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An *in Vivo* Pilot Study Characterizing the New *CYP2A6*7, *8,* and **10* Alleles

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We developed genotyping assays for *CYP2A6*7* (Ile471Thr) and *CYP2A6*8* (Arg485Leu). We found higher allelic frequencies in Japanese and Chinese versus Caucasians and identified an allele in which both substitutions occur together (*CYP2A6*10*). We created a homology model for predicting the impact of allelic variants on enzymatic activity and subsequently tested this *in vivo* in a pilot kinetic study. Consistent with our homology model predictions, we found (i) that *CYP2A6*7* produces an enzyme that has decreased (not inactive) activity for metabolizing nicotine and coumarin; (ii) that *CYP2A6*8* is unlikely to affect catalytic activity *in vivo;* and (iii) that having both substitutions together on an allele (*CYP2A6*10*) dramatically reduces function and may be fully inactive for some substrates. In conclusion, this study identifies, at relatively high frequency in Asians, an allele with decreased activity (may be substrate selective), a fully functional allele, and an allele containing both substitutions in which function is dramatically reduced.

Key Words: **genetic polymorphism; CYP2A6; substrate recognition sites; nicotine; coumarin; smoking.**

Cytochrome P450 2A6 (*CYP2A6*) catalyzes the metabolism of 70–80% of nicotine (NIC) to the inactive metabolite cotinine (COT); it is also the major coumarin 7 hydroxylase. These enzymatic activities can be lost due to genetic polymorphisms resulting in *CYP2A6* poor metabolizers that are homozygous for inactive alleles [(1–3), for review, see (4)]. Several genetic polymorphisms of the *CYP2A6* gene have been described to date. The wild-type alleles, *CYP2A6*1A* and *CYP2A6*1B,* have full CYP2A6 activity. *CYP2A6*2* was found to be a null allele with no activity toward probe substrates (5). In addition to being very rare, the methodologies used in the past for detection of the inactive *CYP2A6*3* allele produced erroneous results, including overestimations of allele frequencies. The C*YP2A6*4* allele is a *CYP2A6* gene deletion (1, 5–9) which lacks activity toward all substrates. *CYP2A6*5* contains a deleterious single nucleotide polymorphism (SNP) in exon 9 leading to a Gly479Val substitution (10). We have also identified a *CYP2A6* gene duplication (*CYP2A6*1X2*) (11). Recently, additional genetic polymorphisms of the *CYP2A6* gene have been reported using *in vitro* expression systems. *CYP2A6*6* which contains a SNP in exon 3 resulting in an Arg128Gln substitution (12) and *CYP2A6*7* and *CYP2A6*8* have the amino acid substitutions Ile471Thr and Arg485Leu, respectively, resulting from nucleotide changes in exon 9 (13). *CYP2A6*9* contains a −48T to G nucleotide substitution in the TATA box of the 5′-flanking region of *CYP2A6* which results in a 50% decrease in transcription (tested *in vitro*) (14).

In the present study, we sequenced several poor and slow metabolizers at all exons of the *CYP2A6* gene. We identified two new polymorphisms of T1412C and G1454T which result in Ile471Thr and Arg485Leu substitutions, respectively; these are the same variants which were recently named *CYP2A6*7* and *CYP2A6*8* (13). We developed allele-specific PCR genotyping assays for *CYP2A6*7* and *CYP2A6*8* (verified by sequencing). We present data demonstrating differences among ethnic groups in the allele frequencies of these two new alleles as well as an allelic variant (*CYP2A6*10*) containing both substitutions. In addition, we provide pilot data indicating differences between the alleles in their ability to metabolize probe substrates nicotine and cotinine.

MATERIALS AND METHODS

*Allele-specific PCR for CYP2A6*7 and CYP2A6*8.* Cosmid DNA from clones 19296, 19019, and 27292 (gratefully received from Dr.

Abbreviations used: NIC, nicotine; COT, cotinine; COU, coumarin; 7-OHCOU, coumarin 7 hydroxylase; PCR, polymerase chain reaction; AS-PCR, allele-specific PCR; dNTP, deoxyribonucleotide triphosphate; SRSs, substrate recognition sites; SNP, single-nucleotide polymorphism; AUC, area under the curve.

