Genetic polymorphism of GSTM1, CYP2E1 and CYP2D6 in Egyptian bladder cancer patients

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Polymorphic changes in the GSTM1, CYP2E1 and the CYP2D6 genes have been reported to be individually associated with increased susceptibility to certain cancers. In the present study, the relationship between genetic polymorphism for these genes and development of urinary bladder cancer among Egyptian patients was investigated. Our results indicate that the frequency of bladder cancer patients with the GSTM1 null genotype is significantly higher than that of the normal controls (86.3 and 47.6%, respectively) with an odds ratio (OR) of 6.97 (95% CL = 1.59–30.57, Fisher’s exact \( P = 0.008 \)). In contrast, our investigation failed to demonstrate any difference in the distribution of CYP2E1 polymorphism between bladder cancer patients and controls as detected by \( PstI \) restriction fragment length polymorphism (RFLP) analysis. RFLP analysis of the CYP2D6 gene revealed a non-significant increase in the number of extensive metabolizers (EM) among the patients compared to the controls (68 versus 48%). However, the EM genotypes enhance the risk further for individuals harboring the GSTM1 null genotype as individuals harboring both the EM and the GSTM1 null genotypes have an odds ratio of 14.0 (95% CL = 1.3–151.4, Fisher’s exact \( P = 0.02 \)) compared to individuals harboring the EM and the GSTM1+/+ genotypes. In conclusion, our results indicate that genetic polymorphism, especially in GSTM1 and CYP2D6 could play an important role as host risk factors for development of urinary bladder cancer among Egyptians.

Introduction

Urinary bladder cancer is a common neoplasm around the world. It is the fifth most common cancer in the United States, accounting for over 51,000 new cases in 1994 (1). In developing countries such as Egypt, it constitutes 30.8% of total cancer incidence and ranks first among all types of cancer reported in males and second only to breast cancer in females (2). Therefore, understanding the etiology and mechanisms for development of this malignancy is of high priority for reduction of human sufferings.

*Abbreviations: OR, odds ratio; NNC, N-nitroso-compounds; PM, poor metabolizer; CYP2D6, cytochrome P450 2D6 gene; RFLP, Dral restriction fragment length polymorphism; GSTM1, glutathione-S-transferase M1; PCR, polymerase chain reaction; EM, extensive metabolizers; HEM, heterozygous extensive metabolizers; CL, confidence limits; BCNU, 3-bis(2-chloroethyl)-1-nitrosourea.

In Egypt, the majority of bladder cancer is associated with *Schistosoma haematobium* infection (2–8). Such infection causes inflammation of the urinary bladder epithelium. It is believed that the induced proliferating cells become more susceptible to mutagenic insults from environmental toxicants such as N-nitroso-compounds (NNC*) (9–11). Continued exposure to toxicants and clonal expansion of the damaged cells in the bladder will increase the likelihood of cancer development (12). Consistent with this hypothesis, NNC have been detected in the urine of schistosoma patients, including those with bladder cancer, at levels significantly higher than in uninfected subjects (10,12,13) and these toxicants have also been demonstrated to induce cancer in schistosoma-infected baboons (9).

Most chemical carcinogens are not capable of inducing genetic damage by themselves but require metabolic activation to electrophilic proximate carcinogens, which can cause mutations. The amount of the ultimate carcinogens produced depends on the action of competing activation and detoxication pathways involving cytochrome P450 and glutathione-S-transferase enzymes (14–16). Genetic differences in these pathways are likely to be a major source of interindividual variations in susceptibility to cancer (17–19). For example, the poor metabolizer (PM) phenotype of the cytochrome P450 2D6 gene (CYP2D6) is associated with reduced bladder cancer susceptibility indicating that the enzyme may be involved in the conversion of procarcinogens to proximate carcinogens (20–22). Although CYP2D6 protein has not been detected in urinary bladder, consistent with this hypothesis, a direct correlation between CYP2D6 genotype and the concentration of the CYP2D6 mRNA was observed in the target bladder mucosa (23). The exact mechanism by which CYP2D6 is involved in schistosomal bladder cancer remains to be characterized.

