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The Contribution of Common *UGT2B10* and *CYP2A6* Alleles to Variation in Nicotine Glucuronidation among European Americans

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Abstract

UDP-glucuronosyltransferase-2B10 (*UGT2B10*) is the primary catalyst of nicotine glucuronidation. To develop a predictive genetic model of nicotine metabolism, the conversion of deuterated (D_2)-nicotine to D_2 -nicotine-glucuronide, D_2 -cotinine, D_2 -cotinine-glucuronide, and D_2 -*trans*-3'-hydroxycotinine were quantified in 188 European Americans, and the contribution of *UGT2B10* genotype to variability in first-pass nicotine glucuronidation assessed, following a procedure previously applied to nicotine C-oxidation. The proportion of total nicotine converted to nicotine-glucuronide (D_2 -nicotine-glucuronide/ (D_2 -nicotine + D_2 -nicotine-glucuronide + D_2 -cotinine + D_2 -cotinine-glucuronide + D_2 -*trans*-3'-hydroxycotinine)) was the primary phenotype. The variant, rs61750900T (D67Y) (minor allele frequency (MAF) = 10%), is confirmed to abolish nicotine glucuronidation activity. Another variant, rs112561475G (N397D) (MAF = 2%), is significantly associated with enhanced glucuronidation. rs112561475G is the ancestral allele of a well-conserved amino acid, indicating that the majority of human *UGT2B10* alleles are derived hypomorphic alleles. *CYP2A6* and *UGT2B10* genotype explain 53% of the variance in oral nicotine glucuronidation in this sample. *CYP2A6* and *UGT2B10* genetic variants are also significantly associated with un-deuterated (D_0) nicotine glucuronidation in subjects smoking *ad libitum*. We find no evidence for further common variation markedly influencing hepatic *UGT2B10* expression in European Americans.

Keywords

UGT2B10; nicotine; cotinine; metabolism; glucuronidation; *CYP2A6*

Introduction

Despite reduced prevalence, tobacco use remains the largest cause of preventable mortality in the US [1]. Smoking phenotypes are highly complex, but they are also strongly influenced by heritable factors [2-4]; therefore genetic studies provide a powerful tool to reveal the underlying biology of nicotine dependence and smoking cessation, which may lead to improved treatments. Genetic associations with DNA variants in the chromosomal region

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19q13, surrounding the nicotine metabolism gene *Cytochrome P450 2A6 (CYP2A6)*, exemplify both the difficulties and opportunities in identifying further genes and pathways related to nicotine dependence and cessation. 19q13 is among the loci most consistently associated with smoking phenotypes, identified in multiple large genome-wide association studies (GWAS) of cigarette consumption, and related pulmonary disease [5-7]. The region also demonstrated the strongest association in a recent candidate gene study of smoking cessation[8].

In most smokers, the primary pathway of nicotine metabolism is CYP2A6-catalyzed 5'-oxidation [9] (Fig. 1). The iminium ion product of this reaction is further oxidized by either CYP2A6 or aldehyde oxidase to cotinine [10], and cotinine is then further metabolized by CYP2A6 to *trans*-3'-hydroxycotinine [11]. There are two additional pathways of nicotine metabolism; flavin monooxygenase (FMO)- catalyzed *N*-oxidation and the UDP-glucuronosyltransferase (UGT)-catalyzed *N*-glucuronidation (Fig. 1). UGT2B10 is an efficient catalyst of the *N*-glucuronidation of both nicotine and cotinine [12]. Nicotine glucuronide typically accounts for <10% of a total nicotine dose excreted, but in some smokers the nicotine-glucuronide may account for up to 40% of the excreted dose [13]. Glucuronide metabolites of nicotine and cotinine together account for on average 25% of the nicotine metabolites excreted in urine, ranging between 0.8 and 84%[13, 14]. An O-glucuronide conjugate of *trans* 3'-hydroxycotinine is excreted, but on average only 20% of the total *trans* 3'-hydroxycotinine is a glucuronide conjugate [9]. The primary metabolite of nicotine in plasma is cotinine, which is typically present at ten times the concentration of nicotine and 3-4x the concentration of 3'-hydroxycotinine [9]. Plasma levels of nicotine *N*-glucuronide have not previously been quantified. We have previously quantified cotinine-*N*-glucuronide indirectly and reported the mean level to be about 10% of the total cotinine [15].

Because the psychoactive properties of nicotine closely correlate with its plasma concentration, dependent smokers titrate nicotine intake to maintain target nicotine blood levels [16-18]. Therefore, heritable differences in nicotine half-life are likely to explain some of the observed differences in cigarettes per day (CPD) and related behaviors between fast and slow nicotine metabolizers and individuals who differ in *CYP2A6* genotype [14, 19-26]. The other pathways of nicotine metabolism may also contribute to smoking behaviors and we have recently found evidence that genetic variation in *FMO3*, the gene encoding the primary catalyst of nicotine-*N*-oxidation, is associated with differences in cigarette consumption[27]. Genetic variation in glucuronidation has been suggested to influence individual variability in total nicotine metabolism [28] and ethnic differences in glucuronidation and in *UGT2B10* genotype [29] are reported to be associated with differences in cigarette consumption[15]; but with the exception of a non-synonymous variant, rs61750900 (D67Y) [12, 15], common variants in *UGT2B10* have not been comprehensively tested. Here we report the results of a controlled nicotine metabolism study performed in 188 European Americans. Nicotine and its metabolites were analyzed in the plasma of smokers and non-smokers orally administered deuterated nicotine to evaluate the effects of *UGT2B10* genotype upon nicotine and cotinine glucuronidation. Our prior investigation that explained ~70% of the variation in first-pass nicotine C-oxidation with *CYP2A6* haplotype[30] demonstrated this as a robust method to determine the relative activities of different alleles of hepatic enzymes regarding metabolism of specific substrates. The primary aim of this study is to detail the association between *UGT2B10* genotype and nicotine glucuronidation by controlling for the indirect effects of *CYP2A6* genotype.

