Expression-Based Discovery of Variation in the Human Glutathione S-Transferase M3 Promoter and Functional Analysis in a Glioma Cell Line Using Allele-Specific Chromatin Immunoprecipitation

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Abstract

Discovery and functional evaluation of biologically significant regulatory single nucleotide polymorphisms (SNP) in carcinogen metabolism genes is a difficult challenge because the phenotypic consequences may be both transient and subtle. We have used a gene expression screening approach to identify a functional regulatory SNP in glutathione S-transferase M3 (GSTM3). Anttila et al. proposed that variation in GSTM3 expression was affected by exposure to cigarette smoke and inheritance of the GSTM1-null genotype. To investigate the mechanism of GSTM3 expression variation, we measured GSTM3 expression in lymphoblast cells from a human Centre d’Etude du Polymorphisme Humain family and observed a low expression phenotype. Promoter sequencing revealed two novel GSTM3 promoter SNPs: A/C and A/G SNPs, 63 and 783 bp upstream of the codon 1 start site, respectively. In this pedigree, the two children homozygous for the −63C/C genotype had 8-fold lower GSTM3 expression relative to the two children with the −63A/A genotype, with no association between A−783G SNP and GSTM3 expression. Further evaluation using genotyped glioma cell lines and with luciferase reporter constructs showed that the −63A allele was associated with lower GSTM3 expression (P < 0.0001 and P < 0.003), RNA pol II chromatin immunoprecipitation was combined with quantitative probed-based allele discrimination genotyping to provide direct evidence of a 9-fold reduced RNA pol II binding capacity for the −63C allele. These results show that the GSTM3 −63C allele strongly affects gene expression in human cell lines and suggests that individuals who carry the low expression allele may be deficient in glutathione transferease catalyzed biological functions. (Cancer Res 2005; 65(1): 99-104)

Introduction

Human glutathione S-transferases (GST) protect cells against toxic insults by catalyzing nucleophilic addition or substitution reactions between glutathione and reactive electrophilic compounds (1–6). The GST gene superfamily has numerous families (7–12), and many have notable polymorphisms that have been shown to alter disease risk (13–15). Because GSTs are important for catalyzed biological functions, their effects on the efficacy of therapeutic treatments (16), characterization of genotypic and phenotypic variation in the GST gene family is important.

GSTM3 is expressed in testis, brain, lung, and lymphocytes as well as in many tumor types (NCI-60; refs. 17–19). A recently described GSTM3 3-bp deletion polymorphism (GSTM3*A/B) has been shown to have a frequency of 0.16 in the Caucasian population (20, 21). This polymorphism was associated with an increased risk of cutaneous basal cell carcinomas and laryngeal carcinomas and interacted with other GST variants in modifying astrocytoma risk but had no effect on pharyngeal carcinoma risk (20–24). Anttila et al. (17) observed variation in GSTM3 expression in lung tissue and proposed a relationship among smoking exposure, GSTM3 expression level, and GSTM1-null genotype.

To explore the mechanism for the Anttila et al. observations of GSTM3 expression variation, we identified human cell lines with low expression GSTM3 phenotypes in a Centre d’Etude du Polymorphisme Humain (CEPH) family and sequenced the GSTM3 promoters of these individuals. Using expression analyses (quantitative real-time PCR and Western blot), luciferase reporter assays and allele-specific chromatin immunoprecipitation (ChIP), we have shown that a GSTM3 promoter polymorphism causes a dramatic reduction in GSTM3 transcription and protein expression in the lymphoblast and glioma cell lines we investigated.

Materials and Methods

Cell Culture. The human lymphoblast cell lines GM07348, GM07349, GM07350, GM07351, GM07353, GM07354, GM07355, and GM07356 (CEPH/UTAH Pedigree 1345) were purchased from Coriell Cell Repositories and were maintained in RPMI 1640 supplemented with 15% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% penicillin-streptomycin (Invitrogen) at 5% CO2 at 37°C. The human glioma cell lines SNB-19, SF-539, and SF-268 were provided by National Cancer Institute and cultured under the same conditions listed above. All cultures were sampled during logarithmic growth phase.

