A novel *CYP2A6* allele, *CYP2A6**23, impairs enzyme function *in vitro* and *in vivo* and decreases smoking in a population of Black-African descent

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Abstract

Objectives—*CYP2A6* is the main enzyme involved in nicotine metabolism in humans. We have identified a novel allele, *CYP2A6**23 (2161C>T, R203C), in individuals of Black-African descent and investigated its impact on enzyme activity and association with smoking status.

Methods—Wildtype and variant enzymes containing amino acid changes R203C (*CYP2A6**23), R203S (*CYP2A6**16) and V365M (*CYP2A6**17) were expressed in *Escherichia coli*. The effect of *CYP2A6**23 *in vivo* was examined in individuals of Black-African descent given 4 mg oral nicotine.

Results—*CYP2A6**23 occurred at an allele frequency of 2.0% in individuals of Black-African descent (N = 560 alleles, 95% confidence interval 0.8%–3.1%) and was not detected in Caucasians (N = 334 alleles), Chinese (N = 288 alleles) or Japanese (N = 104 alleles). *In vitro*, *CYP2A6*.23 had greatly reduced activity towards nicotine C-oxidation similar to *CYP2A6*.17, as well as reduced coumarin 7-hydroxylation. Conversely, *CYP2A6*.16 did not differ in activity compared to the wildtype enzyme. The *trans*-3'-hydroxycotinine to cotinine ratio, a phenotypic measure of *CYP2A6* activity *in vivo*, was lower in *CYP2A6**1/*23 and *CYP2A6**23/*23 individuals (mean adjusted ratio of 0.60, n = 5) compared to *CYP2A6**1/*1 individuals (mean adjusted ratio of 1.21, n = 150) ($p < 0.04$). *CYP2A6**23 trended towards a higher allele frequency in nonsmokers (3.1%, N = 9/286 alleles) compared to smokers (0.7%, N = 2/274 alleles) ($p = 0.06$).

Conclusions—These results suggest the novel *CYP2A6**23 allele impairs enzyme function *in vitro* and *in vivo* and trends toward an association with lower risk of smoking.

Keywords

CYP2A6; genetic polymorphisms; Black-Africans; nicotine; smoking

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Conflicts of interest: Dr. R.F. Tyndale holds shares in Nicogen Research Inc., a company that is focused on novel smoking cessation treatment approaches. None of the data contained in this manuscript alters or improves any commercial aspect of Nicogen, no Nicogen funds were used in this work, and the manuscript was not reviewed by others affiliated with Nicogen.

Introduction

Nicotine is responsible for the majority of the psychoactive and highly addictive properties of cigarettes [1]. In humans, ~80% of absorbed nicotine is converted into cotinine and the majority of this reaction (~90%) is mediated by the hepatic enzyme cytochrome P450 2A6 (CYP2A6) [2, 3]. Cotinine is further metabolized to *trans*-3'-hydroxycotinine and this reaction is entirely mediated by CYP2A6 [4]. CYP2A6 is also the main enzyme metabolizing therapeutic compounds such as coumarin [5], tegafur [6], and SM-12502 [7], and it can bioactivate tobacco smoke procarcinogens such as *N*'-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [8].

Large interindividual and interethnic differences in nicotine metabolism have been reported *in vitro* and *in vivo* (reviewed in [9]). *CYP2A6* is highly polymorphic, with 22 numbered alleles and numerous single nucleotide polymorphisms (SNPs) identified so far (<http://www.cypalleles.ki.se/cyp2a6.htm>). Characterization of *CYP2A6* variants is needed because *CYP2A6* polymorphisms altering nicotine metabolism may be important determinants of smoking behaviors. Several, but not all, studies have found that individuals with *CYP2A6* alleles impairing enzyme activity are less likely to be current smokers [9, 10].

