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Dopamine genes and nicotine dependence in treatment seeking and community smokers

Andrew W. Bergen, Ph.D.^{1,*}, David V. Conti, Ph.D.², David Van Den Berg, Ph.D.², Wonho Lee, Ph.D.², Jinghua Liu, Ph.D.², Dalin Li, Ph.D.², Nan Guo, Ph.D.¹, Huaiyu Mi, Ph.D.¹, Paul D. Thomas, Ph.D.¹, Christina N. Lessov-Schlaggar, Ph.D.³, Ruth Krasnow, M.A.¹, Yungang He, Ph.D.¹, Denise Nishita, B.S.¹, Ruhong Jiang, Ph.D.¹, Jennifer B. McClure, Ph.D.⁴, Elizabeth Tildesley, Ph.D.⁵, Hyman Hops, Ph.D.⁵, Rachel F. Tyndale, Ph.D.⁶, Neal L. Benowitz, M.D.⁷, Caryn Lerman, Ph.D.⁸, and Gary E. Swan, Ph.D.¹

¹SRI International, Menlo Park, CA

²University of Southern California, Pasadena, CA

³Washington University, St. Louis, MO

⁴Group Health Cooperative, Seattle, WA

⁵Oregon Research Institute, Eugene, OR

⁶Centre for Addiction and Mental Health and University of Toronto, Toronto, ON

⁷University of California at San Francisco, San Francisco, CA

⁸University of Pennsylvania, Philadelphia, PA

Abstract

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*Correspondence: Andrew W. Bergen, Ph.D., Molecular Genetics Program, Center for Health Sciences, SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025, USA, Tel: 1 650 859-4618; Fax: 1 650 859-5099; andrew.bergen@sri.com.

Disclosure/Conflict of Interest

Related to THIS WORK. Authors Conti, Van Den Berg, Lee, Liu, Li, Guo, Mi, Thomas, Krasnow, He, Nishita, Jiang, McClure, Tildesley, Hops, Tyndale, Benowitz, Lerman, and Swan declare that all financial and material support for this work was provided by their primary employer. In the past three years, Dr. Bergen has been an employee of the National Cancer Institute. In the past three years, Dr. Lessov-Schlaggar has received compensation for research and professional services from SRI International. Related to ALL OTHER COMPENSATION. Authors Van Den Berg, Lee, Liu, Li, Guo, Mi, Thomas, Krasnow, He, Nishita, Jiang, McClure, and Tildesley declare that, except for income provided from their primary employer, no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional services and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest. In the past three years, Dr. Bergen has been an employee of the National Cancer Institute and has received compensation for professional services from the National Institutes of Health, and from the Price Foundation Ltd. In the past three years, Dr. Conti has been a paid consultant to Pfizer, Inc. In the past three years, Dr. Lessov-Schlaggar has received compensation for research and professional services from SRI International. In the past three years, Dr. Hops has received compensation from the University of Washington for consulting on federally funded projects in adolescent drug use and abuse. In the past three years, Dr. Tyndale has consulted for Novartis, a company that develops and/or markets nicotine dependence medications. Dr. Tyndale is also a shareholder and scientific officer in Nicogen, a company focused on the development of novel smoking cessation treatments. Over the past 3 years Dr. Benowitz has received compensation from pharmaceutical companies marketing or developing smoking cessation medications, including Pfizer, GlaxoSmithKline, Novartis and Aradigm. He has also served as a paid expert witness in litigation against tobacco companies. In the past three years, Dr. Lerman has received compensation and/or research support from GlaxoSmithKline, Pfizer, Astra Zeneca, and Novartis, companies that develop and/or market nicotine dependence medications. In the past three years, Dr. Swan has received compensation from Pfizer.

Supplementary information is available at the *Neuropsychopharmacology* website.

We utilized a cohort of 828 treatment seeking self-identified white cigarette smokers (50% female) to rank candidate gene single nucleotide polymorphisms (SNPs) associated with the Fagerström Test for Nicotine Dependence (FTND), a measure of nicotine dependence which assesses quantity of cigarettes smoked and time- and place-dependent characteristics of the respondent's smoking behavior. 1123 SNPs at 55 autosomal candidate genes, nicotinic acetylcholine receptors and genes involved in dopaminergic function, were tested for association to baseline FTND scores adjusted for age, depression, education, sex and study site. SNP P values were adjusted for the number of transmission models, the number of SNPs tested per candidate gene, and their intragenic correlation. *DRD2*, *SLC6A3* and *NR4A2* SNPs with adjusted P values < 0.10 were considered sufficiently noteworthy to justify further genetic, bioinformatic and literature analyses. Each independent signal among the top-ranked SNPs accounted for ~1% of the FTND variance in this sample. The *DRD2* SNP appears to represent a novel association with nicotine dependence. The *SLC6A3* SNPs have previously been shown to be associated with *SLC6A3* transcription or dopamine transporter density *in vitro*, *in vivo* and *ex vivo*. Analysis of *SLC6A3* and *NR4A2* SNPs identified a statistically significant gene-gene interaction (P=0.001), consistent with *in vitro* evidence that the *NR4A2* protein product (NURR1) regulates *SLC6A3* transcription. A community cohort of N=175 multiplex ever smoking pedigrees (N=423 ever smokers) provided nominal evidence for association with the FTND at these top ranked SNPs, uncorrected for multiple comparisons.

Keywords

dopamine transporter; Fagerström Test for Nicotine Dependence; single nucleotide polymorphism; candidate gene association scan; gene-gene interaction

INTRODUCTION

Cigarette smoking remains prevalent (Kilmer *et al.*, 2008), a risk factor for more than two dozen diseases (CDC, 2003), and is the largest cause of preventable mortality worldwide (Ezzati and Lopez, 2003). Identifying factors influencing smoking will help us understand the nature of this behavior and potentially guide the development of effective interventions for behavior change.

Tobacco dependence is considered to be a multidimensional construct (Hudmon *et al.*, 2003; Russell *et al.*, 1974) and is measured using a variety of questionnaires, including smoking quantity (cigarettes per day), the Fagerström Tolerance Questionnaire (FTQ) (Fagerstrom, 1978), the Heaviness Smoking Index (HSI), consisting of two items from the FTQ (Heatherton *et al.*, 1989), and the Fagerström Test for Nicotine Dependence (FTND), a revision of the FTQ (Heatherton *et al.*, 1991), among other scales. The FTND is one of the most commonly employed measures of nicotine dependence in smoking intervention trials, epidemiological studies, and genetic studies. Scale items assess current smoking level, as well as other time- and place-dependent characteristics of the respondent's smoking behavior. Scores on this six-item scale range from 0 to 10, with higher scores considered indicative of greater nicotine dependence. The additive genetic component of nicotine dependence defined by the FTND has been estimated at 67%, 75% and 40% among

American, Dutch and Finnish twins, respectively (Broms *et al.*, 2007; Maes *et al.*, 2004; Vink *et al.*, 2005). We chose to use the FTND in this study due to the scale's established estimates of heritability and its broad and continued use in the field (Piper *et al.*, 2006).

