Correlation between \textit{UDP-Glucuronosyltransferase} Genotypes and 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanone Glucuronidation Phenotype in Human Liver Microsomes

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\textbf{ABSTRACT}

The nicotine-derived tobacco-specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol, is one of the most potent and abundant procarcinogens found in tobacco and tobacco smoke, and glucuronidation of its major metabolite, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNAL), is an important mechanism for 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone detoxification. Substantial interindividual variability in urinai NNAL glucuronide formation has been observed in smokers and tobacco chewers. To determine whether genetic variations may play a role in this interindividual variability, NNAL-glucuronidating activities were analyzed in 78 human liver microsomal specimens and compared with the prevalence of missense polymorphisms in the two major NNAL-glucuronidating enzymes UGT1A4 and UGT2B7. In vitro assays using liver microsomal specimens from individual subjects demonstrated a 70- and 50-fold variability in NNAL-Gluc and NNAL-O-Gluc formation, respectively, and a 20-fold variability in the ratio of NNAL-N-Gluc: NNAL-O-Gluc formation. Microsomes from subjects with a homozygous polymorphic UGT1A4<sup>24Thr</sup>/UGT1A4<sup>24Thr</sup> genotype exhibited a significantly higher (\(P < 0.05\)) level of NNAL-N-Gluc activity compared with microsomes from subjects with the wild-type UGT1A4<sup>24Pro</sup>/UGT1A4<sup>24Pro</sup> genotype, and a significantly higher (\(P < 0.05\)) number of subjects with liver microsomes having high NNAL-N-Gluc formation activity contained the UGT1A4<sup>24Thr</sup>/UGT1A4<sup>24Thr</sup> genotype. Microsomes from subjects with the homozzygous polymorphic UGT2B7<sup>85Tyr</sup>/UGT2B7<sup>85Tyr</sup> genotype exhibited a significantly lower level (\(P < 0.025\)) of NNAL-O-Gluc activity when compared with microsomes from subjects with the wild-type UGT2B7<sup>85Pro</sup>/UGT2B7<sup>85Pro</sup> genotype, and a significantly (\(P < 0.05\)) higher number of subjects with liver microsomes having low NNAL-O-Gluc formation activity contained the UGT2B7<sup>85Pro</sup>/UGT2B7<sup>85Pro</sup> genotype. These data suggest that the UGT1A4 codon 24 and UGT2B7 codon 268 polymorphisms may be associated with altered rates glucuronidation and detoxification of NNAL \textit{in vivo}.

\textbf{INTRODUCTION}

The nicotine-derived tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNK) is one of the most potent and abundant procarcinogens found in tobacco and tobacco smoke (1, 2). NNK levels in tobacco smoke are 3–15 times higher than that of another major potent carcinogen in tobacco smoke, benzo(a)pyrene (3). NNK induces predominantly lung adenocarcinomas in rodents independent of the route of administration (2). In the Fischer 344 rat, NNK induces pancreatic tumors (4) and, when applied together with the related tobacco-specific nitrosamine, N'-nitrosornornicotine, oral cavity tumors (5). The cumulative dose of 1.8 mg NNK/kg body weight required to produce lung tumors in rodents (6) is similar to the cumulative lifetime dose of 1.6 mg NNK/kg body weight for the average American smoking two packs of cigarettes a day for 40 years (1, 2). NNK is therefore considered to be a likely causative agent for several tobacco-related cancers in humans, including lung, oral cavity, and pancreas (2, 4).