Differences in nicotine metabolism, smoking behaviors and incidence of tobacco-related illnesses have been observed between ethnic groups. Compared to Caucasians, individuals of Black-African descent have a significantly reduced fractional conversion of nicotine to cotinine, reduced metabolic clearance of nicotine to cotinine and higher plasma levels of cotinine for similar cigarette consumption [11, 12]. Together, this suggests reduced nicotine and cotinine metabolism, likely as a result of genetic variations in *CYP2A6*. Interestingly, populations of Black-African descent are also characterized by unique smoking patterns. Despite having a similar prevalence of current smoking as Caucasians [13], populations of Black-African descent report later ages of smoking initiation [14, 15] and lower cigarette consumption [16, 17]. Furthermore, populations of Black-African descent suffer from disproportionately higher rates of most tobacco-related illnesses [17], such as lung cancer [18, 19], despite their lower levels of smoking.

In this study, we identified a novel *CYP2A6* allele (*CYP2A6*23*) with a SNP at 2161C>T (GenBank accession number NG_000008.7) in exon 4 corresponding to an amino acid change R203C in a population of Black-African descent. We discovered this variant while designing a genotyping assay for the previously identified *CYP2A6*16* allele [20], which occurs at the same locus (2161C>A, R203S). Primers were designed for both the C>A and C>T nucleotide change as there had been initial confusion to the specific change found in *CYP2A6*16*. The effect of *CYP2A6*23* on enzyme function was determined *in vitro* using an *Escherichia coli* (*E. coli*) heterologous expression system. We compared it to *CYP2A6*16*, which encodes a different amino acid change at the same position, and to *CYP2A6*17*, a previously characterized reduced-activity allele [21]. Furthermore, we examined the effect of *CYP2A6*23* on nicotine metabolism *in vivo* and its association with smoking status.

Methods

Participants

Adults of Black-African descent were recruited from Toronto, Ontario; detailed descriptions of the population and experimental protocol can be found elsewhere [22]. Briefly, male and female current smokers and tried nonsmokers were recruited. Smokers were defined as smoking 100 lifetime cigarettes and currently smoking at least 5 days of the week while tried nonsmokers had smoked 1–99 lifetime cigarettes but were never regular smokers. Plasma nicotine, cotinine (COT) and *trans*-3'-hydroxycotinine (3HC) levels were measured following administration of an oral nicotine dose (4 mg capsule). The 3HC/COT ratio, calculated from plasma levels collected at 270 minutes after dosing, has been previously verified as an indicator of CYP2A6 activity [23]. Caucasian (n = 167), Chinese (n = 144) and Japanese (n = 52) individuals recruited for a separate study on *CYP2A6* genetic variation and smoking behaviors [10] were also genotyped for *CYP2A6**23. The research protocol was approved and monitored by the University of Toronto Ethics Review Office and the Institutional Review Board Services (Aurora, ON).

Genotyping assay

Blood samples were stored at –20°C, and genomic DNA was extracted and stored at –20°C until use (GenElute Mammalian Genomic DNA Kit, Sigma-Aldrich Co., Mississauga, ON). A novel two-step allele specific polymerase chain reaction (PCR) assay was developed for *CYP2A6**23. A fragment of *CYP2A6* between intron 3 to intron 5 was amplified using the primers: forward (2A6exin3F) 5'–GGC ACT GGC GGT GAG CAG–3' and reverse (2A6in5R) 5'–GGC CTG TGT CAT CTG CCT–3'. The reaction mixture contained: 50 ng of genomic DNA, 1X *Taq* buffer with KCl, 200 µM deoxyribonucleoside triphosphates (dNTPs), 1.5 mM MgCl₂, 125 nM of each primer, 1.25 U of *Taq* Polymerase (MBI Fermentas, Burlington, ON) and H₂O for a total volume of 25 µl. The reaction conditions were as follows: initial denaturation at 95°C for 1 min., denaturation at 95°C for 15 sec., annealing at 58°C for 30 sec., and extension at 72°C for 2 min. for 30 cycles, followed by final extension step at 72°C for 7 min. (MJ Research PCR Cycler PTC 200; Waltham, MA). The product from this first amplification served as the template for a second allele-specific reaction using the primers: forward (2A6in3F) 5'–CTG CCT CCT GGA ATT CTG AC–3' and a reverse primer specific to 2161C (2A6ex42161AWR, 5'–GGA AGA TTC CTA GCA TCA TGC G–3') or to 2161T (2A6ex42161AVR, 5'–GGA AGA TTC CTA GCA TCA TGC A–3'). The reaction mixture contained: 0.8 µl of template, 1X *Taq* buffer with (NH₄)₂SO₄, 1.0 mM of MgCl₂, 75 nM of each primer, 1.25 U of *Taq* Polymerase and H₂O for a total volume of 25 µl. The reaction conditions were: initial denaturation at 95°C for 1 min., denaturation at 95°C for 15 sec., annealing at 57°C for 10 sec., and extension at 72°C for 30 sec. for 20 cycles. The PCR products were separated by electrophoresis on a 1.2% agarose gel stained with ethidium bromide. Subjects had been genotyped for known *CYP2A6* variants (*CYP2A6**1B, *2, *4A & D, *9, *12, *14, *15, *17, *20, *21, *24, *25, *26, *27, *28, *29).