DNA Extraction, Genotyping, and Sequencing. For genotyping, we used a community-based sample of healthy, unrelated (19-53 years old) volunteers of African and European descent from Durham and Chapel Hill, NC (protocol 86E-0037), and placental DNA samples from anonymous full-term, normal, unrelated maternity patients with Asian ancestry were provided by Dr. L. Hsieh (Chang Gung University, Taiwan, China). DNA was isolated from all samples with standard proteinase K digestion followed by phenol-chloroform extraction and ethanol precipitation. Genotyping of the promoter single nucleotide polymorphism (SNP; rs1332018) was carried out using a Taqman allelic discrimination assay (Applied Biosystems, Foster City, CA). The primers used were forward: 5’-GCCGAGGGCGAGTCA-3’ and reverse: 5’-TCGAGGACTAGGGAAACTGTG-3’. The probes used were VIC: 5’-CCCCCTTATGTGGGATA-3’ and FAM: 5’-CCCCCTATGTGGGATATA-3’. The reaction mix included 20 ng genomic DNA.
DNA, 1× primer (0.2 μmol/L)/probe (0.9 μmol/L) mix, and 1× Taqman Universal Master Mix (Applied Biosystems) in a final volume of 10 μL. Thermocycling consisted of a 95°C, 10-minute hold for AmpliTaq Gold activation followed by 40 cycles of 95°C, 15 seconds and 60°C, 60 seconds. VIC and FAM probe signals were recorded post-PCR using an ABI PRISM 7700 instrument (Applied Biosystems). GSTM3 promoter sequencing in lymphoblasts was done using an ABI PRISM BigDye Terminator Cycle Sequencing kit and an ABI PRISM 377 DNA sequencer (Applied Biosystems). Sequences were compared with the GSTM3 consensus (Genbank accession no. AF043105) using the PileUp program of GCG Wisconsin package version 10.3 (Accelrys, San Diego, CA).

**Measurement of GSTM3 Expression Using Real-time PCR.** Total RNA was isolated from cells using a RNeasy Mini kit (Qiagen, Inc., Valencia, CA) treated with DNase and measured for A260/280 nm ratios using a NanoDrop spectrometer (NanoDrop, Rockland, ME). cDNA was generated from 1 μg total RNA using the SuperScript First-Strand Synthesis System (Invitrogen). The cDNA reaction solution was diluted to a final concentration of 1.33 ng/μL. Real-time PCR constituents were 22.5 μL DNA, 1× Taqman Universal Master Mix, and 1× primer/probe mix (Hs00356079, Applied Biosystems) in a 50 μL final volume. Real-time PCR amplification was done using the same conditions as for genotyping, except for the addition of an initial 50°C, 2-minute hold for optimal AmpErase UNG activity. Signals were recorded during PCR using an ABI PRISM 7700 instrument. All gene expression results were normalized to HPRT1 expression. Gene expression was calculated using the ΔCt method (Applied Biosystems).