Materials and methods

Study Subjects

Participants were recruited from the Collaborative Genetic Study of Nicotine Dependence (COGEND)[31], for an oral nicotine metabolism experiment as previously described[30]. All were self-identified as being of European American ancestry, and race was verified using EIGENSTRAT[32]. Sample demographics were previously described[31, 33]. Subjects were given 2 mg of [3',3'-D₂] nicotine (synthesized and purified to >99.4% as described previously[30]) in 4 oz of orange juice. Plasma used in these experiments was collected 240 minutes later and frozen at -20°C until analysis. Subjects who were current smokers were allowed to smoke 2h following the administration of [3',3'-D₂] nicotine. The study complies with the Code of Ethics of the World Medical Association and obtained informed consent from participants and approval from the appropriate institutional review boards.

Nicotine Metabolite Measurements

D₂-nicotine, D₂-cotinine, D₂-*trans*-3'-hydroxycotinine, non-deuterated (D₀)-nicotine, D₀-cotinine, and D₀-*trans*-3'-hydroxycotinine were analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS), as previously described [30, 34]. Plasma concentrations of all but D₀-nicotine have been reported previously [30].

For the quantitation of the nicotine and cotinine *N*-glucuronides, 50 µl plasma was mixed with 450 µl of 1.5 % formic acid in water. Internal standards, nicotine-D₃ *N*-β-D-glucuronide, 2.85 pmol (Toronto Research Chemicals) and cotinine-D₃ *N*-β-D-glucuronide, 1.34 pmol, (Toronto Research Chemicals) were added to each sample. The samples were vortexed and loaded on an Oasis MCX column (10 mg Waters Corporation, Milford, MA) that had been activated by washing with 1 ml methanol then 1ml of 1.5 % formic acid. The columns were washed with 0.5 ml 1.5 % formic acid in water then the glucuronides were eluted with 0.5 ml of 5% ammonium hydroxide in methanol. The eluant was immediately acidified with 5 µl formic acid. Samples were evaporated to dryness under a gentle stream of nitrogen, resuspended in 40 µl of 0.1% TFA in methanol and stored at -20°C until analysis. The LC/MS/MS analysis was similar to that used previously for the quantitation of cotinine [30] except the mobile phase was 80.7% acetonitrile, 18% water, 1.25% formic acid and 0.01% TFA. The mass transitions monitored were as follows: D₀-Nicotine glucuronide (*m/z* 339→163), D₂-nicotine glucuronide (*m/z* 341→165), D₃-nicotine glucuronide (*m/z* 342→166), D₀-cotinine glucuronide (*m/z* 353→177), D₂-cotinine glucuronide (*m/z* 355→179), D₃-cotinine glucuronide (*m/z* 356→180).

The concentrations of the D₀ and D₂ metabolites were calculated from the ratio of the peak area for each metabolite mass-transition to the peak area of its respective D₃-internal standard. The presence of isotopomers of non-deuterated glucuronides with the same nominal mass as the D₂-glucuronides result in a contribution to the D₂-glucuronide SRM signal from non-deuterated glucuronides present in the sample. Using standards, the percent contribution was determined empirically to be 0.54% for nicotine glucuronide and 0.9% for cotinine-glucuronide. These percentages were used to correct for the contribution of D₀-nicotine glucuronide and D₀-cotinine glucuronide to the mass transitions monitored for the analysis of D₂ nicotine glucuronide and D₂-cotinine glucuronide.

The relative recovery for nicotine glucuronide, based on recovery of the internal standard and a standard curve, was 60% to 90%. However, the recovery of cotinine glucuronide was highly variably, and ranged from 0-90%. The mean relative recovery compared to nicotine glucuronide was 42% ± 31% (n=430). For 61 samples the recovery was too low to obtain an

accurate value for the cotinine glucuronides. The limit of quantitation was 0.5 pmol/ml for nicotine glucuronide and 1 pmol/ml for cotinine glucuronide (when recovery was >20%). All samples were analyzed in duplicate in sets of 20 with a positive control. Samples were excluded if the recovery of the internal standard was < 20% or if duplicates did not agree within 10%. The positive control was a smoker's plasma from a previous study in which nicotine and cotinine glucuronide concentrations were determined indirectly as the difference in concentration before and after treatment of the sample with base, which hydrolyzes the glucuronide conjugates releasing the aglycon [15]. The concentrations of nicotine glucuronide and of cotinine glucuronide in the positive control samples were 23.9 ± 2.2 and 91.5 ± 17.1 pmol/ml respectively (n=12).

Genotyping

CYP2A6 genotyping was previously described [30]. All variants with minor allele frequency >0.5% in the exons of *UGT2B10* were genotyped. rs61750900 was previously determined using a custom designed array as part of a larger study[31]. rs61749966, rs112561475, and rs4694358 were genotyped using the KBioscience Competitive Allele Specific PCR genotyping system (KASPar, KBioscience, Hoddesdon, Herts, UK) following standard procedures with custom designed primers (Supplemental Table 1). KASPar assays were set up as 8µl reactions and measured with the 7900HT Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA). rs17146905 was genotyped and rs4694358 genotype was confirmed by Sanger Sequencing using PCR primers that flank the *UGT2B10* 3' UTR, (GATGTGATTGGGTTCTGCT and TTGACAAGGTAGACTTCGAAAGG). All SNP genotypes conformed to Hardy-Weinberg equilibrium. *UGT2B10* copy number was not determined, as copy variation in this gene is reported to be very rare in Europeans (undetected in 180 subjects, i.e. <0.3% frequency[35]). *UGT2B10* haplotypes were determined using PHASE version 2.1.1[19, 20].

