Glucuronidation of Nicotine and Cotinine by UGT2B10: Loss of Function by the *UGT2B10* Codon 67 (Asp>Tyr) Polymorphism

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Abstract

Nicotine, the major addicting agent in tobacco and tobacco smoke, undergoes a complex metabolic pathway, with ~22% of nicotine urinary metabolites in the form of phase II *N*-glucuronidated compounds. Recent studies have shown that UGT2B10 is a major enzyme involved in the *N*-glucuronidation of several tobacco-specific nitrosamines. In the present study, microsomes of UGT2B10-overexpressing HEK293 cells exhibited high *N*-glucuronidation activity against both nicotine and cotinine with apparent K_M's that were 37- and 3-fold lower than that observed for microsomes of UGT1A4-overexpressing cells against nicotine and cotinine, respectively. The K_M of microsomes from wild-type (WT) UGT2B10-overexpressing cells for nicotine and cotinine was similar to that observed for human liver microsomes (HLM) against both substrates. The level of glucuronidated nicotine or cotinine in 112 HLM samples was correlated with *UGT2B10* genotype; the levels of nicotine- and cotinine-glucuronide were 21% to 30% lower in specimens from subjects with the *UGT2B10 (*1/*2)* genotype compared with specimens from subjects with the WT *UGT2B10 (*1/*1)* genotype; a 5- and 16-fold lower level of nicotine- and cotinine-glucuronide formation, respectively, was observed in HLM from subjects with the *UGT2B10 (*2/*2)* genotype. In contrast to the relatively high activity observed for cells overexpressing WT UGT2B10 *in vitro*, little or no glucuronidation was observed for microsomes from cells overexpressing the UGT2B10*2 variant against either nicotine or cotinine. These data suggest that UGT2B10 is the major hepatic enzyme involved in nicotine/cotinine glucuronidation and that the *UGT2B10*2* variant significantly reduces nicotine- and cotinine-*N*-glucuronidation formation and plays an important role in nicotine metabolism and elimination. [Cancer Res 2007;67(19):9024–9]

Introduction

Tobacco smoking causes 500,000 deaths annually in the United States, and nicotine is the single most important pharmacologic agent responsible for tobacco addiction. The most abundant nicotine metabolite is cotinine, which is further metabolized to 3′-hydroxycotinine and other compounds. Nicotine, cotinine, and 3′-hydroxycotinine undergo further phase II detoxification reactions by conjugation with glucuronic acid via catalysis by the UDP-glucuronosyltransferase (UGT) family of enzymes. Up to 31% of nicotine urinary metabolites are in the form of phase II glucuronidated compounds, with nicotine-glucuronide, cotinine-glucuronide, and *trans*-3′-hydroxycotinine glucuronide comprising the majority of these conjugates (1). Both cotinine and nicotine are glucuronidated on the nitrogen of the pyridine ring, and *N*-glucuronidation of both compounds is observed in human liver microsomes (HLM) and in the urine of smokers (2–5). Whereas *N*-glucuronidation of 3′-hydroxycotinine was observed in HLM, only its *O*-glucuronide was detected in the urine of smokers (6).

There is a high correlation between the *in vivo* urinary ratio of nicotine-glucuronide/(unconjugated + nicotine-glucuronide) to the ratio of cotinine-glucuronide/(unconjugated + cotinine-glucuronide) in smokers (7). The *in vivo* urinary nicotine-glucuronide ratio is only moderately correlated with 3′-hydroxycotinine-glucuronide, suggesting that different UGT enzymes are responsible for the glucuronidation of nicotine and cotinine versus 3′-hydroxycotinine.

Previous studies conducted to identify the UGT isoforms that glucuronidate nicotine and cotinine suggested that UGT1A4 was the primary enzyme responsible for the glucuronidation of these compounds (8). Whereas other studies of overexpressed UGT1A4 showed no nicotine and cotinine glucuronidation activity (9, 10), imipramine, a UGT1A4 substrate, was shown to inhibit the glucuronidation of nicotine and cotinine in HLM (10).

