Original article
Genetic polymorphism of glutathione S-transferases M1 and T1 in Egyptian patients with bilharzial bladder cancer

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Abstract

Objective: To assess the influence of glutathione S-transferases M1 and T1 (GSTM1 and T1) genotype on the risk of bladder cancer in patients with urinary bilharziasis.

Materials and methods: This study was designed as a case-control study that involved 60 individuals who were enrolled into 3 equal groups. The first one included patients with bilharzial bladder cancer, the second one had those with nonmalignant urinary bilharziasis, and the last one was the control group. All of the participants were adult males, nonsmokers, and with matched ages. All of them underwent an assessment of the serum level of the total GST concentration and the polymerase chain reaction (PCR) was used for determination of the GSTM1 and T1 genotypes.

Results: The lower most GST enzyme concentration was reported in patients with bilharzial bladder cancer (26 ± 4.4 ng/ml) with significant difference between it and that of the second group (36.8 ± 4.1 ng/ml, \( P < 0.05 \)) and that of the controls (40.4 ± 4 ng/ml, \( P < 0.005 \)). The PCR results have demonstrated that the frequency of combined GSTM1 and T1 genes deletion (M1–ve T1–ve) was significantly higher in cases of bladder cancer (40%) than those of the controls (5%, \( P < 0.005 \)) and those of the second group (10%, \( P < 0.05 \)). The unconditional logistic regression test revealed that patients with urinary bilharziasis and combined GSTM1 and T1 genes deletion are at a significant risk for malignant transformation (OR = 6.3, \( P < 0.05 \)).

Conclusions: Patients with urinary bilharziasis and GSTM1–ve and T1–ve genes might be at increased risk of bladder cancer. However, larger studies are needed for confirmation of these results. © 2010 Elsevier Inc. All rights reserved.

Keywords: Bilharziasis; Bladder cancer; GSTM1; GSTT1; Genetic polymorphism

1. Introduction

In Egypt, the majority of bladder cancer is associated with urinary bilharziasis [1]. It has been reported that schistosoma-induced chronic inflammation and irritation of the urinary bladder are associated with increased incidence of bladder cancer [2]. Inflammatory cells such as macrophages and neutrophils are important sources of endogenous oxygen free radicals that are implicated in the initiation of the malignant changes. These free radicals can induce genotoxic effects such as mutations, sister chromatid exchanges, and DNA strand breaks [3].

Glutathione S-transferases are a multigene family of both cytosolic and membrane bound enzymes, which are widely expressed in mammalian tissues. They exert their effect through catalyzing the conjugation of erythrocyte’s reduced glutathione (GSH) to the products of oxidative stress as reactive oxygen species, electrophilic compounds, and DNA reactive intermediates. This is important for detoxification of xenobiotic and endogenous toxic compounds, protection of tissues from oxidative damage, as well as detoxification of many reactive chemical carcinogens [4].
GST isoenzymes have been assigned to at least eight separate classes designated α (Alpha), μ (Mu), κ (Kappa), ω (Omega), π (Pi), σ (Sigma), θ (Theta), and ζ (Zeta), that are encoded by the GSTA, GSTM, GSTK, GSTO, GSTP, GSTS, GSTT, and GSTZ genes, respectively [5]. Apart from liver enzymes, most of the GST enzymes are also found in extra-hepatic tissues such as muscle, testis, blood, upper aero-digestive mucosa, and urinary bladder [6–8].

Within the GSTM, 5 isoenzymes (M1-M5) have been identified and it was reported that the GSTM1 null genotype is the major factor for reduced total GST activity. GSTM1 gene is located at chromosome 1p13.3 and is involved in polycyclic aromatic hydrocarbons (PAHs) detoxification [9]. The GSTM1 locus is highly polymorphic in the Caucasian population. Besides a null-genotype due to a gene deletion, 2 alleles, GSTM1a and GSTM1b, have been described. Approximately 50% of the Caucasian populations are deficient for the GSTM1 [10].