A decade of linkage analyses of smoking behaviors continues to inform and motivate candidate gene studies in the same family based samples (Li, 2008). Through a combination of genome wide association scans (GWAS) and candidate gene studies, nicotinic receptor candidate genes currently are the leading candidates for influencing responses to cigarette smoking, cigarette smoking quantity and nicotine dependence (Li and Burmeister, 2009). Other candidate gene families, e.g., GABAergic (Agrawal *et al.*, 2008; Beuten *et al.*, 2005; Lou *et al.*, 2007b; Saccone *et al.*, 2007), cell adhesion molecule (Bierut *et al.*, 2007; Gelernter *et al.*, 2006; Uhl *et al.*, 2007; Vink *et al.*, 2009), dopaminergic (Caporaso *et al.*, 2009; Li and Burmeister, 2009; McKinney *et al.*, 2000; Morton *et al.*, 2006), transient receptor potential (Bierut *et al.*, 2007; Caporaso *et al.*, 2009; Saccone *et al.*, 2007; Vink *et al.*, 2009) and nicotine metabolic (Caporaso *et al.*, 2009; Malaiyandi *et al.*, 2006; Saccone *et al.*, 2007) candidate gene families, have also been identified in GWAS and candidate gene studies of smoking behaviors.

A candidate gene SNP genotyping panel focused primarily on neuronal pre-synaptic nicotinic receptors and the dopaminergic pathway was developed to test candidate gene association to nicotine addiction and treatment outcomes by the Pharmacogenetics of Nicotine Addiction and Treatment consortium (Conti *et al.*, 2008), a member of the Pharmacogenetics Research Network (Giacomini *et al.*, 2007). We utilized SNP genotypes from this panel and baseline FTND scores from treatment seeking smokers from two randomized clinical trials (RCTs) of smoking cessation pharmacotherapy, one RCT that randomized participants to bupropion and placebo and one RCT that randomized participants to two forms of nicotine replacement therapy (Lerman *et al.*, 2006), to rank SNPs associated with nicotine dependence. Association analyses of SNPs to smoking cessation outcomes have been performed in these two RCTs previously, e.g., significant association of an insertion deletion SNP at the dopamine D2 receptor gene (*DRD2*) to abstinence has been reported (Lerman *et al.*, 2006). A community ascertained, multiplex ever smoker pedigree cohort was genotyped using the same SNP panel and this genotype data was used to evaluate the significance of association of the top-ranked SNP association findings from the treatment seeking cohort in a second sample. The multiplex ever smoker pedigree cohort consisted of pedigrees with at least three first degree ever-smoking relatives with ever smoking defined as 100 cigarettes smoked lifetime (Swan *et al.*, 2003). Herein we report the ranking of candidate gene SNP associations to baseline FTND score in a cohort of self-identified white treatment seeking smokers, and the significance of association of top-ranked SNPs to FTND in a second cohort of smokers from a cohort of multiplex ever smoker pedigrees.

PARTICIPANTS and METHODS

Treatment seeking smoker cohort

Current daily smokers were recruited for participation into two RCTs of smoking cessation, one evaluating bupropion versus placebo and another evaluating two modalities of

administration of nicotine replacement therapy, as previously described (Lerman *et al.*, 2006). Study sites include Washington, DC, Buffalo, NY and Philadelphia, PA. Inclusion criteria included age ≥ 18 and a recent smoking history of ≥ 10 cigarettes per day, while exclusion criteria included pregnancy or lactation, uncontrolled hypertension, unstable angina, heart attack or stroke within the past 6 months, current treatment or recent diagnosis of cancer, drug or alcohol dependence, current diagnosis or history of a psychiatric disorder, seizure disorder, and current use of bupropion or nicotine-containing products other than cigarettes. Participants were assessed for eligibility, completed a baseline assessment, provided a venous blood sample, were randomized to treatment, and attended at least one clinical visit. In conjunction with the baseline assessment, participants provided demographic information including the Center for Epidemiologic Studies scale for depression symptoms (Radloff, 1977), and completed the FTND. Both genomic DNA and whole genome amplified DNA samples were available from participants in the treatment seeking smoker cohort, and was genotyped using the candidate gene panel as described below and by Conti *et al.* (Conti *et al.*, 2008).

Multiplex ever smoker pedigree cohort

A community ascertained multiplex ever smoker pedigree cohort was selected to evaluate SNPs that were top-ranked in the treatment seeking smoking cohort. These pedigrees were recruited from a longitudinal study that originally recruited N=734 probands 11 to 15 years of age, their parents and siblings, and assessed longitudinal phenotypes of tobacco use (Hops *et al.*, 2000). Pedigrees with three first degree relatives with a smoking history of ≥ 100 cigarettes smoked were selected for recruitment for an integrated study of the genetics of smoking behavior and nicotine metabolism after administration of a family history of tobacco use questionnaire (Swan *et al.*, 2003). After identification and recruitment of probands and family members, a detailed baseline assessment was performed that included sociodemographics, cigarette smoking history, and the FTND at the period of greatest smoking intensity. This analysis utilized smoking history questionnaire data and genomic DNA from N=175 pedigrees. Genomic DNA was extracted from venous whole blood using a standard salt-based precipitation method (Miller *et al.*, 1988), and DNA was genotyped using the same SNP panel as described (Conti *et al.*, 2008).

Candidate gene and SNP selection

Candidate genes for the association analysis of nicotine dependence and for the pharmacogenetic analysis of smoking cessation trials were selected from the presynaptic neuronal nicotinic acetylcholine receptor genes and dopaminergic receptor genes whose proteins mediate the nicotine-stimulated release of dopamine within the mesolimbic system, corpus striatum and prefrontal cortex of the brain and self-administration in animal models of nicotine addiction (Benowitz, 2008). SNP coverage of candidate genes, i.e., the mean (SD) proportion of HapMap Phase II SNPs tagged at an r^2 of > 0.80 by the candidate gene genotyping panel SNPs, is 0.92 (0.10). Additional details on the candidate gene and SNP selection process are provided by Conti *et al.* (Conti *et al.*, 2008).