UGT2B10 was shown recently to exhibit high *N*-glucuronidation activity against several tobacco-specific nitrosamines (TSNA), including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol, *N*′-nitrosonornicotine, *N*′-nitrosoanabasine, and *N*′-nitrosoanatabine.[4] As nicotine and cotinine are structurally related to TSNAs, we hypothesized that UGT2B10 also plays an important role in nicotine and cotinine glucuronidation. The goal of the present study was to examine the activity of UGT2B10 against these agents and study the potential effects of the recently identified functional *UGT2B10* codon 67 polymorphism on nicotine/cotinine glucuronidation activities in UGT2B10-overexpressing cell lines and HLM as a measure of glucuronidation phenotype.

Materials and Methods

[^{14}C]UDPGA (200 mCi/mmol) was purchased from American Radiolabeled Chemicals. The high-performance liquid chromatography (HPLC) scintillation solution, Ecoscint Flow, was purchased from National Diagnostics. Nicotine, cotinine, alamethicin, β-glucuronidase, anti-calnexin antibody, and bovine serum albumin were from Sigma-Aldrich. DMEM, Dulbecco's PBS (minus calcium-chloride and magnesium-chloride), fetal bovine serum, penicillin-streptomycin, and geneticin (G418) were all purchased from Invitrogen. The bicinchoninic acid (BCA) protein assay

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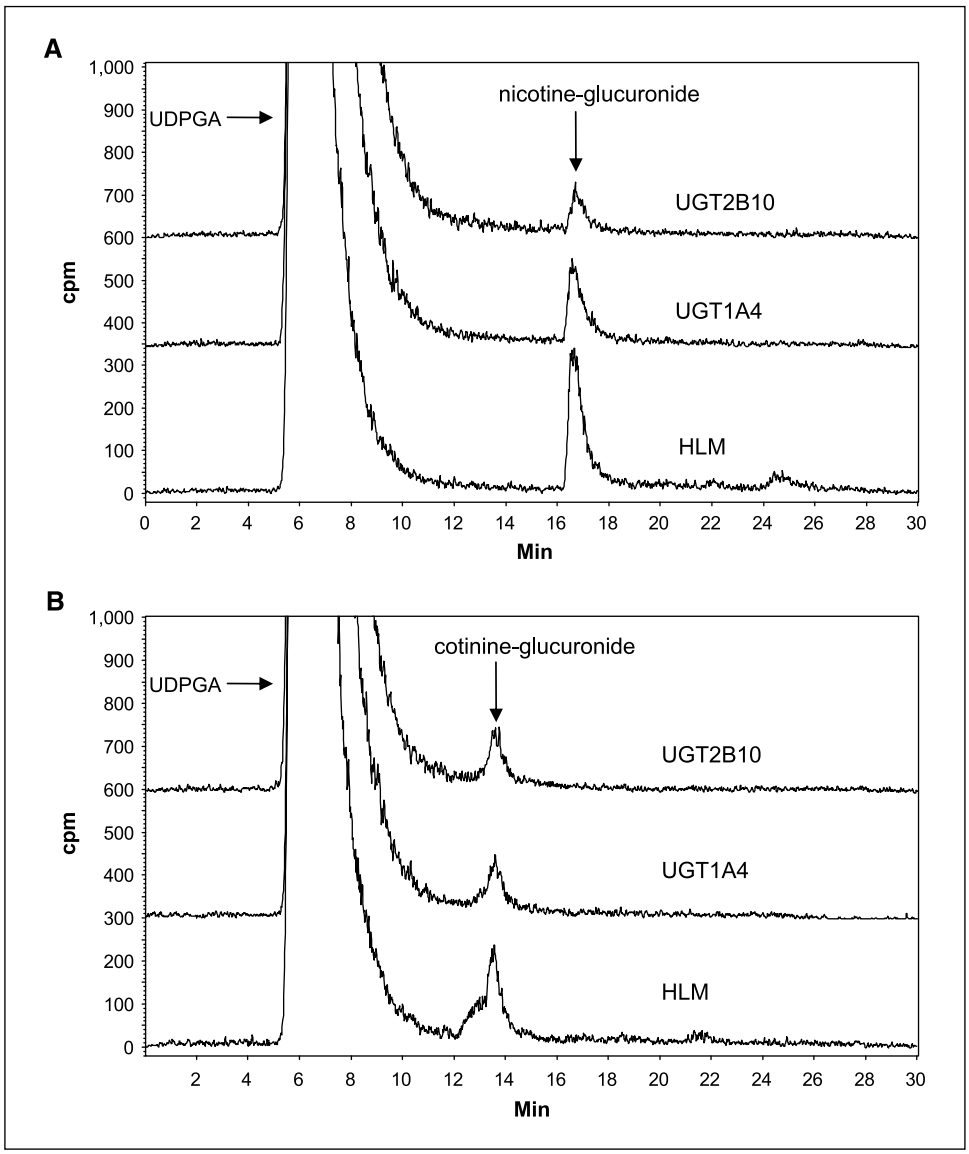

