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Research Article

POLYMORPHIC DELETIONS OF GLUTATHIONE S-TRANSFERASES M1, T1 AND BLADDER CANCER RISK IN ALGERIAN POPULATION

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ABSTRACT

Objective: *Glutathione S-transferase mu 1* (*GSTM1*) and *GST theta 1 (GSTT1)* genes are two xenobiotic metabolizing genes in Phase II of the detoxification process. The polymorphisms of *GSTM1*, *GSTT1* genes, and smoking are involved in many cancers such as bladder cancer. Our aim was to assess the role of smoking status and *GSTM1* and *GSTT1* null genotypes in bladder cancer development in Algerian population.

Methods: The current case–control study included 175 bladder cancer patients and 188 controls matched for age, gender, and ethnic origin. The *GSTM1* and *GSTT1* genotypes were determined by multiplex polymerase chain reaction using blood genomic DNA. Possible associations of stage and grade with the obtained genotypes were also tested.

Results: A significant associations were observed between bladder cancer risk and tobacco smoke (p value: p=1.21E-08), *GSTM1 null* genotype (p=0.018), *GSTT1 null* genotype (p=0.009), and *GSTM1/GSTT1*-double null genotype (p=0.001). The combined effect of smoking and testing deletions increased the risk of bladder cancer and the most important risk was observed among smokers carrying *GSTM1/GSTT1*-double null genotype (p=1.09E-05). No significant association was shown between stage and grade of bladder cancer and the testing genotypes.

Conclusion: This study indicated that smoking, *GSTM1 null*, *GSTT1 null*, *and GSTM1/GSTT1*-double null genotypes individually represent a risk factor for bladder cancer in Algerian population. The interaction smoking gene increased the risk considerably. In fact, it is suggested that patients with cigarette smoking habit and combined *GSTM1* and *T1* genes deletion might be at increased risk of bladder cancer.

Keywords: *Glutathione S-transferase mu 1, Glutathione S-transferase theta 1*, Smoking, Bladder cancer.

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INTRODUCTION

Cancer is currently estimated to be a major cause of deaths worldwide [1]. Urothelial bladder cancer is one of the commonly occurring cancers [2]. It is the 7th most commonly diagnosed cancer in males and the 17th in females worldwide. Global estimates suggest that there are annually 330–400 bladder cancer cases with 123,100 related deaths in the word [3].

In North African men, bladder cancer is the most common cancer after liver and lung cancers, accounting an annual estimated incidence of 11,225 with 5489 deaths [3]. Between 2006 and 2010, the incidence for Algerian men were 10.3/100,000 person-years, this represents 9.1% of the global cancer incidence in Algeria [4].

Bladder cancer can be muscle-invasive or non-invasive and classified as high-grade or low-grade tumor, respectively [5]. The risk of this disease increases with advancing age and most cases are diagnosed above 65 years of age [6]. The incidence is 5–7 times higher in males than in females in Algeria and North Africa [3,4].

It is admitted that the primary prevention of this deadly disease needs first of all understanding the etiology and determining the risk factors.

Smoking is the most well-established risk factor for bladder cancer in both sexes [5,7], with the risk among smokers reported to be approximately fourfold that among non-smokers [7].

The combustion of tobacco releases at least at 69 known carcinogens which have been directly implicated as mutagens causing bladder cancer [8,9].

Following smoking, occupational exposure to aromatic amines and polycyclic aromatic hydrocarbons (PAHs) are other important risk factors [10]. Increasing evidence suggests a significant influence of genetic polymorphism of xenobiotic-metabolizing enzymes which could increase susceptibility to various environmental and clinical conditions. The balance between activation and detoxification of carcinogens affects the amount of DNA damage that occurs in cells [11].

A large family of enzymes has been encoded by glutathione-S-transferases (GSTs) multigene and plays a vital role in the mechanism of cellular detoxification [9,12]. Until now, human cytosolic GST superfamily contains at least 16 genes subdivided into eight distinct classes designated as: (pi), (alpha), (sigma), (mu), (theta), (kappa), (omega), and (zeta), which are encoded by the *GSTA, GST mu (GSTM)*, *GSTK*, *GSTO*, *GSTP*, *GSTS*, *GST theta (GSTT)*, and *GSTZ* genes [11]. They catalyze different reactions with bladder cancer carcinogens such as aminobiphenyls and PAHs [13]. Functional polymorphisms have been identified in the *GSTM1*, *GSTT1*, *GSTP1*, and *GSTA1* [11,14]. In recent years, studies of *GSTs* have revealed new roles for some of the members of this family. It has been demonstrated that *GSTs* of classes (alpha), (mu), and (pi) are involved in cell proliferation, differentiation, and control of cell death through interactions with special signaling proteins [15].