Analysis of the treatment seeking smoker cohort

As described in Conti *et al.*, genotyping of DNA samples from the RCT participants was performed using the GoldenGate™ assay (Illumina, San Diego, CA), with quality control procedures that included automated sample handling protocols and the inclusion of replicate DNA samples to aid in identifying genotyping errors (Conti *et al.*, 2008). The mean genotype concordance rate across 14 replicate DNA samples was 99.0%. As described in Conti *et al.*, 41 SNPs with a genotype call rate of zero and 57 SNPs with a minor allele frequency < 1% from 1528 SNPs (232 ancestry informative markers on the panel), were removed from the analysis dataset. Of the remaining 1430 SNPs, 95% had genotype completion rates of > 97%. Individual call rates were calculated using the entire multiethnic sample (N=1216) and after stratifying by DNA type, i.e. genomic (gDNA) and whole genome amplified (wgaDNA) DNA. Individuals with a call rate less than 90% were removed from the analysis (N=48) for a total of 1091 individuals with gDNA and 90 individuals with wgaDNA. While there was a substantial difference between the call rate distributions for the gDNA samples (99.6% of individuals with a call rate > 95%) in comparison to the wgaDNA samples (85.3% of individuals with a call rate > 95%), inspection of Illumina data for SNPs with drastically discordant SNP call rates between the two DNA types revealed reliable genotyping calls for SNPs with a SNP call rate calculated using only wgaDNA samples of > 80% (Conti *et al.*, 2008). Thus, for 51 SNPs in which the SNP call rate was < 80% for wgaDNA samples, we only report analyses using the individuals with gDNA samples (Conti *et al.*, 2008). Using ancestry informative markers included in the genotyping panel, Conti *et al.* demonstrated little individual admixture within each self-identified ethnic group in the treatment seeking smoker cohort (Conti *et al.*, 2008). We report the results of association analysis to covariate adjusted FTND with 1123 SNPs at 55 autosomal candidate genes (51 contiguous candidate gene regions) in N=821 self-identified white treatment seeking smokers. We choose to limit our reporting to results from autosomal genotypes in order to model SNP association to FTND using a single multivariate model, removing 30 SNPs at three X chromosome candidate genes (FLNA, MAOA and MAOB) from the available genotype dataset. We choose to limit our reporting of analyses to self-identified white participants of the RCTs because of the potential for differential linkage disequilibrium across ethnic groups that might lead to heterogeneity of effect estimates (Conti *et al.*, 2008).

FTND SNP association analysis was based on a generalized linear model (GLM), treating FTND as a quantitative variable, with additive or dominant genetic models, and covariates age, self-reported depression symptoms, education, sex, and site. Covariates included in the GLM were significantly associated (univariate $P < 0.05$) with baseline FTND scores. A GLM including the FTND and covariates was estimated as the base model, and then two GLMs for each SNP using dominant and additive models were estimated. A 1 degree of freedom likelihood ratio test was used to rank SNP GLM results where the P value (P_{LRT}) of the more significant GLM (additive or dominant) is reported. We performed multiple test correction to account for the correlated tests from the estimation of two GLMs (additive and dominant) for each SNP and for the multiple correlated tests from the SNPs within each gene (Conneely and Boehnke, 2007). We report this adjusted P value for correlated tests as P_{ACT} , and selected SNPs with $P_{ACT} < 0.10$ as noteworthy SNPs for further genetic,

bioinformatic and literature based analyses. We evaluated the distribution of FTND scores by SNP genotype via analysis of variance in *post-hoc* analyses of noteworthy SNPs. Genetic interaction was modeled using GMDR (Lou *et al.*, 2007a). Power analyses were performed using Quanto (Gauderman, 2002). Statistical analyses were performed using SAS (SAS for Windows, version 9.1, SAS Institute, Cary, NC) and R (Team, 2003).

Analysis of the multiplex ever smoker pedigree cohort

We evaluated association between SNPs and the FTND in the multiplex ever smoker pedigree cohort previously identified as associated with the FTND in the treatment seeking smoker cohort. FTND data were available on N=425 individual ever smokers (100 cigarettes lifetime) from N=175 nuclear pedigrees. We treated the FTND as an ordinal variable and calculated a covariate-adjusted FTND score using logistic regression (Wang *et al.*, 2006), and utilized this score as the quantitative trait for family based association testing (FBAT) (Schneiter *et al.*, 2005). 16 genotypes inconsistent with Mendelian transmission were deleted prior to FBAT. We performed FBATs using the genetic models suggested by the post-hoc ANOVA analyses of SNP effects on FTND in the treatment seeking smoker cohort, as well as alternative genetic models, i.e., dominant, additive and genotype models, but not recessive models. We did not perform multiple test correction for multiple models or multiple SNPs tested in the FBAT analyses. Genetic interaction of *SLC6A3* and *NR4A2* SNPs in pedigrees was modeled using PGMDR (Lou *et al.*, 2008).

Genomic annotation and visualization

We evaluated the physical location of SNPs in relationship to NCBI Build 35 genomic annotation with Genewindow (Staats *et al.*, 2005), with the UCSC Genome Browser (Hinrichs *et al.*, 2006) and linkage disequilibrium (LD) among SNPs with Haploview 4.0 (Barrett *et al.*, 2005), using genotype data from the treatment seeking smoker cohort or from the CEU HapMap sample (Consortium, 2005).

RESULTS

FTND, covariates and SNPs in the treatment seeking smoker cohort

The treatment seeking smoker cohort consists of 828 self-identified white treatment seeking smokers, with a mean (SD) baseline FTND score of 5.3 (2.1) (Table 1). Participants are about equally divided between college graduates and non-graduates, women and men, and recruitment site. Older age, a higher depression symptom score, lower educational achievement, male sex and two of three sites are significantly associated with higher FTND scores in this cohort (Table 1). The multivariate model indicates that these covariates account for 10% of the variance of FTND in the treatment seeking smoker cohort ($F_{827}=18.01$, $P<0.0001$, $r^2=0.0987$).

73 SNPs at 22 candidate genes were identified in the treatment seeking smoker cohort with a P_{LRT} value < 0.05 (see Table S1 for all SNP GLM results). Five SNPs at three genes (*DRD2*, *SLC6A3* and *NR4A2*) were identified with a $P_{ACT} < 0.10$ (Table 2). These SNPs were evaluated in detail using genomic annotation, literature review and genotyping in a second cohort of multiplex ever smoker pedigrees.

The top-ranked *DRD2* SNP rs10891552 is associated with a ~0.9 unit increase of FTND score and with ~1.3% of FTND variance in an additive or dominant model, as, in this sample, the minor allele homozygote is not observed (Table 3). rs10891552 is located in a linkage disequilibrium (LD) block within the large first intervening sequence (IVS1), but exhibits only weak LD ($r^2 < 0.23$) with SNPs in the local LD block in this sample (Figure S1). There are three additional *DRD2* SNPs (rs2440390, rs4586205 and rs1076562) with P_{LRT} values < 0.05 (Table S1), located an average of 37 kilo base pairs (kbp) 3' of rs10891552 within IVS1 of *DRD2*, and within a LD block that spans IVS1 through the coding region of *DRD2*, but these SNPs have P_{ACT} values ~ 0.6 (Table S1), and rs10891552 has an $r^2 < 0.01$ with these SNPs (Figure S1).

The top-ranked three *SLC6A3* SNPs (rs2975226, rs2652510 and rs2652511) identified as associated with FTND (Table 2) are highly correlated with each other (mean $r^2 \sim 0.97$) in a LD block that extends in this sample from rs12652860, 61 kbp 5' of *SLC6A3* to rs6350 in exon 2 of *SLC6A3*, and are only modestly correlated ($r^2 \sim 0.2$) with SNPs in two 3' LD blocks extending from IVS1 through IVS7 (Figure 1). SNP FTND association model characteristics are similar for the three *SLC6A3* SNPs, i.e., for each of these SNPs, the presence of the minor allele is associated with a ~0.5 unit increase of FTND score and with ~1% of FTND variance in the additive model (Table 3). Inspection of all *SLC6A3* SNP FTND association results (Table S1) identifies rs12652860, also in the 5' linkage block, with a $P_{LRT} < 0.01$ and a $P_{ACT} = 0.20$, and with similar effects on FTND score and variance (data not shown), while the remaining *SLC6A3* SNPs exhibit P_{LRT} values > 0.15 and P_{ACT} values > 0.95 (Table S1). Estimation of haplotypes in the 5' linkage block results in five haplotypes with frequency > 0.01 , with the minor allele at each of the three top-ranked *SLC6A3* SNPs associated with increased FTND found in three haplotypes with combined frequency 39% (Figure 1).