Statistical Analysis

All statistical analyses (t-tests, linear regression) were performed using the software package 'R' (R Foundation for Statistical Computing, Vienna, Austria). Metabolite phenotypes are calculated using measured metabolites in terms of picomoles/ml plasma. For analysis, valid measurements below the limits of detection were assumed to be zero; three of eight subjects with nicotine-glucuronide measurements below the limits of detection were *UGT2B10**2 homozygotes. For regression analyses, genetic variables, such as *CYP2A6**1A or *UGT2B10**2, are treated as number of alleles per subject (0,1 or 2). All *CYP2A6* haplotypes are placed into four categories based on statistically significant differences between them regarding metabolism of nicotine to cotinine [30] i.e. 1) the null alleles *CYP2A6**2, *4, *12 and *38, 2) *CYP2A6**1A, 3) *CYP2A6**9 and 4) all other haplotypes, termed normal alleles. All regression analyses are performed on genetic variables relative to the null *CYP2A6* category. To display and compare the relative nicotine-glucuronidation among groups of subjects after accounting for *CYP2A6* genotype, the residual variance for each subject was determined from the linear model: phenotype ~ (*CYP2A6* Normal alleles) + (*CYP2A6**1A alleles) + (*CYP2A6**9 alleles). The residuals are then displayed for each *UGT2B10* diplotype, such as *UGT2B10**1/*1 or *UGT2B10**1/*2. Direct comparisons between *UGT2B10* diplotype groups were also performed on the residuals for each subject. All t-tests performed were two-sided. Measures of statistical significance are not altered to correct for multiple testing.

Allelic expression study

DNA and RNA extracted from de-identified non-cancerous liver biopsy samples were previously described[36]. cDNAs and genomic DNAs for heterozygotes for the assayed

SNPs were arrayed together in the same 384-well plate in triplicate, and were run on an ABI-7900 real-time PCR system under standard conditions with custom designed assays for rs4694358 (forward primer: GAAGCTGGAAAACCTGATAGATAGGAA; reverse primer: TGTACAGGAAGAAAGAAATCTTGCA; reporter 1: TTGCTGGAATCAGCTGAAGT; reporter 2: TGCTGGAATCAACTGAAGT), and rs61750900 (forward primer: TGGCATCTTCAGCTTCCATTCTTTT; reverse primer: TCAGTTTTAGTTAAAGATGTAGGATAAACTTCAAGTT; reporter 1: ATCCCAACGACTCATC; reporter 2: TGATCCCAACTACTCATC). The relative expression of both alleles for each expression marker was determined by subtracting the smaller cycle threshold (Ct, the number of cycles at which PCR products generated exceed a defined threshold) value of one allele PCR reaction from the larger Ct value of the other allele PCR reaction (dCt). dCt values were obtained as an average of two or three reactions for each cDNA sample and were normalized against the overall average ratio obtained for heterozygous gDNAs for each assay.

Results

UGT2B10 polymorphisms and haplotypes

Subjects (n=188) were analyzed for the five exonic polymorphisms with MAF >0.05% in Europeans [37]. A summary of haplotypes defined by these polymorphisms is presented in Table 1.

Nicotine metabolite measurements

A method was developed to directly quantify nicotine-*N*-glucuronide and cotinine-*N*-glucuronide in plasma. Due to relatively poor recovery of cotinine-*N*-glucuronide, accurate quantitation of this metabolite was possible in only 126 of the genotyped samples. Plasma concentrations of nicotine, cotinine and their glucuronide conjugates are summarized in Table 2. D₂-Nicotine glucuronide was present in plasma at a concentration similar to that of D₂-nicotine, whereas D₂-cotinine glucuronide on average was present at <4% of the D₂-cotinine concentration. In smokers, the mean plasma concentration of D₀-nicotine glucuronide was <20% (Table 1) of the D₀-nicotine concentration. The mean plasma D₀-cotinine glucuronide concentration in smokers was <5% of the cotinine concentration. The difference in D₀-cotinine between males and females was not significant after controlling for weight; but the higher levels of cotinine-glucuronide among smokers was significant (p=0.004). Smokers and current non-smokers in this study do not significantly differ in weight (p=0.5)[30].

The indirect effect of *CYP2A6* genotype upon nicotine glucuronidation

The administration of deuterated nicotine allows for direct measurement of the conversion of nicotine to its metabolites. Although *CYP2A6* is not directly involved in glucuronidation, the large majority of nicotine is metabolized to cotinine by *CYP2A6* in most smokers, and the activity of parallel metabolism pathways can have indirect effects on substrate: metabolite ratios. Therefore we began by testing the relationship between *CYP2A6* genotype and *in vivo* nicotine glucuronidation.

The primary phenotype analyzed here is the fraction of D₂-nicotine equivalents converted to D₂ nicotine-glucuronide, i.e. D₂-nicotine-glucuronide/ (D₂-nicotine + D₂-nicotine-glucuronide + D₂-cotinine + D₂-cotinine-glucuronide + D₂-*trans*-3'-hydroxycotinine) (Figure 2). All results refer to this ratio unless otherwise specified. Additionally, because D₂-cotinine-glucuronide measurements were only available for 126 genotyped subjects, to increase power, D₂-nicotine-glucuronide/ (D₂-nicotine + D₂-nicotine-glucuronide + D₂-cotinine + D₂-*trans*-3'-hydroxycotinine) is also analyzed (Figure 3).