There are two θ class genes, GSTT1 and GSTT2, and a pseudo-gene of the latter has also been reported. The GSTT1 is the most important one, and it catalyzes the conjugation of halomethanes with glutathione in human erythrocytes [11]. Industrial chemicals such as methyl chloride, methyl bromide, dichloromethane, and ethylene oxide are the different substrates for the GSTT enzyme [12].

It was reported that the level serum GST enzyme is related to the genotyping of both GSTM1 and T1 genes as they represent the major part of this family and polymorphism within these genes can result in a complete lose of the enzyme activity [13]. On the other hand, because of the role of these enzymes in detoxification of precarcinogenic materials, the inherited homogenous deletion of GSTM1 (0/0) and GSTT1 (0/0) has been implicated in the pathogenesis of many malignant tumors as oral, laryngeal, cervical, and bladder cancer [7,14–16].

The aim of the present project was to study the role GSTM1 and T1 genes polymorphism as a risk factor for bladder cancer in patients with urinary bilharziasis.

2. Patients and methods

This study was carried out on 60 individuals. All of them were nonsmoker males and above the age of 45 years. These subjects were selected to be included into one of the following 3 groups:

2.1. Group I (bilharzial bladder cancer)

Group I consisted of 20 patients with bilharzial bladder cancer. The diagnosis of this group was achieved through the histopathological examination of bladder biopsies that were obtained by the transurethral resection of suspicious bladder masses.

2.3. Group II (nonmalignant bilharzial bladder)

Group II consisted of 20 patients with urinary bilharziasis with no evidence of bladder cancer. These patients were recruited from the in-patients of the urology department who were scheduled for some endourological procedures that involved bladder visualization [as ureteroscopy and transurethreal resection of the prostate (TURP)]. The diagnosis of urinary bilharziasis was based on the history of repeated bilharzial infestation and anti-bilharzial treatment as well as the presence of bilharzial changes in the cold-punch biopsies taken before the planned endoscopic procedures. Meanwhile, the exclusion of bladder cancer was suggested by the –ve urine cytology and the absence of any malignant changes in the previous biopsies.

2.3. Group III (control group)

Group III consisted of 20 individuals with no evidence of bladder cancer, urinary bilharziasis, or other malignant diseases. All of them were males and were matched to the patients in the previous 2 groups with respect to the age and smoking status. This group was also recruited from the in-patients of the urology department who were scheduled for some endourological procedures that involved bladder visualization. The exclusion of urinary bilharziasis was based on the history of living in non-rural areas with little chance of accidental exposure to bilharzial infestation. In addition, the histopathological examination of the cold-punch biopsies taken before the planned endoscopic procedures showed no evidence of urinary bilharziasis or bladder cancer. Meanwhile, the exclusion of bladder cancer was also suggested by the –ve urine cytology.

All of the participants were subjected to detailed history taking, physical examination, abdomino-pelvic ultra-sonography, and routine laboratory investigations. In addition, all of them had an assessment of the serum level of the total concentration of the GST enzyme by the enzyme linked immunosorbent assay (ELISA) technique. Meanwhile, the genotyping of the GSTM1 and GSTT1 genes was achieved by the PCR technique as described below.

3. GSTM1 and GSTT1 genotyping

3.1. DNA isolation

Four ml of venous blood was withdrawn into an ethylenediaminetetraacetic acid (EDTA) tube. DNA was extracted from whole blood samples using QIAamp DNA blood Muki for DNA extraction (Qiagen, Inc., Valencia, CA) according to the manufacture’s instructions.

3.3. Genotype analysis by the PCR technique

The existence or deletion of the GSTM1 and GSTT1 genes was determined by the PCR amplification using
primer sets lying within the GSTM1 and T1 genes to identify the presence or absence of the gene sequences.