The top-ranked *NR4A2* SNP rs834829 was identified in the initial association analysis as most significantly associated with a dominant model (Table 2), and while both additive and dominant models are significantly associated with increased FTND score, inspection of FTND distributions by genotype in *post-hoc* analysis identifies the heterozygote genotype as significantly associated with increased FTND scores, accounting for a ~0.4 unit increase of mean FTND score and ~0.8% of FTND variance (Table 3). The *NR4A2* SNP associated with FTND is found ~12 kbp 5' of the 5' UTR of the *NR4A2* gene, within a LD block that extends from 5' of the gene, across the entire gene to the 3' flanking region in this sample (Figure S2). Inspection of all *NR4A2* SNP FTND association results (Table S1) identifies rs1150144 and rs1150143, ~2.5 kbp 3' of the 3'UTR, with P_{LRT} values < 0.05 , strong r^2 with rs834829 (0.79 and 0.94, respectively) and modest P_{ACT} values (0.12 and 0.21, respectively).

In *post-hoc* power analyses, the sample size of 821 treatment seeking smokers has power of 82% to detect beta coefficients of 78%, 42% and 43% for minor allele frequencies of 4% (*DRD2*), 29% (*NR4A2*) and 39% (*SLC6A3*), respectively, for genetic effects in a dominant model with an r^2 of 0.01 and the observed FTND mean and standard deviation (Table 1) at an alpha value of 0.05 with a two-sided test.

SLC6A3 NR4A2 interaction analysis

We evaluated gene-gene interaction between rs2652511 at *SLC6A3* and rs834829 at *NR4A2*, using a continuous model of FTND score, adjusted for age, depressive symptoms, education, sex and site in a sample of N=794 individuals with complete FTND, covariate and genotype data. We chose these two SNPs to perform the interaction analysis because the gene product of *NR4A2*, NURR1, is known to be required for *SLC6A3* gene transcription (see Discussion), and because these two SNPs are highly ranked in our GLM results (Table 2). We evaluated training (prediction) accuracy (TA) of three GMDR analyses to predict the distribution of covariate adjusted normalized FTND score within genotype classes for rs2652511, for rs834829, and for both rs2652511 and rs834829 (Figure S2). While each SNP independently predicted FTND score (for rs2652511, TA=0.56, TA Odds Ratio (OR)=1.71, 95CI 1.37–2.14, P<0.0001, cross validation sign test P=0.02; for rs834829, TA=0.56, TA OR=1.64, 95 CI 1.31–2.04, P<0.0001, cross validation sign test P=0.83), only the analysis with both rs2652511 and rs834829 resulted in a highly significant cross validation sign test, in addition to increased training accuracy and odds ratio (TA=0.59, TA OR=2.15, 95CI 1.72–2.70, P<0.0001, cross validation sign test P=0.001).

Multiplex smoking pedigree cohort analysis

FTND score, age, and the proportion of individuals with 16 or more years of education were significantly lower in the multiplex smoking pedigree cohort than in the treatment seeking smoker cohort (all P < 0.0001). Age, depressive symptoms, education and sex exhibited significant association with FTND in the multiplex smoking pedigree cohort in the same direction as in the treatment seeking smoker cohort (Table 4). The multivariate model of covariate association with FTND indicates that these covariates account for 15% of the variance of FTND in the multiplex smoking pedigree cohort ($F_{405}=18.21$, P<0.0001, $r^2=0.1537$). With one exception (rs834829), each of the five top-ranked SNPs in the treatment seeking smoker cohort (Table 2) exhibited nominally significant (P < 0.05) association with FTND in the multiplex smoking pedigree cohort in FBAT analyses uncorrected for multiple testing (Table 5). For rs10891552 (*DRD2*), the minor allele (in additive, dominant or genotype models) was associated with increased FTND. For the *SLC6A3* SNPs, the minor allele (in the genotype model) was associated with increased FTND. For rs834829, the FBAT with the smallest P value was the heterozygote genotype model (P = 0.0511). We evaluated statistical interaction between rs2652511 at *SLC6A3* and rs834829 at *NR4A2* in 411 individuals with FTND scores from N=175 pedigrees using PGMDR (Figure S3). The PGMDR analysis with both rs2652511 and rs834829 had the greatest training (prediction) accuracy, but did not exhibit a significant cross validation sign test P value (TA=0.56, TA OR=1.65, 95 CI 1.24, 2.21, P=0.0006, cross validation sign test P=0.82). The increase in TA over the PGMDR analysis of rs2652511 (TA=0.54, TA OR=1.66, 95 CI 1.15, 2.40, P=0.0063, cross validation sign test P=0.38) was modest. The PGMDR analysis of rs834829 had a non-significant training accuracy estimate and OR (data not shown).

DISCUSSION

This analysis ranked 1123 SNPs at 55 autosomal candidate genes for significance of association to baseline FTND score in 821 self-identified white treatment seeking smokers. We observed five SNPs assigned to three candidate genes with P values adjusted for correlated tests of < 0.10 . We focused our subsequent analyses on these five SNPs to avoid the *post-hoc* exercise of going deeper into the list of ranked SNPs in search of biological coherence. These top-ranked SNPs were found within dopamine candidate genes, with a single association finding each at a dopamine receptor involved in intracellular signaling through multiple pathways (Bonci and Hopf, 2005), at the dopamine transporter, which regulates extracellular dopamine concentration by reuptake of dopamine (Sotnikova *et al.*, 2006), and at an essential regulator of dopaminergic gene transcription and dopaminergic neuron development (Jankovic *et al.*, 2005). The panel of candidate genes was developed using nicotinic acetylcholine receptor and dopaminergic candidate genes, and thus observing top-ranked SNPs at dopaminergic candidate genes is not unexpected.

However, our analysis results do raise the question of why a nicotinic acetylcholine receptor (nAChR) SNP was not identified within the small group of SNPs that met our threshold for follow-up analyses. In our analyses of the treatment seeking smoker cohort, we tested 121 SNPs at nine nAChR genes for association with FTND (Table S1). Three of these nAChR SNPs (*CHRNA4* rs3971872 and *CHRNA2* rs748283 and rs4733065, all several kbp 3' of their assigned gene) exhibited unadjusted P values < 0.05 , but adjusted P values were > 0.10 . We did not observe significant association of two *CHRNA2* SNPs with FTND in this analysis that were previously identified by Conti *et al.* in an age, gender and FTND score adjusted analysis of associated with abstinence in ~ half of the treatment seeking smoker cohort investigated here (Conti *et al.*, 2008). FTND is associated with abstinence in the cohort of treatment seeking smokers analyzed here (data not shown), however, this analysis is focused on baseline nicotine dependence as measured by the FTND in treatment seeking smokers of two RCTs (Lerman *et al.*, 2006).

