ºC and extension at 72ºC were performed for five min each. In order to detect the G→A transition at the junction of intron 3/exon 4 of the CYP2D6 gene, a 334bp fragment that encompasses that spot was obtained by PCR (17). Two primers were used (P1: exon 3: 5´-GCCTTCGCCAA-CCACTCCG-3´; P2: intron 4: 5´-AAATCCTGCTCTTCCGAGGC-3´). Reaction mixture was as described previously, but to a final volume of 50 ml. After an initial denaturation cycle of 10 min at 96ºC, 1 U of Taq DNA polymerase was added and 30 PCR cycles were performed consisting on DNA denaturation at 94ºC for 60 sec, annealing at 60ºC for 30 sec, and extension at 72ºC for 2 min. A final polymerization extension at 72ºC for 10 min was accomplished. The CYP2D6 products were digested at 60ºC over-night with BstNI restriction enzyme. PCR products were separated on an ethidium bromide stained 2% agarose gel for GSTM1, or on a 5% polyacrylamide gel followed by ethidium bromide staining for CYP2D6. Visualization was accomplished with an UV transiluminator. The CYP2D6 normal allele produces two fragments of 105 and 229 bp by

digestion. G→A transition affects the restriction sequence and a unique fragment of 334 bp is observed. Heterozygous individuals display two bands corresponding to the restricted normal allele and a third band from the mutated allele. GSTM1 genotype is termed active when a 230 bp and a 157 bp bands are present and null if the 230 bp is absent. The chi-squared (χ^2) test was used for statistical analyses.

RESULTS

Mean age of the patients at diagnosis was 66.7 years ± 10.6 (standard deviation, SD). Age extremes were 36 and 85, and 71.4% were older than 60 years. Fifty-four (70.1%) tumors were "superficial" (Ta-T1) and 23 (29.9%) were invasive (≥ T2). There were 23 (29.9%) well-differentiated tumors (G1), 37 (48%) moderately differentiated (G2), and 17 (22.1%) poorly differentiated (G3). Among the 54 patients with "superficial" tumors, there was no history of relapses in 32 (63%). Nine (16.7%) had had one relapse, and 11 (20.3%) two or more relapses. There was no case of evolution to invasive tumor at relapse. Thirty-five patients (45.4%) had never smoked, and 42 (54.6%) were currently (22; 28.6%) or had been (20; 26%) smokers. Among the 42 patients with smoking history, the mean pack-years smoked was 43.8 ± 36.6 SD (2-200). The majority (24; 57.2%) were heavy smokers (> 40 pack-year); ten (23.8%) had smoked < 20 pack-year, and 8 (19%) between 20 and 40 pack-year. Seven (9.1%) had history of contact with genotoxics, and 17 (22.1%) recalled excessive alcohol intake. Family history of tumors (1st - 2nd degree relatives) was present in 24 (31.2%) patients, in four cases (5.2%) the tumor being a bladder cancer. The GSTM1 null genotype was detected more commonly in the diseased population altogether (75.3%) than in the healthy individuals (57.1%), and this difference was statistically significant (Table-1). Among patients with invasive tumors, the distribution of the polymorphisms was similar to that of the control group (Table-2). On the other hand, 44 (81.5%) of the 54 patients with "superficial" tumors had the null genotype, an even higher difference to the control group ($\chi^2 = 10.7$, $p = 0.001$; Table-2). Sub-dividing the patients with "superficial" tumors into 2 groups (with and without relapses), the sub-group without relapses demonstrated a higher degree of statistical difference to the controls ($p = 0.005$) than the ones with history of relapses ($p = 0.047$; Table-2). The null genotype was detected in 69.5%, 78.4%, and 76.5% of the patients with tumors well, moderately, and poorly differentiated, respectively. However, statistically significant difference to the control group was achieved only by grouping patients with G2 and G3 tumors ($\chi^2 = 7.64$, $p = 0.006$; Table-2). Patients with "superficial" tumors and "invasive" tumors were marginally different to each other in respect to GSTM1 polymorphisms ($\chi^2 = 3.68$, $p = 0.054$). The comparison of GSTM1 polymorphisms in smokers (current and ex-smokers) and non-smokers revealed that there were more smokers than non-smokers with the null genotype, the difference being statistically significant (Table-3). Further, the sub-division of smokers in 2 groups showed that much more heavy smokers than light smokers had the null genotype

Table 1 *- Comparison of GSTM1 genotypes in patients and healthy individuals*

	GSTM1 +	GSTM1 -	χ^2	p
Control population	82	109	7.79	0.005
Patients	19	58		

Table 2 *- Comparison of GSTM1 genotypes in the control population and in sub-groups of patients, defined in accordance with tumor characteristics*