The major metabolic pathway of NNK in most tissues is carbonyl reduction to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). NNK reduction to NNAL occurs in rodents, monkeys, and humans (2, 7), with an estimated 39–100% of the NNK dose converted to NNAL in smokers (7). NNAL is activated via pathways similar to those observed for NNK and, as with NNK, is a potent lung and pancreatic carcinogen in rodents (2, 4). Previous studies have shown that NNAL is also metabolized to its glucuronide, NNAL-Gluc (2, 7–11). Although the formation of NNAL is not a detoxification pathway for NNK, the glucuronidation of NNAL appears to be an important mechanism for NNK detoxification. This is supported by the fact that the glucuronidation of R-NNAL was significantly greater than S-NNAL after injection into A/J mice, a pattern consistent with the higher tumorigenicity exhibited by S-NNAL in the same experiments (12, 13). In contrast to the relatively high tumorigenicity exhibited by both R- and S-NNAL, NNAL-Gluc is nontumorigenic after s.c. injection into A/J mice (12). In addition, Kim and Wells (14) reported that skin fibroblasts from UDP-glucuronosyltransferase (UGT) family 1-deficient rats were more sensitive to NNK-mediated cytotoxicity.

NNAL glucuronidation can occur at both the carbinol group (NNAL-O-Gluc; Ref. 2, 7–10) and the nitrogen on NNAL’s pyridine ring (NNAL-N-Gluc; Ref. 15). NNAL-O-Gluc formation in human tissues is well characterized and was found to be mediated primarily by the hepatic enzyme, UGT2B7 (10). The identification of NNAL-N-Gluc in human urine has only recently been identified (15), and recent studies have shown that NNAL-N-Gluc formation is mediated exclusively by the hepatic enzyme, UGT1A4 (16). The formation of both NNAL-N-Gluc and NNAL-O-Gluc appear to be important intermediates in the detoxification of NNAL and NNK. The ratio of NNAL-N-Gluc to NNAL-O-Gluc formation was observed to be \(\sim 1.0\) in the urine of smokers (15) and \(\sim 1.7\) in liver microsomes from individual subjects (16), indicating that both of these NNAL glucuronide conjugates were formed in significant amounts in smokers.

Significant interindividual variability in the ratios of both NNAL-Gluc to NNAL (17) and NNAL-N-Gluc to NNAL-O-Gluc (15) was observed in the urine of smokers and snuff users. In addition, significant variability in the levels of NNAL-N-Gluc and NNAL-O-Gluc formation was also observed in \textit{in vitro} assays of human liver microsomal specimens (16). These differences in NNAL glucuronidating capacity may result in a decreased ability to detoxify NNK and suggest that genetic and/or environmental factors may be important determinants of the levels of NNAL-N-Gluc and NNAL-O-Gluc formation observed in smokers and snuff chewers.

Polymorphisms have been previously identified in several \textit{UGT} genes, including UGT1A1, UGT1A6, UGT1A7, UGT1A9, UGT1A10, UGT2B4, UGT2B7, and UGT2B15 (18–25). Among the family of 2B polymorphic variants, the UGT2B15<sup>85Tyr</sup> encoded variant was shown to be associated with an increase in UGT2B15 function (19) and in
preliminary studies has been linked to increased risk of for prostate cancer (26). In studies examining UGT family IA variants, the ‘TATA’ box polymorphism in the promoter region of UGT1A1, commonly associated with Gilbert’s syndrome, is associated with reduced function in the UGT1A1 transcriptional promoter (22) and has been implicated in increased risk for breast cancer (27) and with decreased formation of the glucuronide conjugate of the important benzo(a)pyrene metabolite, BaP-7,8-dihydrodiol(-), in liver microsomes (28). Results from other studies suggest that UGT1A17-specific genetic variants are associated with reduced UGT1A17 metabolic function (23) and are strongly linked to increased risk for orofaryngeal cancer (29), as well as hepatocellular carcinoma (30), whereas a Glu>Lys polymorphism at codon 139 of the UGT1A10 gene has been linked to risk for orofaryngeal cancer (25).

The goal of the present study was to determine whether genetic variations in the two major NNAL-glucuronidating enzymes, UGT1A4 and UGT2B7, could potentially play a role in the inter-individual variability observed in the levels of NNAL-N-Glc and NNAL-O-Glc formation in human liver microsomes (16) and potentially the variability in the ratios of both NNAL-Glc:NNAL and NNAL-N-Glc:NNAL-O-Glc observed in the urine of smokers and snuff users (15, 17). In this article, results are presented demonstrating a correlation between a newly identified polymorphism in codon 24 (Pro->Thr) of the UGT1A4 gene and NNAL-N-Glc formation and the codon 268 (His>Tyr) polymorphism in the UGT2B7 gene and NNAL-O-Glc formation in human liver microsomes.