Figure 1. HPLC analysis of nicotine- and cotinine-glucuronide formation by microsomes of UGT2B10- and UGT1A4-overexpressing cells and HLM. Incubations were done using 40 μg of total microsomal protein for UGTs 1A4- and 2B10-overexpressing cells or 5 μg of total microsomal protein for HLM at 37°C for 2 h with 4 mmol/L [^{14}C]UDPGA and 5 mmol/L substrate. HPLC was done as described in Materials and Methods. *A,* nicotine; *B,* cotinine.

kit was purchased from Pierce. All other chemicals were purchased from Fisher Scientific.

Normal human liver tissue specimens and matching genomic DNA samples from 78 samples were described previously (11). The number of specimens used in the current study was expanded to 112 and obtained in the same manner as the original 78 specimens. All 112 subjects were Caucasians; gender was known for 95 of the 112 subjects, and 41% of these individuals were female. The liver microsomes were prepared using differential centrifugation as described previously (12) and stored (10–20 mg protein/mL) at −80°C. Microsomal protein concentrations were measured using the BCA assay. The cells used for overexpressing UGTs 1A4 and 2B10 have also been described previously (13).[4] Cell microsomes were prepared essentially as described above for liver microsomes by resuspending pelleted cells in TBS [25 mmol/L Tris-HCl (pH 7.4), 138 mmol/L NaCl, 2.7 mmol/L KCl] and subjecting them to three rounds of freeze-thaw before gentle homogenization and differential centrifugation.

Glucuronidation activities of HLM or microsomes from UGT-overexpressing cell lines were determined as described previously[4] after an initial incubation of HLM (2–5 μg protein) or UGT-overexpressing cell microsomes (40 μg protein) with alamethicin (50 μg/mg protein) for 15 min in an ice bath. Incubations (4 μL) were then done at 37°C for 2 h, a time that was found in previous studies to be within the linear range of product formation for the UGTs tested in this study. Reactions were terminated by the addition of equal volume of cold acetonitrile. After centrifugation at 13,000 × *g* for 10 min, the supernatants were diluted to 50 μL with water.

Samples (50 μL) were analyzed for glucuronide formation by HPLC using a Beckman Coulter System Gold 126 Solvent Module HPLC system equipped with an automatic injector (model 508), a UV detector operated at 254 nm (model 166), and a radioactive flow detector with 1,000 μL flow cell (INUS Systems). HPLC was done using a Synergi Fusion-RP-80 4 μm column (4.6 × 250 mm; Phenomenx) and an Aquasil 5 μm C18 analytic column (4.6 × 250 mm; Thermo) in series. The gradient elution conditions were as follows: starting with 100% buffer A [100 mmol/L NH_4Ac (pH 5.0)] for 5 min for nicotine glucuronide formation or for 2 min for cotinine glucuronide formation, a subsequent linear gradient to 78% buffer B (90% acetonitrile, 10% water) over 10 min was done and then maintained at 78% buffer B for 10 min. The elution flow rate was 1 mL/min and the scintillation solution flow rate was 1.5 mL/min. The amount of *N*-glucuronide formed was calculated based on the ratio of the radioactivity of the *N*-glucuronide versus total radioactivity. Nicotine- and cotinine-*N*-glucuronides were confirmed by sensitivity to β-glucuronidase as described previously (14). As controls, glucuronidation assays were regularly done using HLM and untransfected HEK293 microsomes as positive and negative controls, respectively, for glucuronidation activity. Cell line experiments

Table 1. Kinetic analysis of UGT1A4-, UGT2B10-, and HLM-induced glucuronidation of nicotine and cotinine

	Nicotine		Cotinine	
	K_M (mmol/L)	V_{max} (pmol·min^{-1}·mg protein^{-1})	K_M (mmol/L)	V_{max} (pmol·min^{-1}·mg protein^{-1})
UGT1A4	17.5 ± 2.6	133 ± 7	10.3 ± 1.3	71.8 ± 2.5
UGT2B10	0.47 ± 0.26	11.5 ± 2.2	3.5 ± 0.19	25 ± 1.1
HLM	0.93 ± 0.2	643 ± 31	5.5 ± 1.1	1,144 ± 85

NOTE: Data are expressed as mean ± SD for three independent experiments.

were always done in triplicate in independent assays; HLM glucuronidation assays were repeated for 20% of all samples to monitor experimental variation.