DNA sequence analyses

We confirmed the detection of *CYP2A6**23 by our genotyping assay, and determined its haplotype by sequencing all *CYP2A6* exons and exon-intron borders in two heterozygous individuals (*CYP2A6**1/*23) and a homozygous individual (*CYP2A6**23/*23). A 9.2 kb fragment spanning –1.4 kb upstream and 7.8 kb downstream of the +1 ATG start site of *CYP2A6* was amplified from genomic DNA using long-PCR. The primers for long-PCR were: forward (2A65Pr1F) 5'–ACC TAG ACT TAA TCT TCC CGT ATA C–3' and reverse (2A6R0) 5'–AGG TCA TCT AGA TTT TCT CCT ACA–3'. The product was subcloned into pCR®-XL-TOPO plasmid (TOPO® XL PCR Cloning Kit, Invitrogen Canada Inc., Burlington, ON). DNA sequencing was performed with an ABI 3730XL DNA analyzer at the Centre for Applied Genomics (Toronto, ON).

Construction of *CYP2A6* expression plasmids

The bicistronic construct with the full length cDNA of *CYP2A6* and human NADPH-CYP450 reductase (hNPR) inserted into the pCW expression vector (8537 bp) [24] was derived from the cognate monocistronic construct prepared by Soucek *et al.* [25], in which the native *CYP2A6* sequence was changed to encode Ala at the second position with only silent nucleotide changes elsewhere in the N-terminus. The SNPs 2161C>T (*CYP2A6**23), 2161C>A (*CYP2A6**16) and 5065G>A (*CYP2A6**17) were introduced into the expression constructs using the QuikChange® II XL Site-Directed Mutagenesis Kits (Stratagene, La Jolla, CA). Primers used in the mutagenesis reactions were: 2161C>T (5'–AGT TCC TGT CAC TGT TGTGCA TGA TGC TAG GAA TC–3', 5'–GAT TCC TAG CAT CAT GCA CAA CAG TGA CAG GAA CT–3'), 2161C>A (5'–AGT TCC TGT CAC TGT TGAGCA TGA TGC TAG GAA TC–3', 5'–GAT TCC TAG CAT CAT GCT CAA CAG TGA CAG GAA CT–3'), 5065G>A (5'–ATC CAA AGA TTT GGA GAC ATG ATC CCC ATG AGT TTG G–3', 5'–CCA AAC TCA TGG GGA TCA TGT CTC CAA ATC TTT GGA T–3'). A negative control was created using *Bam*HI to remove 802 bp from the 5' end of the *CYP2A6* cDNA (total length of 1485 bp). The products were separated by gel electrophoresis and the remaining plasmid (7735 bp) was extracted and re-ligated. The variant constructs were confirmed by sequencing using an ABI 3730XL DNA analyzer at the Centre for Applied Genomics (Toronto, ON).

