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Variation in P450 oxidoreductase (*POR*) A503V and flavin containing monooxygenase (*FMO*)-3 E158K is associated with minor alterations in nicotine metabolism but does not alter cigarette consumption

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Abstract

Nicotine metabolism rates differ widely, even after controlling for genetic variation in the major nicotine metabolizing enzyme, CYP2A6. Genetic variants in an additional nicotine metabolizing enzyme, flavin containing monooxygenase (*FMO*)-3, and an obligate microsomal CYP-supportive enzyme, cytochrome P450 oxidoreductase (*POR*), were investigated. We examined the impact of *FMO3* E158K and *POR* A503V, before and after stratifying by *CYP2A6* metabolism group. In 130 non-smokers of African descent who received 4 mg oral nicotine, *FMO3* 158K trended towards slower nicotine metabolism in reduced *CYP2A6* metabolizers ($P=0.07$) only, whereas *POR* 503V was associated with faster *CYP2A6* activity (nicotine metabolite ratio) in normal ($P=0.03$), but not reduced, *CYP2A6* metabolizers. Neither *FMO3* 158K nor *POR* 503V significantly altered the nicotine metabolic ratio ($N=659$), cigarette consumption ($N=667$), or urine total nicotine equivalents ($N=418$) in smokers of African descent. Thus, *FMO3* E158K and *POR* A503V are minor sources of nicotine metabolism variation, insufficient to appreciably alter smoking.

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Keywords

Tobacco; smoking; nicotine; metabolism; African Americans; genetic association studies

Introduction

Smoking remains a leading preventable cause of morbidity and mortality [1], with African North Americans displaying disproportionately elevated disease risk despite smoking fewer cigarettes compared to Caucasians [1]. Nicotine is primarily inactivated by CYP2A6 to cotinine (COT), which undergoes further CYP2A6-mediated metabolism to 3'-hydroxycotinine (3HC), and 3HC/COT, a phenotypic measure of CYP2A6 activity, is highly correlated with nicotine clearance [2]. *CYP2A6* variants such as *CYP2A6**2, *4, *9, *12, *17, *20, *23-*28, and *35 (each with ~7% frequency in African North Americans [2-4]) that reduce CYP2A6 activity, lead to lower nicotine clearance and 3HC/COT [3, 4], and influence smoking [2]. Less is known regarding contributions of other enzymes to nicotine clearance, smoking, and tobacco-related disease.

Flavin-containing monooxygenase (FMO)-3 catalyzes hepatic nicotine N'-oxidation, a pathway responsible for ~4-7% of nicotine urinary recovery, but up to 30% in individuals with deleted *CYP2A6* [5], suggestive of a greater reliance on FMO3-mediated nicotine metabolism when CYP2A6 activity is reduced. Variation in *FMO3*, which may impact nicotine metabolism, is associated with altered ratios of COT/(COT + nicotine) and cigarette consumption in Caucasians [5]. We extend this investigation by examining the impact of *FMO3* E158K (rs2266782 G>A), a variant with reduced FMO3 activity (e.g. for benzydamine and ranitidine [reviewed in 6]), on systemic nicotine metabolism and smoking in populations of African descent. Here the FMO3 pathway may be more important for nicotine clearance, relative to Caucasians, due to both higher frequencies of *FMO3* 158K (~48% vs. 39% in Caucasians [reviewed in 6]) and a greater prevalence of reduced activity *CYP2A6* alleles [3, 4].

We also examined the impact of genetic variation in cytochrome P450 oxidoreductase (POR), a coenzyme required for microsomal CYP-mediated drug metabolism [7], on CYP2A6 activity and smoking. POR donates electrons to CYPs, and several variants in *POR* are associated with altered CYP activity, including reduced CYP3A4 and CYP2D6 activity [reviewed in 7]. *POR* A503V (rs1057868 C>T) (~19% frequency in African Americans; ~25-30% in Caucasians [7]), is associated with CYP- and substrate-specific effects on drug metabolism *in vitro* and higher CYP3A-mediated midazolam metabolism *in vivo* [reviewed in 7]. We hypothesized that *POR* 503V will increase nicotine and cotinine metabolism.

