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Common polymorphisms in *FMO1* are associated with nicotine dependence

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Abstract

BACKGROUND—Cigarette smoking and other forms of tobacco use are the leading cause of preventable mortality in the world. A better understanding of the etiology of nicotine addiction may help increase the success rate of cessation and decrease the massive morbidity and mortality associated with smoking.

METHODS—In order to identify genetic polymorphisms that contribute to nicotine dependence, our group undertook a genetic association study including three enzyme families that potentially influence nicotine metabolism: cytochrome P450 enzymes (CYP P450s), flavin monooxygenases (FMOs) and UDP-glucuronosyl transferases (UGTs).

RESULTS—Several polymorphisms in *FMO1* showed association in a discovery sample and were tested in an independent replication sample. One polymorphism, rs10912765, showed association that remained significant after Bonferroni correction (nominal p=0.0067, corrected p=0.0134). Several additional polymorphisms in linkage disequilibrium with this SNP also showed association. Subsequent *in vitro* experiments characterized FMO1 as a more efficient catalyst of nicotine *N*-oxidation than FMO3. In adult humans, FMO1 is primarily expressed in the kidney and is likely to be a major contributor to the renal metabolism and clearance of therapeutic drugs. FMO1 is also expressed in the brain and could contribute to the nicotine concentration in this tissue.

CONCLUSIONS—These findings suggest that polymorphisms in *FMO1* are significant risk factors in the development of nicotine dependence and that the mechanism may involve variation in nicotine pharmacology.

Keywords

FMO1; nicotine dependence; nicotine metabolism

INTRODUCTION

Cigarette smoking and other forms of tobacco use are the leading cause of preventable mortality in the world, and increasing global use of tobacco is expected to cause 175 million deaths between now and the year 2030 [1]. There is variable susceptibility to the addictive

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quality of nicotine, and part of the vulnerability to nicotine addiction is hypothesized to be related to the rate of nicotine metabolism. A number of studies have suggested that polymorphisms in the genes encoding nicotine metabolizing enzymes affect a variety of smoking behaviors including nicotine addiction [2, 3]. In addition, nicotine clearance shows considerable individual variability and a high heritability [4]. Three enzyme families contribute to nicotine metabolism: cytochrome P450 enzymes (P450s), flavin monooxygenases (FMOs) and UDP-glucuronosyl transferases (UGTs). Although the P450 CYP2A6 is considered the primary catalyst of nicotine metabolism, individual variability in nicotine metabolism cannot be explained completely by the known variants in *CYP2A6* [4]. Thus, the other enzyme families such as FMO and UGT may play an important role.

In order to identify genetic polymorphisms that contribute to nicotine dependence, our group undertook a large-scale genetic association study, which consisted of genome wide association and candidate gene studies performed simultaneously [5, 6]. Selection of candidate genes for nicotine dependence was based on several factors, including genes that may affect any aspect of smoking. Genes that potentially influence nicotine metabolism, including *CYP2A6, CYP2B6*, the *FMO* gene family, and the *UGT* gene family, were included in the candidate gene association study. Subsequent to the prior report [6] we have performed additional genotyping in candidate genes and have conducted a formal replication study based on hypotheses generated from the COGEND component of the initial sample and the additional genotyping. Here we report on the association analyses of the nicotine metabolism gene variants with nicotine dependence and follow up in an independent sample.

METHODS

Subjects

Two studies contributed to the genetic analyses. The first study consisted of individuals of European descent recruited from the United States as part of the Collaborative Genetic Study of Nicotine Dependence (COGEND) [5, 6]. This study was approved by the Institutional Review Board (IRB) at each data collection site. The second sample, a replication study, came from the American Cancer Society (ACS) [7]. This research was reviewed and approved by the Emory University IRB. Subjects provided informed consent in both studies.

A total of 1610 individuals of European descent were included from COGEND. Subjects' smoking behavior was assessed using the Fagerström Test for Nicotine Dependence (FTND) [8]. Nicotine dependent cases were defined as current smokers with an FTND score of four or greater, and non-dependent smoking controls were required to have smoked at least 100 cigarettes in their lifetime, but to have never had an FTND score greater than zero [5, 6]. We also tested a quantity-frequency variable, cigarettes per day (CPD). This was based on self reported heaviest smoking and coded as 0 (0–10 cigarettes per day), 1 (11–20 cigarettes per day), 2 (21–30 cigarettes per day), and 3 (31+ cigarettes per day).