Our previous analysis of *CYP2A6* haplotype and nicotine C-oxidation justified dividing all *CYP2A6* alleles in European Americans into four categories with statistically significantly different activities: 1) null alleles *CYP2A6**2, *4, *12, and *38, 2) *CYP2A6**1A associated with reduced mRNA splicing efficiency [36], 3) *CYP2A6**9 associated with lower expression due to variation in the 5' TATA box [38], and 4) all other fully-active alleles (*1B, *1D, *1H, *14, *1X2). The *CYP2A6* null and fully-active allele categories are both highly significantly associated with the nicotine-glucuronide ratio in separate single variable analyses (Table 3). In a combined multivariate model, relative to null *CYP2A6* alleles, the other three allele categories are all highly-significantly associated with reduced glucuronidation (Table 3), and combined *CYP2A6* genotype alone explains 43% ($R^2=0.43$) of the variance in the nicotine-glucuronide : nicotine equivalents ratio ($p=2.4\times 10^{-15}$, $n=126$, Figure 2). *CYP2A6* genotype also explains 37% of the ratio excluding cotinine-glucuronide from the denominator ($p<2\times 10^{-16}$, $n=183$, Figure 3) demonstrating the superior sensitivity of the phenotype that includes the more complete measure of nicotine equivalents.

The relationship between nicotine glucuronidation and *UGT2B10* haplotype indicates that the large majority of *UGT2B10* alleles have reduced function

Four common *UGT2B10* haplotypes are defined by the five exonic variants with MAF 0.5% in Europeans. In a multivariate analysis with *CYP2A6* genotype variables, both *UGT2B10* haplotypes defined by the non-synonymous variants, *UGT2B10**2 and *3 (Table 1), are significantly associated with altered nicotine glucuronidation ($p = 1.0\times 10^{-5}$ and 0.02 respectively, Table 3). The minor allele of rs61750900 (D67Y) is associated with reduced nicotine-glucuronide confirming its previous identification as a *UGT2B10* null allele [12]. Indeed, D₂-nicotine-glucuronide was below the limits of detection in all measured rs61750900 homozygotes ($n=3$). By contrast, the minor allele of rs112561475 (N481D) is associated with significantly increased nicotine-glucuronide. Interestingly, the relatively infrequent minor allele (2%) is the ancestral allele [39], and the more common derived allele is predicted to be 'damaging' by protein structure conservation predictive tools[40, 41]. Direct comparison also shows that diplotypes including *UGT2B10**2 differ significantly from other *UGT2B10* genotypes after controlling for *CYP2A6* genotype (*2/*2 vs. *1/*1 $p<10^{-16}$; *3/*1 vs. *2/*1 $p=0.03$; *3/*1 vs. *2/*2 $p=0.02$; *2/*1 vs. *1/*1 $p=1.0\times 10^{-4}$, Figure 4). Together in a multivariate model, the two non-synonymous variants account for 10% ($R^2=0.10$) of the total variance in the nicotine-glucuronide: nicotine equivalents ratio (D₂-nicotine-glucuronide/ (D₂-nicotine + D₂-nicotine-glucuronide + D₂-cotinine + D₂-cotinine-glucuronide + D₂-trans-3'-hydroxycotinine) $p=3.9\times 10^{-4}$, $n=126$).

The second-most common haplotype (*UGT2B10**1B), is defined by a variant in the 3'-UTR, rs4694358-C (Table 1). With either nicotine glucuronidation phenotype (Table 3) *UGT2B10**1B does not significantly differ from the other reference haplotype (*UGT2B10**1A). In multivariate analyses including the *CYP2A6* variables, *UGT2B10**2 and *UGT2B10**3, the parameter estimate for *UGT2B10**1B is -0.001 ± 0.003 , $p=0.7$ for the primary phenotype.

We also repeated the multivariate analysis of *UGT2B10* haplotypes with the primary phenotype in *CYP2A6* normal homozygotes only. In this subset of subjects which are assumed to have near uniform *CYP2A6* activity, the *UGT2B10**2 allele is also significantly associated with reduced nicotine glucuronidation (estimate= -0.011 ± 0.003 , $p=7.9\times 10^{-5}$); the association between the *UGT2B10**3 allele and glucuronidation is not statistically significant in this group (estimate= 0.006 ± 0.006 , $p=0.25$). Together, the two non-synonymous *UGT2B10* variants account for 20% ($R^2=0.20$) of the total variance in the primary phenotype in *CYP2A6* normal homozygotes.

***CYP2A6* and *UGT2B10* genotype together account for the majority of variance in oral nicotine glucuronidation**

In a previous investigation of *CYP2A6* we identified a weak association between the metabolism of nicotine to cotinine and smoking status, along with a more robust association between faster metabolism and female sex, consistent with prior reports [42-44]. Here we do not find sex significantly associated with nicotine glucuronidation, either treated individually ($p=0.64$), or in a multivariate model with *UGT2B10* and *CYP2A6* variables ($p=0.45$). Current smoking status is not significantly associated with the primary phenotype ($p=0.1$), although there is a suggestion of an association with greater nicotine glucuronidation using the phenotype D_2 -nicotine-glucuronide/ (D_2 -nicotine + D_2 -nicotine-glucuronide + D_2 -cotinine + D_2 -*trans*-3'-hydroxycotinine) ($p=0.045$, Table 3). Altogether an optimum multivariate model including genetic variants in *UGT2B10* and *CYP2A6* with smoking status, explains 53% of the variance in the primary phenotype ($R^2=0.53$ Table 3).

