Genetic Monitoring of Human Polymorphic Cancer Susceptibility Genes by Polymerase Chain Reaction: Application to Glutathione Transferase μ

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Several genes involved in the metabolism of carcinogens have been found to be polymorphic in human populations and are associated with increased risk of cancer at some sites. This study focuses on the polymorphic enzyme glutathione transferase μ (GT μ). Smokers with low lymphocyte GT μ activity are at an approximately 2-fold higher risk for lung cancer and an approximately 3-fold higher risk for stomach and colon adenocarcinomas. Recent cloning and sequencing of the *GST1* gene has allowed the development of convenient genotyping methods based on restriction fragment length polymorphisms (RFLP) or the polymerase chain reaction (PCR). The *GST1* polymorphism has been shown to be a deletion of the gene locus. To detect the presence or absence of the gene we amplified exons 4–5 and/or exons 6–7 of the *GST1* gene by PCR. PCR amplification produced bands of 215-bp or 273-bp from individuals with one or two copies of the *GST1* allele and no band if the individual was homozygously deleted (0/0). In the exon 6–7 PCR, we co-amplified a 268-bp portion of the β-globin gene as an internal reference standard for quantitative analysis of product yield. This allowed homozygote individuals (+/+) to be distinguished from heterozygotes (+/0).

We have compared the *GST1* genotype to lymphocyte GT μ activity measured on *trans*-stilbene oxide (TSO) in the lymphocytes of 45 individuals. Low GT μ activity (< 67 pmole/min/10^7 cells) was strongly associated (24/24) with the *GST1* 0/0 genotype. With the exception of one individual, activities greater than 67 pmole/min/10^7 were associated with the presence of the *GST1* allele (20/21). Individuals with the highest GT–TSO activity were found to be homozygous for *GST1* (+/+), while heterozygotes (+/0) generally had lower activity, suggesting a gene dosage effect in lymphocytes. The allele distribution among four sampled populations varied considerably. In a North Carolina population, 51% (65/127) were *GST1* 0/0, and this finding is consistent with those of other studies based on phenotypic analysis. In three smaller cohorts, the *GST1* 0/0 genotype was observed to occur in: 30% (14/47) of Finnish foundry workers, 33% (18/54) of Georgia dye workers, and 62% (74/120) of Taiwanese placental samples. In the future, we shall investigate the mechanistic link between polymorphisms in carcinogen metabolism genes and interindividual variation in measures of DNA damage, such as DNA adducts and *hprt* mutation frequency.

Introduction

Metabolic activation and detoxification pathways for carcinogens mediate the initial steps in environmental carcinogenesis. Genetic differences in these pathways are likely to be a major source of interindividual variation in susceptibility to cancer (*1,2*). Several genes involved in the metabolism of carcinogens have been found to be polymorphic in human populations, and specific alleles are associated with increased risks of cancer at various sites (*1–4*).

This study focuses on the polymorphic enzyme glutathione transferase μ (GT μ). Smokers with low lymphocyte GT μ activity are at approximately 2-fold higher risk for lung cancer, particularly adenocarcinoma (*3*). In addition, there is a 3-fold higher risk of stomach and colon adenocarcinoma among individuals with no detectable GT μ enzyme, as determined by starch gel electrophoresis (*4*). The GT μ enzyme catalyzes the conjugation of glutathione to epoxides of polycyclic aromatic hydrocarbons, aflatox-

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ins, and other compounds. In general, this is a detoxification reaction and may be an important protective system against exposure to these agents (*5–7*). In most human studies, the GT μ polymorphism has been characterized at the phenotypic level, either by measuring enzyme activity related to a specific chemical (i.e., *trans*-stilbene oxide [TSO]) or by isolating and identifying isozymes (*3,4*). Enzyme assays on isolated lymphocytes can be logistically difficult and technically cumbersome when applied to population studies, and they require substantial quantities of blood (10–30 mL).

Recent cloning and sequencing of the gene for GT μ (*GST1* gene) has allowed the development of convenient genotyping methods based on restriction fragment length polymorphisms (RFLP) or the polymerase chain reaction (PCR) (*8,9*). PCR approaches are rapid and reliable, and one can use DNA prepared from small numbers of cells (10^2–10^6 cells, or 0.001–1.0 mL blood). These characteristics are well suited to population studies involving large numbers of samples and limited quantities of biologic material (blood or tissue).

Our laboratory is studying the distribution of high- and low-risk alleles for cancer susceptibility in various exposure groups and ethnic populations. We are looking for associations between genotype and tumor incidence at a variety of sites, and also specific measures of DNA damage such as mutation frequency and DNA adducts. In this paper, we describe PCR approaches for detecting the *GST1* gene deletion, report our comparison of PCR results with lymphocyte GT μ enzyme activity measurements, and also report preliminary data on the distribution of *GST1* alleles in some ethnic and geographic populations.