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Linda Ashworth, Human Genome Center, Liverpool, CA) containing *CYP2A6, CYP2A7,* and *CYP2A13,* respectively (15), were used to establish the *CYP2A6* gene specificity of the primers. Allele-specific PCR assays to genotype *CYP2A6*7* and *CYP2A6*8* alleles were developed based on a two-step PCR method. In the first PCR the primers are selective for the *CYP2A6* gene, using a forward primer in exon 8 [2A6ex8F, 5′-CAC TTC CTG AAT GAG AAG G-3′ (10)] and reverse primer in the 3′ flanking region (2A6R3, 5′-GGA ATA GGT GCT TTT TAA GAA TC-3′). The reaction mixture contained 50 ng genomic DNA, 0.125 μM of each primer, 200 μM of each deoxyribonucleotide triphosphates (dNTP), 1.2 mM $MgCl_2$, and 1.25 U of *Taq* polymerase (Gibco BRL, Life Technologies, Burlington, Ontario, Canada) in total volume of 25 μl. The PCR conditions for the first amplification consisted of initial denaturation at 95°C for 1 min, following by 30 cycles of denaturing at 95°C for 15 s, annealing at 50°C for 20 s, and extension at 72°C for 1 min. following by final extension at 72°C for 7 min. Amplifications were performed by a MJ Research PCR cycler PTC 200. Genomic *CYP2A6, CYP2A7,* and *CYP2A13* DNA from the cosmid clones as well as genomic DNA from individuals homozygous for the gene deletion (*CYP2A6*4/*4*) were always used in the first PCR amplification to confirm gene specificity of the PCRs and primer pairs.

The PCR product (1272 bp) from the first amplification was subsequently used as a template in the second allele-specific PCRs for the *CYP2A6*7* and *CYP2A6*8* alleles. The allele-specific forward primers for detection of the **7* wild-type alleles (2A6*7-wt in exon 9, 5′-CTC CCA GTC ACC TAA GGA CAT-3′) and for the **7* variant allele (2A6*7-variant in exon 9, 5′-CTC CCA GTC ACC TAA GGA CAC-3′), were described by Ariyoshi (13). The allele-specific forward primers for detection of the *8 wild-type (2A6*8-wt in exon 9, 5′-GCT TTG CCA CGA TCC CAC G-3′) and for the *8 variant allele (2A6*8-variant in exon 9, 5′-GCT TTG CCA CGA TCC CAC T-3′), or those for the **7* allele, were used in combination with a nested reverse primer (2A6R2 in 3′UTR, 5′-AAA ATG GGC ATG AAC GCC C-3′). The 25-μl PCR mixture consisted of 0.8 μl the first PCR product, 0.125 μM each primer, 100 μM dNTPs, 1.0 mM $MgCl_2$, and 0.3 U of *Taq* DNA polymerase. The reaction conditions were as follows: an initial denaturation at 95°C for 1 min, followed by 17 cycles of denaturation at 95°C for 15 s, annealing at 67°C for 20 s and extension at 72°C for 45 s, before a final extension at 72°C for 7 min for *CYP2A6*7.* The same conditions were used for *CYP2A6*8* except that annealing was performed at 59°C for 20 s and 20 cycles were performed. PCR products (15 μl loaded, 439 bp for *CYP2A6*7* and 402 bp for *CYP2A6*8*) were analyzed on a 1.2% agarose gel stained with ethidium bromide.

DNA sequencing. Genomic DNA samples with genotypes of **1/*1* and **4/*10* [alleles containing both the **7* and the **8* nucleotide changes are designated **10* (http://www.imm.ki.se/CYPalleles/cyp2a6.htm)] were amplified by PCR using *CYP2A6*-selective forward and reverse primers (2A6ex8F and 2A6R3) and conditions from the first amplification step of the genotyping assay. This step allowed sequencing of the **10* allele alone, as the **4* allele is not amplified under these conditions. Sequencing for exon 9 was performed on an ABI 373 automatic sequencer from both by the DNA Sequencing Facility, the Centre for Applied Genomics, Hospital for Sick Children (Toronto, Canada).

Homology model and alignments. A homology model was constructed based on the CYP2C5 crystal structure, pdb file 1DT6 of Williams *et al.* (16), with the Modeller program of Sali (17). Alignments were done with clustalW (18).

Allele frequencies among ethnic groups. Genotyping assays for established (**2, *4,* and **1×2*) and new (**7* and **8*) alleles (19) were used to assess *CYP2A6*7* and *CYP2A6*8* allelic frequencies among three ethnic groups. Inclusion in an ethnic group required all four grandparents to be reported as being from that ethnic group (11).

Kinetic study. Participants for the kinetic study were recruited by advertisement or word of mouth and signed a consent form approved by the Research Ethics Board of Sunnybrook and Women's Health Science Center, University of Toronto. Of a total of 14 subjects (9 females and 5 males), 12 were Japanese nonsmokers, one was a Japanese smoker (*CYP2A6*1/*1*) and one was a Chinese nonsmoker (*CYP2A6*1/*8*). Smokers in the study ($N = 1$) were instructed to abstain from smoking from midnight the night before a study day. Compliance with the instructions was confirmed by measurement of breath carbon monoxide (Micro II Smokelyzer, Bedfont Scientific Ltd., Upchurch, England). Subjects were genotyped for *CYP2A6* for **2, *4, *7,* and **8* and the duplication (**1×2*) alleles (5, 9, 11). Only individuals ($N = 14$) with **1, *4, *7,* and **8* alleles were included in the kinetic study.