Materials and methods

Study 1. Nicotine pharmacokinetics, following 4 mg oral nicotine, was studied in African Canadians [8]. The area under the plasma nicotine-concentration time curve (AUC) was estimated over 360 minutes, and 3HC/COT was determined at 270 minutes [8]. Participants were excluded if they had unreliable kinetic or *CYP2A6* genetic data. The final sample utilized kinetic data from 130 non-smokers, and cigarette consumption data from 133

smokers. Study 2. Total nicotine equivalents were determined as the molar sum of nicotine and nine major metabolites in urine, corrected for creatinine, in 534 African American light smokers (10 cigarettes/day) [9]. DNA was extracted from blood samples for genotyping *CYP2A6* [8], *FMO3* and *POR*. Demographic characteristics of participants were published previously [8, 9].

CYP2A6 genotyping was performed using validated two-step gene and allele-specific PCR [8]. *CYP2A6* variants predicted to reduce *CYP2A6* activity included: *2, *4, *9, *17, *20, *23, *25-*28, and *35 [3, 4, 10]. Rare alleles *12 and *24 were also genotyped in study 1 [3]. Individuals with 1 variant were grouped as “reduced *CYP2A6* metabolizers”; individuals without were grouped as “normal *CYP2A6* metabolizers”. *FMO3* E158K and *POR* A503V were genotyped using allele-specific TaqMan SNP genotyping assays (Applied Biosystems, Foster City, USA) with ~99% call confidence. Studies were approved by the University of Toronto ethics board, Institutional Review Board, University of Kansas human subjects committee, and the University of California San Francisco human subjects committee.

Hardy-Weinberg equilibrium (HWE) was tested using chi-square tests. Shapiro-Wilk tests were used to determine data normality. Mann-Whitney U tests (two-tailed) were used to compare outcomes between two genotype groups. Analyses were performed using SPSS, version 21 (IBM, Armonk, USA).

Results

FMO3 158K frequencies were 41.9% and 45.9% in Study 1 and Study 2, respectively. *POR* 503V frequencies were 16.5% and 17.8% in Study 1 and Study 2, respectively. *FMO3* E158K and *POR* A503V frequencies, consistent with previous reports [6, 7], were in HWE.

We first examined associations between *FMO3* E158K (G>A) variation and nicotine pharmacokinetics in Study 1 non-smokers that received oral nicotine, using plasma nicotine AUC as the outcome measure. No association between *FMO3* E158K and nicotine AUC was observed in all subjects or in normal *CYP2A6* metabolizers (Figure 1A, B). However, in reduced *CYP2A6* metabolizers, there was a trend toward higher nicotine AUC in the *FMO3* GA/AA vs. GG group (mean=2.0 vs. 1.4 µg/ml·min, respectively; P=0.07) (Figure 1C).

In contrast to *FMO3*, *POR* likely influences nicotine metabolism indirectly through supporting *CYP2A6* activity. Therefore we used 3HC/COT, a marker of *CYP2A6* activity, to assess associations between *POR* A503V and nicotine metabolism. No association between *POR* A503V and 3HC/COT was observed in all subjects or in reduced *CYP2A6* metabolizers (Figure 1D, F). However, in normal *CYP2A6* metabolizers, 3HC/COT was higher in the *POR* CT/TT relative to CC group (mean=0.25 vs. 0.18, respectively; P=0.03) (Figure 1E), suggesting faster *CYP2A6* activity supported by the *POR* T allele. This was insufficient to significantly alter nicotine AUC in the *POR* CT/TT relative to CC group (mean=0.9 vs. 1.3 µg/ml·min, respectively; P=0.21). When examining baseline plasma 3HC/COT from 659 smokers, no association with *POR* A503V was observed in the total group (P=0.63), or in reduced (P=0.89) or normal (P=0.66) *CYP2A6* metabolizers. Similar