Based on recent association studies of nicotinic receptor SNPs and nicotine dependence, the lack of nAChR SNPs among the top-ranked SNPs in this study may be assumed to be the result of a lack of statistical power and/or differences between the treatment seeking smokers in our study and the smokers in other recent population based and clinically based studies. Our treatment seeking smoker cohort was ~19 and ~13 fold smaller than two GWAS studies that identified the chr15q25.1 nAChR locus as associated with heavy smoking (≥ 25 cigarettes per day (CPD) versus < 5 CPD) in population based and clinical based samples originally recruited for other genetic studies (Berrettini *et al.*, 2008), and with cigarettes smoked per day in smokers originally recruited for other genetic studies (Thorgeirsson *et al.*, 2008). Our treatment seeking smoker cohort was ~3.4 and ~2.3 fold smaller than two candidate gene association studies of the chr15q25.1 nAChR locus and FTND that characterized risk and protective haplotypes in three cohorts of current and former smokers, one of which was composed of treatment seeking smokers (Weiss *et al.*, 2008), and a study that evaluated association of SNPs at 348 candidate genes, including 40 of the candidate genes investigated in this study, to a population based sample of non-dependent and dependent ever smokers (Saccone *et al.*, 2007). While our treatment seeking

smoker sample was much smaller than prior studies, our noteworthy SNPs at dopaminergic candidate genes accounted for ~1% of FTND variance. Thorgeirsson *et al.* (Thorgeirsson *et al.*, 2008) estimated that ~0.7% of the variance of cigarettes smoked per day is explained by the chr15q25.1 variant (rs1051730) identified in the Icelandic population sample of 10,995 smokers used in their study. rs1051730 was tested in our treatment seeking smoker cohort for association with FTND with non-significant unadjusted and adjusted P values (0.47 and 0.99, respectively). Finally, our sample of treatment seeking smokers exhibited a normal distribution of FTND scores, rather than a dichotomized distribution utilized by some investigators, i.e., selected for extremes of the CPD or nicotine dependence distributions from a larger population or clinically based sample. Our results and the ascertainment differences between our treatment seeking cohort and previously studied cohorts raise the possibility that dopaminergic candidate genes may exert a greater influence on nicotine dependence among treatment seeking smokers with average nicotine dependence scores than do the chr15q25.1 nicotinic receptor candidate genes. Additional recent GWAS in population based and clinically ascertained populations have identified a wide variety of candidate genes as significantly associated with smoking behaviors in addition to nicotinic receptor candidates (Bierut *et al.*, 2007; Caporaso *et al.*, 2009; Uhl *et al.*, 2007; Uhl *et al.*, 2008; Vink *et al.*, 2009), suggesting that many factors, including sample ascertainment, sample size, genotyping platforms and analyses approaches, may influence the ranking of specific candidate genes or candidate gene families in association analyses of smoking behaviors.

We evaluated association of dopaminergic SNPs identified in the treatment seeking smoker cohort in an independent cohort of non-treatment seeking ever smokers from multiplex ever smoking pedigrees that were significantly younger, with significantly less education and with significantly lower FTND scores than the individuals in the treatment seeking smoker cohort. We also evaluated the biological coherence of these findings by performing interaction analyses among SNPs from two genes with *a priori* evidence for biological interaction, which revealed a significant *SLC6A3 NR4A2* interaction in the treatment seeking smoker cohort, although we failed to observe a significant gene-gene interaction in the multiplex smoker pedigree cohort. The sample size of the multiplex ever smoker pedigree cohort, about half that of the treatment seeking smoker cohort, may have limited our ability to observe statistically significant interaction in these pedigrees. Below, we attempt to place our top-ranked association findings of *DRD2*, *SLC6A3* and *NR4A2* SNPs to the FTND in the context of the nicotine dependence susceptibility candidate gene association literature.

DRD2

This study has identified one *DRD2* SNP (rs10891552) among 52 SNPs tested at the *ANKK1* and *DRD2* loci as worthy of followup evaluation (SNPs from the two regions were tested together because SNPs at these genes are in LD). There are three extant studies that have evaluated association with nicotine dependence measures and *DRD2* SNPs (Gelernter *et al.*, 2006; Huang *et al.*, 2008; Saccone *et al.*, 2007). Saccone *et al.* tested 41 SNPs at the two genes for association with nicotine dependence and rs4245150, 69 kbp 5' of *DRD2*, was identified as significant with a FDR < 0.40 level (Saccone *et al.*, 2007). Huang *et al.*, 2008 (Huang *et al.*, 2008) tested 23 SNPs at the two genes for association with nicotine

dependence in the Mid South Tobacco Family sample and identified one *ANKK1* SNP (rs2734849, Ex8+475, H490R) associated with nicotine dependence in African-American pedigrees after multiple test correction. Gelernter *et al.* (Gelernter *et al.*, 2006) tested 43 SNPs at these two genes and two additional flanking genes (*TTC12* and *NCAMI*) for association to both DSM-IV and FTND nicotine dependence and identified one, four and five SNPs that exhibited significant association to DSM-IV nicotine dependence in African-American pedigrees, European-American pedigrees and both sets of pedigrees combined, however, no SNPs were considered to be significantly associated to FTND after multiple test correction. Inspection of r^2 in the HapMap CEU sample between the SNP associated with FTND in this study and SNPs found associated with nicotine dependence in Saccone *et al.*, Huang *et al.*, and Gelernter *et al.*, identified no substantial LD ($r^2 < 0.01$ for six such SNPs). P_{ACT} values for SNPs declared significantly associated with nicotine dependence by Huang *et al.* (Huang *et al.*, 2008) and Gelernter *et al.* (Gelernter *et al.*, 2006) were non-significant (for five such SNPs, P_{ACT} values > 0.98). rs10891552 does not appear to be in LD with flanking *DRD2* SNPs (Figure 1), or with *DRD2* SNPs associated with nicotine dependence in other studies (Gelernter *et al.*, 2006; Huang *et al.*, 2008; Saccone *et al.*, 2007), and those other SNPs do not exhibit association in this study. The association of rs10891552 in the cohort of treatment seeking smokers thus represents a novel finding of association of a *DRD2* SNP to nicotine dependence and the cohort of multiplex smoking pedigrees provides nominal support for this association in a second sample.