The replication study consisted of 2844 participants in the American Cancer Society CPS-II Nutrition Cohort (ACS). Again, only individuals of European descent were included. Though nicotine dependence was not assessed using standard tools, proxy phenotypes of heavy smoking cases and light smoking controls were developed which correspond to nicotine dependent smokers and non-dependent smokers as detailed elsewhere [7]. All subjects reported smoking more than 100 cigarettes lifetime. Heavy smoking cases smoked at least 30 cigarettes/day for at least five years. In contrast, controls smoked for at least one year during their lifetime. Because different smoking questions were asked in the assessments, somewhat different thresholds were used. In the 1982 and 1992 surveys, control subjects were selected based on reporting fewer than 5 cigarettes per day, and in the

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1997 survey, subjects were chosen if reporting smoking fewer than 10 cigarettes per day (the lowest amount in this survey).

Genotyping

Genotyping was performed on a variety of platforms. Genotyping on the discovery sample was performed by Perlegen using custom high-density oligonucleotide arrays on the Affymetrix platform [5, 6]. A total of 35,382 SNPs were individually genotyped. Of these, 3,713 SNPs were selected for exon-based coverage of a variety of candidate genes [6]. An additional 31,669 SNPs were selected for individual genotyping based on the results of a preliminary genome wide association study (GWAS) using pooled samples [5].

Additional genotyping on the COGEND sample was performed by the Center for Inherited Disease Research (CIDR) using an Illumina Golden Gate custom assay. This genotyping included SNPs for improved coverage of the candidate genes. From this genome wide association, candidate gene, and improved coverage genotyping, we selected 194 SNPs that cover the genes of interest: 16 SNPs in *CYP2A6* and *CYP2B6*; 64 SNPs in the *FMO* gene family; and 114 SNPs in the *UGT* gene family. Complete details are presented in Supplementary Table 1.

For the ACS sample, genotyping was performed by CIDR using an Illumina Golden Gate custom assay for 1536 SNPs. This genotyping was for follow up of the genome wide association and candidate gene study in COGEND. Of the 194 SNPs genotyped in the COGEND samples, 5 SNPs were selected for replication testing of the nicotine metabolizing genes in the ACS sample.

We applied a standard cleaning protocol to all platforms to insure high quality genotyping results. We required SNPs to have a call rate of at least 98%, a HWE p-value greater than 10^{-4} for all subjects as well as cases and controls considered separately, and a minor allele frequency of 1% or greater as measured in the entire sample.

In vitro FMO1 catalyzed nicotine metabolism

To determine the extent to which human FMO1 metabolizes nicotine, the kinetic parameters of FMO1 catalyzed nicotine *N*-oxidation were compared to the FMO3 catalyzed reaction. Human FMO1 or FMO3 Supersomes™(BD Bioscience Woburn MA) were incubated with [5′-^{3}H]-(S)- nicotine (10 – 2000 μM, S.A = 0.01 – 3 μCi/nmol), and an NADPH generating system in 50 mM potassium phosphate buffer, pH 8.5 for 10 to 30 min at 37 °C with shaking. Microsomal protein concentrations ranged from 0.04 to 0.08 μg/ml and the reaction was stopped by the addition of 15 % TCA (30 μl). The samples were analyzed for total nicotine N-oxidation by reverse phase HPLC with radio-flow detection on System 1. To determine the ratio of *cis* to *trans* nicotine *N*-oxide, the *N*-oxide peak was collected from System 1 and analyzed on System 2. Standard *cis* and *trans* nicotine–*N*-oxide were co-injected with all samples. HPLC System 1 consisted of a Gemini C18 column (Phenomenex, Torrance, CA) with a mobile phase of 20 mM ammonium bicarbonate, pH 10.5 (A) and acetonitrile (B). The mobile phase was held at initial conditions 99% A: 1% B for 5 min followed by a gradient to 30 % B in 25 minutes. Flow rate was 1 ml/min. Nicotine eluted at 29.4 min and the nicotine *N*-oxides eluted at 10.0 min. HPLC System 2 consisted of a Luna C18 column (Phenomenex, Torrance, CA) using an isocratic mobile phase consisting of 0.2% TFA in water. The flow rate was 0.7 ml/min. c*is*-Nicotine *N*-oxide eluted at 12.0 minutes and *trans* *N*-oxide at 13.6 minutes.