As in our previous investigation of nicotine-C-oxidation [30], to demonstrate the robustness of the parameter estimates in the resultant predictive model of nicotine glucuronidation (Table 3), subjects were stratified by smoking status and the parameter estimates generated from current smokers only ($n=72$) were used to predict the phenotype in current non-smokers ($n=58$), and vice-versa, and similarly in males ($n=61$) and females ($n=70$). Parameter estimates for *CYP2A6* and *UGT2B10* (Table 3) variables based on measurements made only in current smokers predicted phenotype in non-current (former) smokers with an adjusted R^2 of 0.48, and equivalent estimates in non-current smokers predicted phenotype in current smokers with an adjusted R^2 of 0.53. The power of estimates based on each group to predict phenotype in the other indicates that the relative influence of genotypes does not differ between smokers and non-current smokers. Similarly, parameters estimated in males predicted phenotype in females with an adjusted R^2 of 0.47, and vice-versa with an adjusted R^2 of 0.46.

Variables associated with cotinine glucuronidation and *ad libitum* nicotine glucuronidation

Cotinine, the primary initial metabolite of nicotine, is also glucuronidated to cotinine-glucuronide, as well as oxidized to *trans*-3'-hydroxycotinine; prior evidence indicates that the primary catalysts of these conversions are also *UGT2B10* and *CYP2A6* respectively. We therefore tested those variables significantly associated with deuterated nicotine glucuronidation with regard to a metric of cotinine glucuronidation: D_2 -cotinine-glucuronide / (D_2 -cotinine + D_2 -cotinine-glucuronide + D_2 -*trans*-3'-hydroxycotinine). The relative size and direction of the effects of these variables upon cotinine glucuronidation paralleled their effects upon nicotine glucuronidation (Tables 3 & 4). Together, genetic variants in *UGT2B10* and *CYP2A6*, and smoking status explain 15% ($R^2=0.15$ Table 4) of the variance in this cotinine-glucuronidation metric.

To determine the contribution of these variables to nicotine glucuronidation derived from *ad libitum* smoking we also pursued a metric of nicotine glucuronidation based on the non-deuterated metabolites of nicotine we had measured, i.e. D_0 -nicotine-glucuronide / (D_0 -nicotine + D_0 -nicotine-glucuronide + D_0 -cotinine + D_0 -cotinine-glucuronide + D_0 -*trans*-3'-hydroxycotinine)). Again, the relative size and direction of the effects of these variables upon *ad libitum* nicotine glucuronidation paralleled their effects upon oral nicotine glucuronidation (Tables 3 & 5). Altogether, genetic variants explain 37% of the variance ($R^2=0.37$ Table 5).

Differences in allelic mRNA expression among *UGT2B10* haplotypes

Allele-specific gene expression assays performed in heterozygous tissues can robustly determine the relative mRNA expression levels of two alleles by avoiding confounding

factors that affect total gene expression such as sample quality, diet or disease. Thus the relative expression of different alleles may be demonstrated in fairly small numbers of subjects heterozygous for assayable exonic SNPs. We were interested to find evidence of further common polymorphism in the *UGT2B10* locus that might influence gene function by mechanisms unrelated to the amino acid sequence, i.e. differences in mRNA transcription, splicing efficiency, or stability. Therefore ninety-nine European American liver samples were genotyped for the SNPs rs4694358 and rs61750900 (Table 1). Among these individuals, we identified nineteen rs4694358 heterozygotes and fifteen rs61750900 heterozygotes. None were heterozygous for both SNPs. In both groups, TaqMan allelic expression assays revealed relatively little heterogeneity among differences in relative allelic expression; the total range of dCt (the difference in the number of cycles at which PCR products generated from each allele exceed a defined threshold) was 0.49 for rs4694358 and 0.72 for rs61750900, corresponding to 1.4 and 1.6-fold differences in mRNA levels respectively (Figure 5). For comparison, in a recent study in which we confirmed differences in allelic expression among *CYP2A6* haplotypes associated with a modest difference in nicotine metabolism, the range of dCt equaled 2.1, or a 4.3-fold difference in mRNA levels[36]. Therefore, we conclude that there is no evidence for further common variation in the *UGT2B10* locus in European Americans associated with functionally-relevant differences in *UGT2B10* allelic mRNA expression.

Discussion

Variation in nicotine metabolism and nicotine metabolism genes has been associated with differences in cigarette consumption [15, 19, 22, 27, 45], smoking cessation [26] and liability for smoking-related disease [7, 46]. Although the majority of nicotine metabolism in most smokers is catalyzed by one enzyme, *CYP2A6*, there is evidence that other nicotine metabolism pathways may significantly impact smoking behavior[15, 27]. Nicotine glucuronidation has been shown to vary widely among subjects as well as among ethnic groups [13, 29, 47]. Therefore, to assess the contribution of nicotine glucuronidation to total nicotine metabolism, we quantified the plasma concentration of the glucuronide conjugates of both nicotine and cotinine in subjects who had been administered D₂-nicotine. These subjects have been previously analyzed for *CYP2A6* genotype and *CYP2A6*-generated nicotine metabolites [30].