SLC6A3

There are three published studies investigating *SLC6A3* SNPs for association with nicotine dependence (Ling *et al.*, 2004; Saccone *et al.*, 2007; Segman *et al.*, 2007). Ling *et al.* (Ling *et al.*, 2004) evaluated association of a 3' UTR SNP (rs27072) with nicotine dependence and age of smoking onset in a sample (668 individuals, 99% male, from 253 sibships) of severely nicotine dependent smokers (mean (SD) FTND = 8.1 (2.1)). Ling *et al.* did not identify a significant association with nicotine dependence, but did identify significant association with smoking onset 18 years of age, in the subsample of severely dependent smokers with a FTND score 8 (Ling *et al.*, 2004). Segman *et al.* evaluated association of four *SLC6A3* SNPs (located in the promoter (rs2652510), IVS1, Exon 9, and Exon 15) and the 3' VNTR polymorphism, located in exon 15, to smoking initiation and to dichotomized nicotine dependence in a sample of 390 female undergraduate students, 242 of whom who had smoked daily for at least one year and 148 of whom had never smoked (Segman *et al.*, 2007). The 242 daily smokers were dichotomized into a group with FTQ scores of 6 (N=127) and a group with scores 4 (N=115). After multiple test correction, Segman *et al.* did not observe significant association between any SNP and either smoking initiation or nicotine dependence. Segman *et al.* did identify significant association to both phenotypes with a haplotype composed of the 3' VNTR and a SNP in the same (15th) exon (Segman *et al.*, 2007). Saccone *et al.* tested five *SLC6A3* SNPs located in the 3'UTR (3 SNPs), exon 12, and exon 13; none of these SNPs were associated with dichotomized FTND (Saccone *et al.*, 2007).

In contrast to these two studies evaluating association between *SLC6A3* SNPs and nicotine dependence, there have been many studies focused on assessing association between a 3'

polymorphic variable number of tandem repeat (VNTR) locus found in exon 15 of the *SLC6A3* gene (Vandenbergh *et al.*, 1992) and smoking behaviors, including smoking status (Jorm *et al.*, 2000; Lerman *et al.*, 1999; Sabol *et al.*, 1999; Segman *et al.*, 2007; Timberlake *et al.*, 2006; Vandenbergh *et al.*, 2002), adolescent smoking progression (Audrain-McGovern *et al.*, 2004; Laucht *et al.*, 2008), smoking cessation (David *et al.*, 2007; David and Munafo, 2008; Han *et al.*, 2008; Lerman *et al.*, 2003; O'Gara *et al.*, 2007; Styn *et al.*, 2008; Swan *et al.*, 2007; Ton *et al.*, 2007), and affective, behavioral and brain responses to smoking (Brody *et al.*, 2006; Erblich *et al.*, 2004; Erblich *et al.*, 2005; Franklin *et al.*, 2008; Newberg *et al.*, 2007; Perkins *et al.*, 2008; Yang *et al.*, 2008). Only one of these studies has evaluated a sample of smokers for potential association of the *SLC6A3* 3' VNTR with nicotine dependence (Lerman *et al.*, 1999). Lerman *et al.* evaluated four smoking behaviors in a sample of 289 smokers and 233 controls; association of 3' VNTR genotypes with FTND in the sample of 289 smokers was not observed (Lerman *et al.*, 1999). Lerman *et al.* did identify significant association with smoking status in the entire sample, and with age of initiation and quitting history in the subsample of smokers (Lerman *et al.*, 1999).

The *SLC6A3* 3' VNTR polymorphism has been associated with dopamine transporter expression differences *in vivo* via single photon emission computed tomography of the radioligand ¹²³I-2β-carbomethoxy-3β-4(iodophenyl)tropane (β-CIT) for measurement of striatal dopamine transporter binding potential (Heinz *et al.*, 2000; Martinez *et al.*, 2001; van Dyck *et al.*, 2005), and with differences in *SLC6A3* gene expression via quantitative polymerase-chain reaction analysis of RNA *ex vivo* in lymphocytes (Mill *et al.*, 2002) and in *post-mortem* human brain (Brookes *et al.*, 2007; Mill *et al.*, 2002). The presence of the 9 repeat allele of the 3' VNTR has generally been associated with reduced dopamine transporter binding potential or reduced levels of *SLC6A3* transcripts. However, the *SLC6A3* promoter and 5' UTR region and the 3' VNTR region (exon 15) are in very modest linkage disequilibrium in the sample investigated in this study (Figure 1), and in other samples (Greenwood and Kelsoe, 2003; Kelada *et al.*, 2005).

SLC6A3 promoter SNPs have been associated with dopamine transporter expression *in vivo* via [¹¹C] cocaine positron emission tomography (PET) (Drgon *et al.*, 2006), *in vitro* via expression constructs (Greenwood and Kelsoe, 2003; Kelada *et al.*, 2005; Sacchetti *et al.*, 1999) and *ex vivo* in *post mortem* human brain via [³H] carboxyfluorotropane binding studies (Drgon *et al.*, 2006). In studies of promoter sequence constructs, Sacchetti *et al.* found that a proximal promoter construct that includes rs2975226 drives expression up to 150 times greater than an expression construct without this proximal promoter sequence, while *SLC6A4* promoter sequences further 5' serve to repress transcription (Sacchetti *et al.*, 1999). Greenwood and Kelsoe evaluated promoter constructs that differed by promoter sequence and by allelic content (Greenwood and Kelsoe, 2003). Promoter expression constructs that include the very proximal promoter and 5'UTR sequence, including rs2975226, have greater reporter activity than expression constructs that include more 5' promoter and/or more 3' sequence, as observed by Sacchetti *et al.* (Sacchetti *et al.*, 1999). However, promoter constructs that included the minor allele of common promoter SNPs, e.g., at rs2652510, within the core or extended promoter constructs, exhibited reduced reporter activity *in vitro* (Greenwood and Kelsoe, 2003). Another analysis of expression

constructs of the *SLC6A3* promoter and more 3' sequence spanning 7391 basepairs evaluated transcription levels for six haplotypes that included rs2975226, rs2652511 and rs2652510, as well as other *SLC6A3* promoter SNPs (Kelada *et al.*, 2005). Expression constructs containing the minor alleles at these three SNPs in the promoter haplotype exhibited significantly reduced transcription *in vitro* (Kelada *et al.*, 2005). *in vivo* [¹¹C] cocaine PET and *ex vivo* [³H] carboxyfluorotropane binding studies indicate that the minor allele at rs2975226 is associated with reduced dopamine transporter binding (Drgon *et al.*, 2006). Thus, four studies of *SLC6A3* promoter SNPs including *in vitro*, *in vivo* and *ex vivo* designs showed significantly reduced expression with the minor allele of rs2975226, rs2652510 and/or rs2652511 (Drgon *et al.*, 2006; Greenwood and Kelsoe, 2003; Kelada *et al.*, 2005; Sacchetti *et al.*, 1999).