Methods

Enzyme Assay

Peripheral blood from healthy individuals was collected into Leucoprep (Becton–Dickinson) tubes, and lymphocytes were isolated by the standard procedure described in the product literature. Lymphocytes were assayed immediately for GT activity or frozen at −80°C for up to 4 weeks. Liu et al. (*7*) reported that freezing had no impact on GT–TSO activity. GT activity toward TSO was measured by the procedure of Seidegard and Pero (*10*), with slight modifications as described by Liu et al. (*7*).

Genotype Analysis by Polymerase Chain Reaction

DNA was extracted from 10^5–10^7 isolated lymphocytes using the method of Miller et al. (*11*) and resuspended in sterile distilled H_2O or buffer (10 mM Tris, 1 mM EDTA) and frozen until use. PCRs were carried out separately for exons 4–5 and 6–7. DNA (~100 ng) from approximately 2×10^4 cells was added to a PCR mix composed of 30 pmole of each primer, 200 μmole deoxynucleotide triphosphates, 1 unit *Taq* polymerase (Amplitaq, Perkins-Elmer Cetus, Norwalk, CT) and PCR buffer composed of 16.6 mM $(NH_4)_2SO_4$, 50 mM β-mercaptoethanol, 6.8 μM EDTA, 67 mM Tris (pH 8.8), 80 μg/mL bovine serum albumin and 1.6 mM $MgCl_2$ in a volume of 30 μL. In a Perkin–Elmer Cetus Thermal Cycler, the reaction mixture was denatured at 94°C for 3 min and subjected to 25 cycles of 94°C for 1 min; 59°C for 1 min and 72°C for 1.5 min. A final 72°C extension for 5 min was performed. Samples were electrophoresed on 3:1 Nusieve Agarose gels and checked for the presence of bands. PCRs for exons 6–7 were also carried out in the presence of 10-pmole primers for the β-globin gene (Perkin-Elmer Cetus); following electrophoresis, the ethidium bromide-stained gel was photographed and a negative was prepared. Densitometry was carried out on the photographic negative with an LKB Gel Scan II scanning laser densitometer, and the ratio between band intensities of the β-globin and *GST1* PCR products was characteristic of the homozygous (0.9–1.3) or heterozygous genotype (0.5–0.8). For all analyses, positive and negative control reactions were run in parallel to check for reagant contamination.

Results and Discussion

The GT μ polymorphism was shown to be a deletion of the entire *GST1* gene locus by Siedegard et al. (*9*). In order to detect the GT μ sequence polymorphism by PCR, we used oligonucleotide primers that amplify exons 4–5 (*8,12*) or exons 6–7 of the *GST1* gene. PCR amplification with these primer sets produced 273-bp or 215-bp bands on agarose gels from individuals with one or two copies of the *GST1* allele, and no visible band if the individual was homozygously deleted.

The exon and intervening intron regions of the *GST1* gene that were amplified by PCR show nearly 100% homology with other functional and psuedo-GST μ loci (*13–15*). In our hands, the PCR primer sequences of Comstock et al. (*8*) for exons 4–5 often produced spurious PCR fragments with lengths of approximately 100 bp or approximately 265 bp, which appeared in samples from individuals with no GT μ activity. These primers may not adequately discriminate between the *GST1* locus and the highly homologous human muscle-specific *GST4* locus or the testis-specific GT μ locus, neither of which is polymorphic (*13,15*). Because of this problem, primers for exons 6 and 7 were designed to detect the *GST1* locus specifically. In PCRs for exons 6 and 7, primers for the β-globin gene locus (Perkins–Elmer Cetus) were also included, for the following reasons. First, individuals with the deleted *GST1* genotype produce no PCR product; co-amplification of β-globin provides a convenient internal positive control and allows relative quantification of template DNA in the reaction. This is particularly important for samples with only small amounts of DNA, where a technical failure to amplify would be falsely interpreted as homozygous deletion. Second, the β-globin PCR product was also used as a reference standard for quantitative analysis of the product yield from the *GST1* amplification. This approach was used by Neubauer et al. (*16*), who demonstrated that by optimizing reagent concentrations the relative copy numbers of two or more co-amplified genes could be determined by