The DNA of each sample was introduced into a reagent mixture containing an excess of deoxynucleoside S-triphosphates (d NTPs), biotinylated primers, and thermostable DNA polymerase (Taq). The primers should amplify the region surrounding the point of polymorphism. By heating, the 2 strands of the DNA helix were separated (denaturation) in order to expose the target sequences to the primers. These primers were complementary to the regions flanking the target sequence. Therefore, upon cooling to a specific temperature, the primers would bind to their specific sequence (annealing). At another temperature and using d NTPs, the thermostable DNA polymerase would extend the annealed primers along the target template (extension). In this way, a biotinylated exact copy of the template sequence was produced after 1 cycle, thus yielding a multifold amplified biotinylated target sequence.

Briefly, the PCR was carried out in a 50 μl mixture containing 100 ng DNA and a master mix of PCR buffer containing 1.5 mM MgCl₂, 250 mM d NTPs, the thermostable DNA polymerase (Taq) polymerase (Taq Platinum; Gibco BRL, Gaithersburg, MD), and 0.8 μM of each primer.

The paired primers for GSTM1 were:

- 5'-GAA CTC CCT GAA AAG CTA AAG C-3' and 5'-GTT GGG CTC AAA TAT ACG GTG G-3'

The paired primers for GSTT1 were:

- 5'-TTC CTT ACT GGT CCT CAC ATC TC-3' and 5'-TCA CCG CAT GAT GGC CAC CA-3'

The paired primers for β-globulin were:

- 5'-ACA CAA CTG TGT TCA CTA GC-3' and 5'-CAG CTG CAT CCA CGT TCA CC-3'

β-Globulin was used as an internal control, confirming successful PCR amplification to ensure that the GSTM1-null and GSTT1-null were due to deletion of GST alleles and not due to failure of the PCR. Amplification was carried out in thermocycler (model 9600; Perkin-Elmer/Cetus, Langen, Germany). It consisted of 35 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute and extension at 72°C for 1 minute. The GSTM1 fragment was 230 bp, the GSTT1 fragment was 480 bp, and the β-globulin fragment was 110 bp in size.

4. Statistical analysis

Because the total GST enzyme concentration does not have a normal distribution, we used the Mann-Whitney U test to compare the results of the different groups. The frequencies of the null-genotype in tumor patients and other groups were compared using the χ² test. The association between the genotypes and the bladder cancer risk was examined by using the unconditional logistic regression to calculate the odds ratio (OR) and the 95% confidence interval (CI). Values of $P < 0.05$ were considered statistically significant. All of these studies were performed by using the computer software SPSS for Windows, ver. 10 (Chicago, IL).

5. Results

This study was carried out on 60 individuals who were stratified into 3 groups. The age of the participants ranged from 46.7 to 63.5 years with no significant difference among the 3 groups ($P = 0.7$). In the group of bilharzial bladder cancer, 60% of cases (12 patients) had transitional cell carcinoma (TCC) and the rest had squamous cell carcinoma (Sq CC). In the second group, there were 4 patients with ureteral stones and the others had benign prostatic hyperplasia (BPH), and all of them had a histological evidence of bilharzial bladder. In the control group, 5 cases had ureteral stones and the rest had BPH with no evidence of urinary bilharziasis in all of them.

The mean value of the total GST enzyme concentration of each group is demonstrated in Table 1, and our statistical analysis revealed that patients with bilharzial bladder cancer had a significantly lower concentration than that of the other 2 groups ($P < 0.005$, $P < 0.05$). Meanwhile, there was no significant difference between patients with TCC and those with Sq CC in this regard ($P = 0.11$). On the other hand, the enzyme concentration of patients with nonmalignant bilharzial bladder was less than that of the control group but the difference was statistically insignificant (Table 1).

In an attempt to gain insight into the molecular basis of the reduction of the serum level of GST enzymes in our patients, GSTM1 and T1 genotyping was done. These PCR results have shown 4 categories of such genes in these cases (M1–ve T1–ve, M1–ve T1–ve, M1–ve T1–ve, M1–ve T1–ve) with the –ve variety indicating the null type of this gene (Fig. 1). In addition, when we studied the frequencies of these categories in the 3 groups we observed that the frequency of combined GSTM1 and T1 genes deletion (M1–ve T1–ve) was significantly higher in cases of bladder cancer (40%) than those of the controls (5%, $P < 0.05$) and those of the second group (10%, $P < 0.05$) (Table 1). Meanwhile, there was no statistically significant difference in the frequency of this combined genes deletion between the controls and those with nonmalignant urinary bilharziasis ($P = 0.54$) (Table 1, Fig. 2).