Genetic polymorphism in CYP2E1 is another contributing cancer risk factor since this enzyme activates a number of small molecular weight compounds including NNC (24–27), which are known bladder carcinogens. A Dral restriction fragment length polymorphism (RFLP) of CYP2E1 is associated with an increased risk of lung cancer in Japanese (28). This finding, however, is not confirmed in Western populations (29–31). The difference may be due to ethnic differences in distribution of the mutated gene. Furthermore, it is not known if polymorphism in this enzyme may constitute a potential risk factor in schistosomal bladder cancer.

While oxidation by CYP450 enzymes is primarily regarded as the phase-I activating process in carcinogenesis (32), most phase-II metabolizing enzymes, e.g. glutathione-S-transferase M1 (GSTM1) are considered to be predominantly protective enzymes since they detoxify a number of reactive chemical carcinogens (16,33). The gene is polymorphic in humans, and the deficiency in enzyme activity is caused by an inherited homozygous deletion of the gene (GSTM1 0/0) (34–36). With the exception of a recent report (37), many studies indicate
that GSTM1 deficiency is correlated with bladder cancer (38–40). No reports, however, have been found in the literature regarding GSTM1 gene deficiency and schistosomal bladder cancer risk.

In the present study, the genetic alterations in GSTM1, CYP2E1 and CYP2D6 genes were investigated in an Egyptian bladder cancer population. Our results indicate that genetic polymorphisms in GSTM1 and CYP2D6 but not CYP2E1 could be important host risk factors for urinary bladder cancer among Egyptians.

Materials and methods

Study subjects

The target group consisted of 22 Egyptian bladder cancer patients (18 male and 4 females) (mean age ± SD = 58.6 ± 8.3). As part of the medical survey, all study subjects were interviewed to obtain their demographic data and health and smoking history. Among these patients, 19 (86.3%) had clinical or self-reported history of schistosomiasis. Their bladder cancers were histologically verified. The control group consisted of 21 normal individuals (13 males and 8 females) who were matched to the cases with respect to the range of age and gender and smoking history. There was no history of schistosoma infection nor malignancies. To avoid confounding factors due to ethnic differences in GSTM1 (41), CYP 2D6 (42) and GSTP1 (43), only Egyptians were included in the study.

Chemicals and reagents

All primers used for polymerase chain reaction (PCR) analysis were custom synthesized by the Recombinant DNA Laboratory, Sealy Center for Molecular Sciences, University of Texas Medical Branch, Galveston, TX. All other chemicals and reagents were from commercial sources and were of the highest purity available as described in each experimental procedure.

DNA Isolation

Normal and tumor bladder tissues were removed from bladder cancer patients by cystoscopy or surgery at the National Cancer Institute, Cairo, Egypt. Peripheral blood samples were obtained from normal controls. DNA was isolated from normal or tumor bladder tissues of patients and from peripheral blood lymphocytes of controls by a non-organic DNA extraction procedure as previously described (43) or by the conventional phenol–chloroform extraction method (44). Isolated DNA was resuspended in Tris–EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.2). Isolated DNA and frozen tissues were sent by courier express mail from Egypt to our laboratory in the United States. Upon arrival, samples were stored at −70°C until use.

Genotype analysis by polymerase chain reaction (PCR)