Putative UGT2B10-catalyzed nicotine- and cotinine-glucuronide peaks were collected by HPLC essentially as described above, with collected fractions dried and resuspended in methanol. An Applied Biosystems 4000 Q Trap LC/MS/MS mass spectrometer was used to characterize individual glucuronides as described previously (8).

RFLP analysis was done using the *Hin*fI restriction enzyme as described previously to detect the *UGT2B10* codon 67 polymorphism.[4] Genotyping was done for subjects from whom liver specimens were obtained and for a subset (n = 784) of healthy subjects serving as controls recruited as part of a case-control study conducted at the H. Lee Moffitt Cancer Center (Tampa, FL) from 2000 to 2003 (15).

Kinetic constants were determined using Prism version 4.01 software (GraphPad Software). The rate of nicotine- and cotinine-glucuronide formation in HLM was compared by gender and *UGT2B10* codon 67 genotype [homozygotes, heterozygotes, and wild-type (WT)] by trend test and Student's t test using SPSS statistical software (version 15.0, SPSS, Inc.).

Results

To determine whether UGT2B10 exhibited activity against nicotine and cotinine similar to that for UGT2B10 against TSNAs,[4] glucuronidation assays using microsomes prepared from a WT UGT2B10-overexpressing cell line were done. UGT2B10 exhibited significant *N*-glucuronidation activity against both nicotine and cotinine (Fig. 1). A putative glucuronide peak was observed by HPLC for assays using UGT2B10-overexpressing microsomes at retention times of 16.6 and 13.5 min for nicotine (Fig. 1*A*) and cotinine (Fig. 1*B*), respectively, times that were identical to that observed for UGT1A4 and HLMs. Both peaks were sensitive to treatment with β-glucuronidase (data not shown), indicating the presence of a glucuronide conjugate. Whereas previous studies have indicated that glucuronidation activity for UGT1A4 against both nicotine and cotinine was only observed in incubations done at pH 8.9 (8), glucuronidation activity was observed for microsomes from both UGT2B10- and UGT1A4-overexpressing cells when assays were done at either pH 7.4 or pH 8.9 in the present study (data not shown). The putative UGT2B10-catalyzed nicotine- and cotinine-glucuronide peaks were analyzed by tandem mass spectrometry and were shown to fragment to a mass of 163 and 177, respectively (data not shown), which is consistent with that observed in previous studies (8).

Kinetic analysis was done for UGT2B10- and UGT1A4-overexpressing cell lines. Microsomes from UGT2B10-overexpressing cells were significantly more active as determined by *in vitro* K_M assessment than microsomes from cells overexpressing UGT1A4