The kinetic study was single blind. NIC (4 mg) or COU (50 mg) was given orally on two separate days. NIC was prepared from the bitartrate salt (Sigma Chemical) with the 4 mg oral dose calculated as the free base fraction of 12.3 mg salt. NIC capsules contained additional lactose as filler. COU capsules were prepared by the Pharmacy at Women's College Hospital, containing a mixture of COU powder (50 mg, Fluka Chemika) and lactose monohydrate (BDH Inc.) as a filler. On both kinetic study days, blood samples (10 ml each) were taken at 0, 10, 20, 30, 45, 60, 75, 90, 120, 180, 270, and 360 min, centrifuged for plasma, frozen, and analyzed for NIC, COT or 7 hydroxy-coumarin (7-OHCOU) by HPLC (3, 20, 21). Analytical sensitivity was <0.5 ng/ml for NIC, 10 ng/ml for COT and 15 ng/ml of 7-OHCOU. NIC and COT measurements were essentially as described by Sellers *et al.* (22); 7-OHCOU was measured using the method described by Egan *et al.* (21). The internal standard for the NIC and COT assay was 5-methyl NIC (1500 ng/ml) and for the COU assays was 7 amino-4 methyl COU (606 ng/ml). Baseline corrections were performed using a NIC half-life of 90 min and a COT half-life of 20 h.

Statistics. Compliance with the Hardy–Weinberg equations was tested by χ^2 test. Differences in plasma drug AUCs between genotype groups were tested using *t* tests.

RESULTS

*Identification of the CYP2A6*7 and CYP2A6*8 Polymorphism and Development of Genotyping Assays*

We identified many new and novel genetic variants by sequencing the exons of the *CYP2A6* gene in several poor and slow metabolizers. Here we reported on two of them (and the two in combination). DNA sequencing revealed two new exon 9 nucleotide substitutions of the T1412C and G1454T resulting in an Ile471Thr substitution (*CYP2A6*7*) and an Arg485Leu substitution (*CYP2A6*8*), respectively. We developed a two-step allele-specific PCR assays for detection of the *CYP2A6*7* and the *CYP2A6*8* alleles. Figure 1A illustrates the *CYP2A6* gene-specificity of the first step; amplification occurred from samples containing *CYP2A6* cosmid DNA (as well as genomic DNA samples) but no amplification occurred from cosmid DNA containing *CYP2A7* or *CYP2A13.* Figures 1B and 1C illustrate the allele-specificity of the second steps for the *CYP2A6*7* allele and the *CYP2A6*8* allele, respectively. The one individual identified as heterozygous for the *CYP2A6*4* allele (gene deletion) had both *CYP2A6*7* and *CYP2A6*8* indicating that it contained both substitutions on one allele (designated *CYP2A6*10*). These results were confirmed by sequencing of DNA

