

Convergent Evidence that Choline Acetyltransferase Gene Variation is Associated with Prospective Smoking Cessation and Nicotine Dependence

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The ability to quit smoking is heritable, yet few genetic studies have investigated prospective smoking cessation. We conducted a systems-based genetic association analysis in a sample of 472 treatment-seeking smokers of European ancestry after 8 weeks of transdermal nicotine therapy for smoking cessation. The genotyping panel included 169 single-nucleotide polymorphisms (SNPs) in 7 nicotinic acetylcholine receptor subunit genes and 4 genes in the endogenous cholinergic system. The primary outcome was smoking cessation (biochemically confirmed) at the end of treatment. SNPs clustered in the *choline acetyltransferase* (*ChAT*) gene were individually identified as nominally significant, and a 5-SNP haplotype (block 6) in *ChAT* was found to be significantly associated with quitting success. Single SNPs in *ChAT* haplotype block 2 were also associated with pretreatment levels of nicotine dependence in this cohort. To replicate associations of SNPs in haplotype blocks 2 and 6 of *ChAT* with nicotine dependence in a non-treatment-seeking cohort, we used data from an independent community-based sample of 629 smokers representing 200 families of European ancestry. Significant SNP and haplotype associations were identified for multiple measures of nicotine dependence. Although the effect sizes in both cohorts are modest, converging data across cohorts and phenotypes suggest that *ChAT* may be involved in nicotine dependence and ability to quit smoking. Additional sequencing and characterization of *ChAT* may reveal functional variants that contribute to nicotine dependence and smoking cessation.

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INTRODUCTION

Chronic exposure to nicotine, the addictive chemical in tobacco smoke, produces neuroadaptive changes that promote continued smoking (Buisson and Bertrand, 2002). Even with the most effective pharmacotherapies, only one in four smokers are able to quit (Schnoll and Lerman, 2006). Evidence for the heritability of nicotine dependence and smoking cessation (Li *et al*, 2003; Xian *et al*, 2003) has led to intensive efforts to identify susceptibility genes for these complex traits.

Nicotine binds to neuronal nicotinic acetylcholine receptors (nAChRs) in the mesolimbic-cortical reward pathway (Nestler, 2005), pointing to nAChRs as attractive candidates for genetic investigations of nicotine dependence. Data from genome-wide and candidate gene-based association studies have identified single-nucleotide polymorphisms (SNPs) in the *CHRNA5/CHRNA3/CHRNA4* gene cluster as associated with smoking rate and nicotine dependence (Saccone *et al*, 2007; Thorgeirsson *et al*, 2008). However, these SNPs have not been related consistently to smoking cessation (Baker *et al*, 2009; Breitling *et al*, 2009; Conti *et al*, 2008), supporting the premise that risk of developing dependence and the ability to quit once dependence has been established may represent two different but genetically overlapping phenotypes (Heath and Martin, 1993). Prospective assessment of smoking cessation among individuals intending to quit represents a more refined dependence phenotype for genetic association studies, as

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well as a powerful approach to identify novel therapeutic targets for developing more effective therapies for smoking cessation (Breitling *et al*, 2009).

We used a candidate gene panel focused on nAChRs in the endogenous cholinergic system to examine associations with prospective smoking cessation and nicotine dependence. The primary 'discovery' cohort included 472 smokers of European ancestry receiving open-label transdermal nicotine therapy, the most widely used treatment in the United States (Jonk *et al*, 2005) and Europe (West *et al*, 2005). We extended a previous systems-based genetic study of smoking cessation (Conti *et al*, 2008) by including genes involved in acetylcholine (ACh) synthesis and transport. Such genes may also contribute to nicotine dependence because ACh (released by cholinergic neurons) binds to presynaptic nAChRs, thereby influencing dopamine neurotransmission (Exley and Cragg, 2008). A set of nominally significant SNPs identified in the discovery cohort was tested in an independent family-based community sample of non-treatment-seeking smokers of European ancestry to replicate associations with nicotine dependence.

