Glutathione *S*-Transferase-μ (*GSTM1*) and -θ (*GSTT1*) Genotypes in the Etiology of Prostate Cancer[1]

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Abstract

The glutathione *S*-transferases (GSTs) are involved in the metabolism of numerous potential prostate carcinogens. Common homozygous germ-line deletions exist in the genes that encode GST-μ (*GSTM1*) and GST-θ (*GSTT1*) and preclude enzyme expression. To evaluate whether *GSTM1* and/or *GSTT1* contribute to prostate cancer (CaP) etiology, we studied 237 incident CaP cases and 239 age- and race-matched controls. The probability of having CaP was increased in men who had nondeleted (functional) genotypes at *GSTT1* (odds ratio, 1.83; 95% confidence interval, 1.19–2.80) but not *GSTM1* (odds ratio, 1.07; 95% confidence interval, 0.74–1.55). No interaction of these genes in CaP etiology was observed. GST-θ is highly expressed in the prostate and can produce genotoxic effects upon exposure to specific carcinogens. These results suggest that *GSTT1* is associated with CaP risk.

Introduction

The GSTs[3] μ (*GSTM1*; chromosome 1p13.3) and θ (*GSTT1*; chromosome 22q11.2) catalyze the conjugation of glutathione to numerous potentially genotoxic compounds, including aliphatic aromatic heterocyclic radicals, epoxides, or arene oxides. Glutathione *S* conjugates, particularly those formed by the activity of GST-θ, may also produce genotoxic intermediates (1–3). *GSTM1* and *GSTT1* have distinct structures, kinetic properties, and substrate specificities (4, 5). Common homozygous deletions exist at *GSTM1* in ~40–60% of the United States Caucasian population (6) and at *GSTT1* in ~20–30% of the United States Caucasian population (6). Absent GST enzyme in individuals who have these deletions may result in poorer elimination of electrophilic carcinogens, which may result in increased risk of somatic mutation leading to tumor formation. Alternatively, *GSTT1* is expressed at high levels in the prostate (3), suggesting that the activation of compounds to genotoxic intermediates by *GSTT1* could increase CaP risk. We hypothesize that CaP risk may be modified by *GSTM1* and/or *GSTT1*. To evaluate this hypothesis, we undertook a case-control study to evaluate the role of *GSTM1* and *GSTT1* in CaP etiology.

Materials and Methods

Study Subjects and Data Collection. A sample of 237 incident CaP cases was identified through Urological Oncology Clinics at the HUP between September 1994 and April 1998. Case status was confirmed by medical records review using a standardized abstraction form. Men were excluded from this study if they reported having exposure to finasteride (Proscar) at the time of their CaP diagnosis. Patients who were nonincident cases (*i.e.,* those diagnosed more than 12 months before the date of study ascertainment) or had a prior diagnosis of cancer at any site were also excluded. The mean age of diagnosis was 60.0 years (SD, 6.0 years) with a range of 42–82 years.

The 239 controls studied here were men attending HUP general medicine clinics. These clinics see a patient population that is demographically similar to those seen in the Urological Oncology clinics at HUP. These men were ascertained concurrently with the CaP cases (*i.e.,* between September 1994 and April 1998). Controls were excluded from this study if they ever had an abnormal PSA test (*i.e.,* $\geq$4 ng/dl), if they had ever had an abnormal digital rectal examination, if they had a previous cancer diagnosis, or if they reported having had exposure to finasteride (Proscar) at the time of study ascertainment. Controls were frequency matched to cases on age and race. However, because the frequency matching was approximate, analyses adjusted for age and race were also undertaken to account for residual variation due to these factors. The mean age of these men at the time of their clinic visit was 61.0 years (SD, 8.4 years), with a range of 40–91 years.