It was reported that both *GSTM1* and *GSTT1* genes exhibit deletion polymorphisms. Homozygous deletions of these genes resulted in null genotypes which results in a lack of enzyme activity [9,12]. This information suggests that these mutations may interfere and affect the susceptibility to many malignant tumors as cervical, laryngeal, oral, and bladder cancer [9,12].

Positive association of *GSTM1* and *GSTT1* deletion polymorphism and bladder carcinoma was found among many populations [16]. However, data related to a genetic status of *GSTM1* and *GSTT1* and their association to bladder cancer is not available in Algerian population. Therefore, the aim of this current study was first to examine the association between *GSTM1* and *GSTT1* genes variants and bladder cancer risk through a case–control study in Algerian population. Then, we evaluated the differential as well as the combined effect of selected variants according to tobacco smoking in the onset and development of bladder carcinoma. Finally, we tried to establish an association between these two studied genes and clinicopathologic characteristics of bladder cancer.

METHODS

Subjects

The study population consisted of 175 patients with bladder cancer and 188 healthy controls. Cases were recruited between September 2014 and May 2016 from Central Hospital University of Tizi-Ouzou (Urology department) and Daksi renal Clinic of Constantine (Urology department). All were from North of Algeria, aged between 22 and 90 years. More than 92% of them were men. All patients were confirmed by clinical histopathology and staged according to the tumor/node/metastasis staging system of the Union International Contre le Cancer (UICC; 1997). Tumors were graded according to the World Health Organization 1973 classification.

The control group consisted of unrelated healthy subjects without a history of malignant disease who were approximately matched for gender proportion, geographic origin, and age range, to those in the case group. A detailed questionnaire was elaborated and used to obtain study information's, and under informed consent, peripheral blood samples were collected into tubes with EDTA (pH 8).

DNA extraction

Genomic DNA was extracted from peripheral leukocytes using sodium chloride (NaCl) method. The quality and quantity of DNA were determined, respectively, by agarose gel electrophoresis and spectrophotometric analysis using nanodrop. DNA was diluted to 10 ng/ml and stored at −20°C for further use as a template.

Genotyping of the *GSTM1* and *GSTT1* using multiplex polymerase chain reaction (PCR)

The screening of *GSTM1* and *GSTT1* genes was performed by multiplex PCR methodology. Briefly, the PCR was carried out in 25 µl mixture containing 50 ng of DNA using DNA Polymerase Kit: HotStarTaq DNA polymerase-QIAGEN (4 µl buffer ×1, 8 mM of deoxynucleosides S-triphosphates, 2 U/µl Taq polymerase, and 2.5 mM of each primer).

The paired primers for *GSTM1* were:

5'-GAACTCCCTGAAAAGCTAAAGC-3' and 5'-GTTGGGCTCAAATATACGGT GG-3' [12].

The paired primers for *GSTT1* were:

5'-TTCCTTACTGGTCCTCACATCTC-3' and 5'-TCACCGGATCATGGCCACCA-3' [12].

The paired primers for *β-globulin* were:

5'-ACACAACTGTGTTCACTAGC-3' and 5'-CAACTTCATCCACGTTCACC-3' [12].

β-globulin was used as an internal control, confirming successful PCR amplification to ensure that the *GSTM1 null* and *GSTT1 null* were due to the deletion allele of the *GSTs* and not because of the failure of the PCR.

Cycling conditions were as follows: Initial preheat 95°C for 10 min, 35 cycles of 94°C for 1 min, 60°C for 45 s, and 72°C for 1 min. The final extension was done at 72°C for 10 min. The amplified products were subjected to electrophoresis on 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. The *GSTM1* fragment was 230 bases pairs (bp), the *GSTT1* was 480 bp, and the *β*-globulin fragment was 110 bp in size.

Statistical analysis

Relative risks were estimated by calculating the odds ratios (OR) with 95% confidence intervals (CI) at the 0.05 significance level. OR were calculated using R software version 3.2.3. $p<0.05$ were considered statistically significant.

RESULTS

Characteristics of the study population

Relevant characteristics of cases and controls are given in Table 1. There are more male than female cases (92.6% vs. 7.4%) with a sex ratio of 12:1. The mean age of bladder cancer patients and controls were similar, 60.77±13.21 years (range 22–90) and 59.56±14.02 years (range 22–89), respectively. 80% of bladder cancer patients were more than 50 years of age. Smoking was more widespread among patients with bladder cancer 122/175 (69.7%) than controls 74/188 (39.4%) which it recalled that they had been taken randomly (p=1.21E-08). Of the 175 cancer patients, 16% had tumors with Grade I, 25.1% Grade II, and 58.9% Grade III. 19.4% had a Ta stage, 48.6% had a T1, 23.4% had a T2, and 8.6% had a T3 stage or more.