relationships were observed for analyses of CT individuals alone, as were seen for the CT/TT group. Moreover, in 44 Caucasian livers with wild type *CYP2A6*, there was no association between *POR* A503V and Vmax (mean=30.2 vs. 30.7 nmol/mg protein/hour in *POR* CC (N=22) vs. CT/TT (N=22), respectively; P=0.45) or Km (mean=55.6 vs. 58.7 μ M in *POR* CC vs. CT/TT, respectively; P=0.81) in nicotine C-oxidation assays [described in 10]. We also found no significant relationship between *POR* rs17148944 (G>A), associated with reduced *POR* mRNA expression [11], and nicotine AUC (mean=1.5 vs. 1.2 μ g/ml·min in *POR* GG (N=118) vs. GA (N=12), respectively; P=0.58) or 3HC/COT (mean=0.16 vs. 0.21 in GG (N=118) vs. GA (N=12), respectively; P=0.15) in African Canadian non-smokers.

Regression analyses (number of minor allele copies entered as the predictor) indicated that *FMO3* E158K accounted for <1% and ~4% of the variation in nicotine AUC in normal and reduced *CYP2A6* metabolizers, respectively, while *POR* A503V accounted for ~6% and ~1% of the variation in 3HC/COT in normal and reduced *CYP2A6* metabolizers, respectively.

In contrast to the modest impact of *FMO3* E158K and *POR* A503V on nicotine metabolism, *CYP2A6* was strongly associated with differences in nicotine AUC and 3HC/COT. Relative to normal *CYP2A6* metabolizers, reduced *CYP2A6* metabolizers displayed 50% higher nicotine AUC (mean=1.8 vs. 1.2 μ g/ml·min, respectively; P<0.001) and 40% lower 3HC/COT (mean=0.12 vs. 0.20, respectively; P<0.001).

When examining relationships between *FMO3* E158K (G>A) and *POR* A503V (C>T) with cigarettes smoked per day (CPD) and urine nicotine equivalents (reflecting daily nicotine intake), a trend toward lower CPD was noted in the *FMO3* GA/AA group, relative to the *FMO3* GG group (mean=7.5 vs. 10.1, respectively; P=0.05) among African Canadian reduced *CYP2A6* metabolizers, but this was not replicated in the African American smokers (Table 1). No other associations were observed between *FMO3* E158K and *POR* A503V with CPD, and neither SNP was associated with differences in total nicotine equivalents (Table 1).

Discussion

Our findings suggest *FMO3* E158K and *POR* A503V modestly influence systemic nicotine metabolism within *CYP2A6* sub-groups, but do not appreciably alter CPD or daily nicotine intake. The trend toward lower CPD with *FMO3* 158K in African Canadian reduced *CYP2A6* metabolizers was not replicated in African American smokers. While these latter smokers consumed ~10 CPD (a clinical trial inclusion criteria), their total nicotine equivalents values are similar to those reported in Caucasian heavier smokers [1], suggesting similar levels of nicotine intake due to greater smoking intensity.

In European American heavy smokers (~20-25 CPD) variation in *FMO3* haplotype was reported to be associated with CPD, although the effect (~3 CPD difference) was restricted to individuals predicted to have faster *CYP2A6* activity [5]. Discrepancies between these findings and ours may reflect differences in study design related to *CYP2A6/FMO3*

grouping strategies, haplotype vs. allele assessments, and/or heterogeneity in study populations attributable to ethnicity and heaviness of smoking.

Six human *FMO* genes exist, encoding five functional FMO enzymes [6]. FMO1 is the major fetal hepatic FMO isoform, but in adults is expressed in extra-hepatic tissues, likely including brain [12]. FMO1 also catalyzes nicotine metabolism [12], and variation in *FMO1* is associated with nicotine dependence, perhaps through altered brain metabolism of nicotine [12]; further study of associations between this enzyme family and smoking behaviors may be warranted.

In conclusion, *FMO3* E158K and *POR* A503V do not substantially impact nicotine metabolism in African North Americans, consistent with the lack of effect on daily smoking or nicotine intake. Although African Americans smoke fewer cigarettes than Caucasians, they appear to achieve similar levels of exposure to nicotine and experience a disproportionately higher prevalence of tobacco-related diseases [1]. Future investigations probing the genetic and environmental determinants of tobacco consumption and tobacco-related diseases among populations of African descent are warranted.