MATERIALS AND METHODS

Chemicals and Materials. UDPGA, D₃,2-lysophosphatidyl choline palmital C16:0, and β-glucuronidase were purchased from Sigma (St. Louis, MO). NNAL was obtained from Toronto Research Chemicals (Toronto, Ontario, Canada). ¹⁴C-UDPGA (specific activity: 300 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO), TaqDNA polymerase (HotMaster) was purchased from Perkin-Elmer Biosystems (Foster City, CA), and HPY188III Stul and FokI were purchased from New England Biolabs (Beverly, MA). Baculosomes overexpressing UGT1A4 was purchased from BD Biosciences (Chicago, IL), whereas Genetecin was purchased from Medi-atech (Herndon, VA).

Tissues and Cell Lines. Normal human liver tissues and genomic DNA specimens were provided by the Tissue Procurement Facility at the H. Lee Moffitt Cancer Center from individuals (n = 78) undergoing surgery for resection of hepatocellular carcinoma, with matching tissue and DNA obtained from each subject. Tissue samples were quick-frozen at -70°C) with 2 h after surgery. Liver microsomes were prepared through differential centrifugation as described previously (31) and were stored (10–20 mg protein/ml) at -70°C, with total protein concentrations measured using the BCA assay (Pierce Corporation, Rockford, IL). Demographic data were collected by medical chart review for corresponding individuals from whom liver specimens were obtained. All subjects were Caucasian, 44% were female, and the average age of these subjects was 64 years. All protocols involving the analysis of tissue specimens were approved by the Institutional Review Board at the University of South Florida and in accordance with assurances filed with and approved by the United States Department of Health and Human Services.

The HK293 cell line overexpressing the rat UGT2B1 has been previously described (32) and was grown to 80% confluence in DMEM supplemented with 4.5 mM glucose, 10 mM HEPES, 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin and maintained in 700 µg/ml Genetecin for selection of UGT overexpression in a humidified incubator in an atmosphere of 5% CO₂. Cells were grown to 80% confluence before the preparation of cell homogenates as described previously (16).

NNAL Glucuronidation Assays. The rate of NNAL glucuronidation by liver microsomes was determined after a preincubation with D₃,2-lysophosphatidyl choline palmital C16:0 (0.1 mg/mg protein) for 10 min at 4°C as described previously (16). Briefly, liver microsomes (0.5 mg of protein) were incubated (100 µl of final volume) in 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, D₃,2-lysophosphatidyl choline palmital C16:0 (0.1 mg/mg protein), 4 nm ¹⁴C-UDPGA (1 µCi/100 µl reaction volume), and 5 mM NNAL at 37°C for 2 h. Reactions were terminated by the addition of 1/10 volume of 0.3 n Ba(OH)₂/0.3 v ZnSO₄ on ice. The precipitate was removed by centrifugation, and the supernatant was subjected to solid phase extraction on an Oasis HLB 3 ml reverse phase cartridge (Waters, Milford, MA) activated with acetonitrile and equilibrated with solvent A (0.05 n NH₄OAc, pH 7.0). After loading onto the cartridge, the sample was washed with 1 ml of solvent A and eluted with 0.5 ml of acetonitrile. The acetonitrile was evaporated, the resulting sample dilute to 110 µl with water, and the sample was analyzed for glucuronidated NNAL metabolites by high-pressure liquid chromatography with radioflow detection using the following system: a Waters Associates dual-pump (model 510) HPLC system (Milford, MA), equipped with an automatic injector (WISP model 710B), a UV detector operated at 254 nm (model 440), and a radioactive flow detector (IN/US Systems, Fairfield, NJ). High-pressure liquid chromatography was performed using a 5-µ Aquasil C18 column (4.6 x 250-mm; Thermo Hypersil-Keystone, Bellefonte, PA) with gradient elution at 1 ml/min using the following conditions: 5 min with 100% solvent A; a linear gradient for 10 min to 30% solvent B (100% methanol); a subsequent linear gradient to 50% solvent B for 10 min; and a final linear gradient to 100% solvent B for 5 min. The column was washed for 10 min with 100% solvent B and regenerated for 15 min with 100% solvent A. For quantification of NNAL-N-Glc and NNAL-O-Glc formation in liver microsomes, assays were repeated for randomly selected samples (n = 21) to assure the validity and reproducibility of our assay system. ¹⁴C-NNAL-Gluc peaks were confirmed by relative retention time compared with that observed for NNAL-N-Glc and NNAL-O-Glc purified from in vitro assays with UGT1A4- and UGT2B1-overexpressing baculosomes or cell homogenates (10, 16). In some cases (n = 5), glucuronidated NNAL peaks were confirmed by sensitivity to Escherichia coli β-glucuronidase as described previously (10, 16, 33).