Risk factor, medical history, and CaP diagnostic information was obtained by using a standardized questionnaire and review of medical records. Information collected included CaP occurrences in first- and second-degree relatives, personal history of benign prostatic hyperplasia and vasectomy, previous cancer diagnoses, demographic information such as race, educational level, and occupation, and CaP screening history. All study subjects provided informed consent for participation in this research under a protocol approved by the Committee for Studies Involving Human Subjects at the University of Pennsylvania.

***GSTM1* and *GSTT1* Genotype Analysis.** Genomic DNA for the present study was self-collected by each study subject using sterile cheek swabs (Cyto-Pak Cytosoft Brush; Medical Packaging Corp., Camarillo, CA) and processed using a protocol

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[3] The abbreviations used are: GST, glutathione *S*-transferase; CaP, prostate cancer; HUP, Hospital of the University of Pennsylvania; PSA, prostate-specific antigen; OR, odds ratio; CI, confidence interval.

Table 1 Descriptive characteristics of the case-control sample

Trait	Controls	Cases	Comparison
Mean age in years (SD)	61.0 (8.4)	60.0 (6.0)	$\chi^2 = 0.058$, $df = 1$, $P = 0.809$[a]
Range	40–91	42–82	
Race			
Caucasian	203 (84.9%)	204 (86.1%)	Fisher's Exact test, $P = 0.902$
African American	29 (12.1%)	28 (11.8%)	
Other	7 (2.9%)	5 (2.1%)	
Mean PSA in ng/dl (SD)[b]	1.2 (0.9)	15.8 (49.3)	$\chi^2 = 234.03$, $df = 1$, $P < 0.0001$[a]
Range	0–3.9	0.2–590.0	
Family history of CaP			
No	181 (75.7%)	138 (58.2%)	Fisher's Exact test, $P = 0.0002$
Yes	40 (16.7%)	73 (30.8%)	
Unknown/Not reported	18 (7.5%)	26 (11.0%)	

[a] From Kruskal-Wallis χ^2 approximation.
[b] PSA was measured at time of diagnosis in cases and at most recent clinic visit in controls.

modified from Richards *et al.* (7). Briefly, the swab brush was placed inside a 1.5-ml microcentrifuge tube, and 600 μl of 50 mM NaOH was added. The closed tube was vortexed for 10 min and then heated at 95°C for 10 min. Finally, 120 μl of 1 M Tris (pH 8.0) were added, after which the brush was removed and discarded. The resulting biosample was used for PCR-based genotype analyses.

The complete gene deletion at *GSTM1* was determined by using a PCR-based assay that modified the protocol of Davies *et al.* (8). Four primer sequences were used to amplify *GSTM1*. The *β-globin* gene was amplified to serve as a positive internal control. These primers were GME4, 5′-CTG CCC TAC TTG ATT GAT GGG; GMN5, 5′-CTG GAT TGT AGC AGA TCA TGC; Beta-Globin 1, 5′-CAA CTT CAT CCA CGT TCA CC; and Beta-Globin 3, 5′-CAT GGT GCA TCT GAC TCC T. The PCR reaction mix consisted of 5 μl of 10× buffer [100 mM Tris-HCl (pH 9), 500 mM KCl, 1% (v/v) Triton, and 15 mM $MgCl_2$), 1 μl of 10 mM deoxynucleotide triphosphates, 5 μl each of the four primers at 5 μM concentration, 10 μl of template DNA, and 0.3 μl of Taq polymerase in 13.7 μl of double-distilled H_2O, for a reaction volume of 50 μl. The PCR temperature profile consisted of 1 cycle of 94°C for 3 min and 1 cycle of 82°C for 1 min, at which time the Taq polymerase was added to the reaction mixture. This was followed by 30 cycles of 94° for 1 min, 55°C for 1 min, and 72°C for 2 min, ending with an elongation step at 72°C for 10 min. Visualization of the amplification products was accomplished on a 2% agarose gel after staining with ethidium bromide.