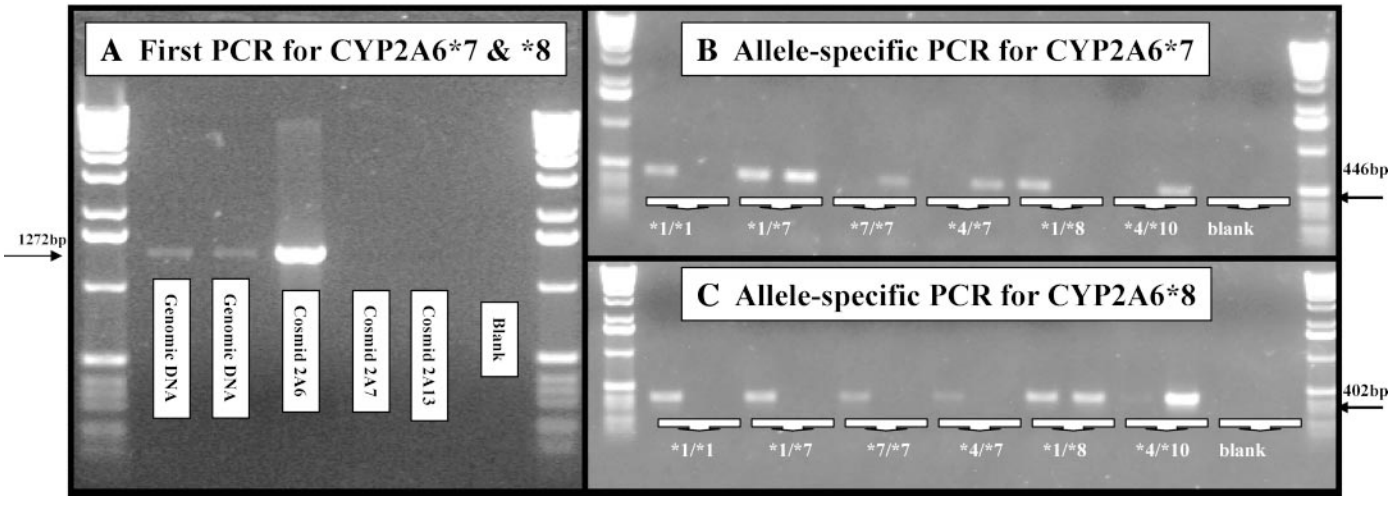

FIG. 1. Typical results from the *CYP2A6*7* and *CYP2A6*8* genotyping assays. (A) Products from the first step of PCR amplification. Lanes 1 and 2 contained genomic DNA; lanes 3, 4, and 5 included cosmid DNA controls of the *CYP2A6*, *CYP2A7*, and *CYP2A13* genes, respectively. Lane 6 contains PCR amplification from a sample without DNA (blank). (B and C) Results of the second allele-specific PCR step for *CYP2A6*7* and *CYP2A6*8*, respectively, for various genotypes. The first lane in each pair (indicated by the bar) of lanes was amplified using the wild-type forward primer, while the second lane in each pair was amplified using the variant-specific forward primer. Typical genotyping results for individuals with a *CYP2A6*1/*1*, *CYP2A6*1/*7*, *CYP2A6*7/*7*, *CYP2A6*1/*8*, or *CYP2A6*4/*10* genotype, are shown in lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, 11, and 12, respectively. Note that in the absence of a second allele (e.g., heterozygous for the *4 deleted allele), both the *7 and *8 genotyping predict homozygosity in the individuals genotyped as CYP2A6*4/*10 (verified by sequencing).

samples with the genotypes of the **1/*1* and **4/*10* from both directions in exon 9 of the *CYP2A6* gene. The sequence information indicated homozygosity throughout the sequence from the **4/*10* DNA (only the **10* nondeleted allele is amplified and sequenced) providing evidence of the coexistence of the **7* and **8* substitutions on the same allele now designated *CYP2A6*10*.

Homology Model and Alignment

To investigate whether the *CYP2A6*7* and *CYP2A6*8* substitutions would be predicted to alter enzyme–substrate interactions, we constructed a homology model based on the *CYP2C5* crystal structure (16). The substrate [S]-NIC was docked into the active site of the CYP2A6 homology model in such a way so as to make the substrate overlay with the binding mode of acetaminophen with CYP2A6, which is based on NMR relaxation studies (23). No attempt was made to optimize the docked structure except to place the 5′ hydrogen atoms of NIC close to the heme iron. This binding orientation is shown in Fig. 2. The homology model reveals that the residues in question, I471 and R485, are part of a loop region in which F480 points into the active-site and could interact with the substrate. However, the residues in question are not part of the active-site and instead appear to be in a region that would stabilize the tertiary structure of this substrate recognition region.

Allele Frequencies among Ethnic Groups

As expected the allele frequencies differed substantially among the ethnic groups. The *2, *4, and *1×2 allelic frequencies were similar to those previously observed. The frequencies of both new alleles were similar in Japanese and Chinese with *CYP2A6*7* a little higher in Japanese (Table 1). The compound allele *CYP2A6*10*, containing both *CYP2A6*7* and **8*, is found at a low frequency in both Chinese and Japanese. Neither *CYP2A6*7*, *CYP2A6*8*, nor *CYP2A6*10* (*7 and *8 combination allele) was found in 602 Caucasian alleles tested. The genotypes for all three ethnic groups, for the 6 alleles (Table 1), were not significantly different than predicted by the Hardy–Weinberg law indicating no evidence for selection. While the frequency of the *7 heterozygotes is in accordance with Hardy–Weinberg law, they are found as *4/*7 heterozygotes suggesting that the assay may preferentially detect homozygous *7 genotypes (with or without the deleted allele). Our sequencing does not support this and we have found *1/*7 individuals in other studies suggesting that it is less likely to be the assay itself. This suggests the lack of *1/*7 individuals in this study is either a question of power (due to low allele frequency and small populations) or it may be real and due to an unknown cause(s).