NNAL glucuronide standards were prepared as described above using UGT2B1-overexpressing cell line homogenates (1.5 mg of protein) or UGT1A4-overexpressing baculosomes (1 mg of protein).

PCR Amplifications, Sequencing, and Genotyping Analysis. For sequencing analysis, PCR amplification of UGT1A4 exon 1 was performed using a sense primer (1A4S1; 5'-GCCCATACAGGAAAGGCTG-3') corresponding to nucleotides 180 through 162 relative to the translation start site in UGT1A4 exon 1 (GenBank accession no. AF297093) and an antisense primer (1A4AS2; 5'-CTCTCAAAGTTTATCTGTTATAAAGA-3') corresponding to nucleotides 983 through 1008 relative to the UGT1A4 translation start site. PCR amplifications were routinely performed in a 50-µl reaction volume containing 100–250 ng of purified genomic DNA in 1X HotMaster TaqBuffer with 0.05 mM each of deoxynucleotide triphosphates, 20 pmol of both sense and antisense UGT1A4 primers, and 2.5 units of TaqDNA polymerase. The PCR mixture underwent the following incubations in a GeneAmp 9700 thermocycler (Perkin-Elmer Biosystems): 1 cycle of 94°C for 2 min; 41 cycles of 94°C for 20 s; 59°C for 10 s; and 65°C for 45 s, followed by a final cycle of 7 min at 70°C. PCR amplification integrity of all samples was confirmed by electrophoresis on 8% PAGE gels that were subsequently stained with ethidium bromide and examined under UV-light using a computerized photo-imaging system (AlphaImager 2000; α Innotech Corp., San Leandro, CA).

For dideoxy sequencing, PCR products were purified after electrophoresis in 1% agarose using the QIAEX II gel extraction kit (Qiagen, Valencia, CA). Dideoxy sequencing was performed at the DNA Sequencing Facility in the Department of Genetics at the University of Pennsylvania School of Medicine, using the same sense and antisense primers as were used for PCR amplification as well as an internal antisense primer (1A4AS2; 5'-CACACAACCTACT-GAAGGG-3') corresponding to nucleotides +368 through +387 relative to the UGT1A4 translation start site.

UGT1A4 and UGT2B7 polymorphisms were assessed by PCR-RFLP analysis. PCR amplifications were performed essentially as described above using the following primers: 1A4S1 and 1A4AS2 for UGT1A4 (producing a PCR-amplified product of 567 bp), and 2B7S1 (sense; 5'-TGCTTACATATTCTAACC-3') and 2B7AS1 (antisense; 5'-TCTCTGAAATTCTGACT-3') for UGT2B7 (producing a PCR-amplified product of 580 bp). PCR annealing temperatures were at 59°C and 58°C for UGT1A4 and UGT2B7, respectively. The UGT1A4 codon 24 polymorphism was examined by digestion with the HPY188III restriction enzyme (37°C, overnight), the UGT1A4 codon 48 polymorphism by digestion with the Stul restriction enzyme (37°C, overnight).
RESULTS