	GSTM1 +	GSTM1 -	χ^2	p
Control population	82	109		
Patient sub groups:				
Ta/T1 tumor	10	44	10.7	0.001
≥ T2 tumor	9	14	0.12	0.73
Ta/T1, relapsing	4	16	3.90	0.047
Ta/T1, no relapses	6	28	7.75	0.005
G 1	7	16	1.32	0.25
G 2/3	12	42	7.64	0.006

Table 3 - *Relationship between smoking habits and GSTM1 polymorphism*

	GSTM1 +	GSTM1 -	χ^2	p
Non-smokers	13	22	5.36	0.02
Smokers	6	36		
Smokers < 40 PY	5	13	4.68	0.03
Smokers ≥ 40 PY	1	23		

(Table-3). Patients with family history of tumors were significantly more prone to have the null phenotype than patients without ($\chi^2 = 5.01$, $p = 0.02$). CYP2D6 polymorphisms frequency in the patients was not significantly different from that of the control population (Table-4). No significant differences were

Table 4 - *Comparison of CYP2D6 genotypes in patients and healthy individuals*

	EM	HET	PM	χ^2	p
Control population	61	31	7	2.22	0.32
Patients	37	15	1		

EM = extensive metabolizer, HET = heterozygote, PM = poor metabolizer

detected even after sub-division of the diseased population in sub-groups according to tumor characteristics (Table-5). There was a strong positive association between invasiveness and poor

Table 5 - *Comparison of CYP2D6 genotypes in the control population and in sub-groups of patients, defined in accordance with tumor characteristics*

	EM	HET	PM	χ^2	p
Control population	61	31	7		
Patient sub groups:					
Ta/T1 tumor	24	8	-	3.25	0.19
≥ T2 tumor	11	4	1	0.85	0.30
Ta/T1, relapsing	12	3	-	2.30	0.31
Ta/T1, no relapses	12	5	-	1.40	0.49
G 1	12	4	-	1.70	0.42
G 2/3	23	8	1	1.33	0.51

EM = extensive metabolizer, HET = heterozygote, PM = poor metabolizer

differentiation of the tumor (4.3% of G1, 24.3% of G2 and 76.5% of G3 tumors were invasive; $\chi^2 = 25.32$, $p = 0.0001$). On the other hand, no significant association between tumor differentiation or tumor invasiveness and smoking habits was detected.

DISCUSSION

The results of the present study strongly suggest that lack of activity of the GSTM1 gene is associated with the occurrence of bladder cancer. These results were achieved by comparing the genetic polymorphisms detected by PCR in a diseased population and in a control population, matched for ethnic and geographic origin. This requisite is essential, as polymorphisms show inter-ethnic, and even inter regional variations (12). In this study, absence of GSTM1 activity was detected in 57.1% of our control population, a value higher than the reported in several other studies (3,7,9,10,13), although identical to others (3,18). Patients with invasive (≥ T2) tumors demonstrated a GSTM1 null genotype frequency similar to controls: 60.9% and 57.1%, respectively. Division of the patients with "superficial" tumors in those with and without history of relapses revealed a stronger statistically significant difference to controls in the no relapses group (Table-2). However, given that 80% of the patients with history of relapses showed the null phenotype versus only 57.1% of the control population, it is likely that it was the small number of patients with history of relapses that have prevented the achievement of a stronger statistical significance in this group. Okkels et al., in a case control study involving 234 patients, detected association between GSTM1 deficiency and bladder tumors only in the group with "prevalent benign" tumors (meaning Ta or Tis (in situ) tumors with a long evolution) (9). Although not directly comparable, in both studies the association of GSTM1 null genotype with bladder tumor was more apparent in the group with less aggressive tumors. This distinction between patients with more or less aggressive tumors may be in accordance with recent studies that demonstrated distinct molecular defects in these different types of tumor, and that point to

different genetic pathways in the evolution from normal epithelium to "superficial" or invasive tumors (19). Our study design precludes for estimating the odds of developing bladder tumor in individuals exposed to risk factors such as tobacco smoke or alcohol. However, once smokers demonstrated a significantly higher frequency of the GSTM1 null genotype than non-smokers, and that the heavier the smoking history was, the higher was the probability of being GSTM1 null (Table-3), our data agree with previous studies suggesting that the GSTM1 null genotype increases the risk for bladder cancer only in individuals who smoke (9). Like others (3,6,11), we observed that variability in CYP2D6 activity does not seem to be relevant in respect to bladder tumor development. Anwar et al. found that the extensive metabolizer genotype increased the risk further in individuals with the GSTM1 null genotype (7), but the combined analysis of our results does not sustain this conclusion.

CONCLUSION

The GSTM1 null genotype seems to be associated with bladder tumor occurrence, particularly "superficial" tumors (Ta/T1), and this association is stronger in individuals with exposure to tobacco smoke. CYP2D6 gene does not seem to play any significant role in bladder tumor development.

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