**Measurement of GSTM3 Protein Level Using Western Blot.** Cells were harvested and incubated (30 minutes on ice) in lysis buffer after centrifugation (13,000 rpm, 15 minutes), proteins in the supernatants were quantified (Bio-Rad Laboratories, Hercules, CA). Cell lysates (10 μg) were denatured (80°C, 15 minutes), loaded onto an 18% Tris-glycine gel (Invitrogen), run on an XCell SureLock Mini-Cell (Invitrogen), and transferred onto a 0.2 μm nitrocellulose membrane (Invitrogen). The membrane was rinsed (PBS with 0.05% Tween), blocked (1% bovine serum albumin in PBS), rinsed again, and incubated in primary rabbit anti-human GSTM3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). A no-antibody immunoprecipitation served as a negative control. Following precipitation, protein-DNA complexes were washed and eluted from the antibody by adding elution buffer (Upstate Biotechnology). Protein-DNA cross-links were determined by 1% agarose Tris-borate EDTA gel electrophoresis. Samples were centrifuged (13,000 rpm, 10 minutes) and the supernatant was diluted 10-fold in ChIP dilution buffer (Upstate Biotechnology) to a 2 mL final volume. For each sample, 20 μL supernatant was kept as input/starling material. Immunoprecipitations were done by incubating (4°C, 30 minutes) 2 mL diluted supernatant in 80 μL salmon sperm DNA/protein A agarose 50% slurry with agitation and then by centrifugation (2,000 rpm, 3 minutes). The supernatant was incubated (4°C, overnight) in 3 μg RNA pol II antibody (Santa Cruz Biotechnology, Santa Cruz, CA). A no-antibody immunoprecipitation served as a negative control. Following precipitation, protein-DNA complexes were washed and eluted from the antibody by adding elution buffer (Upstate Biotechnology). Protein-DNA cross-links were quantified (Bio-Rad Laboratories, Hercules, CA). The membrane was stripped and similarly blotted with β-actin antibody.

**Preparation of pG3 Vector Constructs.** A fragment of the GSTM3 5′ untranslated region, −948 to +3 nucleotides (relative to codon 1 start, Genbank accession no. AF043105), was generated by PCR amplification using genomic DNA from GM07354 cells (−783AA/−63AA). PCR was done using Expand High-Fidelity PCR System (Roche Molecular Diagnostics Corp., Indianapolis, IN) according to the manufacturer's instructions. The primers used were forward: 5′-AGAAGAATCGTGTTGACGATGGAATGGTG-3′ and reverse: 5′-AGAAGAAGATCTCTAGTGAGGAGGCTTTATACCC-3′. The PCR product was purified and inserted into the firefly luciferase reporter plasmid, pG3-enhancer vector (Promega, Madison, WI), in the reporter plasmid. In pGL3, the four possible haplotypes for the A−783G and A-63C sites were generated using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The primers used were forward: 5′-CCGCCCTTATGTGTTGATAAAGCCCTCTC-3′ and reverse: 5′-GAGGGGCTTTATACCCcAATAAAGGGGGG-3′ for A−783G site and forward: 5′-GATATTTTCCATTAAAGCCTGACTGTG-3′ and reverse: 5′-CCATATGTCCAgGATTAGGGGAAATAA-3′ for A-63C site. Sequences were verified using the dye terminator cycle sequencing method on an ABI PRISM.

**Transient Transfection.** The human glioma cell line SF-268 was plated in six-well plates at a density of 2 × 10⁴ cells per well and incubated at 37°C in RPMI 1640 containing 15% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen) for 24 hours. After washing the cells with PBS, the cells were cotransfected (37°C, 4 hours) with 3 μg pGL3-enhancer construct, 0.3 μg pRL-TK (for normalization of luciferase expression), and 6 μg Transfectam (Promega) in 500 μL RPMI 1640 without fetal bovine serum and then incubated (37°C, 48 hours) with fresh medium. The pRL-TK, pGL-3 control, and pGL-3 empty vectors were used to establish transfection conditions. Cells were washed with PBS twice and harvested after incubation (2 hours, room temperature) in 500 μL passive lysis buffer (Promega) with agitation. Luciferase activity was determined using 20 μL cell lysate aliquots. The lysates were mixed with 100 μL luciferase firefly assay reagent and read on a model TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) every 0.5 second for 25 seconds in a 96-well plate. Luciferase activity was normalized to the intensity of pRL-TK (Promega) and expressed as relative light units. All luciferase assays were carried out in triplicate.