Significant variability in total urinary NNAL-Gluc and urinary NNAL-N-Gluc:NNAL-O-Gluc ratios were observed between individual subjects in previous studies (15). To determine whether variability in NNAL-Gluc formation exists for human liver specimens from individual subjects, NNAL glucuronidation assays were performed for microsomes prepared from liver specimens from 78 different subjects. Significant variability in the ratio of NNAL-N-Gluc:NNAL-O-Gluc was observed for liver microsomes from individual subjects, with a ratio range of 0.16–3.2 (Table 1). The mean (±SD) NNAL-N-Gluc:NNAL-O-Gluc ratio for the 78 specimens was 1.61 ± 0.59. Representative chromatograms demonstrating this variability are shown in Fig. 1. Significant variability was also observed between liver microsomal specimens from individual subjects in terms of absolute levels of glucuronidated metabolites, with a range of 0.48–25.5 and 0.27–21.5 pmol·mg microsomal protein⁻¹·min⁻¹ for NNAL-N-Gluc and NNAL-O-Gluc, respectively.

Previous studies have shown that the hepatic enzyme, UGT1A4, is the only known UGT that exhibits NNAL-N-Gluc congluturonidating activity (16). To determine whether there are polymorphisms in the UGT1A4 gene that could contribute to the variability in NNAL-N-Gluc levels observed in liver microsomes from different individuals, dideoxy sequencing was performed on genomic DNA from liver specimens from the 10 subjects whose liver microsomes exhibited the five highest and five lowest levels of NNAL-N-Gluc formation. The family IA locus codes for 8 known functional UGT enzymes and is composed of divergent and individually regulated exon 1 sequences that transcribe for mRNAs that are alternatively spliced onto the 5’-end of the sequence encoded by the common UGT exons 2–5 region. Because UGT mRNA consists of a unique region encoded by exon 1 and a region encoded by exons 2–5 that is common for all family IA UGTs, only UGT1A4 exon 1 was examined in this analysis. In order to examine additional polymorphism at codon 68 (CCG→CCA), two misense polymorphisms were identified by DNA sequencing analysis; one at codon 24 (CCC→ACC) resulting in an amino acid change from Pro→Thr, and one at codon 48 (TTG→GTG) resulting in an amino acid change from Leu→Val (Fig. 2A). Both polymorphisms were identified in the high-activity NNAL-N-glucuronidating group and were confirmed by RFLP analysis, with the RFLP pattern consisting of three bands (266, 246, and 55 bp) for the UGT1A448Val variant and two bands (512 and 55 bp) for the wild-type UGT1A448Pro allele (Fig. 2B) and one band (567 bp) for the UGT1A448Pro variant and two bands (319 and 248 bp) for the wild-type UGT1A448Pro allele (Fig. 2C).

To determine whether there is a potential link between UGT1A4 genotype and the variability in NNAL-N-Gluc activity observed in human liver microsomes, RFLP analysis was used to screen for the codon 24 polymorphism using DNA from the 78 liver specimens previously analyzed for NNAL glucuronidation. Eight (10%) of the 78 subjects were heterozygous for the UGT1A448Thr variant, whereas an additional 2 subjects (3%) exhibited the homozygous polymorphic UGT1A448Thr/UGT1A448Thr genotype (Table 2). The allelic frequency for the UGT1A448Thr variant in this cohort was 0.077. Two of the 78 subjects were heterozygous for the UGT1A448Val variant, whereas an additional subject exhibited the homozygous polymorphic

Table 1 NNAL-N-Gluc and NNAL-O-Gluc formation in microsomes prepared from the livers of 78 individual subjects