GSTT1 genotypes were generated using a modification of the protocol of Pemble *et al.* (9). Four primers were used to amplify *GSTT1* and a β-globin positive internal control. These primers were: GSTT1–1, 5′-TTC CTT ACT GGT CCT CAC ATC TC-3′; GSTT1–2, 5′-TCA CCG GAT CAT GGC CAG CA-3′; β-globin-1, 5′-CAA CTT CAT CCA CGT TCA CC-3′; and β-globin-2, 5′-GAA GAG CCA AGG ACA GTT AC-3′. The reaction mixture included 2.5 μl of 10× PE Buffer II (Perkin-Elmer), 0.25 μl of 100× BSA, 1.0 μl of 25 mM Mg^{2+}, 0.5 μl of 10 mM deoxynucleotide triphosphates, 2.5 μl each of the four 5 μM primers, 6.0 μl of template DNA, and 0.3 μl of Taq polymerase in 4.45 μl double-distilled H_2O, for a total reaction volume of 25 μl. The PCR temperature cycling profile consisted of 1 cycle of 93°C for 2 min 20 s and 1 cycle of 82°C for 1 min, at which time the Taq polymerase was added to the reaction mixture. This was followed by 20 cycles of 93°C for 1 min, 60°C with a decrease of 0.5°C per cycle for 1 min, and 72°C for 1 min, 15 cycles of 93°C for 1 min, 50°C for 1 min, 72°C for 1 min, and a final extension step of 72°C for 7.5 min. Visualization of the amplification products was accomplished on a 4% agarose gel after staining with ethidium bromide.

Analytical Methods. Four genotype classes were considered for analysis: (*a*) homozygous deletion genotypes at *GSTM1* (denoted *GSTM1–0* or "functional" *GSTM1*) were compared with genotypes for which at least one none-deleted allele was present (denoted *GSTM1-1* or "non-functional" *GSTM1*); (*b*) the same definition was used to distinguish *GSTT1–0* and *GSTT1–1* genotypes. The *GSTM1–1* and *GSTT1–1* genotypes did not distinguish between those individuals who were homozygous for a nondeleted allele and those who were heterozygous for a nondeleted allele; (*c*) an interaction genotype was defined in which an individual had both *GSTM1–0* and *GSTT1–0* (denoted *GST-0*). Individuals who had at least one nondeleted allele at either *GSTM1* or *GSTT1* were denoted *GST-1*; and (*d*) an interaction genotype was defined in which an individual had either *GSTM1–0* or *GSTT1–0* (denoted *GST1–0*). Individuals who had inherited nondeleted alleles at both *GSTM1* and *GSTT1* were denoted *GST1–1*.

Descriptive analyses for discrete traits were carried out using contingency table methods and χ^2 or Fisher's Exact test statistics. Means or medians were used to summarize continuously distributed traits, and nonparametric Kruskal-Wallis statistics were used to compare these values across groups (*e.g.*, between cases and controls). Genotype disease associations were undertaken using unconditional logistic regression. Analyses considered the effect of genotype unadjusted for potential confounders and adjusted for age (at time of diagnosis in cases or time of study ascertainment in controls) and race (coded as a discrete variable with three levels: Caucasian, African American, or other). All *P* values were based on two-sided hypothesis tests.

Results

Table 1 presents a description of the sample population. Consistent with most previous reports, mean PSA levels were significantly higher in cases than controls, and cases were significantly more likely to have a family history of CaP than controls.

We observed 118 (25%) *GSTT1–0* and 220 (47%) *GSTM1–0* genotypes (Table 2). We also observed 53 (11%) subjects with homozygous deletion genotypes at both loci (*i.e.*, *GSTT1–0* and *GSTM1–0)*, 240 (51%) subjects with a deletion genotype at one locus (*i.e.*, *GSTT1–0* or *GSTM1–0)*, and 176