Expression of *CYP2A6* constructs in *E. coli*

The constructs encoding *CYP2A6* and hNPR were expressed in *E. coli* as described previously [26]. DH5α cells (Invitrogen, Burlington, ON) were transformed with wildtype and variant constructs and a starter culture was grown in Luria-Bertani medium containing ampicillin (100 µg/ml) overnight at 37°C with shaking at 200 rpm. The starter culture was diluted (1:100) in 100 ml of terrific broth containing ampicillin (100 µg/ml), 1.0 mM thiamine, and 0.5 mM δ-aminolevulinic acid. The cultures were incubated at 30°C with shaking at 120 rpm for 4–6 hours, with induction initiated by the addition of 1.0 mM isopropyl β-D-thiogalactopyranoside. The cultures were incubated for a further 19–22 hours at 30°C and shaking at 120 rpm. Membrane fractions were prepared as described in [26] with minor modifications. Briefly, the pelleted bacteria was resuspended in buffer (50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, pH 7.4) and digested with lysozyme (0.25

mg/ml) for 60 min. The spheroplast was pelleted, sonicated, and centrifuged at $12,000 \times g$ for 12 min. The supernatant was centrifuged for $110,000 \times g$ for 90 min, and the pellet containing the membrane fraction was resuspended in 1.15% KCl and stored at -80°C until use. All constructs were initiated for expression concurrently and processed for immunoblotting and activity at the same time. Batch processing was performed to reduce construct to construct differences in degradation.

Immunoblotting

Total amount of membrane protein was measured by the Bradford protein assay (Bio-Rad Labs, Mississauga, ON) and the amount of CYP2A6 protein in the membrane preparations was determined by immunoblotting as previously described [27]. A standard curve was constructed using CYP2A6 expressed by a baculovirus-infected insect cell system (BD Gentest, San Jose, CA). The bacteria membrane preparations and baculovirus-expressed CYP2A6 were serially diluted to establish linear range of detection.

Enzyme assays for nicotine and coumarin

Nicotine C-oxidation and coumarin 7-hydroxylation were determined as previously described [28, 29]. Briefly, the reaction mixture contained 20 nM of CYP2A6, 20 nM of expressed cytochrome b_5 (Invitrogen, Burlington, ON), 50 mM Tris-HCl buffer (pH 7.4) and substrate. Two substrate concentrations of nicotine (30 and 300 μM) or coumarin (5 and 50 μM) were initially used to screen for catalytic activity. Subsequently, full kinetic analyses were performed with nicotine, our main substrate of interest, ranging from 1–500 μM . Mouse liver cytosol (1.2 mg protein/ml) was added to the nicotine C-oxidation reaction as a source of aldehyde oxidase; this was added in excess so that CYP-mediated oxidation would be rate-limiting [28, 30]. The mixture was pre-warmed for 2 min. and the reaction was initiated by the addition of 1 mM NADPH. The mixture was incubated at 37°C for 45 min. for nicotine C-oxidation and 30 min. for coumarin 7-hydroxylation, and the reaction was stopped with 4% Na_2CO_3 . The amount of cotinine and *trans*-3'-hydroxycotinine formed were detected by high-pressure liquid chromatography (HPLC) as previously described [28].

To detect 7-hydroxycoumarin formation, 5 μl of 20% (w/v) of trichloroacetic acid was added to the samples. 10 μl of 4-hydroxycoumarin (1 mg/ml) was also added as an internal standard. The samples were centrifuged at 13,000 rpm for 10 min. and 100 μl of the supernatant was analyzed by HPLC. Coumarin and its metabolite 7-hydroxycoumarin were separated on the ZORBAX SB C18 Column (250×4.6 mm I.D.; particle size, 5 μm) from Agilent Technologies Inc. (Mississauga, ON) at a flow-rate of 1 ml/min., with a mobile phase of acetonitrile, water and acetic acid (25 : 75 : 0.1, v/v) and detected at 315 nm. The concentrations of coumarin and 7-hydroxycoumarin were determined from standard curves created by spiking drug-free bacterial membrane preparations with known amounts of coumarin (0.2–10 $\mu\text{g/ml}$) and 7-hydroxycoumarin (50–1000 ng/ml). The within-day precision were 2.4–12.8% for coumarin and 2.6–8.3% for 7-hydroxycoumarin, and the between-day precision were 4.2–7.7% for coumarin and 5.9–13.7% for 7-hydroxycoumarin.

Bioinformatics

Linkage disequilibrium of *CYP2A6*23* to other genotyped *CYP2A6* variants was determined using Haploview (version 3.32) [31]. We used SIFT and PMUT, bioinformatics programs that predict whether nonsynonymous SNPs will affect enzyme function or structure based on physicochemical properties and evolutionary conservation of the amino acid changes, to examine how *CYP2A6*23* may be affecting enzyme function [32, 33].