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
<th>Range</th>
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<tr>
<td>NNAL-N-Gluc formation (pmol · mg⁻¹ · min⁻¹)</td>
<td>10.5 ± 5.8</td>
<td>0.48–25.5</td>
</tr>
<tr>
<td>NNAL-O-Gluc formation (pmol · mg⁻¹ · min⁻¹)</td>
<td>6.7 ± 3.3</td>
<td>0.27–21.5</td>
</tr>
<tr>
<td>Ratio of NNAL-N-Gluc to NNAL-O-Gluc</td>
<td>1.6 ± 0.59</td>
<td>0.16–3.21</td>
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</table>

Fig. 1. High-pressure liquid chromatography analysis of NNAL-Gluc formation by human liver microsomes. Human liver microsomes (0.5 mg of protein) were incubated at 37°C for 2 h with 5 μmol NNAL and 4 μmol [14C]-UDPGA as described in “Materials and Methods.” A, [14C]-labeled NNAL-N-Gluc and NNAL-O-Gluc standards. B and C, [14C]-labeled metabolites from incubations using human liver microsomal specimens 13 and 30, respectively. D, [14C]-labeled metabolites from incubations using liver microsomal specimen 13 after subsequent incubation with β-glucuronidase.

overnight), and the UGT2B7 codon 268 polymorphism by digestion with FokI (37°C, 2 h), using 5–15 μl of PCR product and 5 units of restriction enzyme. Digestions were electrophoresed on 8% PAGE gels that were subsequently stained with ethidium bromide and examined under UV light.

Statistical Analysis. Bivariate analysis included the χ² test or Fisher’s exact test (used as appropriate) for examining differences in genotype frequencies and the Student’s t test for the examination of continuous variables (i.e., levels of NNAL-Gluc formation). The χ² test for trend was used for the analysis of levels of NNAL-Gluc formation for multiple genotypes.
UGT1A4<sup>48Val</sup>/UGT1A4<sup>48Val</sup> genotype (Table 2). The allelic frequency for the UGT1A4<sup>48Val</sup> variant in this cohort was 0.026.

A significant (P < 0.05) increase in NNAL-N-Gluc formation was observed in liver microsomes from individuals who had at least one UGT1A4<sup>24Thr</sup> variant allele as compared with liver microsomes from individuals who were wild-type for the UGT1A4 codon 24 polymorphism (Table 2). Although a significant increase in NNAL-N-Gluc formation was not observed in liver microsomes from individuals who were heterozygous for the UGT1A4<sup>24Thr</sup> allele as compared with liver microsomes from individuals who were wild-type for the UGT1A4 codon 24 polymorphism, a significant (P = 0.035) trend toward increasing liver microsomal NNAL-N-glucuronidating activity was observed when the number of UGT1A4<sup>24Thr</sup> variant alleles increased in individual subjects. When liver microsomes were stratified at the median for levels of NNAL-N-Gluc formation activity (Table 3), a significantly (P < 0.02) higher number of subjects with a variant UGT1A4<sup>24Thr</sup> allele were observed in the high-activity group (8 of 39 subjects, including the 2 subjects with the homozygous polymorphic UGT1A4<sup>24Thr</sup>/UGT1A4<sup>24Thr</sup> genotype) as compared with the low-activity group (2 of 39 subjects; Table 3). A significant (P < 0.01) association was also observed when comparing the total number of UGT1A4<sup>24Thr</sup> variant alleles in the high-activity versus low-activity groups (Table 3). Although both of the subjects homozygous for the UGT1A4<sup>24Thr</sup> variant allele were in the high-activity group, a separate analysis comparing subjects who were homozygous polymorphic versus homozygous wild-type for the UGT1A4 codon 24 polymorphism could not be adequately performed because of the low number of subjects in the homozygous polymorphic group. No association was observed between UGT1A4 genotype and NNAL-N-Glut:total NNAL-Gluc formation (Table 2). Because of the low prevalence of the UGT1A4 codon 48 polymorphism, an assessment of liver microsomal NNAL-N-Glut glucuronidation activity versus UGT1A4 codon 48 genotype could not be performed in the present studies.