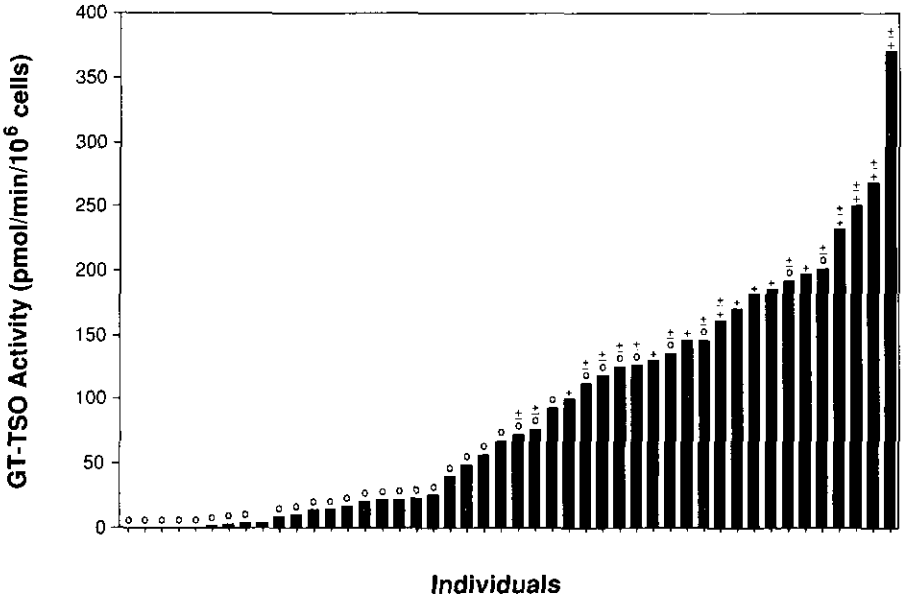

FIGURE 1. Agarose gel electrophoresis of polymerase chain reaction products from co-amplification of *GST1* exons 6–7 (215-bp band) and β-globin gene (268-bp band). Lane 3 was homozygously deleted (0/0); lanes 1, 4, and 5 were heterozygotes (+/0); and lane 2 was homozygous for the *GST1* gene (+/+). Marker lane is *Hinc*II digest of ΦX174 DNA, bands are 1057-bp, 770-bp, 612-bp, 495-bp, 392-bp, 341-bp, 297-bp, 210-bp, and 162-bp.

quantifying their PCR product ratios. This allowed us to distinguish homozygous wild-type from heterozygous individuals.

Figure 1 demonstrates the band pattern on a 4% 3:1 NuSieve agarose gel that resulted from use of this method. The 268-bp band found in all samples (lanes 1–5) was the PCR product from the β-globin gene amplification. Amplification of *GST1* exons 6–7 and the intervening intron produced the 215-bp fragment that was present in lanes 1, 2, 4, and 5 but was absent in lane 3. Lane 3 shows the homozygously deleted genotype. Quantification of the band intensities in each lane by scanning laser densitometry revealed the β-globin:*GST1* ratios for lanes 1–5 to be 0.65, 0.98, 0.0, 0.66, and 0.54. The β-globin:*GST1* ratios for lanes 1, 4, and 5 were characteristic of heterozygotic individuals, while the higher ratio (0.98) in lane 2 was characteristic of homozygotes.

We have compared the PCR-determined *GST1* genotype to GT μ enzyme activity measured on TSO in the lymphocytes of 45 individuals and found good agreement between these methods. The results are displayed on Figure 2. Bars represent lymphocyte GT–TSO activity for each individual; above each bar is the PCR result (0 = *GST1* deleted; +/0 = one copy of *GST1*; +/+ = two copies of *GST1*). The distribution of enzyme activity appears as a continuum from low to high values. Individuals with GT–TSO activities less than 67 pmole/min/10^7 cells were consistently found to have the homozygously deleted genotype by the PCR assay (24/24). With the exception of one individual, subjects with activities greater than 67 pmole/min/10^7 cells had at least one *GST1* allele. The continuous distribution of enzyme activity shown in Figure 1 is different from that found by Seidegard et al. (*9,10*), who described a steplike distribution with low (< 25 pmole/min/10^7 cells) and high (> 600 pmole/min/10^7 cells) activities but no intermediate value. The difference may be due to methodological differences in lymphocyte preparation.

The four individuals with the highest GT–TSO activity (> 230 pmole/min/10^7 cells) were homozygous (two copies of the gene) for *GST1* by the quantitative PCR assay. *GST1* heterozygous individuals with activities of 72–145 pmole/min/10^7 cells had a single copy of the *GST1* gene. These results are consistent with a gene dosage effect; however, the relationship between *GST1* copy number and enzyme activity breaks down in the 150–200 pmole/min/10^7 cells range, possibly owing to assay variation or to intraindividual variation. The gene dosage effect that we observed suggests that *GST1* is expressed at a basal, constitutive level in lymphocytes. On-going studies may reveal whether gene dosage and level of enzyme activity affect DNA adduct levels or mutation frequency in lymphocytes.