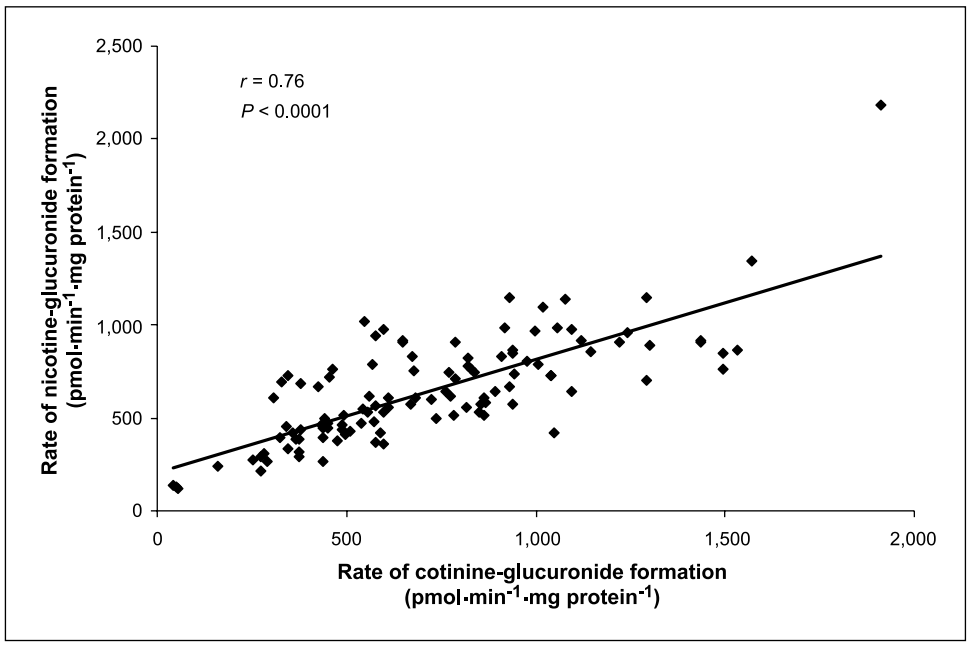

Figure 2. Correlation between rate of nicotine- and cotinine-glucuronide formation in HLM. Glucuronidation assays were done as described in the legend of Fig. 1 for 112 HLM specimens as described in Materials and Methods.

against both nicotine and cotinine (Table 1). The apparent K_M values for nicotine and cotinine were 37- and 3-fold lower, respectively, for UGT2B10 than for UGT1A4. The higher affinity exhibited by UGT2B10 was observed whether assays were done at pH 7.5 or pH 8.9, with >200-fold lower K_M observed for both UGTs 2B10 and 1A4 against nicotine in glucuronidation assays done at pH 8.9 (data not shown). The apparent K_M's for UG2B10 against both nicotine and cotinine approached that observed for HLMs (Table 1).

Previous studies showed that the glucuronidation of nicotine was inhibited by imipramine in HLM (10). To determine whether imipramine is a substrate and potential competitive inhibitor of UGT2B10, glucuronidation assays similar to those described above for nicotine and cotinine were done with imipramine as substrate. Imipramine was shown to be a substrate for UGT2B10 in the present study, with an apparent K_M of 49 μmol/L. This compared with a K_M of 763 μmol/L for UGT1A4 (data not shown).

Recent haplotyping studies have indicated the presence of a single missense polymorphism at codon 67 of the *UGT2B10* gene that is highly correlated with UGT2B10 glucuronidation activity against TSNAs.[4] In an analysis of 784 healthy Caucasian subjects, the prevalence of the *UGT2B10*2* variant allele was 9.1%. The prevalence of the *UGT2B10* codon 67 polymorphism in this population was consistent with that expected by Hardy-Weinberg equilibrium (P = 0.266).

Similar to that observed for TSNAs, the glucuronidation of nicotine by the UGT2B10^{67Tyr} variant was barely detectable *in vitro*; no detectable glucuronidation activity was observed for the UGT2B10^{67Tyr} variant against cotinine. This contrasts with the relatively high level of activity observed for WT UGT2B10^{67ASP} against both compounds (Table 1). Due to its overall low activity, a K_M could not be ascertained for the UGT2B10^{67Tyr} variant against either substrate.

To determine whether an association was observed for the UGT2B10 codon 67 polymorphism and the glucuronidation of nicotine and cotinine in HLM, the formation of nicotine- and cotinine-glucuronide was examined in a series (n = 112) of microsomal specimens prepared from normal human liver tissue from individual subjects. The rate of nicotine-glucuronide formation was strongly correlated (r = 0.76) with cotinine-glucuronide formation in this series of HLM (Fig. 2). No significant difference in levels of nicotine- or cotinine-glucuronide formation was observed in HLM after stratification of the entire population by gender. As shown in Fig. 3, there was a significant (P < 0.01) trend toward decreased glucuronidation activity against both nicotine and cotinine in HLM from subjects with an increasing number of the variant *UGT2B10* allele (termed the *UGT2B10*2* allele). Eighty-two percent (n = 92) of the subjects for whom HLMs were analyzed were homozygous WT *UGT2B10 (*1/*1)*. There was a significant