GSTs genotypes, bladder cancer susceptibility, and interactions with smoking

GSTs genotypes frequencies and their relation to bladder cancer risk

GSTM1 null genotype (M1-) was more frequent than *GSTT1 null* genotype (T1−). Their frequencies were 57.1% and 39.4%, respectively, in bladder cancer patients compared to 44.7% and 26.6% respectively in healthy controls (Table 2). These results show a significant association between the *GSTM1 null* genotype and risk of bladder cancer (p=0.018). *GSTT1 null* genotype was also associated with greater risk toward this type of cancer (p=0.009).

Combined *GSTM1* and *GSTT1* genotypes and bladder cancer risk

The analysis of combined *GSTM1* and *GSTT1* genotypes revealed that all combinations were significantly associated with increased bladder cancer risk. As presented in Table 2, our results show clearly that the combinations between *GSTM1* and *GSTT1* genotypes increase the OR values and decrease the p value. The highest OR was observed for *GSTM1/GSTT1*-double null genotype (OR=2.9; CI: 1.44–6; p=0.001).

Association of GSTs genotypes and smoking status with bladder cancer risk

The stratification of patients and controls according to *GSTs* genotypes and tobacco status is depicted in Table 2. The results indicated any statistically significant association between *GSTM1 null*, *GSTT1 null* genotypes, or possible combined *GSTM1/GSTT1* genotypes with bladder cancer risk among never smoker group.

However, among current or prior smokers group, all tested genotypes were revealed as risk genotypes except for combined *GSTM1 positive/ GSTT1 positive* (p=0.17).

It is important to note that the interaction of smoking and one *GSTs null* genotypes increase the risk considerably, (OR=6.7; CI: 2.8–16.8; p=1.33E-06) for *GSTM1 null* genotype and (OR=6.9; CI: 2.3-22.7; p=0.0001) for *GSTT1 null* genotype. Smokers with *GSTM1/GSTT1*-double null genotypes have a most important risk for developing bladder cancer (OR=7.9; CI: 2.8–24.4; p=1.09E-05) when compared to a reference group of non-smokers with the *GSTM1 positive/GSTT1* positive.

Relationship between GSTs genotypes and clinicopathological parameters

The associations of *GSTM1*, *GSTT1*, or combined *GSTM1/GSTT1* genotypes with tumor grades or stages of bladder cancer were not seen (data not shown).

Table 1: Characteristics of the study population

Parameters	Controls (%)	Cases (%)	OR	CI	p value
Gender					
Male	169 (89.9)	162 (92.6)	-	-	0.37
Female	19 (10.1)	13 (7.4)	-	-	
Age (years)					
<50	43 (22.9)	35 (20)	-	-	0.5
≥50	145 (77.1)	140 (80)	-	-	-
Mean age (range)	59.56±14.02 (22–89)	60.77±13.21 (22–90)	-	-	-
Smoking status					
Never smoker	114 (60.6)	53 (30.2)	-	-	-
Current or prior smoker	74 (39.4)	122 (69.7)	3.55	229–5.48	1.21E-08**
Grade					
G1	-	28 (16)	-	-	-
G2	-	44 (25.1)	-	-	-
G3	-	103 (58.9)	-	-	-
TNM stage					
Ta	-	34 (19.4)	-	-	-
T1	-	85 (48.6)	-	-	-
T2	-	41 (23.4)	-	-	-
T3 or more	-	15 (8.6)	-	-	-
Recurrences					
No	-	83 (47.4)	-	-	-
Yes	-	92 (52.6)	-	-	-
Cystectomy after	-	9 (5.1)	-	-	-
Evolution					

**: P<0.01. TNM: Tumor/node/metastasis, OR: Odds ratios, CI: Confidence intervals

Table 2: *GSTM1, GSTT1* genotypes, and combined genes-smoking effect on bladder cancer susceptibility