**ChIP Assay.** We have used an approach similar to Knight et al. (25), combining a ChIP protocol in heterozygous −63AC (SNB-19) cells with Taqman quantitative genotyping. SNB-19 cells (3 × 10⁴) were used in 10 independent experiments carried out with a ChIP procedure modified from Upstate Biotechnology (Lake Placid, NY). Cells were fixed using 1% formaldehyde and incubated (37°C, 10 minutes). Fixation was stopped with 125 mMol/L glycine (37°C, 5 minutes). Cells were washed, pelleted (4°C, 2,000 rpm, 4 minutes), and lysed with 200 μL SDS lysis buffer (Upstate Biotechnology) containing the protease inhibitors. The lysate was sonicated (20 minutes, six times) using a Sonicator 3000 (Misonix, Farmingdale, NY) at 90% of output level, resulting in an average of 500-bp DNA length, determined by 1% agarose EDTA gel electrophoresis. Samples were centrifuged (13,000 rpm, 10 minutes) and the supernatant was diluted 10-fold in ChIP dilution buffer (Upstate Biotechnology) to a 2 mL final volume. For each sample, 20 μL supernatant was kept as input/starling material. Immunoprecipitations were done by incubating (4°C, 30 minutes) 2 mL diluted supernatant in 80 μL salmon sperm DNA/protein A agarose 50% slurry with agitation and then by centrifugation (2,000 rpm, 3 minutes). The supernatant was incubated (4°C, overnight) in 3 μg RNA pol II antibody (Santa Cruz Biotechnology, Santa Cruz, CA). A no-antibody immunoprecipitation served as a negative control. Following precipitation, protein-DNA complexes were washed and eluted from the antibody by adding elution buffer (Upstate Biotechnology). Protein-DNA cross-links were quantified (Bio-Rad Laboratories, Hercules, CA). The membrane was stripped and similarly blotted with β-actin antibody.

**Figure 1.** Real-time PCR analysis of GSTM3 mRNA expression in CEPH13145 family. A, pedigree of CEPH1345 family. B, GSTM3 mRNA expression. The amount of GSTM3 mRNA was calculated relative to a standard curve of reference RNA and the HPRT1 gene in the same cDNA sample. Columns, mean from triplicate measurements; bars, SD. Each lymphoblast cell line was evaluated with three independent experiments.
reversed in 0.2 mol/L NaCl by heating (65°C, 4 hours). The protein was digested in 40 μg/mL proteinase K (45°C, 1 hour, Qiagen), 0.01 EDTA, and 0.40 mol/L Tris-HCl (pH 6.5). DNA was extracted with phenol-chloroform, precipitated with ice-cold 100% ethanol, and resuspended in 50 μL Tris-EDTA. DNA concentrations were estimated using a NanoDrop method according to the manufacturer’s directions.

Quantitative Allelic Discrimination Assay. Quantitative allelic discrimination of the A→63C SNP was done using an ABI PRISM 7700 with the same primer and probe sets used for genotyping. A standard curve was created using −63AA and −63CC genomic DNA mixtures at varying percentages (0%, 10%, 20%, …, 100%). PCR constituents and thermocycling were the same as for A→63C genotyping, except that 11.25 μL immunoprecipitated DNA (estimated to be −10 ng) in a 25 μL final volume and 36 cycles were used. VIC and FAM signals were recorded post-PCR using an ABI PRISM 7700.

Statistical Analysis. The results for gene expression, transient transfection, and allele-specific ChIP assays are given as mean ± SD of at least triplicate measurements. The significance of the results was determined using the Student’s t test at α = 0.05.

Results and Discussion

Phenotype-Directed Discovery of GSTM3 Promoter SNPs. SNPs can alter protein function (coding SNP) or affect gene expression regulation (rSNP), and these effects may lead to interindividual differences in drug metabolism or disease susceptibility (26–31). Although numerous projects have focused on coding SNPs, few biologically directed approaches for identifying and evaluating function of rSNPs are known (32). In this work, we used a gene expression screening approach to discover a functional rSNP in GSTM3. Anttila et al. (17) reported that the GSTM1-null (GSTM1*0) genotype was associated with GSTM3 expression.