Table 2 Comparisons of *GSTM1* and *GSTT1* genotypes in cases and controls

Risk factor	Controls	Cases	OR (95% CI)	
			Unadjusted	Adjusted[a]
GSTM1-0	110 (47.6%)	110 (46.6%)	1.0[b]	1.0[b]
GSTM1-1	121 (52.4%)	126 (53.4%)	1.04 (0.72–1.50)	1.07 (0.74–1.55)
GSTT1-0	72 (31.2%)	46 (19.8%)	1.0[b]	1.0[b]
GSTT1-1	159 (68.8%)	186 (80.2%)	1.83 (1.20–2.80)	1.83 (1.19–2.80)
GST-0[c]	31 (13.3%)	22 (9.3%)	1.0[b]	1.0[b]
GST-1	202 (86.7%)	214 (90.7%)	1.49 (0.84–2.66)	1.53 (0.85–2.73)
GST1-0[d]	151 (65.9%)	134 (57.8%)	1.0[b]	1.0[b]
GST1-1	78 (34.1%)	98 (42.2%)	1.42 (0.97–2.07)	1.46 (1.00–2.14)

[a] OR adjusted by multiple logistic regression for age and race.
[b] Reference group.
[c] *GST-0* is defined as *GSTM1-0* and *GSTT1-0*; *GST-1* is defined as *GSTM1-1* or *GSTT1-1*.
[d] *GST1-0* is defined as *GSTM1-0* or *GSTT1-0*; *GST1-1* is defined as *GSTM1-1* and *GSTT1-1*.

(38%) subjects with no deletion genotype at either locus (*i.e.*, *GSTT1–1* and *GSTM1–1*).

The relationship of *GSTM1* and/or *GSTT1* genotype with case status is presented in Table 2. The *GSTM1* genotype distribution did not significantly differ between CaP cases and controls. However, *GSTT1–1* genotypes were significantly more common among cases than controls (adjusted OR_{GSTT1}, 1.83; 95% CI, 1.19–2.80). When *GSTM1* and *GSTT1* were simultaneously considered, cases were more likely to have at least one *GSTM1–1* or *GSTT1–1* genotype with an adjusted OR_{GST1} of 1.46 and 95% CI of 1.00–2.14. There was no statistically significant association of having homozygous deletions at both loci (OR_{GST}, 1.53; 95% CI, 0.85–2.73) with CaP case status, perhaps due to limitations in statistical power of this sample.

We also evaluated whether one locus modified the effects of the other locus by fitting models that contained both *GSTM1* and *GSTT1*. The adjusted OR effects at each locus did not change substantially from those estimates obtained from the univariate genotype models. The adjusted OR effect associated with *GSTM1* controlling for *GSTT1* and other covariates was 1.06 (95% CI, 0.73–1.55), whereas the *GSTM1* estimate not controlling for *GSTT1* was 1.07 (95% CI, 0.74–1.55; Table 2). Similarly, the adjusted OR associated with *GSTT1* controlling for *GSTM1* and other covariates was 1.84 (95% CI, 1.20–2.83), whereas the *GSTT1* estimate not controlling for *GSTM1* was 1.83 (95% CI, 1.19–2.83; Table 2). To further evaluate the independence of *GSTM1* and *GSTT1* genotypes, we also compared the distribution of *GSTM1* and *GSTT1* among cases only. In 95 *GSTM1–0* cases, 18 (19%) were *GSTT1–0*. Among 94 *GSTM1–1* cases, 17 (18%) were *GSTT1–0* (Fisher's Exact test, $P > 0.99$). This implies the effect of each of these genes on CaP is independent (*e.g.*, additive) rather than interactive (*e.g.*, multiplicative).

Compared with *GSTM1–1/GSTT1–0* genotypes (the referent class), individuals with *GSTM1–0/GSTT1–0* genotypes were not at increased risk (OR, 1.15; 95% CI, 0.55–2.43), whereas individuals with *GSTM1–1/GSTT1–1* and *GSTM1–0/GSTT1–1* genotypes were at increased risk (OR, 2.04; 95% CI, 1.13–3.68; and OR, 1.89; 95%CI, 1.04–3.42, respectively). These results further indicate that an increase in risk occurred only among those with a *GSTT1–1* genotype. Therefore, we conclude that CaP risk may be increased by *GSTT1–1* (*i.e.*, the presence of GST-θ enzyme), and that this effect is largely independent of *GSTM1*.