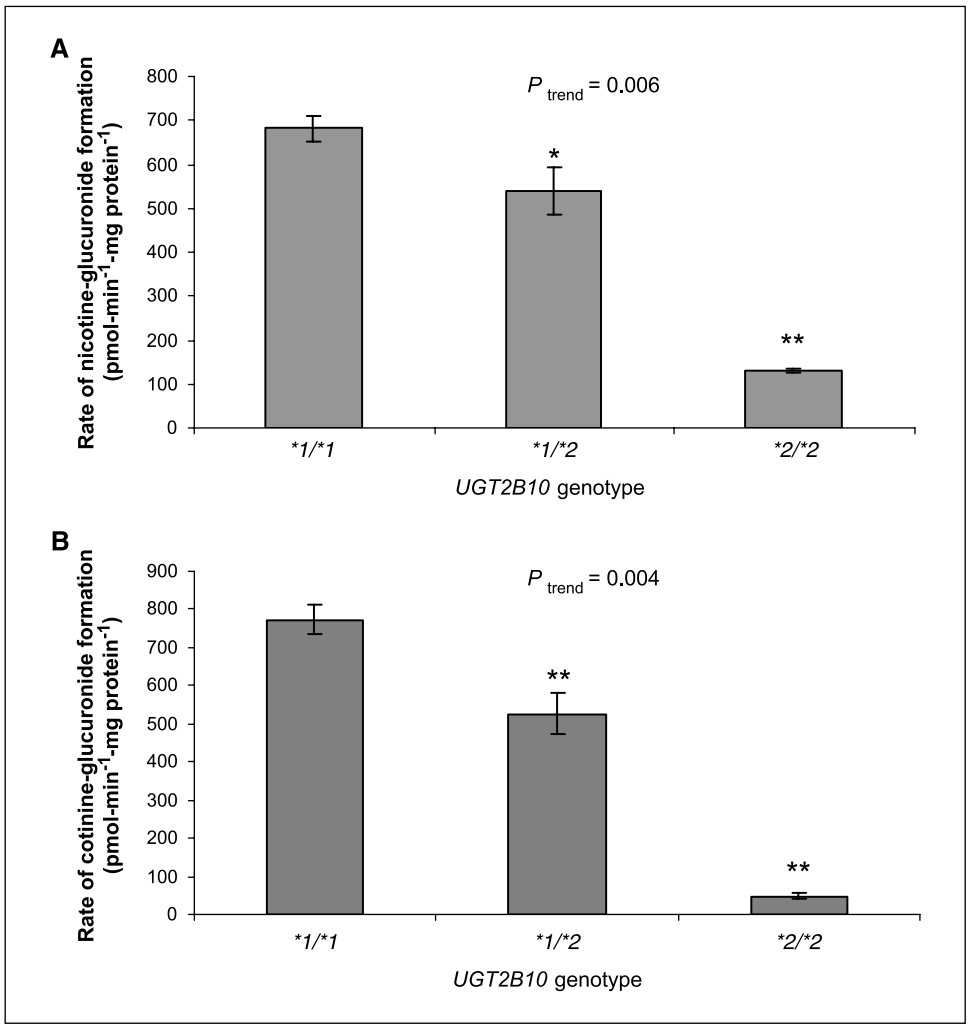

Figure 3. Nicotine-*N*-glucuronide (*A*) and cotinine-*N*-glucuronide (*B*) formation in HLM from subjects stratified by copy number of *UGT2B10*2*. The number of subjects within each genotype group of 0, 1, and 2 copies of the *UGT2B10*2* allele are 92, 18, and 2, respectively. *Columns,* mean of glucuronide formation; *bars,* SE. *, P < 0.05; **, P < 0.01, a significant decrease in rate of nicotine- or cotinine-glucuronide formation was observed when compared with the *UGT2B10 (*1/*1)* genotype group.

($P < 0.05$) 21% decrease in nicotine glucuronidation activity in HLM from subjects with the heterozygous *UGT2B10 (*1/*2)* genotype ($n = 18$) and a significant ($P < 0.001$) 5-fold decrease in activity in HLM from subjects homozygous for the *UGT2B10*2* allele ($n = 2$), compared with subjects with the homozygous WT *UGT2B10 (*1/*1)* genotype. Similarly, a significant ($P < 0.001$) 30% decrease in cotinine glucuronidation activity was observed in HLM from subjects with the heterozygous *UGT2B10 (*1/*2)* genotype and a significant ($P < 0.001$) 16-fold decrease in activity in HLM from subjects with the homozygous *UGT2B10 (*2/*2)* genotype, compared with subjects with the homozygous WT *UGT2B10 (*1/*1)* genotype. Of the 95 subjects for whom gender was known, 44%, 33%, and 0% of the subjects exhibiting the *UGT2B10 (*1/*1)*, *UGT2B10 (*1/*2)*, and *UGT2B10 (*2/*2)* genotypes, respectively, were female. There was no association between levels of nicotine- or cotinine-glucuronide formation and *UGT2B10* genotype after stratification by gender.