Previous studies have shown that the hepatic enzyme, UGT2B7, is the major UGT responsible for the O-glucuronidation of NNAL (10). A polymorphism resulting in a His<sup>→</sup>Tyr amino acid change at codon 268 of the UGT2B7 gene has been previously described (34) and was shown to be the only missense polymorphism present in the UGT2B7 gene after a screening of the entire UGT2B7 gene in 39 Caucasian individuals (24). Similar to that described above for the UGT1A4

<table>
<thead>
<tr>
<th>UGT1A4 genotype</th>
<th>No. of subjects</th>
<th>NNAL-N-Glut formation&lt;sup&gt;a&lt;/sup&gt; (pmol · mg&lt;sup&gt;-1&lt;/sup&gt; · min&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>NNAL-N-Glut/total NNAL-Glut</th>
<th>Ratio</th>
<th>P&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>Pro&lt;sup&gt;2&lt;/sup&gt;/Pro&lt;sup&gt;2&lt;/sup&gt;</td>
<td>68</td>
<td>10.0 ± 5.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Referent</td>
<td>0.59 ± 0.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Referent</td>
</tr>
<tr>
<td>Pro&lt;sup&gt;2&lt;/sup&gt;/Thr&lt;sup&gt;2&lt;/sup&gt;</td>
<td>8</td>
<td>13.2 ± 5.5</td>
<td>0.13</td>
<td>0.56 ± 0.10</td>
<td>0.98</td>
</tr>
<tr>
<td>Thr&lt;sup&gt;2&lt;/sup&gt;/Thr&lt;sup&gt;2&lt;/sup&gt;</td>
<td>2</td>
<td>16.3 ± 4.1</td>
<td>0.12</td>
<td>0.67 ± 0.13</td>
<td>0.33</td>
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<tr>
<td>Pro&lt;sup&gt;2&lt;/sup&gt;/Thr&lt;sup&gt;2&lt;/sup&gt; + Thr&lt;sup&gt;2&lt;/sup&gt;/Thr&lt;sup&gt;2&lt;/sup&gt;</td>
<td>10</td>
<td>13.9 ± 5.2</td>
<td>0.047</td>
<td>0.61 ± 0.10</td>
<td>0.66</td>
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</table>

<sup>a</sup> Levels of NNAL-N-Glut formation determined for 78 subjects from whom UGT1A4 genotype could be obtained.

<sup>b</sup> A significant (P < 0.001) trend towards increasing NNAL-N-Glut formation was observed with UGT1A4 genotypes containing increasing numbers of variant UGT1A4<sup>24Thr</sup> alleles as determined by trend test.

<sup>c</sup> P-values determined by Student’s t test, with comparisons made versus wild-type UGT1A4 Pro<sup>2</sup>/Pro<sup>2</sup> genotype.

<sup>d</sup> Numbers represent means ± SD for three experiments.
codon 24 polymorphism and NNAL-N-Gluc phenotype, a RFLP assay was developed to determine whether there is a significant link between UGT2B7 genotype and the variability in NNAL-O-Gluc activity observed in human liver microsomes from the 78 subjects screened in this study. This assay resulted in a RFLP pattern consisting of three bands (458, 76, and 46 bp) for the UGT2B7*268Tyr variant and four bands (346, 112, 76, and 46 bp) for the wild-type UGT2B7*268His (Fig. 2D). Of the 74 subjects informative for UGT2B7 genotype, 38 (51%) were heterozygous for the UGT2B7*268Tyr/UGT2B7*268Tyr genotype (Table 4). The allelic frequency for the UGT2B7*268Tyr variant in this cohort was 0.50, which is similar to that observed previously for Caucasians (35). A significant ($P < 0.005$) decrease in NNAL-O-Gluc formation was observed in liver microsomes from individuals who were heterozygous for the UGT2B7 codon 268 polymorphism (Table 4). The levels of NNAL-O-Gluc formation in liver microsomes were similar for individuals who were wild-type for the UGT2B7 codon 268 polymorphism. Although significant differences in UGT2B7 genotype prevalence were not observed when liver microsomes were stratified into quartiles for levels of NNAL-O-Gluc formation activity (results not shown), a significantly ($P < 0.005$) higher number of subjects with the homozygous polymorphic UGT2B7*268Tyr/UGT2B7*268Tyr genotype were observed in the lowest activity group (5 of 18 subjects) as compared with subjects from the highest activity group (1 of 18 subjects) when liver microsomes were stratified into quartiles for levels of NNAL-O-Gluc formation activity (Table 5).