Statistics

The catalytic activities of the *in vitro* expressed wildtype and variant enzymes were compared using one-way ANOVA with the Bonferroni correction for post-hoc analyses. In ethnic groups where the allele frequency of *CYP2A6*23* was zero, the score method was used to calculate confidence intervals [34]. A comparison of the *CYP2A6*23* allele frequencies between ethnic groups, differences in the distribution of the *CYP2A6*23* allele in nonsmokers versus smokers, and the Hardy-Weinberg equilibrium were calculated using Fisher's Exact Test. Consistent with previous studies, gender [35] and smoking status [36, 37] were found to affect the rate of nicotine metabolism in our population of Black-African descent, as indicated by the 3HC/COT ratio [22]. Thus, the metabolic ratio was adjusted by dividing the value from each individual with the overall mean from their respective group (e.g. female nonsmokers, male nonsmokers, female smokers, male smokers). The effect of genotype on the adjusted 3HC/COT ratio was examined by a two-tailed independent t-test. A multivariate linear regression model was also used to examine the impact of *CYP2A6*23* genotype on the unadjusted 3HC/COT ratio while controlling for the effect of smoking status and gender. The unadjusted 3HC/COT ratio was not normally distributed according to the Kolmogorov-Smirnov test and was log-transformed for the regression model. All statistical analyses were performed using SPSS (version 14.0 for Windows) and GraphPad Prism (version 2.0 for Windows).

Results

*CYP2A6*23* was found in populations of Black-African descent

Among the individuals of Black-African descent genotyped for *CYP2A6*23* (n = 280), nine heterozygous and one homozygous individuals were found. Thus, *CYP2A6*23* occurred at an allele frequency of 2.0% (95% confidence interval 0.8–3.1%, Table 1); genotype frequencies did not deviate from Hardy-Weinberg equilibrium ($p = 0.821$). Five of the ten individuals with *CYP2A6*23* had other *CYP2A6* variants (Table 2); *CYP2A6*23* was not found in linkage disequilibrium with any of the other *CYP2A6* variants genotyped in this population when analyzed with Haploview (version 3.32) [31], and no other nonsynonymous SNPs were found in linkage with *CYP2A6*23* in the samples sequenced. *CYP2A6*23* was not detected in Caucasian (N = 334 alleles), Chinese (N = 288 alleles) or Japanese individuals (N = 104 alleles) (Table 1). The allele frequency of *CYP2A6*23* in individuals of Black-African descent significantly differed from Caucasians and Chinese ($p < 0.05$, Table 1).

CYP2A6*23 reduced enzyme activity towards nicotine and coumarin *in vitro*

The specific content of CYP2A6 was similar between CYP2A6.1 (0.11 pmol CYP2A6/μg membrane protein), CYP2A6.16 (0.12 pmol CYP2A6/μg of membrane protein) and CYP2A6.17 (0.12 pmol CYP2A6/μg membrane protein), although CYP2A6.23 was expressed at lower levels (0.07 pmol CYP2A6/μg membrane protein) (Fig. 1A). The negative construct did not produce any detectable CYP2A6 protein. Subsequent enzyme assays with the wildtype and variant constructs were performed using equivalent amounts of CYP2A6 (20 nM).

We initially screened the activities of the wildtype and variant constructs towards nicotine and coumarin at two concentrations (Fig. 1B, C). There was a significant difference in activities of the constructs at 30 μM of nicotine ($F = 29.0$, $p < 0.001$), 300 μM of nicotine ($F = 35.9$, $p < 0.001$), and 50 μM of coumarin ($F = 28.3$, $p < 0.01$). Both CYP2A6.23 and CYP2A6.17 had significantly lower activity towards nicotine compared to CYP2A6.1 at 30 μM ($p < 0.01$) and 300 μM ($p < 0.001$) of substrate. CYP2A6.23 also had significantly reduced activity towards 50 μM of coumarin ($p < 0.01$) while CYP2A6.17 retained similar activity as the wildtype enzyme. CYP2A6.16 had similar activities as CYP2A6.1 towards nicotine and coumarin at both concentrations tested (Fig. 1B, C).