The glucuronide conjugates of cotinine and nicotine have been quantified in the urine of smokers in many studies [13, 15, 28, 30]. However, in all cases quantitation was by an indirect method, which determines nicotine and cotinine concentrations before and after treatment with β -glucuronidase. The same method has been used to measure plasma cotinine glucuronide concentrations in two studies [15, 48]. In those studies about 10% of plasma cotinine was conjugated, compared to the 4-5% reported here for D₂ and D₀ cotinine. In contrast, the glucuronide metabolite accounted for more than 40% of the total D₂-nicotine and 15% of the D₀-nicotine. Although, the plasma concentration of nicotine glucuronide is significantly lower than the concentration of the products of *CYP2A6*-catalyzed metabolism, cotinine and *trans* 3'-hydroxycotinine, it accounts for a significant proportion of the total circulating nicotine and might serve as a reservoir for free nicotine.

Our analyses demonstrate that the majority of variation in nicotine glucuronidation in European Americans following oral nicotine administration can be predicted by a few non-synonymous polymorphisms in the *UGT2B10* gene, in addition to the large indirect influence of variation in the *CYP2A6* gene. This study focused on variants in the *UGT2B10* exons. Non-exonic variants that might alter *UGT2B10* gene function were not investigated, but the small differences in allelic expression among common haplotypes demonstrated from human liver derived cDNAs indicate that it is unlikely that further common variants

occur among Europeans with a marked impact upon UGT2B10 function. Undetectable levels of glucuronidated nicotine among the *UGT2B10**2 (D67Y) homozygotes in this study also strongly indicates that other UGT enzymes do not significantly contribute to hepatic nicotine glucuronidation, despite the modest reported *in vitro* nicotine metabolism activities of other UGTs [49, 50].

Our results confirm prior evidence that *UGT2B10**2 (D67Y) is a null allele lacking all nicotine glucuronidation activity. A surprising result of this study however is the apparently greater activity of the infrequent (MAF = 2%) *UGT2B10**3 (N481D) allele when compared to the *UGT2B10* reference allele previously assumed to possess full activity. Sequence similarity in primates and the high conservation of amino acid 481 across other species indicates that the less-frequent (MAF = 2%) *3 allele is in fact the ancestral allele despite the fact that the hypomorphic reference allele comprises the vast majority of *UGT2B10* alleles in both European and African Americans [37]. Examples exist of metabolism enzyme gene variants detrimental to enzymatic function that have become ubiquitous in some but not all ethnic groups [51, 52]; this allele may serve as an example of a ‘damaging’ mutation that has come to predominate in most ethnic groups without reaching fixation.

This is the first study to investigate nicotine glucuronidation using orally-administered deuterated nicotine in both smoking and smoking-abstinent subjects. The effect of current smoking upon the parallel nicotine metabolism pathway, C-oxidation, has been controversial, with published evidence supporting both inductive [53, 54] and inhibitory effects [55]. Our prior investigation of nicotine C-oxidation in this sample indicated a clinically insignificant induction of nicotine C-oxidation associated with current smoking [30]. Here we detect a potential weak induction of nicotine glucuronidation and a relatively robust induction of cotinine glucuronidation associated with current smoking. CYP2A6 activity is also reportedly induced by estrogen[56], and this is believed to explain the consistent difference in nicotine C-oxidation between men and women[42-44]. We find no evidence for a similar sex difference in nicotine glucuronidation efficiency.

To our knowledge, this is the first study to examine the influence of genetic variation upon metabolism that includes polymorphisms in genes contributing to two metabolic pathways competing for the same substrate and utilizing direct measurement of both relevant metabolites. The comprehensiveness of this analysis is key because, although the effects of *UGT2B10* haplotypes upon nicotine metabolism appear to be robust, the majority of variation in nicotine glucuronidation is still explained by polymorphism in a gene, *CYP2A6*, that does not encode a glucuronosyltransferase enzyme. In light of this large indirect influence upon levels of measurable glucuronidated nicotine metabolites, it would be difficult to properly assess the association between nicotine-glucuronidation and variation in *UGT2B10* without accounting for *CYP2A6* genotype, and statistical power to detect the effects of infrequent alleles like *UGT2B10**3 would be greatly reduced. Overall our results demonstrate the value of thoroughly determining the contribution of genotype to functional variation in one gene or pathway to act as a covariate to detect the effects of further genetic variation upon another related pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Nicotine metabolism pathways with primary associated metabolic enzymes.

Distribution of Nicotine Glucuronidation

Figure 2.

The Distribution of deuterated (D_2)-nicotine-glucuronide/ (D_2 -nicotine + D_2 -nicotine-glucuronide + D_2 -cotinine + D_2 -cotinine-glucuronide + D_2 -*trans*-3'-hydroxycotinine) measured 4 hours after oral D_2 -nicotine administration among European American subjects displayed as a cumulative histogram color-coded by *CYP2A6* diplotype class. White = normal/normal (n=69), Yellow = normal/intermediate (n=34), Green = intermediate/intermediate (n=8), Blue = normal/null (n=10), Purple = intermediate/null (n=4), Red = null/null (n=1). Normal haplotypes are *1B, *1D, *1H and *14. Intermediate haplotypes are *1A and *9. Null haplotypes are *2, *4, *12 and *38. Genotype is not further differentiated.

Distribution of Nicotine Glucuronidation

Figure 3.