NR4A2

There are three published reports associating *NR4A2* polymorphisms with substance dependence (Ishiguro *et al.*, 2002; Nielsen *et al.*, 2008; Saccone *et al.*, 2007). Ishiguro *et al.* identified rs34884856 in the promoter (-2920), and a biallelic CA dinucleotide repeat sequence in the 3' UTR of the *NR4A2* gene and identified a significant haplotypic association with alcohol dependence in a sample of 171 individuals with a DSM-IV diagnosis of alcohol dependence and 161 unscreened individuals (Ishiguro *et al.*, 2002). Nielsen *et al.* identified rs1405735, 45kbp 5' of *NR4A2*, as the fourth ranked candidate gene SNP from an *a priori* candidate gene genotypic association analysis nested within a case:control GWAS of heroin dependence with 99 individuals meeting Federal criteria for methadone maintenance and 99 individuals screened for past and current history of drug use genotyped for this SNP (Nielsen *et al.*, 2008). Information on the smoking status of cases and controls was not provided by Nielsen *et al.* and Ishiguro *et al.* (Ishiguro *et al.*, 2002; Nielsen *et al.*, 2008). Saccone *et al.* tested two SNPs at *NR4A2* (rs12803 and rs834835, located in the 3' UTR and IVS1, respectively) for association with dichotomized FTND, however, neither exhibited significant association with nicotine dependence (Saccone *et al.*, 2007). The level of LD (r^2) in the HapMap CEU sample between rs834829 and rs1405735, rs12803 and rs834835 is 0.12, 0.49 and 1.0, respectively. The *NR4A2* SNP (rs34884856) tested by Ishiguro *et al.* (Ishiguro *et al.*, 2002) was not genotyped in the HapMap project.

NURR1, the protein product of the *NR4A2* gene, is an orphan nuclear receptor transcription factor (Law *et al.*, 1992) expressed in dopaminergic neurons (Zetterstrom *et al.*, 1996), required for the development and maintenance of dopaminergic neurons (Saucedo-Cardenas *et al.*, 1998; Zetterstrom *et al.*, 1997), and coexpressed with tyrosine hydroxylase and the dopamine transporter (DAT) within striatal neurons (Cossette *et al.*, 2005). Together with DAT, NURR1 is decreased in expression in dopamine neurons of cocaine (Bannon *et al.*, 2002) and heroin (Horvath *et al.*, 2007) abusers, suggesting that DAT and NURR1 are involved in the adaptation of the striatal dopaminergic system in addiction, as well as in normal development. NURR1 regulates DAT expression using sites in the promoter region ~1000 basespairs 5' of the transcription start site of *SLC6A3* (Sacchetti *et al.*, 1999; Sacchetti *et al.*, 2001). The finding that *NR4A2* variation is associated with nicotine, heroin and alcohol dependence is of great interest because it suggests that genetic variation at NURR1 may influence the development and course of substance dependence generally and

not just for a single substance (Goldman and Bergen, 1998). The *SLC6A3-NR4A2* interaction identified in this study is consistent with what is known regarding the development and maintenance of dopaminergic neurons by *NURR1* (Zetterstrom *et al.*, 1997) and the regulation of the *SLC6A3* gene by *NURR1* (Sacchetti *et al.*, 2001). A challenge now will be to identify the specific biological interaction between the *NURR1* protein and the *SLC6A3* promoter that increases the risk for nicotine dependence.

Conclusion

In this study, the minor alleles at *DRD2* SNP rs10891552, *SLC6A3* SNPs rs2975226, rs2652510 and rs2652511, and *NR4A2* SNP rs834829 are top-ranked in association with increased FTND scores among both treatment seeking and community smokers. The *DRD2* SNP appears to be a novel association with nicotine dependence. There is abundant existing information on the three *SLC6A3* promoter SNP SNPs identified in this study demonstrating association with *SLC6A3* expression. One hypothesis generated by this study and the extant literature is that the minor alleles of these *SLC6A3* promoter SNPs result in increased nicotine dependence severity among smokers via reduced expression of the *SLC6A3* gene. Hypotheses on whether *SLC6A3* promoter variation influences risk for nicotine dependence among individuals naïve to tobacco smoke, or whether the primary influence is on *DAT* expression during smoking initiation or during regular smoking, are not addressed by this analysis. Finally, evidence from this study and the existing drug dependence literature suggests that *NR4A2* is a candidate susceptibility gene influencing nicotine, alcohol and opioid dependence. Our interaction analysis suggests that some component of this influence occurs through interactive effects with variation at *SLC6A3*.

The primary limitation of this candidate gene association study with the FTND is the modest sample size of treatment seeking smokers. A larger sample or a similarly sized sample oversampled for extreme FTND scores from a larger sample would have had greater power to detect SNPs associated with FTND (Schork *et al.*, 2000). To limit the reporting of false positives, we used adjustment for testing of additive and dominant models and for testing of multiple SNPs at a candidate gene to rank SNPs in the treatment seeking smoker cohort. This adjustment does not adjust for the number of genes evaluated. We used a second multiplex ever smoker pedigree cohort to evaluate the top-ranked SNPs, although this cohort had substantial demographic and behavioral differences from the treatment seeking smoker cohort, suggesting that its value in this analysis is supportive in nature. With these limitations, noteworthy SNPs associated with FTND in the treatment seeking smoker cohort at three dopaminergic genes were associated with FTND in a second multiplex ever smoker pedigree cohort. For *SLC6A3* and *NR4A2* SNPs, supportive evidence for association to substance dependence is available in the genetic epidemiological literature (Li and Burmeister, 2009; Nielsen *et al.*, 2008), and for the *SLC6A3* SNPs, there is abundant evidence for functional effects of these noteworthy SNPs.

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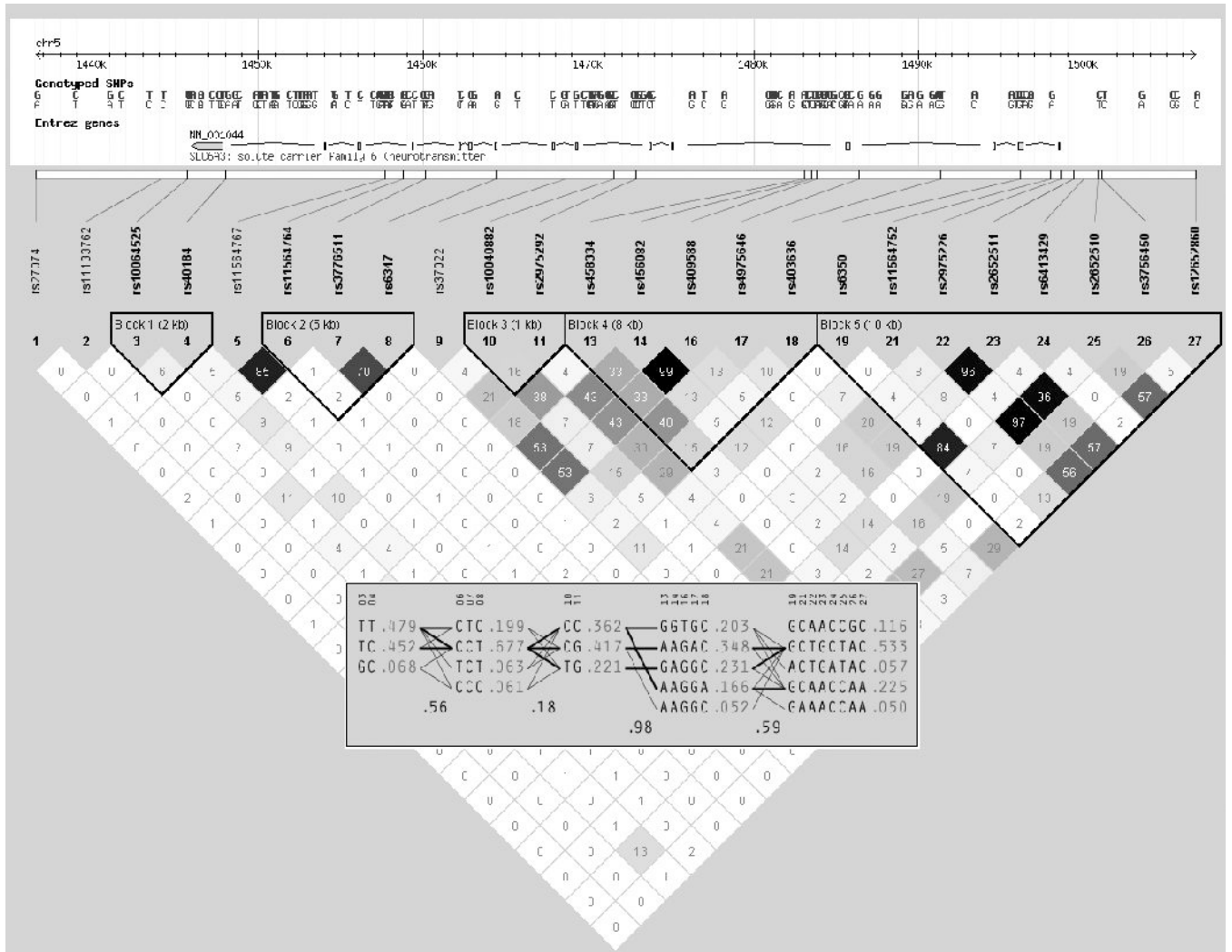