Discussion

We report that men who do not have homozygous deletions at *GSTT1* (*i.e.*, whose GST-θ protein is presumably expressed and functional) are at increased CaP risk. Although our results are largely hypothesis generating, these findings are consistent with knowledge that GST-θ produces genotoxic metabolites in response to specific exposures (1–3). There is substantial evidence that glutathione *S* conjugates derived from halogenated alkanes, halogenated alkenes, hydroquinones, quinones, aminophenols, and other compounds can cause cellular and DNA damage (reviewed in Ref. 10). Many of these compounds are commonly found as occupational exposures, and some are known carcinogens. Glutathione metabolic intermediates from ethylene dibromide, 1,2,3,4-diepoxybutane, methyl bromide, epibromohydrin, 1,3-dichloroacetone, and methylene chloride are mutagenic (1, 2). Epidemiological evidence has provided further support that these compounds may be involved in prostate carcinogenesis. For example, cellulose triacetate-fiber workers exposed to methylene chloride may have a significantly elevated CaP mortality (11). Other occupations with increased CaP risk include those that may involve exposure to halogenated alkanes or alkenes, such as the rubber industry (12). However, there has been little consistency in these reports (13), and these potentially carcinogenic exposures may be found in a wide range of exposures. *GSTT1* is expressed at high levels in the prostate (3), suggesting that the activation of specific carcinogenic compounds to genotoxic intermediates by *GSTT1* may occur in the prostate itself. Thus, our results support the inference that carcinogen activation by *GSTT1* may play a role in prostate carcinogenesis.

Information about biological differences in the activity of *GSTT1* relative to *GSTM1* may further help to identify pathways involved in prostate carcinogenesis. There does not appear to be a single class of chemical compounds that is associated with *GSTM1* or *GSTT1* metabolism. Despite functional similarity, the two loci also have different substrate specificities. These include metabolism of benzo(*a*)pyrene, styrene-7,8-oxide, and *trans*-stilbene oxide by *GSTM1*, and of epoxybutanes, ethylene oxide, halomethanes, and methyl bromide by *GSTT1*. Therefore, it is plausible that *GSTT1* metabolizes a compound or set of compounds distinct from those metabolized by *GSTM1*, which could elucidate specific pathways of prostate carcinogenesis.

Although *GSTT1* is the most recently identified and one of

the lesser studied of the GST multigene family (14), a great deal is known about the activity of the GSTs in general. In addition to metabolism of chemical carcinogens, described above, the GSTs metabolize steroid hormones, compounds found in the diet, and other agents potentially involved in CaP etiology. The GSTs are thought to be involved in the intracellular transport of steroid hormones (15) and are involved in the isomerization of androst-5-ene-3,17-dione to androst-4-ene-3,17-dione, the immediate precursor of testosterone (16). The hypothesis that presence of GST-θ may be involved in hormone-mediated prostate carcinogenesis requires additional investigation. The GSTs are also involved in the metabolism of numerous dietary compounds. For example, dietary fat consumption can result in an increase in GST activity (17), and dietary lipids are substrates for GST metabolism (16). Somatic hypermethylation of deoxycytidine residues in CpG islands in the 5′ regulatory region of *GSTP1* eliminates GST-π expression in the majority of prostate tumors (18) and prostatic intraepithelial neoplasia (19). Harries *et al.* (20) have reported case-control study results that suggest germ-line variability at *GSTP1* is associated with CaP risk. These results provide additional support for the hypothesis that the GST family may be acting on multiple levels of prostate carcinogenesis. Therefore, the GSTs may play a role in prostate carcinogenesis by modulating the metabolism of androgens or other exposures.