We then performed full kinetic analyses on nicotine, our main substrate of interest. CYP2A6.23 and CYP2A6.17 had reduced activity towards nicotine *in vitro* while CYP2A6.16 had similar activity as CYP2A6.1 (Fig. 2). The apparent K_m values did not significantly differ between the wildtype and variant constructs ($F = 3.0$, $p = 0.083$), though it trended towards higher values for CYP2A6.17 ($p = 0.09$, Table 3). However, there was a significant difference in V_{max} ($F = 35.2$, $p < 0.001$) and V_{max}/K_m ($F = 19.0$, $p < 0.001$) between the constructs. V_{max} was significantly lower for CYP2A6.23 ($p < 0.001$) and CYP2A6.17 ($p < 0.01$) compared to CYP2A6.1. Likewise, V_{max}/K_m was significantly lower for CYP2A6.23 ($p < 0.001$) and CYP2A6.17 ($p < 0.01$) compared to CYP2A6.1. There was no significant difference in V_{max} and V_{max}/K_m between CYP2A6.23 and CYP2A6.17, or between CYP2A6.16 and CYP2A6.1.

CYP2A6*23 decreased the rate of nicotine metabolism *in vivo* in a population of Black-African descent

The 3HC/COT is a validated phenotypic measure of CYP2A6 activity and rates of nicotine metabolism [23], with previous studies finding a significant association between the ratio and *CYP2A6* genotype [38–40]. Because the 3HC/COT ratio did not differ significantly from *CYP2A6**1/*1 individuals in this population of Black-African descent, the wildtype group (*1/*1) included individuals with the *CYP2A6**1B allele. The adjusted 3HC/COT ratio was significantly lower in individuals with at least one *CYP2A6**23 allele and no other variants ($n = 5$) in comparison to *CYP2A6**1/*1 individuals ($n = 150$) ($p < 0.04$, Fig. 3). A gene-dose effect was observed such that *CYP2A6**1/*23 heterozygous individuals had ~40% loss in enzyme activity compared to *CYP2A6**1/*1 individuals, while one *CYP2A6**23/*23 homozygous individual did not produce any 3HC (Fig. 3). A similar impact of *CYP2A6**23 was observed in a multivariate linear regression model with the log (3HC/COT) as the dependent variable and including smoking status and gender as predicting

variables ($R^2 = 0.355$, $p < 0.04$). It is notable that the five individuals with *CYP2A6*23* in addition to other genetic variants also had significantly lower 3HC/COT ratios compared to the wildtype group ($p < 0.02$, Table 2).

***CYP2A6*23* was associated with a lower likelihood of being a current adult smoker**

*CYP2A6*23* trended towards a higher frequency in nonsmokers (3.1%, $N = 9/286$ alleles) compared to smokers (0.7%, $N = 2/274$ alleles) ($p = 0.06$, Fig. 4). Individuals with *CYP2A6*23* were approximately 4–5 times less likely to be smokers when all individuals with *CYP2A6*23* were taken into account (odds ratio = 0.23). The magnitude of the effect was the same when analyses were restricted to individuals with only *CYP2A6*1/*1*, *CYP2A6*1/*23*, and *CYP2A6*23/*23* (odds ratio = 0.18, $p = 0.11$).

Discussion

We have identified a novel *CYP2A6* allele, *CYP2A6*23*, which is found in populations of Black-African descent but not in Caucasians, Chinese and Japanese. Two recently described alleles, *CYP2A6*17* and *CYP2A6*20*, have also been identified exclusively in this population [21, 41]. These genetic variants may help explain the reduced rates of nicotine metabolism in individuals of Black-African descent compared to Caucasians [11, 12].