GSTM1 genotyping

The presence or absence of the GSTM1 gene was determined using a modification of a differential PCR approach described previously (38). Briefly, 50–100 ng of isolated DNA was added to a PCR reaction mixture composed of 30 pmol of each GSTM1 primer G1-5′-GAA CTC CCT GAA AAG CTA AAG C and G2-5′-GTT GGG CTC AAC TAT ACG CCT G. 10 pmol of each β-globin gene primers used as internal positive control (β1-5′-CAA CCT CAT CCA GGT TCC TCA CG and β2-5′-GAG CCA AGG ACA GGT AC), 200 μM of each dNTPs and 5 U of AmpliTaq DNA polymerase (Roche Molecular Systems, Inc., Branchburg, NJ, USA) in a total volume of 50 μl. Following an initial denaturation at 94°C for 2 min, 30 cycles of PCR were carried out at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. A final polymerization step of 72°C for 4 min is added at the end. All PCR products were then digested with three units of Drdl (New England Biolabs, Beverly, MA) and 2 U of ScrFI (New England Biolabs, Beverly, MA) in the presence of 100 μg/ml BSA and digestion buffer (10 mM NaCl, 5 mM Tris–HCl, 1 mM MgCl2, 0.1 mM DTT, pH 7.9) at 37°C under oil for 3 h. 50 μl of the reaction mixture was electrophoresed on an ethidium bromide stained 3% agarose gel. The bands were then analyzed on an ethidium bromide stained 1.5% agarose gel. The presence or absence of coamplification of GSTM1 (215 bp) and β-globin (268 bp) were then determined using a modification of a differential PCR approach described (43) or by the conventional phenol–chloroform extraction method (44). Isolated DNA was resuspended in Tris–EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.2). Isolated DNA and frozen tissues were sent by courier express mail from Egypt to our laboratory in the United States. Upon arrival, samples were stored at −70°C until use.

Results

GSTM1 genotyping

As shown in Figure 1, the differential PCR method adopted in our study allowed an internal standard-controlled classification of the different GSTM1 genotypes (GSTM1 +/+, +/0 and 0/0). In our study, only one out of 22 bladder cancer cases and one out of the 21 controls were found to be heterozygous for GSTM1 (GSTM1 +/0). Since GSTM1 +/0 and GSTM1 +/+ genotypes were reported to possess similar enzyme expression (38), these individuals were grouped among those
Ten controls (48%) were classified as extensive metabolizers (EM) and one patient was either homozygous for the bp deletion mutation (PM1) or heterozygous for the gene deletion and mutation 2 (PM2). The remaining two PM controls were PM3. Although the distribution of EM genotypes in patients (n = 15) was not statistically different from that of healthy controls (n = 10) (Fisher’s exact P = 0.145), an OR of 2.36 (95% CL = 0.68–9.9) reflected a modest increase in risk associated with EM genotypes (Table I). The same observation was true for the distribution of PM genotypes, where no statistical difference between patients and controls was detected although a modest under representation in the number of PM patients relative to the controls was observed (7 patients versus 11 controls) (Table I). There was no difference in the allelic frequency in EM and PM genotypes among patients and controls (Table I).

**Combined polymorphisms and bladder cancer risk**

To further elucidate the genetic factors associated with cancer risk, the role of combined genetic polymorphisms in the CYP2D6 and GSTM1 genes was investigated. Four genetic combinations were possible and individuals were classified into four groups as follows: PM/GSTM1++/++ individuals (group A), PM/GSTM1 0/0 individuals (group B), EM/GSTM1+++/++ individuals (group C) and EM/GSTM1 0/0 individuals (group D). The OR and the Fisher’s exact-test were calculated to estimate the relative risk associated with each genetic combination (Table II). The results indicated that EM with the GSTM1 null genotype (group D) are at a significantly higher relative risk to develop bladder cancer than EM individuals harboring the GSTM1++/+ genotype (group C) (OR = 14; 95% CL = 1.3–51.4; Fisher’s exact P = 0.02). Furthermore, there was an increased relative risk associated with EM/GSTM1 0/0 genotypes (group D) relative to PM/GSTM1+++/++ genotypes (group A) (OR = 8.4; 95% CL = 1.26–56.03; Fisher’s exact P = 0.02). In contrast, there was no significant increase in relative risk associated with the other genetic combinations compared to each other (group B compared to group A, group D compared to group B, group B compared to group C and group A compared to group C).

**Discussion**

The ability to characterize polymorphic genes involved in metabolism of carcinogens has opened up a new approach for human cancer risk assessment (52–54). In the present work, CYP2D6, CYP2E1 and GSTM1 were studied as indicators of metabolizers. Six of them were EM and four were HEM. The remaining 11 controls were PM; four were PM1 and five were PM2. The remaining two PM controls were PM3.
The majority of our patients had a history of schistosomal infection. The assumption that alkylating agents such as N-nitrosamines may be targets for the glutathione-S-transferase μ protective effect (38).