When we stratified all of the participants (60 individuals) according to their genotyping (4 categories), our statistical analysis revealed that patients with combined GSTM1 and T1 genes deletion (category D) had the lowest enzyme activity (Table 2).

To find the relation between the genotyping and the risk of bladder cancer in patients with urinary bilharziasis, we used the unconditional logistic regression test to compare the genetic pattern of the group of bilharzial bladder cancer...
with that of patients with nonmalignant urinary bilharziasis. Statistical analysis showed that patients with bilharzial bladder and combined GSTM1 and T1 genes deletion (category D) had a 6 times increased risk of bladder cancer compared to those with GSTM1-ve and T1-ve genes (category A, reference category), and this increase was found to be statistically significant ($P < 0.05$, OR = 6.3, 95% CI = 1.02–36.8) (Table 3).

### 6. Discussion

Free radicals can cause DNA damage and play a significant role in the pathogenesis of malignant diseases. On the other hand, the GST enzymes have an important role in detoxification of these hazardous compounds. However, a wide variation in human GST activity has been observed, and this is attributed to the common polymorphism of the genes responsible for these enzymes. In addition, there are obvious discrepancies between the different ethnic groups in the genotyping of the GST family. It was shown that the rate of homozygous deletion of GSTM1 among healthy Africans, Europeans, Asian, Indians, Chinese, Japanese, Koreans, Filipinos, Samoans, and Hispanics ranged from 31% to 88% [17].

Meanwhile, the different studies in the literature have shown that individuals with very low levels of these enzymes are at increased risk for many malignant diseases such as oral, laryngeal, and esophageal cancers [7,14]. The potential role of GST enzyme activity and their gene polymorphism for bladder cancer susceptibility is less certain, particularly in Egyptian patients with chronic endemic bilharziasis.

In the present case, control study, we compared the group of bilharzial bladder cancer to the group of nonmalignant urinary bilharziasis to find out if the genetic predisposition could play a role in the development of this serious complication. We included also 20 healthy controls to det-
ect if the chronic bilharzial infestation can affect the GST enzyme level. Meanwhile, because tobacco smoking is an important risk factor for development of bladder cancer, all of the participants in this project were nonsmokers.

Because hereditary changes in the genotyping are the basis of life-long changes in the phenotype, we investigated the GSTM1 and T1 genes as well as the total GST enzyme concentration. We studied only the GSTM1 and T1 genes because their enzymes constitute the major part of this family and the serum level of the GST enzyme is usually related to the activity of these 2 genes [13]. This molecular genetic testing has the advantage of being able to unequivocally distinguish between GSTM1, GSTT1, and other glutathione transferase gene family members and can reveal the inherited basis of the changes in the enzyme activity. Meanwhile, to evaluate the role of genetic polymorphism in health effects, the combined genetic polymorphism of different genes should be taken into consideration. Therefore, we investigated both GSTM1 and GSTT1.

In the present study, we have demonstrated that the level of GST concentration in the group of patients with bilharzial bladder cancer was significantly lower than that of the control group and those of nonmalignant urinary bilharziasis. In addition, our results have shown that the lower most level of GST concentration was associated with the combined homozygous deletion of GSTM1 and T1 genes (GSTM1–ve, T1–ve). These results are in agreement with the result of Hayes et al. and Rebbeck who showed that this homozygous deletion is associated with reduction or complete loss of the enzyme activity [11,13]. Meanwhile, our statistical analysis revealed that patients with this combined homozygous deletion (GSTT10/0, GSTM10/0) were at increased risk of bilharzial bladder cancer (OR = 6.3, P < 0.05). We have shown that 10% of the control group had the GSTM1/GSTT1 null genotype, compared with 35% of the bladder cancer group. This is consistent with the results of Abdel-Rahman et al. who reported this combined gene deletion in 8.8% of healthy Egyptians [18]. The previous results of Anwar WA et al.,