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Analysis

Our statistical design is based on an initial discovery stage in the COGEND sample where we identify nominally significant polymorphisms, followed by a formal replication stage in an independent sample using the Li and Ji method to control for multiple testing [9]. Although some research suggests that a joint analysis is more powerful than replication [10], this is based on a genome wide association study where a large proportion of the samples are genotyped in stage 1 and a large proportion of the SNPs are genotyped in stage 2. In the present circumstance, we have a candidate gene study in stage 1 and a substantially larger stage 2 sample with very limited genotyping.

First, we verified self reported ethnicity in both the COGEND and ACS samples using the programs Structure and EIGENSTRAT [11, 12].

Association testing for nicotine dependence (or heavy versus light smoking) was performed using logistic regression with covariates. The model included an indicator variable for gender, age as a linear covariate, and the SNP genotype coded as 0, 1 or 2 based on the number of minor alleles (corresponding to a multiplicative effect). The p-value for each SNP was a 1-degree of freedom likelihood ratio test generated by comparing the full model to a model without the SNP covariate term. Association testing for CPD in the COGEND sample was performed using linear regression with an indicator variable for gender and age as a linear covariate.

In order to determine the appropriate correction for multiple testing in the replication sample, we used the method of Li and Ji [9]. This method uses an eigenvalue decomposition of the SNP correlation matrix to compute the effective number of tests (M_{eff}). The method is implemented in an updated version of SNPSpD [13]. We also computed LD bins and tagging SNPs for the bins. A bin is a collection of correlated SNPs with one or more "tag SNPs." Tag SNPs have the property of having a correlation (r^2) of 0.8 or greater with all SNPs in the bin.

RESULTS

Association tests in the discovery sample revealed 10 SNPs with an uncorrected p-value less than 0.05 (Table 2). The associated genes were *FMO1, FMO3*, and *FMO4,* which cluster on chromosome 1, and *UGT2A1, UGT2A2*, and *UGT2A3*, which cluster on chromosome 4. Several of the associated SNPs are correlated. We did not identify any variants in *CYP2A6* that were associated with nicotine dependence. Results for all SNPs are in the Supplementary Table 1. Had we used the CPD association test to select SNPs for genotyping in the replication sample, we would have selected 6 SNPs, including 5 of the 10 SNPs identified through case/control association (Table 2).

To test for replication in the independent ACS sample, five SNPs (two correlated signals) were followed up with genotyping (Table 2). By genotyping only five SNPs, we reduce the multiple testing issues and focus only on the best candidates. Only two correlated SNPs, rs10912675 and rs7877, were associated ($p < 0.05$) in the replication sample that compared heavy and light smokers.

To further aid in the interpretation of the significance of the results, we determined the number of effective tests based on the correlation between the SNPs. The results of the eigenvalue decomposition reveal that of the 194 SNPs in the discovery sample, there are 89 effective independent tests. In the replication sample, the 5 SNPs provide 2 effective independent tests.

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To follow up the significant association with the SNP in *FMO1*, we characterized the metabolism of nicotine by the extrahepatic enzyme FMO1 and compared it to FMO3-catalyzed metabolism. Interestingly, FMO1 metabolized nicotine more efficiently than did FMO3. FMO1 followed classic Michaelis-Menten kinetics with a K_M of 1.2 mM and a V_{max} of 35 nmol/min/mg protein (1 nmol FAD/mg protein). However, at a concentration of 2 mM nicotine the rate of FMO3-catalyzed *N*-oxidation was still increasing linearly. At 1.2 mM nicotine the FMO3-catalyzed rate of *N*-oxidation was 9.2 nmol/min/mg protein (0.87nmol flavin/mg protein), 3.8-fold lower than the rate of FMO1. FMO1-catalyzed metabolism generated both *cis* and *trans* nicotine-*N*-oxide (in a 55:45 ratio) whereas the product of FMO3-catalyzed metabolism was predominately (>95%) *trans*-nicotine *N*-oxide.