**DISCUSSION**

UGTs are a superfamily of enzymes that glucuronidate many xenobiotics and endogenous compounds (36). Both the O- and N-glucuronide forms of NNAL-Glc have been observed in the urine of smokers (15, 17, 37), never-smokers exposed to environmental tobacco smoke (38, 39), as well as tobacco chewers (15, 40), and both glucuronides were shown to be formed in human liver microsomes (10, 16). Significant interindividual variability in the ratios of both NNAL-Glc:NNAL (17, 37) and NNAL-N-Gluc:NNAL-O-Gluc (15) was observed in the urine of smokers and snuff users, suggesting that individuals may differ greatly in their ability to detoxify NNK and forming different NNAL-glucuronides. In addition, variability in the levels of NNAL-N-Gluc and NNAL-O-Gluc formation was also observed in *in vitro* assays of human liver microsomal specimens (16). In the present study, large differences in the ratio of NNAL-N-Gluc:NNAL-O-Gluc, as well as in the absolute levels of NNAL-N-Gluc and NNAL-O-Gluc were observed in *in vitro* assays of a series of liver samples.
expression of UGT1A4 in either the aerodigestive tract or lung (42), a hepatic UGT1A4 (or UGT2B7) genotype-associated effect on overall NNK detoxification and cancer risk cannot be excluded for these organ/tissue sites. Additional studies examining the role of UGT2B7 and UGT1A4 genotype on susceptibility to tobacco-related cancers will be necessary to better evaluate the role of UGT gene variations on cancer risk.

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REFERENCES


Corrections

In the article by A-K. Olsson et al., titled “A Fragment of Histidine-Rich Glycoprotein Is a Potent Inhibitor of Tumor Vascularization,” which appeared in the January 15, 2004 issue of Cancer Research (pp. 599–605), the color contrast in Figure 6B was insufficient to illustrate the authors’ findings. The correct figure appears below.

In the article by D. Wiener et al., titled “Correlation between UDP-Glucuronosyltransferase Genotypes and 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanone Glucuronidation Phenotype in Human Liver Microsomes,” which appeared in the February 1, 2004 issue of Cancer Research (pp. 1190–1196), the titular phenotype should have been identified as butanol. The correct title is “Correlation between UDP-Glucuronosyltransferase Genotypes and 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanol Glucuronidation Phenotype in Human Liver Microsomes.”

Fig. 6. Paxillin in focal adhesions is affected by histidine-rich glycoprotein (HRGP) treatment. A, cells were treated with HRGP (100 ng/ml), as indicated, and paxillin was immunoprecipitated from the cell lysate. Immunoblotting was performed for paxillin (top panel) and phospho-tyrosine (middle panel). To verify equal loading, the cell lysate was blotted for β-actin (bottom panel). IP, immunoprecipitation. B, bovine adrenal cortex capillary endothelial cells treated with vascular endothelial growth factor (VEGF, 10 ng/ml), fibroblast growth factor (FGF)-2 (10 ng/ml), and HRGP (100 ng/ml) as indicated were fixed after 10 min and stained with anti-paxillin antibody (green). Nuclei (blue) were stained by Hoechst 33342. Bar indicates 20 µm.
Correlation between *UDP-Glucuronosyltransferase* Genotypes and 4-(MethylNitrosamino)-1-(3-Pyridyl)-1-Butanone Glucuronidation Phenotype in Human Liver Microsomes

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