The Distribution of deuterated (D_2)-nicotine-glucuronide/ (D_2 -nicotine + D_2 -nicotine-glucuronide + D_2 -cotinine + D_2 -*trans*-3'-hydroxycotinine) measured 4 hours after oral D_2 -nicotine administration among European American subjects displayed as a cumulative histogram color-coded by *CYP2A6* diplotype class. White = normal/normal (n=102), Yellow = normal/intermediate (n=51), Green = intermediate/intermediate (n=10), Blue = normal/null (n=17), Purple = intermediate/null (n=5), Red = null/null (n=3). Normal haplotypes are *1B, *1D, *1H and *14. Intermediate haplotypes are *1A and *9. Null haplotypes are *2, *4, *12 and *38. Genotype is not further differentiated.

nicotine glucuronidation after controlling for CYP2A6 genotype

Figure 4.

UGT2B10 diplotypes associated with nicotine glucuronidation (deuterated (D₂)-nicotine-glucuronide/ (D₂-nicotine + D₂-nicotine-glucuronide + D₂-cotinine + D₂-cotinine-glucuronide + D₂-*trans*-3'-hydroxycotinine) after accounting for *CYP2A6* genotype. Data points are the residual values for each subject after applying the model including three *CYP2A6* variables (*CYP2A6* normal alleles, *CYP2A6**1A, and *CYP2A6**9). The boxplots provide summaries of the data distributions for each group of (n) subjects defined by *UGT2B10* diplotype. A box represents the interquartile range, which includes 50% of values. The line across the box indicates the median. The whisker lines extend to the highest and lowest values that are within 1.5x the interquartile range. Further outliers are marked with circles.

Figure 5. *UGT2B10* relative allelic expression in rs4694358 (n=19) and rs61750900 (n=15) heterozygous liver cDNAs. Data points are the difference in Ct (the number of cycles at which products generated exceed a defined threshold) between PCR reactions for one allele minus the other normalized against the average ratio obtained for all heterozygous gDNAs for each assay. 0.0 equals no difference.

Table 1*UGT2B10* Haplotypes

Haplotype name	rs61749966 (N37N)	rs61750900 (D67Y)	rs112561475 (N481D)	rs17146905 (3'-UTR)	rs4694358 (3'-UTR)	alleles	frequency
*1A	T	G	A	A	T	288	76.6%
*1B	T	G	A	A	C	42	11.2%
*2A	C	T	A	G	T	36	9.6%
*3	T	G	G	A	T	8	2.1%
*1C	T	G	A	G	T	1	0.3%
*2B	C	T	A	A	T	1	0.3%

Polymorphic sites analyzed are given at the top of each column by rs number, and further relevant description. Haplotypes are ordered by frequency. For convenient reference in the text, haplotype names are designated by the authors based on previously published nomenclature.

Table 2

Plasma concentrations of D₂-nicotine, D₂-nicotine-glucuronide, D₂-cotinine, D₂-cotinine-glucuronide, D₀-nicotine, D₀-nicotine-glucuronide, D₀-cotinine, D₀-cotinine-glucuronide and D₀-3OH-cotinine^a

	D ₂ -nicotine	D ₂ -nicotine-glucuronide	D ₂ -cotinine	D ₂ -cotinine-glucuronide	D ₂ -3OH-cotinine	D ₀ -nicotine	D ₀ -nicotine-glucuronide	D ₀ -cotinine	D ₀ -cotinine-glucuronide	D ₀ -3OH-cotinine
All Subjects (192 ^b /131 ^c)	5.1 ±3.5	3.9 ±2.3	136 ±37.4	4.6 ±2.6	13.7 ±8.9					
Males (92/61)	5.1 ±2.8	3.7 ±2.0	117±28.5	3.9 ±2.4	10.0 ±5.4					
Females (100/70)	5.2 ±4.1	4.2 ±2.6	152 ±36.5	5.1 ±2.6	17.0 ±10.0					
Current smokers ^d										
All (103/72)	5.0 ±3.6	4.1 ±2.4**	133 ±39.2	5.1 ±2.9***	13.9 ±9.4	186 ±86.5	30.9 ±31.2	1890 ±1080	87.9 ±68.6	558.3 ±349.8
Male (53/32)	4.6 ±2.8	4.0 ±2.1	115 ±29.0	4.8 ±2.8	10.8 ±6.2	196 ±93.6	36.1 ±39.5	2020 ±1180	95.8 ±80.8	579.3 ±388.8
Female (50/40)	5.4 ±4.3	4.4 ±2.7	150 ±40.4	5.4 ±3.0	17.1 ±11.0	175 ±77.7	25.3 ±17.5	1750 ±951	81.5 ±57.2	536.9 ±308.0
Non-current smokers										
All (88/58)	5.3 ±3.4	3.6 ±2.2**	139 ±35.2	3.9 ±1.9***	13.4 ±8.4					
Male (38/28)	5.7 ±2.8	3.2 ±1.8	118±28.3	2.9 ±1.4	8.7 ±3.8					
Female (50/30)	4.9 ±3.8	4.0 ±2.4	154±32.3	4.8 ±1.8	17.0 ±9.1					

^aMeasures taken from plasma collected 240 min following the oral administration of deuterated nicotine (D₂-nicotine) and analyzed by LC/MS/MS. Values are expressed as means ± standard deviation in pico-moles/ml. *trans*, 3'-hydroxycotinine (3-OHcotinine).

^bNumber of subjects with measures of D₂-nicotine, D₂-nicotine-glucuronide, D₂-cotinine, D₂-*trans* 3'-hydroxycotinine, D₀-nicotine, D₀-nicotine-glucuronide, D₀-cotinine, and D₀-*trans* 3'-hydroxycotinine, includes subjects without genotypes who were not further analyzed.

^cNumber of subjects with measures of D₂-cotinine-glucuronide, and D₀-cotinine-glucuronide, includes subjects without genotypes who were not further analyzed.