Discussion

This is the first report examining the role of *UGT2B10* in nicotine metabolism. Whereas both UGTs 1A4 and 2B10 exhibited *N*-glucuronidation activity in the present study, UGT2B10 exhibited significantly greater activity against both nicotine and cotinine than UGT1A4. Like UGT1A4, UGT2B10 is a hepatic enzyme (16). The fact that the K_M of liver microsomes for nicotine and cotinine approached the K_M's observed for UGT2B10 against these two compounds *in vitro* suggests that UGT2B10 is the dominant enzyme involved in the glucuronidation of these compounds in liver. This is supported by the fact that the *UGT2B10* codon 67 polymorphism effectively reduces overall liver glucuronidation of both nicotine and cotinine to 5- and 16-fold, respectively, in subjects homozygous for the $UGT2B10^{67Tyr}$ variant. In addition, the high correlation between nicotine- and cotinine-glucuronide formation observed in HLM in this study indicates that the two substrates are glucuronidated by the same enzymes, primarily UGT2B10. The fact that imipramine was a good substrate for UGT2B10 is consistent with the fact that imipramine was able to block nicotine and cotinine glucuronidation in HLM in previous studies (10). Although *O*-glucuronidation of 3′-hydroxycotinine is important in the overall nicotine metabolism pathway, its *N*-glucuronide has not been detected in smokers' urine (3) and has therefore not been screened in the present study.

A previous report showed that UGT1A4-overexpressing baculosomes are active against both nicotine and cotinine, with activity observed only at pH 8.9; no activity was observed at physiologic pH (8). *N*-glucuronidation activity was observed against both compounds when assays were done at pH 7.4 as well as pH 8.9 in the present study. This contrast is likely due to the different source of overexpressed UGT1A4 in the two studies (baculosomes versus overexpressing mammalian cells). Baculosomal UGTs may lack the necessary post-translational modifications necessary for optimal activity for all UGTs (17), and this may be the case for UGT1A4 against these substrates. The fact that UGT1A4 was considered inactive in a second study (10) is likely due to methodologic issues including assay sensitivity.

The prevalence of the *UGT2B10*2* variant allele is relatively high in Caucasians (9.1%). Therefore, this polymorphism may have an important overall effect on nicotine metabolism and potentially nicotine addiction. In the urine of smokers, up to 5% of absorbed nicotine is metabolized to form nicotine-glucuronide and up to 10% remains as unmetabolized nicotine (1, 18). Therefore, an increase in overall unconjugated nicotine could occur due to the resulting near-inactivation of the UGT2B10 enzyme in people homozygous for the $UGT2B10^{67Tyr}$ variant. In addition, cotinine-glucuronide comprises up to 17% of the total urinary nicotine metabolites in smokers. Therefore, subjects with the $UGT2B10^{67Tyr}$ variant would also have significantly higher levels of cotinine. This would also likely result in higher absolute levels of nicotine in subjects with one or more low-activity *UGT2B10* alleles assuming the rate of metabolism of nicotine to cotinine and the rate of metabolism of cotinine to *trans*-3′-OH-cotinine remains constant.

The effects of this polymorphism could be most pronounced, however, in subjects with a deleterious *CYP2A6* polymorphism because this is the major hepatic enzyme involved in metabolism of nicotine to its nonaddictive metabolite, cotinine. It has been shown that ~20% of Caucasians and African Americans as well as 70% of Asians exhibit a poor CYP2A6 metabolizing enzyme phenotype (activity decreased by at least 25%; ref. 19). For example, in smokers homozygous for the *CYP2A6*4* deletion allele and who therefore have no active CYP2A6 enzyme, the levels of urinary nicotine-glucuronide and unconjugated nicotine were shown to be as high as 45% and 55%, respectively, of total absorbed nicotine (20). In subjects with a poor CYP2A6 metabolizing enzyme phenotype, the levels of nicotine would likely increase to even higher internal levels if that individual also had one or more *UGT2B10*2* variant alleles. Therefore, the *UGT2B10* codon 67 polymorphism could be an important modifier of nicotine metabolism and addiction. Further studies examining urinary nicotine metabolites in smokers of different combined *CYP2A6* and *UGT2B10* genotypes will be required to better assess these possibilities.

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