DISCUSSION

This large study of nicotine dependent smokers and non-dependent smokers tested the hypothesis that variants in nicotine metabolizing genes are associated with nicotine dependence. This hypothesis is based on the theory that nicotine metabolism, which varies between individuals, can influence smoking behaviors and alter the risk of developing nicotine dependence. We studied polymorphisms in genes that potentially metabolize nicotine: *CYP2A6, CYP2B6*, the *FMO* gene family, and the *UGT* gene family.

A number of polymorphisms in *FMO* gene family members showed association with nicotine dependence in our discovery sample. We followed 5 of these polymorphisms into a replication sample of heavy and light smokers and a significant association remained with 2 of these SNPs. Two highly correlated SNPs in *FMO1* (rs7877, rs10912675) show significant association in the replication sample after Bonferroni correction (nominal p-values 0.0192 and 0.0067, respectively; corrected p-values 0.0384 and 0.0134, respectively). In both samples, these SNPs have similar risks of developing nicotine dependence or heavy smoking (OR of 0.77 and 0.80 for nicotine dependence, respectively; OR of 0.87 and 0.85 for heavy smoking, respectively). These two SNPs are located in the 3′ UTR and 5′ UTR of *FMO1*, respectively, suggesting a potential role for regulation of gene expression. These SNPs are not known to be in high LD with any exonic variants and their exact function is unclear. Had we used association tests on the CPD measure in the initial sample (more directly comparable to the phenotype in the replication sample), we would have followed up many of the same SNPs and would have tagged the bin that shows replication.

Because human FMO1 was not known to metabolize nicotine, we determined the catalytic efficiency of nicotine *N*-oxidation by this enzyme. *In vitro* experiments established that human FMO1, like pig FMO1 [14] catalyzes the non-stereospecific N-oxidation of nicotine. Contrary to conventional wisdom, FMO1, an extrahepatic enzyme in humans, is a better catalyst of nicotine *N*-oxidation than the hepatic enzyme, FMO3. Although the K_M of FMO1 is relatively high, it is lower than that of FMO3. We also note that the K_M for CYP2A6 is 140 μM, also well above the plasma concentration of nicotine [15].

In adult humans, FMO1 is an extrahepatic enzyme with relatively high levels of expression in the kidney and shows moderate inter-individual variability in protein levels [16]. FMO1 is likely to be a major contributor to renal metabolism and clearance of therapeutic drugs [16]. However, we report here that FMO1-catalyzed nicotine metabolism results in the formation of approximately equal amounts of *cis* and *trans* nicotine *N*-oxide, whereas only *trans* nicotine *N*-oxide has been detected in the urine of smokers [14]. This suggests that renal FMO1 does not contribute significantly to the formation of nicotine *N*-oxide excreted by smokers. However, FMO1 may play a role in nicotine metabolism in other extrahepatic tissues. FMOs are expressed in the human brain [17] and may contribute to the level of nicotine present in this organ. One of the two or more FMOs present in the human brain has

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been purified and partially characterized [18]. This brain FMO, based on substrate specificity, is likely FMO1 because it catalyzes the *N*-oxidation of imipramine, an FMO1 mediated reaction [19]. If FMO1 activity catalyzed the *N*-oxidation of nicotine in the brains of smokers, the nicotine *N*-oxide formed could serve as a substrate pool available for reduction back to nicotine. The reduction of the *N*-oxide of tertiary amines has been suggested to play a role in the pharmacology of both imipramine and tamoxifen [20, 21].