We screened GSTM1 genotype and GSTM3 expression in lymphoblast cell lines from a CEPH family (CEPH-1345) containing parents heterozygous for the GSTM1*1/*0 null genotype and children with various combinations of the GSTM1 alleles. These individuals were all homozygous for the GSTM3*A allele (the GSTM3*B allele is a 3-nucleotide deletion in intron 6). No relationship between GSTM1 genotype and GSTM3 expression was observed; however, several children displayed GSTM3 low expression phenotypes (Fig. 1). GSTM3 promoter sequencing in these children revealed two SNPs, A→783G and A→63C, relative to the start of codon 1. The individuals homozygous for the −63CC genotype had 8-fold lower expression compared with those with the −63AA genotype (P < 0.0003). Mean GSTM3 expression in the two parents and two other children, all −63AC heterozygotes, was 1.6-fold lower than the −63AA children, suggesting a recessive mode of action for this SNP. GM07350 displays somewhat lower expression than the other three heterozygous cell lines, possibly due to the presence of the −783 G allele. However, this was not consistent across all cell lines.

Genotyping of GSTM3. To estimate the frequencies of the GSTM3 A→63C genotypes in human populations, allele discrimination genotyping was carried out on samples from European American, African American, and Taiwanese individuals. Table 1 shows the genotype and allele frequencies in these groups. Among those of European descent, the C allele was common (0.44) and had a high frequency, relative to individuals of African (0.17) or Asian (0.14) descent. Of note, the GSTM1*0 null allele is also common and occurs at similar frequencies in these groups (European, 0.49; African, 0.38; ref. 14). However, despite the relative close proximity (~50 kb) of GSTM3 to the GSTM1*0/*1 deletion polymorphism, the GSTM3 −63C allele can occur on the same chromosome with either GSTM1*1 or GSTM1*0 allele (all four haplotypes are present), suggesting that these polymorphisms are not strongly in linkage.

GSTM3 Expression in Glioma Cell Lines. We also examined the NCI-60 tumor cell line gene expression database (http://genomewww.stanford.edu/nci60/) and observed GSTM3 expression variability across the various tumor types. This finding suggested that the −63C SNP might also affect GSTM3 expression in cancer cell lines. Because GSTM3 is highly expressed in brain tissue, we obtained three glioma cell lines, SNB-19, SF-268, and SF-539, from the National Cancer Institute Developmental Therapeutics Program. Protein levels determined by Western blotting with GSTM3 antibody (Fig. 2A) were consistent with values determined by the Developmental Therapeutics Program. Both SNB-19 and SF-268 cells showed higher GSTM3 protein levels than those of SF-539, which was undetectable. Real-time PCR was used to determine GSTM3 mRNA expression levels (Fig. 2B) and these results were consistent with the Western blot data. SF-539, homozygous −63C/C, had 9-fold lower expression relative to SF-268, homozygous −63A/A (P < 0.0001; Fig. 2B). SNB-19, heterozygous −63A/C, expressed similar GSTM3 mRNA levels (1.3-fold lower) compared with SF-268 cells (Fig. 2B). These genotype-phenotype results mirrored those observed in our lymphoblast lines. Taken together, these data suggest that the absence of GSTM3 mRNA and protein is the consequence of the −63C SNP in the proximal GSTM3 promoter and is not due to other post-transcriptional processes.