GSTM1 enzyme would also play a protective role in the bladder by preventing the formation of NNC. The increased susceptibility of the GSTM1 null individuals may result from impaired detoxication of reactive oxygen species (59), such as those involved in the formation of NNC. Indeed, it has been reported that NNC can be synthesized endogenously by enzymatic reactions as well as by activated macrophages and neutrophils resulting from inflammatory processes such as those accompanying schistosomal infection (60). The latter two cell types generate, via nitric oxide synthase, the nitric oxide radical that is involved in the formation of carcinogenic nitrosamines (61). Consistent with this hypothesis, it has been suggested that GSTM1 null individuals would be more susceptible to oxidative stress-induced damage during inflammation, a process that may progress to malignancy particularly following free-radical mediated activation of potential procarcinogens (62).

In contrast to the association between GSTM1 null genotype and increased risk to bladder cancer, our study failed to demonstrate an association between CYP2E1 PstI polymorphism and increased risk for the disease. This CYP2E1 polymorphism has not been studied extensively in relation to bladder cancer risk.
duce more of such alkylating reactive intermediates than poor metabolizers would be more at risk of developing schistosoma-associated bladder cancer since they would produce proximate carcinogens (20-22). It is logical to assume that constitutive drug metabolizing enzyme activity is a major determinant for the risk of developing bladder cancer (23). In view of the reported role of CYP2D6 in the conversion of proximate carcinogens to proximate carcinogens (20-22) it is logical to assume that extensive metabolizers would be more at risk of developing schistosoma-associated bladder cancer since they would produce more of such alkylating reactive intermediates than poor metabolizers.

The results of our study indicate an ethnic difference in the allelic frequency of the CYP2D6 genotypes compared to that previously reported in the literature for Caucasians, Chinese, Zimbabwe and Black Americans (for review see Reference 42). Among our Egyptian control population studied, 48% were classified as extensive metabolizers (EM and HEM) as compared to a reported range of 56-94% extensive metabolizers in different ethnic populations (42). This implies an over-representation of the PM genotypes in our control population and 43% of all PMs in our bladder cancer patients. This value is significantly higher than the percentage of PM1 reported in the literature (42,70-73). This unusual observation is unlikely to be due to technical error. Using the same technique for CYP2D6 polymorphism analysis in our laboratory we observed a similar allelic distribution in a North American population compared to that reported in the literature (unpublished data). In addition, the results from our internal PCR controls is consistent with expectations. Nevertheless, a larger population needs to be studied in order to rule out the chance confounding factor.

The majority of the reports found in the literature have focused on the study of polymorphism of a single gene in relation to cancer risk without considering variations in other metabolizing enzymes genes. In an effort to elucidate what constitutes the highest risk subpopulation, investigators have recently studied the risk for cancer in individuals harboring multiple polymorphisms (74). The results of our study indicate that there is a 7-fold increase in risk for development of bladder cancer among individuals harboring the GSTM1 null genotype than individuals who harbor the GSTM1 +/+ genotype (OR = 6.97; 95% CL = 1.59–30.57). The risk is further increased in the presence of the EM genotype (OR = 14; 95% CL = 1.3–151.4 for EM with GSTM1 0/0 versus EM with GSTM1 +/+). In addition, the relative risk in EM individuals harboring the GST null genotype compared to PM harboring the GST +/+ genotype is also increased (OR = 8.4; 95% CL = 1.26–56.03). Studies are ongoing in our laboratory with expanded populations and with additional polymorphic genes to better define the relationship between genetic predisposition and bladder cancer. Effort is made to recruit additional non-schistosomal bladder cancer patients as well as non-cancer patients with schistosoma infection. This would allow an assessment of the role of the genotypes in bladder cancer without confounding by the suspected promoting agent. Our study clearly supports the use of combined genotypic analysis of different metabolizing genes for the estimation of individual risk for cancer development.

In conclusion our results indicate that CYP2E1 does not seem to play a major role in schistosomal bladder cancer. Although our studied population is small, our data indicate that polymorphisms for CYP2D6 and GSTM1 genes should be considered as important risk modifiers considered in the development of schistosomal bladder cancer among Egyptians and should be further investigated.

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References