We have demonstrated that *CYP2A6*23* (2161C>T, R203C) impairs both nicotine C-oxidation and coumarin 7-hydroxylation *in vitro*. *CYP2A6.23* had a significantly reduced $V_{\max}$ towards nicotine, and the intrinsic clearance ($V_{\max}/K_m$) was reduced to 19% of the wildtype enzyme. This is in agreement with the observation that *in vivo*, the 3HC/COT ratio is reduced in individuals with the *CYP2A6*23* allele. *CYP2A6*23* may be affecting enzyme function through alteration in substrate binding. Molecular modeling indicates Arg203 may be important in the orientation of Phe209, a residue critical for coumarin binding and possibly involved in nicotine binding [42, 43]. In addition, *CYP2A6.23* had a lower level of expression *in vitro* compared to the other constructs used in this study, which may also contribute to the lower activity observed *in vivo*.

In contrast to *CYP2A6*23* (2161C>T, R203C), *CYP2A6*16* (2161C>A, R203S) did not appear to affect enzyme function. Our *in vitro* data suggests *CYP2A6.16* has similar rates of nicotine and coumarin metabolism as the wildtype enzyme. Furthermore, Nakajima *et al.* recently reported that *CYP2A6*16* did not affect rates of nicotine metabolism *in vivo* [44]. These data suggests the functional impact of *CYP2A6*23* (Cys203) differs from that of *CYP2A6*16* (Ser203). The wildtype residue (Arg203) is positively charged and hydrophilic while Cys203 is neutral and hydrophobic and Ser203 is neutral and hydrophilic. Furthermore, the bioinformatics program SIFT and PMUT [32, 33] predicted the amino acid change in *CYP2A6*23* as not tolerated while *CYP2A6*16* was predicted to be benign.

Interestingly, *CYP2A6*23* impaired nicotine C-oxidation to at least the same extent as *CYP2A6*17* both *in vitro* and *in vivo*. Similar to expressed *CYP2A6.23*, *CYP2A6.17* had a significantly reduced $V_{\max}$ towards nicotine, with $V_{\max}/K_m$ reduced to 30% of the wildtype enzyme. This is in agreement with a previous study where *CYP2A6.17* expressed in *E. coli* did not alter K_m but reduced $V_{\max}$ towards nicotine, with $V_{\max}/K_m$ reduced to 40% of the

wildtype construct [21]. *In vivo*, the 3HC/COT ratio in heterozygous individuals for *CYP2A6*17* is reduced to approximately 55% of wildtype activity, while homozygous individuals of *CYP2A6*17* had approximately 9% of wildtype activity. This is consistent with previous studies using the COT/NIC ratio as a biomarker [44], and together these data strongly suggest the *CYP2A6*17* allele results in reduced nicotine metabolism. We also observed that the impact of *CYP2A6.17* is substrate-dependent such that it did not differ in coumarin 7-hydroxylation compared to the wildtype enzyme. Accordingly, a previous study found *CYP2A6.17* expressed in *E. coli* had an increased K_m but no change in V_{max} towards coumarin [21].

A trend was observed where individuals with *CYP2A6*23* were less likely to be current adult smokers. Several case-control studies have associated *CYP2A6* genetic variations leading to impaired activity with a lower likelihood of smoking [45–47], though negative findings have also been reported [48–50]. A greater understanding of *CYP2A6* genetic variation through identification and characterization of novel variants, particularly among different ethnic groups, will allow for better replication of genetic case-control association studies.

In summary, we have discovered a novel *CYP2A6* allele, occurring predominantly in a population of Black-African descent, which impairs enzyme activity *in vitro* and *in vivo* and may be associated with a lower risk of smoking. An understanding of the genetic factors that contribute to nicotine pharmacokinetics in populations of Black-African descent is important given their unique smoking patterns and higher incidence of tobacco-related illnesses.

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A) Immunoblot

B) Nicotine C-oxidation

C) Coumarin 7-hydroxylation

Figure 1. CYP2A6.23 had substantially reduced catalytic activity towards nicotine and coumarin *in vitro*