Functional Analysis of GSTM3 Promoter Haplotypes. To evaluate relative promoter activity of the −A783G and −A63C alleles, we amplified ~900 bp of the GSTM3 promoter (containing the −783A/−63A haplotype) and cloned this fragment into a pGL3-enhancer vector upstream of the luciferase reporter. After transient transfection into SF-268 cells, strong luciferase expression was observed for the −783A/−63A vector compared with an empty pGL3-enhancer vector (Fig. 3). Using in vitro mutagenesis, we generated luciferase reporter constructs containing the four possible

Table 1. Genotyping and allele frequencies among Taiwanese, African Americans, and European Americans

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<th>A/A, n (%)</th>
<th>A/C, n (%)</th>
<th>C/C, n (%)</th>
<th>% Allele frequency (C)</th>
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<td>33 (30.8)</td>
<td>2 (1.9)</td>
<td>17</td>
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<tr>
<td>European Americans</td>
<td>175</td>
<td>57 (32.6)</td>
<td>82 (46.9)</td>
<td>36 (20.6)</td>
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Note: Hardy-Weinberg equilibrium test indicates that all populations were in equilibrium.
were evaluated in three independent experiments. Significant differences were observed in luciferase expression in SF-268, SF-539, and SNB-19 cell lines. The -783A and -63A haplotypes and measured luciferase expression activity in SF-268 cells (Fig. 3). Relative to the -783A/-63A allele, both -63C containing alleles reduced expression. The -783A/-63C allele reduced luciferase expression 4-fold from 9.4 ± 1.36 to 2.36 ± 0.3 (P < 0.0004). The -783G/-63C allele was reduced 2.7-fold. The -783G polymorphism also reduced luciferase expression but to a lesser extent (1.6-fold lower in the -783G/-63A haplotype relative to the -783A/-63A haplotype; P < 0.02). These observations indicate that the A-63C site is more important for promoter activity than the A-783G site. We hypothesized that the A-63C site, which is 4-nucleotide upstream of the TATA box, could alter the binding of RNA pol II and examined this possibility using ChIP.

**Allele-Specific ChIP Assay.** ChIP can be used to precipitate DNA sequences directly bound to cellular proteins in vivo (33). Immunoprecipitation of the cross-linked transcription complex has been used to study specific molecular events occurring in transcription, and Knight et al. (25) showed that ChIP could be used in an allele-specific format. We modified this approach and quantified the relative abundance of the A-63C GSTM3 alleles in immunoprecipitated DNA from a A-63C heterozygous cell line (SNB-19) using Taqman-based allelic discrimination genotyping.

We validated the quantitative genotyping component by measuring the VIC (−63A) and FAM (−63C) probe signals in cell line DNA with the three possible A-63C genotypes (−63A/A, −63C/C, and −63A/C) and in genomic DNA mixtures of varying −63A/A and −63C/C DNA percentages (0%, 10%, 20%, ..., 100%). The DNA mixture experiment showed a linear relationship (r² = 0.93) between allele composition and VIC/FAM ratios (Fig. 4A). VIC/FAM ratios for the homozygous −63A/A and −63C/C DNA samples were 2.82 ± 0.41 and 0.28 ± 0.03 (Fig. 4B), respectively, and the heterozygote −63A/C cell line was 0.93 ± 0.02. Furthermore, the VIC/FAM ratio observed from a heterozygote −63A/C cell line, GM01032A (0.93 ± 0.02), was not significantly different from the 50%/50% allele mixture experiment (1.17 ± 0.04). VIC and FAM signals were linear down to at least 1 ng genomic DNA per reaction (data not shown). This low detection limit and low variability indicated that this method would permit quantitative allelic discrimination in small quantities of immunoprecipitated DNA and would allow multiple analyses from the same RNA pol II-precipitated sample.

For allele-specific ChIP experiments, heterozygous −63A/C SNB-19 cells were cross-linked, lysed, sonicated, and then mixed with RNA pol II antibody and protein A agarose beads to precipitate DNA from actively transcribed promoters. SNB-19 input DNA (DNA extracted from sonicated cells with no further treatment) produced a VIC/FAM ratio of 1.04 ± 0.02 (Fig. 4C), consistent with VIC/FAM ratio of the other −63A/C heterozygous cell line, GM01032A (0.93 ± 0.02). These values represent the expected equal representation of the two alleles in the genomic DNA of heterozygous cells and are the reference value for comparison with the ChIP samples.