### Table 2

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Category</th>
<th>No.</th>
<th>Enzyme concentration (ng/ml)</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1+ve T1+ve</td>
<td>A</td>
<td>27</td>
<td>31–69</td>
<td>49 ± 2.2</td>
</tr>
<tr>
<td>M1+ve T1–ve</td>
<td>B</td>
<td>6</td>
<td>15.6–68</td>
<td>26.4 ± 8.4</td>
</tr>
<tr>
<td>M1–ve T1+ve</td>
<td>C</td>
<td>16</td>
<td>17–67</td>
<td>28.4 ± 4</td>
</tr>
<tr>
<td>M1–ve T1–ve</td>
<td>D</td>
<td>11</td>
<td>7–20</td>
<td>11.6 ± 1.5</td>
</tr>
</tbody>
</table>

Man Whitney test for comparison of enzyme concentration:
- Between category A and C: U = 59.5 – P = 0.00.
- Between category A and D: U = 0.00 – P = 0.00.
- Between category B and C: U = 29.5 – P = 0.17.
- Between category B and D: U = 11.5 – P = 0.03.
- Between category C and D: U = 11 – P = 0.00.

### Table 3

Genotype as a risk factor for bilharzial bladder cancer

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Bih. bladder cancer no. (%)</th>
<th>Bilharzial bladder no. (%)</th>
<th>OR</th>
<th>95% CI for OR</th>
<th>Sig. (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1+ve T1+ve</td>
<td>7 (35%)</td>
<td>11 (55%)</td>
<td>2.29</td>
<td>1.02–5.82</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>M1+ve T1–ve</td>
<td>2 (10%)</td>
<td>2 (10%)</td>
<td>1.00</td>
<td>0.00–1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>M1–ve T1+ve</td>
<td>3 (15%)</td>
<td>5 (25%)</td>
<td>1.50</td>
<td>0.37–7.23</td>
<td>0.69</td>
</tr>
<tr>
<td>M1–ve T1–ve</td>
<td>8 (40%)</td>
<td>2 (10%)</td>
<td>4.00</td>
<td>1.00–15.62</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Gracia-Closas M et al., Georgiou et al., and Engel LS et al. showed that the GSTM1 –ve patients are more susceptible to bladder cancer [19–22]. In addition, it was shown that the GSTM1 null genotype increased the risk of bladder cancer in the German, Turkish, Tunisian, and Argentinean populations [23–26]. On the contrary, in a study by McGrath et al., the results showed no statistically significant association between the GSTM1 null genotype and bladder cancer [16].

Published results concerning the GSTT1 polymorphism and bladder cancer are less consistent. In a study by Hung RJ et al., the authors showed that patients with GSTT1 null genotype were at increased risk of bladder cancer [27]. In studies conducted in Slovakian and Egyptian population, statistically significant risk of bladder cancer for GSTT1 null genotype was observed [28–30]. However, the study by McGrath M et al. demonstrated that there is no association between GSTT1 genotyping and increased susceptibility to bladder cancer [16].

Lastly, in studies by Moore et al., Saad et al., and Abdel-Rahman et al., it was shown that the combination of GSTM1 and GSTT1 null genotype is associated with a significantly higher risk for developing bladder cancer [26,29,30]. Meanwhile, in a study by Srivastava et al. the combination of GSTT1 and M1 deletion showed an increased tendency of increased risk of bladder cancer in Indian population, although this difference was not statistically significant compared with the controls [31].

In conclusion, our results suggested that patients with urinary bilharziasis and a GSTM1–ve, T1–ve genotyping might be at increased risk of bladder cancer. Consequently, we have to keep an eye on this group of patients for early detection of malignant transformation. In addition, gene therapy may have a role in the future for correction of this genetic defect to prevent the transformation. However, we have to admit that, given our small sample size, we had limited power to detect small to moderate effects. Therefore, further studies should be done on a larger scale for confirmation of our results.

### References