We have reported OR effects associated with *GSTT1* that are relatively small (<2), as may be expected for genes involved in the metabolism of a myriad of carcinogenic compounds. However, because a large proportion of the population has a functional allele *GSTT1*, the population-attributable risk associated with *GSTT1–1* genotypes may be large. For example, we can assume a *GSTT1–1* frequency in the United States population to be $P = 0.76–0.85$ (Ref. 6), and an adjusted OR effect of the functional state of 1.83 (Table 2). We can then estimate the percent attributable risk of CaP due to *GSTT1–1* using the formula $100\% \times P\,(\mathrm{OR} - 1)/[P\,(\mathrm{OR} - 1) + 1]$ (Ref. 21). The resulting attributable risk range is 39–41%. Although these estimates are at best rough approximations, they suggest that a sizable proportion of CaP may be explained by the effect of *GSTT1–1* genotypes.

We also considered a number of potential study limitations. The observed *GSTT1–0* frequency in controls (0.31; Table 2) was somewhat higher than that reported in other U.S. populations (0.15–0.24; Ref. 6). This higher estimate may be explained by the fact that our sample included Asian and African Americans, who have higher rates of *GSTT1–0* than United States Caucasians (6). Among Caucasian controls, the *GSTT1–0* frequency was 0.24. This value is within the range reported previously and suggests that the present results cannot be explained by an elevated *GSTT1–0* frequency in controls. Our correction for race as a confounder in multiple logistic regression models (Table 2) also minimizes the potential for population stratification as an explanation for the present findings. It is unlikely that genotyping errors could account for an elevated proportion of *GSTT1–0* in controls but not cases. Positive internal and external controls were used in all assays to limit the possibility of PCR failures being typed as a homozygous deletion genotype. All genotype readings were done blinded to case status. Therefore, it is unlikely that genotype errors induced significant bias in the OR estimates reported in Table 2. The analyses were not adjusted for multiple comparisons, because only eight hypothesis tests were undertaken. However, a simple Bonferroni correction of our significance levels can be applied, $P^* = p/n$, where p is the standard P cutpoint of 0.05, n is the total number of tests conducted, and P^* is the adjusted P level for assessing statistical significance given the multiple tests. The corrected P required to conclude statistical significance using this correction would be 0.006. The P associated with the *GSTT1* association in Table 2 was 0.005. Therefore, even with a very stringent correction for multiple hypothesis tests, we can conclude that there is a significant association between *GSTT1* and prostate cancer.

Misclassification of case or control status could also have influenced the inferences of this study. Cases were all confirmed to have disease by medical records review, and it is highly unlikely that cases were misclassified. No control reported ever being told they had a diagnosis of CaP, nor were controls who had any history of elevated PSA or abnormal digital rectal exam included in this study. Medical records review confirmed that no control was diagnosed with CaP. Controls could have had latent disease that was not detectable by either PSA and/or digital rectal exam, or the disease may have arisen between the time these tests had been done and time of ascertainment, but without the patient's knowledge. This misclassification might bias the OR toward the null hypothesis. If that were the case, the true OR effects might be higher those reported here. If we assume that 10% of controls had a latent (undetected) CaP at the time of their participation in this study, then the unadjusted OR associated with *GSTM1* would be essentially the same (1.04) as that estimated using the available data (Table 2), and the *GSTT1* OR estimate would be 1.73 (compared with 1.83; Table 2). Although it is highly unlikely that as many as 10% of controls were misclassified, the OR estimates assuming this misclassification rate are not substantially different than those reported in Table 2.

As described above, GST-θ is highly expressed in the prostate and can produce genotoxic effects upon exposure to specific carcinogens. The GSTs role in steroid hormone metabolism may be an additional pathway through which *GSTT1* may be acting in prostate carcinogenesis. Although not providing any information about the mechanism of action, our results do indicate that men who have a nondeleted (presumably functional) *GSTT1* genotype, and can therefore express prostate-specific GST-θ, may be at increased risk of CaP. By identifying specific compounds that may be metabolized by *GSTT1*, novel pathways of prostate carcinogenesis may be identified.

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