To determine the relative binding of RNA polymerase to the −63A and −63C promoter alleles, we quantified the presence of each of the alleles in the DNA recovered from the ChIP procedure. Figure 4D displays the mean VIC/FAM ratios obtained from quantitative

![Figure 2. Analysis of GSTM3 expression level in glioma cell lines. A, Western blot of GSTM3 using 10 μg cell lysates from SF-268, SF-539, and SNB-19. B, real-time PCR analysis of GSTM3 mRNA expression in SF-268, SF-539, and SNB-19. Columns, mean from triplicate measurements; bars, SD. Expression measurements for each cell line were evaluated in three independent experiments.](image-url)

![Figure 3. Functional analysis of the four GSTM3 promoter haplotypes. A, fragments of human GSTM3 promoter regions were constructed in pGL3-enhanced luciferase (Luc) reporter plasmid. pGL3 plasmids were transiently cotransfected with pRL-TK into SF-268 cells, and luciferase activities were analyzed after 48 hours of treatment. B, columns, mean from triplicate measurements of three independent experiments; bars, SD.](image-url)
protein A agarose beads with no antibody present showed baseline VIC and FAM signal levels (data not shown), indicating an absence of non-specifically precipitated GSTM3 promoter DNA. These allele-specific ChIP results are consistent with both gene expression (Figs. 1 and 2) and luciferase reporter assays results (Fig. 3). It is interesting to note that the magnitude of the allele difference for the allele-specific ChIP assays and the mRNA expression assays are similar (~9-fold difference), although results for the luciferase reporter assays showed a smaller difference (~4-fold).

Allele-specific ChIP is a powerful technique for assessing functional differences between promoter alleles. This method provides in vivo relative determination of transcription factor loading on an endogenous gene promoter under normal regulatory control in a chromosomal environment. This approach may reflect the "true" biological differences between promoter alleles in vivo and as such could prove to be more biologically relevant than the usual allele comparisons using dual luciferase reporter gene constructs. A previous study by Knight et al. (25) determined relative RNA pol II binding to tumor necrosis factor α promoter by combining ChIP with primer extension/matrix-assisted laser desorption/ionization time-of-flight mass spectrometry—based genotyping (Sequenom, San Diego, CA). In the present study, we modified the Knight et al. approach by combining RNA pol II ChIP with allele quantification using a 5’ nuclease-based allelic discrimination assay (Taqman). As there are many thousands of laboratories with instruments capable of using the 5’ nuclease chemistry, this approach should be more accessible than the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry method.

**Biological Impact of the A→63C Polymorphism in the GSTM3 Promoter.** GSTM3 is the ancestral gene in the GSTU gene family (2), and several biological functions have been shown. Like other GSTU proteins, GSTM3 catalyzes the conjugation of numerous toxic or carcinogenic compounds to glutathione, and low levels of GSTM3 expression in the cell might lead to greater amounts of cellular DNA damage. It has also been suggested that GSTM3 expression could affect sensitivity to chemotherapeutic agents. In addition, GSTM3 was identified recently as a cytosolic glutathione-dependent prostaglandin E2 synthase in human brain (34), suggesting an endogenous role for GSTM3 protein levels in regulation of prostaglandin signaling pathways. Because prostaglandin E2 plays an important role in cell proliferation signaling, GSTM3 expression variation could have an impact on growth or cell proliferation during tumorigenesis in the brain or perhaps in other GSTM3-expressing tissues.

In summary, we have shown that the GSTM3 A→63C SNP has a major impact on regulation of GSTM3 expression in vivo. GSTM3 has several potentially important biological functions, and the effect and prevalence of a SNP in multiple ethnic populations suggests the possibility of a significant role in carcinogenesis or tumor biology. To test this hypothesis, we have initiated studies examining the relationship between this GSTM3 SNP and the risk of brain, lung, and testicular cancer.

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References

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