Genotypes	Controls (%)	Cases (%)	OR	CI 95%	p value
GSTM1					
M1+	104 (55.3)	75 (42.9)	-	-	-
M1−	84 (44.7)	100 (57.1)	1.65	1.09–2.5	0.018
GSTT1					
T1+	138 (73.4)	106 (60.6)	-	-	-
T1−	50 (26.6)	69 (39.4)	1.8	1.15–2.8	0.009**
GSTM1/GSTT1					
M1+/T1+	76 (40.4)	40 (22.9)	-	-	-
M1+/T1−	28 (14.9)	35 (20)	2.36	1.2–4.67	0.004**
M1-/T1+	62 (33)	66 (37.7)	2.02	1.2–3.39	0.007**
M1-/T1−	22 (11.7)	34 (19.4)	2.9	1.44–6	0.001**
GSTM1-smoking					
Never smoker					
M1+	58 (30.9)	28 (16)	-	-	-
M1−	56 (29.8)	25 (14.3)	0.9	0.45–1.86	0.87
Current or prior smoker					
M1+	46 (24.5)	47 (26.9)	2.1	1.1–4.1	0.01**
M1−	28 (14.9)	75 (42.9)	5.5	2.84–10.9	3.87E-08**
GSTT1-smoking					
Never smoker					
T1+	81 (43.1)	31 (17.7)	-	-	-
T1−	33 (17.6)	22 (12.6)	1.7	0.8–3.6	0.1
Current or prior smoker					
T1+	57 (30.3)	75 (42.9)	3.4	2–6	6.96E-06**
T1−	17 (9)	47 (26.9)	7.1	3.4–15.4	6.3E-09**
GSTM1/GSTT1-smoking					
Never smoker					
M1+/T1+	38 (20.2)	14 (8)	-	-	-
M1+/T1−	20 (10.6)	14 (8)	1.88	0.7–5.2	0.2
M1-/T1+	43 (22.9)	18 (10.3)	1.1	0.5–2.8	0.8
M1-/T1−	13 (6.9)	7 (4)	1.4	0.4–5	0.56
Current or prior smoker					
M1+/T1+	38 (20.2)	26 (14.9)	1.8	0.8–4.4	0.17
M1+/T1−	8 (4.3)	21 (12)	6.9	2.3–22.7	0.0001**
M1-/T1+	19 (10.1)	48 (27.4)	6.7	2.8–16.8	1.33E-06**
M1-/T1−	9 (4.8)	27 (15.4)	7.9	2.8–24.4	1.09E-05**

GSTM1 positive (M1+), *GSTM1 null* (M1−), *GSTT1 positive* (T1+), and *GSTT1 null* (T1−), (**): *P*<0.01. *GSTM1: Glutathione S-transferase mu 1, GSTT1: Glutathione S-transferase theta 1*, OR: Odds ratios, CI: Confidence intervals

DISCUSSION

We have undertaken this current study to investigate the combined effect of *GSTM1*, *GSTT1* genes polymorphism, and smoke on bladder cancer susceptibility in Algerian population.

Our case–control study revealed, first in bladder cancer group that the incidence is 12 times higher in males than in females (sex ratio 12:1). This difference in incidence is greater than those reported in previous studies among North Africans [3] and Algerian population [4]. We have also shown that cigarette smoking was a significant risk factor for bladder cancer risk (OR=3.55; CI: 2.9–5.48; p=1.21E-08). More than 50% of our cases are smokers. A similar observation was made by Burger *et al.* [8]. Several previous studies in the world demonstrated the increased risk of bladder cancer in smokers [5,7,16,17].

Bladder cancer is the result of the variable combination of two determinants: Endogenous or constitutional factors and exogenous or environmental factors [6]. Genetic differences between detoxification systems may cause an increase in susceptibility to environmentally induced bladder cancer [18]. Thus, the GSTs are multigene family-related isozymes that involved in the detoxification of carcinogens, including PAHs present in tobacco smoke [6,9,12,19]. It was noted that *GSTM1 null* genotype is the most important factor for the reduction of total GST activity [20]. The polymorphism in the *GSTT1* gene loci is also caused by a gene deletion and leads to a virtual absence of enzyme activity in individuals with the null genotype [11]. *GSTM1* and *GSTT1* gene polymorphisms have been associated with a wide variety of cancers including bladder cancer [9].

It is reported that the prevalence of *GSTM1 null* genotype varies widely from 31% to 88% in relation to ethnic origin [21]. This frequency in our studied population was 44.7% for healthy controls and 57.1% for cases which approach substantially to that given for Tunisian and Egyptian populations [22-24]. Deletion of *GSTT1* loci was higher in bladder cancer patients than in controls (39.4% vs. 26.6%, respectively). These proportions were so different to that compared to other ethnic groups [25].

To investigate the impact of metabolic polymorphism in modulating bladder cancer risk susceptibility, we have compared genotypic frequencies of studied genes between individuals carrying the wild-type (*GSTM1* positive, *GSTT1* positive) and individuals at risk (*GSTM1 null*, *GSTT1 null*).