Kinetic Study

Following an oral administration of NIC, individuals with a homozygous wild-type genotype (*CYP2A6*1/*1*) demonstrated significantly lower plasma levels of NIC and higher levels of the CYP2A6-mediated NIC metabolite COT (Figs. 3 and 4) relative to the other genotype groups tested. An individual who was homozygous for the *7 allele (*CYP2A6*7/*7*) had intermediate levels of

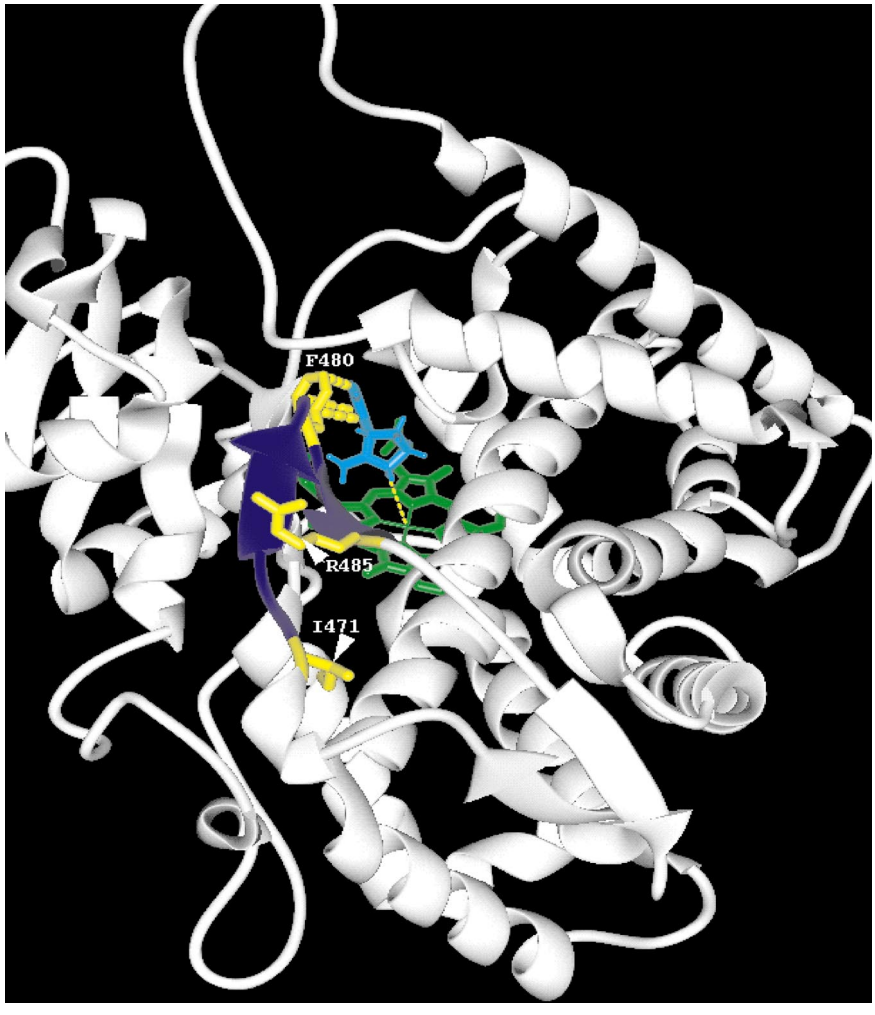

FIG. 2. [S]Nicotine binding to a homology model for CYP2A6. F480, I471, and R485 are shown in yellow. The region between I417 and R485 is shown in blue. F480 is predicted to be the major substrate interaction residue in SRS-6 and is shown interacting with nicotine. Both I471 and R485 are at the hinge region that controls the conformation of SRS-6, but do not directly interact with the substrate.

plasma NIC and COT formation but was clearly able to metabolize COU. The two Japanese subjects with the *7 alleles in combination with a gene deletion (*CYP2A6*4/*7*) demonstrated similar NIC plasma levels to those observed in individuals which were homozygotes null (*CYP2A6*4/*4*, $N = 3$). These subjects produced intermediate amounts of COT and readily formed 7-OH COU. One subject with a *CYP2A6*1/*8* genotype was indistinguishable from those homozygous for wild-type alleles (*CYP2A6*1/*1*) for NIC levels, COT production and COU metabolism. The study also contained an individual with the compound genotype (*CYP2A6*4/*10*). This individual demonstrated activity similar to those fully null (*CYP2A6*4/*4*) with markedly higher levels of plasma NIC and reduced COT formation as well as reduced COU metabolism (Fig. 3). Mean 6-h plasma AUCs for NIC, COT, and 7-OHCOU were compared among genotypes (Fig. 4). The data indicate that the *CYP2A6*7* allele reduces NIC to COT metabolism but is not inactive while the *CYP2A6*8* allele does not appear to alter activity. However both NIC and COU metabolism were dramatically reduced in the individual containing the allele with both *7 and *8 (*CYP2A6*4/*10*) (Fig. 4).