There are some limitations to this study that need to be noted. While our work highlights the potential role of *FMO1* in the development of nicotine dependence, heavy smoking, and nicotine metabolism, we have not shown an association of the polymorphisms in *FMO1* with nicotine metabolism. Nor have we demonstrated a biological mechanism by which these polymorphisms contribute to nicotine metabolism. Secondly, although the SNPs associated with nicotine dependence lie in *FMO1,* highly correlated SNPs span the entire cluster of *FMO* genes, indicating that causal variant(s) may be in one of the other *FMO* genes. In addition, this study was undertaken in subjects of European descent and these findings may not generalize to other populations. This is important because it is known that nicotine metabolism varies between populations [22, 23]. Finally, we note that enzyme was not highlighted in three recent meta-analyses of smoking behavior [24–26]. Our finding may therefore be a replicated false positive. However, this discrepancy could also be due to heterogeneous sample selection, different assessment such as current cigarettes per day, coverage from different platforms, and a relatively small effect size for the variants in FMO1.

In this study, we did not find common variants in *CYP2A6* associated with nicotine dependence. However, our coverage of *CYP2A6* is minimal, including only two SNPs, neither of which are known to alter CYP2A6 activity. Our negative association does not rule out the importance of *CYP2A6* in the development of nicotine metabolism. Furthermore, although the other nicotine metabolizing genes received better coverage, most rare and some common variants were not tagged.

In summary, the association of polymorphisms in *FMO1* with nicotine dependence and heavy smoking along with the demonstration that this enzyme is a catalyst of nicotine *N*-oxidation suggest that polymorphisms in *FMO1* may be significant risk factors in the development of nicotine dependence. The mechanism by which this enzyme plays a role in nicotine dependence is unclear; however the role of brain metabolism in the pharmacology of nicotine warrants further study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Characteristics of subjects in the COGEND and ACS samples

	COGEND N=1610	**ACS N=2844**
Sex		
Male, N (%)	602 (37%)	1162 (41%)
Female, N (%)	1008 (63%)	1682 (59%)
Status		
Case, N (%)	813 (50%)	1458 (51%)
Control, N (%)	797 (50%)	1386 (49%)
Age in Years		
Mean (SD)	36.3 (5.4)	69.7 (6.8)
Range	25–45	49–90

COGEND = Collaborative Genetic Study of Nicotine Dependence

ACS=American Cancer Society

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Table 2

Association results for SNPs in nicotine metabolizing genes with Nicotine Dependence. COGEND sample (N=1610) and ACS sample (N=2844). UGT2 A1/A2 refers to the UGT2A1/UGT2A2 cluster. Genes taken from NCBI "GeneView." SNP positions from GRCh37.

SNP	Chr	Position	Gene	Minor Allele Frequency	COGEND P-Value	OR (95% CI)	COGEND CPD P-Value	Minor Allele Frequency	ACS P value	OR (95% CI)
rs1736560	1	171059150	FMO3	0.30	0.0307	1.18 (1.02 – 1.38)	0.0668			
rs4433435	1	171223951	FMO1	0.40	0.0094	0.83 (0.72 – 0.95)	0.0288			
rs10912675*	1	171227216	FMO1	0.28	0.0065	0.80 (0.68 – 0.94)	0.0578	0.27	0.0067	0.85 (0.75 – 0.96)
rs742350^	1	171250044	FMO1	0.14	0.0019	0.72 (0.58 – 0.88)	0.0083	0.14	0.2205	0.91 (0.78 – 1.06)
rs1126692^	1	171252287	FMO1	0.14	0.0014	0.71 (0.58 – 0.88)	0.0065	0.14	0.2160	0.91 (0.78 – 1.06)
rs7877*	1	171254890	FMO1	0.28	0.0011	0.77 (0.65 – 0.90)	0.0128	0.27	0.0192	0.87 (0.77 – 0.98)
rs16864387^	1	171283843	FMO4	0.14	0.0013	0.71 (0.58 – 0.88)	0.0070	0.14	0.2037	0.90 (0.89 – 1.05)
rs7682207#	4	69771283	UGT2A3	0.13	0.0432	0.80 (0.64 – 0.99)	0.1146			
rs12651295#	4	69771935	UGT2A3	0.13	0.0390	0.80 (0.64 – 0.99)	0.0981			
rs3775783	4	70463854	UGT2 A1/A2	0.20	0.0362	0.82 (0.69 – 0.99)	0.1889			

*,^, #These SNPs are correlated (r^2) 0.8 or greater.

COGEND = Collaborative Genetic Study of Nicotine Dependence

CPD = Cigarettes per day

ACS=American Cancer Society