Preliminary results suggest that GT–TSO activity in the liver (which is 10-fold higher than in lymphocytes) does not differ between heterozygous and homozygous genotypes (*GST1* +/0, or +/+). This may indicate that for detoxification reactions mediated through the liver, the homozygous genotype (*GST1* +/+) is not more protective than the heterozygous genotype (*GST1* +/0). Because of the high GT μ expression level in the liver, however, any deleterious effect of the homozygous deleted genotype (*GST1* 0/0) should be more apparent.

One individual with the homozygous deleted genotype had unusually high activity and was assayed on three

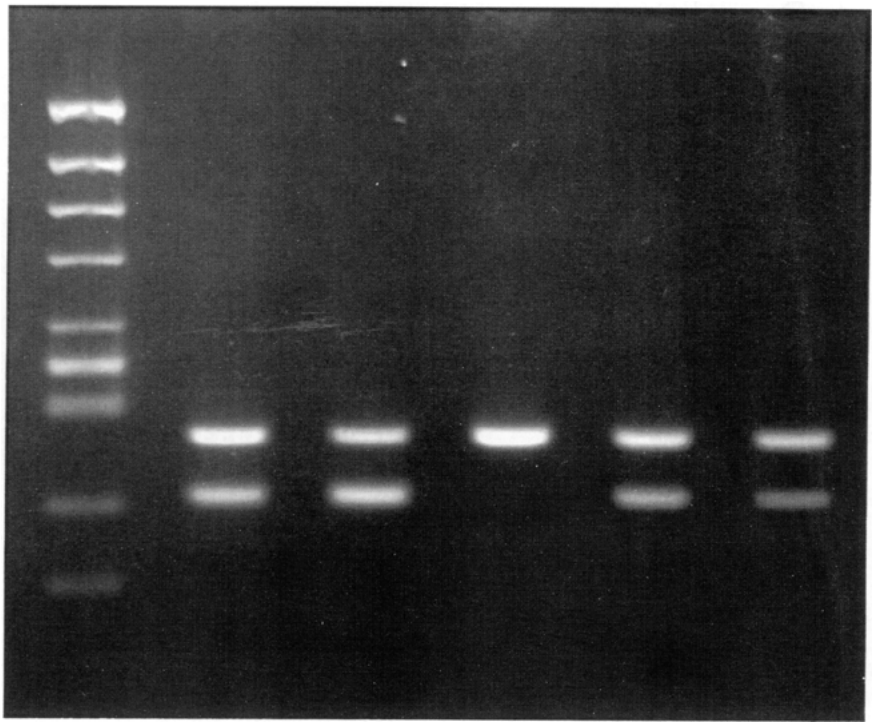

FIGURE 2. Distribution of lymphocyte glutathione–*trans*-stilbene oxide (GT–TSO) enzyme activity. Labels above bars indicate result of polymerase chain reaction assay for *GST1* (+ = *GST1* present; +/0 = one copy *GST1*; +/+ = two copies of *GST1*; 0 = *GST1* deleted). Values are averages of one to four individual determinations.

separate occasions approximately 3 months apart on freshly isolated cells, producing values of 144.3, 127.8, and 4.7 pmole/min/10^7 cells (mean = 92.6 pmole/min/10^7 cells). The high activity (and variability) for this individual and the significant activity (10–67 pmole/min/10^7 cells) for other *GST1*-deleted individuals suggests that other enzymes, possibly other GTs that are known to be expressed at high levels in lymphocytes, may interfere with the GT–TSO assay. If the high-activity individuals possess large amounts of other GTs, it is plausible that there may be some overlapping substrate specificity.

Published reports based on phenotypic methods suggest that 30–60% of U.S., European, and Asian populations have the homozygously deleted *GST1* genotype (*4,6*). In our genotyping studies, the allele distribution among four sampled populations varied considerably but was consistent with previous reports. In a population in North Carolina, we observed that 51% (65/127) were *GST1* homozygously deleted. In three smaller cohorts, however, the percentages with the homozygously deleted genotype were: Finnish foundry workers, 30% (14/47); Georgia (USA) dye workers, 33% (18/54); Taiwanese, 62% (74/120). The divergent allele frequency distributions for these population samples may be related to ethnic or geographic patterns or to small sample size; however, if these distributions are real, significant differences in risk may exist among regional and ethnic groups for adverse health effects from exposure to polycyclic aromatic hydrocarbons.

The overall approach of our genotyping program is to investigate the link between polymorphisms in genes for carcinogen metabolism and interindividual variation in measures of DNA damage, mutation frequency, and cancer incidence. In addition to studying the GT μ polymorphism, we are also genotyping for the debrisoquine hydroxylase, *N*-acetyl transferase, and aryl hydrocarbon hydroxylase polymorphisms. This genotypic analysis should permit a clearer understanding of the relationship between polymorphisms of genes involved in carcinogen metabolism and individual susceptibility to the mutagenic and carcinogenic actions of specific chemical exposures.

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