^dCurrent smokers includes all subjects with mean undeuterated cotinine > 2 ng/ml, excluding one subject using the nicotine patch.

* males vs. females, p = 0.45 corrected for weight

** current smokers vs. non-current smokers, p = 0.09

*** current smokers vs. non-current smokers, p = 0.004

Table 3

Variables predicting D₂-nicotine glucuronidation individually, and when included in a multivariate regression model

Phenotype	Percent of total D ₂ -nicotine converted to nicotine-glucuronide ^a (n= 126 subjects/ 252 chromosomes)				Percent of D ₂ -nicotine (excluding cotinine-glucuronide) converted to nicotine-glucuronide ^b (n= 188 subjects/ 376 chromosomes)		
	n ^c	Modeled individually		multivariate model		n ^c	multivariate model
Variable		R ²	p	Parameter estimate	p		p
<i>CYP2A6</i> normal alleles ^d	182	0.20	7.5×10 ⁻⁸	-0.040 ± 0.004	<2×10 ⁻¹⁶	272	<2×10 ⁻¹⁶
<i>CYP2A6</i> *1A	37	0	0.3	-0.033 ± 0.004	7.1×10 ⁻¹²	53	3.1×10 ⁻¹³
<i>CYP2A6</i> *9	17	0	0.3	-0.033 ± 0.005	5.7×10 ⁻⁹	23	4.8×10 ⁻⁸
<i>UGT2B10</i> *2 (67Y)	26	0.08	4.6×10 ⁻⁴	-0.014 ± 0.003	1.0×10 ⁻⁵	39	1.1×10 ⁻⁵
<i>UGT2B10</i> *3 (481D)	6	0.02	0.07	0.014 ± 0.006	0.02	8	0.047
Current smoking ^e	72	0	0.5	0.004 ± 0.003	0.1	103	0.045
Total adjusted R ² optimum model				0.53		0.46	

Results of linear regression analyses treating *CYP2A6* null alleles (*CYP2A6**2, *4, *12 and *38) and *UGT2B10**1 as the reference. Genetic variables are coded as number of haplotypes per subject (0,1,2). Negative parameter estimates indicate reduced nicotine glucuronidation relative to the reference genotype.

^aDeuterated (D₂)-(nicotine-glucuronide / (nicotine + nicotine-glucuronide + cotinine + cotinine-glucuronide + *trans*-3'-hydroxycotinine))

^bDeuterated (D₂)-(nicotine-glucuronide / (nicotine + nicotine-glucuronide + cotinine + *trans*-3'-hydroxycotinine))

^c number of alleles

^d all *CYP2A6* alleles excluding *CYP2A6**1A, *9 and assumed null alleles *CYP2A6**2, *4, *12 and *38.

^e Current smoking status as stratified by a mean D₀-cot >2ng/ml.

Table 4

Variables predicting D₂-cotinine glucuronidation^a following oral D₂-nicotine administration in a multivariate regression model (n=126 subjects)

Variable	<i>b</i> n	Parameter estimate	p
<i>CYP2A6</i> normal alleles ^c	182	-0.012 ± 0.005	0.02
<i>CYP2A6</i> *1A	37	-0.012 ± 0.006	0.04
<i>CYP2A6</i> *9	17	-0.015 ± 0.007	0.03
<i>UGT2B10</i> *2 (67Y)	26	-0.012 ± 0.004	0.003
<i>UGT2B10</i> *3 (481D)	6	0.016 ± 0.008	0.06
Current smoking ^d	72	0.010 ± 0.004	0.006
Total adjusted R ²		0.15	

Results of linear regression analyses treating *CYP2A6* null alleles (*CYP2A6**2, *4, *12 and *38) and *UGT2B10**1 as the reference. Genetic variables are coded as number of haplotypes per subject (0,1,2). Negative parameter estimates indicate reduced nicotine glucuronidation relative to the reference genotype.

^aDeuterated (D₂)-(cotinine-glucuronide / (cotinine + cotinine-glucuronide + *trans*-3'-hydroxycotinine))

^bnumber of alleles

^call *CYP2A6* alleles excluding *CYP2A6**1A, *9 and assumed null alleles *CYP2A6**2, *4, *12 and *38.

^dCurrent smoking status as stratified by a mean D₀-cot >2ng/ml.

Table 5

Variables predicting *ad libitum* D₀-nicotine glucuronidation^a in a multivariate regression model in current smokers

Variable	<i>b</i> n	Parameter estimate	p
<i>CYP2A6</i> normal alleles ^c	88	-0.009 ± 0.003	0.003
<i>CYP2A6</i> *1A	16	-0.007 ± 0.003	0.02
<i>CYP2A6</i> *9	6	-0.007 ± 0.004	0.09
<i>UGT2B10</i> *2 (67Y)	11	-0.007 ± 0.002	0.0007
<i>UGT2B10</i> *3 (481D)	2	0.012 ± 0.005	0.02
Total adjusted R ²		0.37	

Results of linear regression analyses treating *CYP2A6* null alleles (*CYP2A6**2, *4, *12 and *38) and *UGT2B10**1 as the reference. Genetic variables are coded as number of haplotypes per subject (0,1,2). Negative parameter estimates indicate reduced nicotine glucuronidation relative to the reference genotype.

^aNon-deuterated (D₀)-(nicotine-glucuronide / (nicotine-glucuronide + nicotine + cotinine + cotinine-glucuronide + *trans*-3'-hydroxycotinine))

^b number of alleles

^c all *CYP2A6* alleles excluding *CYP2A6**1A, *9 and assumed null alleles *CYP2A6**2, *4, *12 and *38.