DISCUSSION

Our group and Ariyoshi *et al.* (13) have identified the exon 9 *CYP2A6* gene variants *CYP2A6*7* and *CYP2A6*8*, containing T1412C (Ile471Thr) and G1454T (Arg485Leu) substitutions, respectively. In this study, we developed two-step allele-specific PCR assays for genotyping *CYP2A6*7* and *CYP2A6*8;* the results were verified with sequencing. In addition, there was no co-amplification of the *CYP2A7* and *CYP2A13* in either PCR step for either allele (Fig. 1). Of note, the T1412C and G1454T variations are not found in either *CYP2A7* (Accession No. M33317) or *CYP2A13* (Accession No.

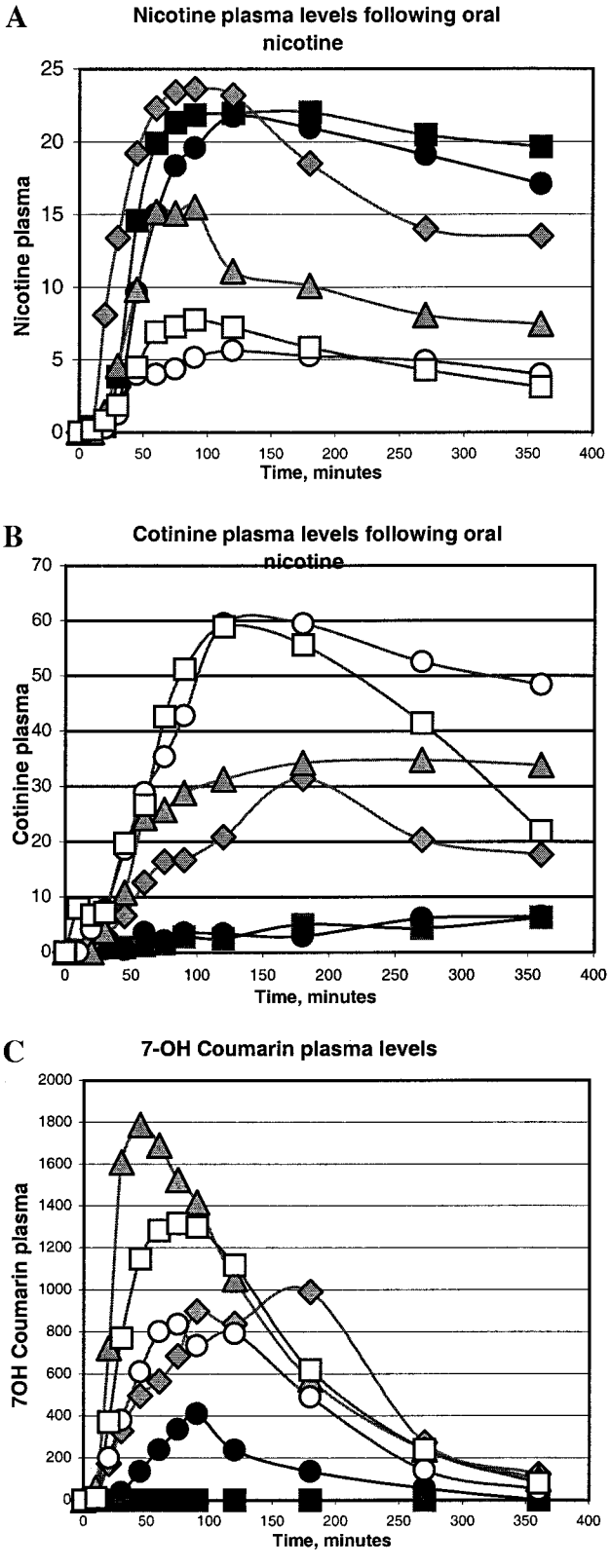

FIG. 3. Plasma levels of NIC, COT or 7-OHCOU in subjects with differing *CYP2A6* genotypes. (A) NIC plasma levels following a 4-mg oral dose. (B) COT plasma levels following a 4-mg oral NIC dose. C. 7-OHCOU plasma levels. The mean plasma levels for those with *CYP2A6*1/*1* (□, $N = 6$), *CYP2A6*4/*7* (◇, $N = 2$), *CYP2A6*4/*4* (■, $N = 3$), *CYP2A6*4/*10* (●, $N = 1$), *CYP2A6*7/*7* (△, $N = 1$), and *CYP2A6*1/*8* (○, $N = 1$) are shown.