Figure 1.
SLC6A3 SNPs, LD and haplotypes, treatment seeking smoker cohort.

Table 1

Treatment Seeking Smoker Cohort Covariate Associations with the FTND

Variable	N	FTND M (SD)	r, t or F ¹	p ²	t ³	p ⁴
Age ⁵	828	5.3 (2.1)	0.19	<0.0001	5.84	<0.0001
Depressive symptoms ⁶	828	5.3 (2.1)	0.09	0.008	2.67	0.0077
Education						
16 years	410	4.9 (2.2)	5.58	<0.0001	4.37	<0.0001
<16 years	418	5.7 (2.1)				
Sex						
Female	420	5.1 (2.2)	3.03	0.003	3.47	0.0006
Male	408	5.6 (2.2)				
Site						
Buffalo	260	5.5 (2.1)	8.51	0.0002	2.89	0.0039
Philadelphia	258	5.6 (2.1)				
Washington	310	4.9 (2.2)				

¹ Univariate association estimate.² Univariate P.³ Multivariate association estimate.⁴ Multivariate P.⁵ Mean (SD) age is 45.5 (11.5) years.⁶ Mean (SD) CESD score is 11.8 (8.5).

Table 2

Top Ranked SNPs Associated with the FTND in the Treatment Seeking Smoker Cohort

SNP	Gene	Genomic ¹	Major ²	Minor ³	MAF ⁴	Model ⁵	Beta ⁶	SE ⁷	P _{LRT} ⁸	P _{ACT} ⁹
rs10891552	DRD2	IVS1+12127	A	T	0.04	Add	0.87	0.27	0.0014	0.037
rs2975226	SLC6A3	-2303	T	A	0.39	Add	0.31	0.10	0.0029	0.057
rs2652510	SLC6A3	-4547	T	C	0.40	Dom	0.45	0.15	0.0026	0.081
rs834829	NR4A2	-14150	G	A	0.29	Dom	0.37	0.15	0.0117	0.091
rs2652511	SLC6A3	-3076	G	A	0.39	Dom	0.44	0.15	0.0033	0.097

¹ Genomic, location of SNP relative to gene.² Major, major or referent allele.³ Minor or variant allele.⁴ MAF, minor allele frequency.⁵ Models are Additive (Add) and Dominant (Dom).⁶ Coefficient of genotype term of GLM model. A positive value implies association of the minor allele with increased FTND.⁷ SE, standard error of Beta.⁸ Likelihood ratio test P value.⁹ P value adjusted for correlated tests.

Unadjusted FTND Distributions by Genotype in the Treatment Seeking Smoker Cohort

Table 3

SNP	Major	Mean (SD)	Heterozygote	Mean (SD)	Minor	Mean (SD)	P_{F^2}	r^2
rs10891552	754	5.27 (2.18)	64	6.20 (2.10)	-	-	0.001	1.32
rs2975226	289	5.02 (2.20)	345	5.49 (2.15)	123	5.65 (2.22)	0.006	1.32
rs2652510	296	5.19 (2.21)	377	5.47 (2.15)	132	5.59 (2.19)	0.030	0.87
rs834829	405	5.18 (2.12)	334	5.58 (2.23)	69	5.12 (2.29)	0.010	0.82
rs2652511	300	5.06 (2.21)	377	5.49 (2.13)	124	5.59 (2.20)	0.016	1.04

I Genetic model of ANOVA is dominant or additive for rs10891552, heterotic for rs834829, and additive for *SLC6A3* SNPs. The genotype(s) associated with increased FTND scores can be inferred by inspection.

Table 4
 Multiplex Ever Smoker Pedigree Cohort Covariate Associations with the FTND

Variable	N	FTND M (SD)	r or t ¹	P ²	t ³	P ⁴
Age ⁵	423	3.9 (2.7)	0.24	<0.001	6.87	<0.0001
Depressive symptoms ⁶	410	3.9 (2.7)	0.11	0.022	3.65	0.0003
Education						
16 years	110	3.3 (3.0)	3.07	0.002	4.93	<0.0001
<16 years	312	4.2 (2.5)				
Sex						
Female	219	3.6 (2.7)	2.43	0.015	2.83	0.0048
Male	206	4.3 (2.6)				

¹ Univariate association estimate.

² Univariate P.

³ Multivariate association estimate.

⁴ Multivariate P.

⁵ Mean (SD) 39.4 (13.6).

⁶ Mean (SD) 11.9 (9.4)

Table 5

FBAT of the FTND in the Multiplex Ever Smoker Pedigree Cohort

SNP	Gene	Model ¹	Frequency ²	Pedigrees ³	Z ⁴	P _{FBAT} ⁵
rs10891552	DRD2	A/D & G	0.11 & 0.21	15	2.301	0.0214
rs2975226	SLC6A3	G	0.17	42	3.065	0.0022
rs2652510	SLC6A3	G	0.11	47	3.059	0.0022
rs834829	NR4A2	G	0.32	67	1.951	0.0511
rs2652511	SLC6A3	G	0.10	42	2.856	0.0043

¹ FBAT genetic model: A=Additive, D=Dominant, G=Genotype;

² allele or genotype frequency of minor allele model tested;

³ number of informative pedigrees;

⁴ normalized Z score, $(S-E(S))/\text{Var}(S)$, where a positive Z implies association of the genotype with a higher FTND score;

⁵ P value of FBAT.