Our results indicate that in Algerian population the *GSTM1 null* genotype confers statistically a significant correlation for overall risk for bladder cancer (OR=1.65; CI: 1.09–2.5; p=0.018). This finding is consistent with combined results of 26 studies represented in the meta-analysis of Zhang *et al.* [26], including 5029 bladder cancer cases and 6680 controls. Other more recent studies carried out in India and Pakistan have given the same result [16,27]. Among all studied ethnic groups, the association between *GSTM1 null* genotype and risk of bladder cancer was observed in Asians and Caucasians [26]. Conversely, in Tunisian and Egyptian populations results suggested that this polymorphism had no effect on risk of bladder carcinogenesis [24,28-30]. Moreover, only one Egyptian study recorded this association, but it presented a limitation of effective which was too low [31].

We also investigated the association of *GSTT1* and bladder cancer. As for the *GSTM1 null* genotype, statistical test shows an association between the *GSTT1 null* genotype and urinary bladder cancer (OR=1.8, CI: 1.15–2.8; p=0.009). Our results were in agreement with a number of other studies presented in a meta-analysis of Gong *et al.* [19] who reported this association in Caucasians. However, no dramatically increased risk was found for other populations including North Africans [12,19,24,28,29,32], except for the study of Saad *et al.* [30] who suggested an increased risk with *GSTT1null* genotype in the Egyptian population.

Patients with combined homozygous deletion (*GSTM1 null/GSTT1 null*) have a greater risk (OR=2.9; CI: 1.44–6; p=0.001) compared to those harboring only one deletion. This is in accordance to the results mentioned in the meta-analyses of Gong *et al.* [19]. This observation can be explained by the fact that the presence of the combined homozygous deletion of *GSTM1* and *GSTT1* genes is associated with reduction or complete loss of the enzyme activity [12,13]. In this context, we can suggest that the double mutated genotype may play an important role in the susceptibility of bladder cancer.

In this study, we have also compared genotypic frequencies of studied genes combined with smoking status between non-smokers carrying wild type as a reference group and individuals at risk: Smokers with one or two genetic risk factors. About *GSTM1 null* genotype, we found that risk is present only in smokers. However, for *GSTT1 null* genotype, the risk is most important in smokers than no smokers group.

The simultaneous association of both null *GSTM1* and *GSTT1* genotypes with cigarette smoke status gives a highest probability to develop bladder cancer.

This combination smoking gene is biologically plausible because *GSTM1* and *GSTT1* genes are involved in Phase II detoxification of carcinogens PAHs found in cigarette smoke. Anyone of the two null *GSTM1* or *GSTT1* genotypes causes reductions of enzymatic activity [12,13].

Among non-smokers group, the exposure to these carcinogens is much less important than in smokers group. Therefore, the null genotypes of *GSTM1* and *GSTT1* genes will have a less dangerous effect on non-smokers group. However, in smokers, continuous exposure makes the process of detoxification that involves interventions of enzymes encoded by *GSTM1* and (or) *GSTT1* genes very important. Rouissi *et al.* [29] have noted an increased risk after the combination of many factors including *GSTM1 null*, *GSTT1 null* genotypes, and smoking. We concluded that the simultaneous association of risk factors gives a high probability to develop bladder cancer as shown by increased OR value presented in our study (OR=7.9).

On the other hand, the results of this current study indicate that there were no statistically significant associations between the clinical stages and histological grades of tumors and the frequencies of genotypes. Our findings concur with many previously published studies [11,20,32].

CONCLUSION

In conclusion, our data suggest in Algerian population that *GSTM1 null* and *GSTT1 null* genotypes increase the risk for urinary bladder carcinoma. The combination of the two gene polymorphisms represents a greater risk. A strong association was found between genetic deletions of *GSTM1*, *GSTT1*, and bladder cancer among smokers. The most important risk was shown in smokers carrying double deletions of studied genes. An absence of association between *GSTs* genotypes, stage, and grade of tumors was registered. However, owing to the relatively smaller sample size, further studies on larger series of patients are required to confirm the present findings.

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AUTHORS' CONTRIBUTIONS

All of the authors mentioned in the article have contributed to this research work. A Hireche: Protocol/project development, data collection or management, data analysis, and Manuscript writing/

editing. N Chaoui-Kherouatou: Protocol/project development, data analysis, Manuscript writing/editing, and final correction of the manuscript. A Ribouh: Protocol/project development and data collection or management. N Abadi: Protocol/project development. MJ Shi: Data analysis. D Satta: Protocol/project development and final correction of the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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