TABLE 1

Allele and Genotype Frequencies among Different Ethnic Groups

Genotype	Japanese % ($N = 63$)	Chinese % ($N = 114$)	Caucasian % ($N = 301$)
*1/*1	57.1 (36)	77.2 (88)	94.4 (284)
*1/*1 × 2	0.0 (0)	0.9 (1)	1.3 (4)
*1/*8	3.2 (2)	7.0 (8)	0.0 (0)
*1/*7	0.0 (0)	0.0 (0)	0.0 (0)
*7/*7	3.2 (2)	1.8 (2)	0.0 (0)
*1/*2	0.0 (0)	0.0 (0)	2.3 (7)
*1/*4	19.0 (12)	11.4 (13)	2.0 (6)
*4/*7	6.3 (4)	0.9 (1)	0.0 (0)
*4/*10	3.2 (2)	0.9 (1)	0.0 (0)
*4/*4	7.9 (5)	0.0 (0)	0.0 (0)
Allele	% ($N = 126$)	% ($N = 228$)	% ($N = 602$)
*1 × 2 (Y)	0.0 (0)	0.4 (1)	0.7 (4)
*2	0.0 (0)	0.0 (0)	1.2 (7)
*4	22.2 (28)	6.6 (15)	1.0 (6)
*7	6.3 (8)	2.2 (5)	0.0 (0)
*8	1.6 (2)	3.5 (8)	0.0 (0)
*10	1.6 (2)	0.4 (1)	0.0 (0)
Pooled	31.7	12.7	2.9

U22028) gene sequences. Thus, it is unlikely that these variants are transferred from these neighboring genes through crossover events as has been speculated for other alleles (10, 11).

These two new allelic variants are located in, or close to, SRS-6 region, a region that is highly conserved in the *CYP2A*, *CYP2B*, and *CYP2F* subfamilies (24). As seen in Fig. 2, residues I471 and R485 are part of a loop region in which F480 points into the active-site and could interact with the substrate. However, the residues in question are not part of the active site and instead appear to be in a region that would stabilize the tertiary structure of this substrate recognition region. It is most likely that the variants destabilize the tertiary structure of SRS-6, resulting in a change in the structure of the active site. Interestingly, the residue that aligns with I471 in 2A6 is always a isoleucine or a leucine in the 12 other human enzymes of the 2 family for which complete sequences are available. Thus, mutation to a polar amino acid such as threonine might be expected to change the conformation. Furthermore, the residues at 485 are arginine, proline or serine in the human 2 family enzymes. Thus, mutation to leucine might be expected to alter the local secondary structure since leucine has very different secondary structure preferences than arginine, proline and serine (25). Since the two amino acids in questions are at either