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Figure 1. Association of *FMO3* E158K and *POR* A503V variation with nicotine metabolism in African Canadian non-smokers that received 4 mg oral nicotine

One hundred and thirty adult African Canadian non-smokers received 4 mg oral nicotine and were genotyped for *CYP2A6*, *FMO3* E158K, and *POR* A503V. Nicotine AUC according to *FMO3* E158K (G>A) genotype group (GG vs. GA + AA) is shown in the total group (A), in individuals with normal *CYP2A6* genotype (B), and in individuals with reduced *CYP2A6* genotype (C). The 3HC/COT ratio according to *POR* A503V (C>T) genotype group (CC vs. CT + TT) is shown in the total group (D), in individuals with normal *CYP2A6* genotype (E), and in individuals with reduced *CYP2A6* genotype (F). All P values indicated were derived from Mann-Whitney U tests comparing *FMO3* GG and *FMO3* GA + AA groups, or comparing *POR* CC and *POR* CT + TT groups. The number of participants fluctuates slightly between *FMO3* and *POR* genotype analyses, reflecting the number of participants for whom the allele was successfully genotyped and included in the analyses.

Table 1

Cigarette consumption according to *FMO3* E158K (G>A) genotype and *POR* A503V (C>T) genotype in African Canadian and African American smokers

Genotype Group	Total group	Normal <i>CYP2A6</i>	Reduced <i>CYP2A6</i>
<u>Mean cigarettes per day ($\pm$ SD; N) in Study 1 African Canadian Smokers (N=133^a)</u>			
<i>FMO3</i> E158K			
GA + AA	9.4 (5.7; 85)	10.5 (6.5; 53)	7.5 (3.3; 32)
GG	9.3 (5.0; 46)	8.9 (5.0; 29)	10.1 (5.2; 17)
P value ^b	.81	.30	.05
<i>POR</i> A503V			
CT + TT	9.0 (5.4; 47)	9.3 (5.8; 31)	8.5 (4.6; 16)
CC	9.5 (5.4; 86)	10.2 (6.1; 53)	8.4 (4.0; 33)
P value ^c	.41	.29	.97
<u>Mean cigarettes per day ($\pm$ SD; N) in Study 2 African American Smokers (N=534^a)</u>			
<i>FMO3</i> E158K			
GA + AA	7.9 (2.5; 369)	8.1 (2.5; 193)	7.8 (2.5; 176)
GG	7.9 (2.6; 161)	8.1 (2.9; 73)	7.8 (2.4; 88)
P value ^b	.84	.93	.85
<i>POR</i> A503V			
CT + TT	7.8 (2.5; 160)	8.1 (2.6; 78)	7.5 (2.4; 82)
CC	8.0 (2.5; 373)	8.1 (2.6; 191)	7.9 (2.5; 182)
P value ^c	.31	.87	.20
<u>Mean total nicotine equivalents^d ($\pm$ SD; N) in Study 2 African American Smokers (N=418^a)</u>			
<i>FMO3</i> E158K			
GA + AA	59.1 (60.9; 292)	60.5 (40.7; 153)	57.6 (77.4; 139)
GG	56.5 (45.7; 122)	58.4 (58.1; 56)	54.9 (32.0; 66)
P value ^b	.88	.45	.26
<i>POR</i> A503V			
CT + TT	54.3 (36.7; 128)	54.4 (37.5; 64)	54.1 (36.2; 64)
CC	60.2 (63.5; 289)	62.5 (48.8; 148)	57.9 (76.1; 141)
P value ^c	.50	.22	.73

^aThe number of participants fluctuates slightly between *FMO3* and *POR* genotype analyses, reflecting the number of participants for whom the allele was successfully genotyped and included in the analyses

^bDerived from Mann-Whitney U test (*FMO3* GG group vs. *FMO3* GA + AA groups)

^cDerived from Mann-Whitney U test (*POR* CC group vs. *POR* CT + TT groups)

^dMeasured in nmol/mg creatinine