A) Immunoblot shows the expression of CYP2A6 protein from the wildtype and variant constructs. The amount of total membrane protein loaded is as labeled and ranged from 0.5–2.0 µg for CYP2A6.1, CYP2A6.16 and CYP2A6.17, while CYP2A6.23 was loaded at 0.75–4.0 µg. Detection of CYP2A6 constructs was linear by immunoblotting at lower amounts loaded, and was used to calculate the amount of CYP2A6 detected by comparison to the standard (not shown). **B)** CYP2A6.17 and CYP2A6.23 have reduced cotinine formation from nicotine (30 and 300 µM) while CYP2A6.16 had similar activity as CYP2A6.1. **C)** CYP2A6.23 had reduced 7-hydroxycoumarin formation from coumarin (5 and 50 µM) while CYP2A6.16 and CYP2A6.17 had similar activity as CYP2A6.1. No product formation was found using the negative construct. The data is presented as mean ± SEM for nicotine C-oxidation (n = 3) and coumarin 7-hydroxylation (n = 2). * $p < 0.01$, ** $p < 0.001$ when compared to CYP2A6.1.

Figure 2. CYP2A6.17 and CYP2A6.23, but not CYP2A6.16, have reduced *in vitro* nicotine C-oxidation

A representative plot is shown, and the curve was fitted to the Michaelis-Menten equation using non-linear regression in GraphPad Prism (version 2.0).

Figure 3. *CYP2A623 decreased the rates of nicotine metabolism *in vivo*, as measured by the 3HC/COT ratio**

The number of individuals in each genotype group is shown in parentheses on the x-axis. The data is presented as mean ± SD. Individuals with other *CYP2A6* genetic variants (*CYP2A6**2, *4A & D, *9, *12, *14, *15, *17, *20, *21, *24, *25, *26, *27, *28, *29) were excluded from the wildtype and *CYP2A6**23 genotype groups.

Figure 4. Individuals with the *CYP2A623 allele trended to having a lower likelihood of being current smokers**

The allele frequency of *CYP2A6**23 was calculated from the 280 genotyped individuals in the study, including 143 nonsmokers and 137 smokers.

Table 1Allele frequency of *CYP2A6*23* by ethnicity

Ethnicity	Allele frequency (%)	Total # of alleles	95% Confidence Intervals	p-values ^a
Black-African descent	2.0	560	0.8–3.1%	---
Caucasian	0	334	0–1.1%	0.01
Chinese	0	288	0–1.3%	0.02
Japanese	0	104	0–3.6%	0.23

^aThe allele frequency of *CYP2A6*23* in each ethnic group was compared against the value found in individuals of Black-African descent.

Table 2

*CYP2A6**23 genotype groups and their mean adjusted 3HC/COT ratio

Allele	Genotype	n	Mean adjusted 3HC/COT	SD	% of wildtype	p-values ^a
*23	*1/*1	150	1.210	0.634	100	0.04
	*1/*23	4	0.756	0.540	62.4	
	*23/*23	1	0	0	0	
*17	*1/*1	150	1.210	0.634	100	<0.001
	*1/*17	19	0.672	0.468	55.5	
	*17/*17	3	0.109	0.095	9.1	
More than one variant	*1/*1	150	1.210	0.634	100	0.02
	*17/*23	2	0.259	0.366	21.4	
	*20/*23	2	0.555	0.512	45.8	
	*9/*23	1	1.032	0	85.2	

^aThe adjusted 3HC/COT ratio was compared between wildtype individuals (*CYP2A6**1/*1) to other genotype groups combined using a two-tailed independent t-test.

Table 3

Kinetic parameters of CYP2A6 wildtype and variant constructs for nicotine

	K_m (μM) ^a	V_{max} ($\mu mol \cdot min^{-1} \cdot \mu mol$ CYP2A6 ⁻¹)	V_{max}/K_m ($nL \cdot min^{-1} \cdot \mu mol$ CYP2A6 ⁻¹)
CYP2A6.1	58.3 ± 6.5	5.5 ± 0.5	97.3 ± 11.6
CYP2A6.16	73.6 ± 6.1	5.1 ± 0.2	71.4 ± 7.7
CYP2A6.17	88.9 ± 6.8	2.6 ± 0.2 *	29.6 ± 4.2 *
CYP2A6.23	77.7 ± 10.4	1.4 ± 0.2 **	18.4 ± 2.3 **

^a Kinetic parameters were calculated using non-linear regression in GraphPad Prism (version 2.0). Data is presented as mean ± SEM of parameters calculated from three or four independent experiments.

* $p < 0.01$,

** $p < 0.001$ when compared to CYP2A6.1.