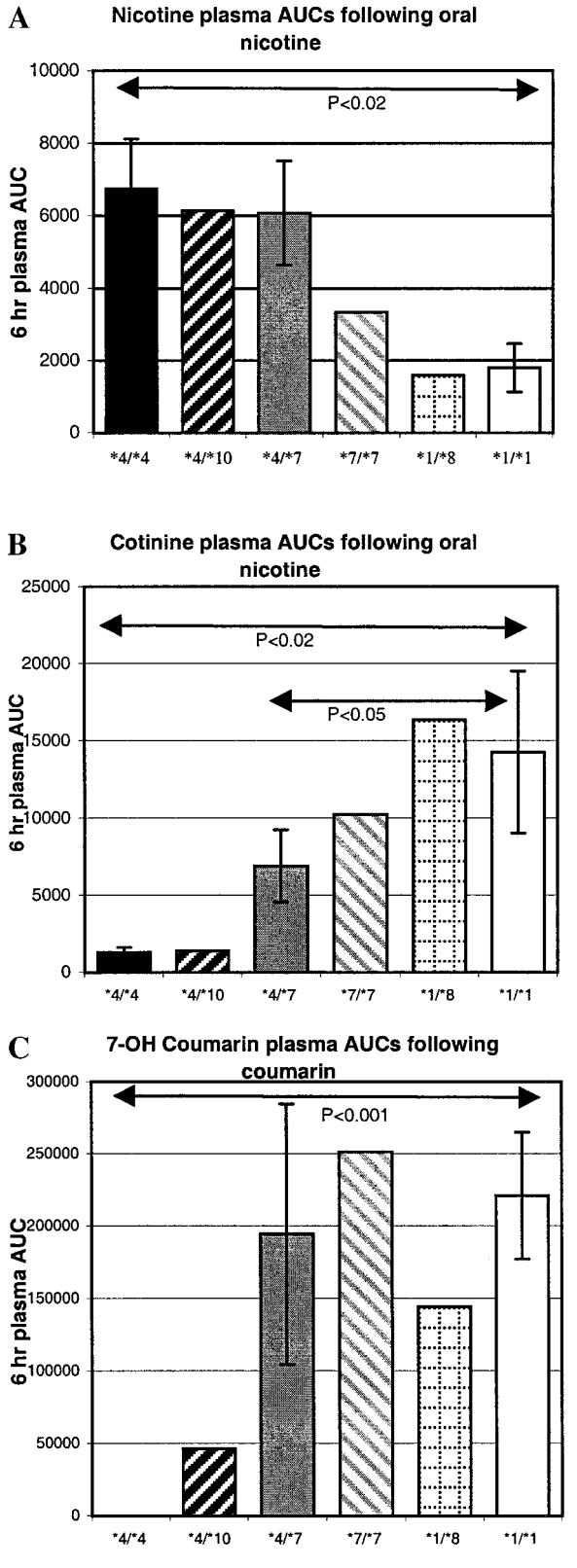

FIG. 4. Area under the plasma concentration curves for subjects of differing *CYP2A6* genotype. (A) NIC plasma AUCs following a 4-mg oral dose. (B) COT plasma AUCs following a 4-mg oral NIC dose. C. 7-OHCOU plasma AUCs. AUCs ± standard deviation for those with *CYP2A6*1/*1* ($N = 6$), *CYP2A6*4/*7* ($N = 2$), and *CYP2A6*4/*4* ($N = 3$) are shown. Statistically significant differences ($P < 0.05$ unless otherwise indicated) are indicated by values over the group of comparison.

end of the turn (see Fig. 2) it would be likely that the combination of mutations at these two locations would act synergistically. Finally, an altered conformation of SRS-6 would likely have differential effects on various substrates since this area is relatively distant from the active oxygen species. Thus, smaller substrates, such as coumarin, are likely to be affected less than larger substrates, such as nicotine.

To elucidate the effects of the *CYP2A6*7* and *CYP2A6*8* variants on enzyme activity, NIC and COU oxidation were measured *in vivo.* Our results suggest that the *CYP2A6*7* allele encodes an enzyme which has reduced metabolic activity toward NIC resulting in decreased production of COT. Our data indicates that this allele is able to make 7-OHCOU from COU. This is the first *in vivo* characterization of a *CYP2A6* allele, *CYP2A6**7, which has reduced but not inactive metabolic activity, which may show substrate specificity (i.e., decreased for NIC while fully functional for COU or other substrates). These data are analogous to the *in vitro* expressed *CYP2A6**7 which displayed reduced activity toward NIC, but not COU (13); however it is clear that more subjects with this allele will need to be studied before firm conclusions can be reached. In contrast *CYP2A6*8* appears to be indistinguishable from the activity of the wild-type allele. It is of particular interest that the *7 and *8 substitutions were found together on one allele designated as *10; this combination appeared to dramatically reduce the function of the resulting enzyme activity toward both NIC and COU. These data are consistent with the predictions made by our homology model (Fig. 2).

*CYP2A6*7* is found at greater frequency among the Japanese suggesting that the impact of this allele on metabolism and smoking may be most important for this ethnic group. The *CYP2A6*8* allele also demonstrated differing frequencies among ethnic groups however its impact *in vivo* on metabolism may be insignificant; this remains to be clarified. Of note, the combination of the *CYP2A6*7* and *CYP2A6*8* substitutions on the same allele designated as *CYP2A6*10* was relatively rare, but given its substantial impact on reducing metabolic activity these data demonstrate the need to identify all three allelic variants prior to extrapolating the genotype to *in vivo* kinetics. Our data suggest that *CYP2A6* alleles exist which may be fully functional for COU and have decreased or no activity toward other substrates such as NIC. The number of *CYP2A6* poor (or decreased) metabolizers for alternative substrates such as NIC may actually be higher than predicted by frequency estimations made with COU as the *in vivo* probe.

In conclusion, techniques involving homology modeling, sequence alignment and *in vivo* assessment of individuals with genetic variants increases our ability to understand and predict P450 structure–function relationships, to determine the "hot spot" or key residues involved, and to pinpoint the importance of specific residues on enzymatic activity. Individuals possessing *CYP2A6*7*, an Ile471Thr substitution, appear to have decreased rates of NIC metabolism while no little or no effect on COU metabolism was observed. Our data also indicate that the *CYP2A6*8* (Arg485Leu substitution) is unlikely to affect catalytic activity *in vivo*. In addition, we provide evidence for the first *CYP2A6* variant containing multiple amino acid substitutions (i.e., both the *7 and *8 substitutions); the combination allele (*CYP2A6*10*) appears to decrease activity of the enzyme to a much greater extent than either allelic substitution alone. Further identification and characterization of *CYP2A6* variant alleles will provide a better understanding of the role that genetic polymorphisms in this gene locus have on NIC metabolism, smoking behaviors, and risks for tobacco-related cancers.

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