MATERIALS AND METHODS

Smoking Cessation Discovery Cohort

Sample. Treatment-seeking smokers were screened at the University of Pennsylvania from 2004 to 2008. Inclusion criteria included ages 18–65 and a smoking rate of ≥ 10 cigarettes per day. The exclusion criteria were: DSM IV Axis I psychiatric or substance abuse disorder (based on the Structured Clinical Interview-Non-Patient) (Spitzer *et al*, 1990), current use of psychotropic medications, and pregnancy or lactation. In the full cohort of 571 trial participants, there were 472 smokers of self-reported European ancestry. In all, 42% of participants were female and 34% were college graduates. The mean age was 45 years ($SD = 10.4$), with an average smoking duration of 29.5 years ($SD = 10.9$), and average cigarettes smoked per day of 21.9 ($SD = 9.1$). The mean Fagerström test for nicotine dependence (FTND) score was 5.24 ($SD = 2.2$), with a median of 5.

Procedures. The study protocol was approved by the University of Pennsylvania institutional review board. Participants completed assessments of demographics, current smoking rate, and nicotine dependence assessed with the FTND (Heatherton *et al*, 1991). After a pre-quit counseling session, transdermal nicotine therapy was initiated on a target quit date, which occurred 2 weeks later. All participants received brief behavioral counseling sessions at weeks 1, 2, and 4 of treatment (Schnoll *et al*, 2009). Self-reported smoking was assessed using the timeline follow-back procedure (Brown *et al*, 1998), and biochemically verified with a carbon monoxide (CO) breath sample. The primary outcome was biochemically confirmed 7-day point-prevalence abstinence at the end of treatment. As per convention (SRNT, 2002), participants who reported smoking within 7 days before the assessment ($n = 136$), failed to provide a CO sample ($n = 140$), or provided a CO > 10 p.p.m. ($n = 9$) were considered nonabstinent.

SNP selection. The genes included those coding for nAChRs, choline acetyltransferase (*ChAT*), acetylcholinesterase (*AChE*), choline transporter (*CHT*), and vesicular acetylcholine transporter (*VChAT*). The Illumina Assay Design Tool (www.illumina.com) identified all SNPs within, or 10 kb up- or down-stream from, the 11 targeted genes. The resulting list was filtered for Illumina designability rank of 1 and minor allele frequency (MAF) > 0.1 ; one SNP from any pairs separated by < 60 bp was chosen by design score prioritization to prevent interference in the multiplex genotyping assay. The filtered SNPs provided multiple markers distributed throughout each targeted gene region (Supplementary Table S1), generating a high-resolution panel for detecting haplotype structures in the study cohort. The panel was shown on existing HapMap linkage disequilibrium (LD) maps to identify any previously known Caucasian LD blocks not covered by SNPs in the panel. In such cases, the design filters were relaxed to include at least two SNPs within the relevant LD block. Non-synonymous coding SNPs and SNPs identified in previous association studies were included. The final custom Illumina GoldenGate array included 169 SNPs in 11 candidate genes and 359 ancestry informative markers and technical control SNPs found in the pre-designed Illumina DNA Test Panel (Supplementary Tables S1 and S2). The Illumina 'SNPscore' file containing all the annotations for the SNPs tested in the panel at the time of design is available by request.

Genotyping. GoldenGate 768-plex genotyping assays were performed in the Sentrix Array Matrix format. Failed SNP assays ($n = 18$) and DNA samples with low call rates ($n = 65$ out of 571) were removed from the data set after confirming replicate concordance. There were no significant deviations from Hardy-Weinberg equilibrium (HWE) using an adjusted cut-off of $p = 9.4 \times 10^{-5}$ to account for 528 SNPs tested based on Bonferroni correction. Thirty-four SNPs with MAF < 0.05 in this cohort were excluded. Potential population stratification in this European ancestry sample was analyzed with a multi-dimensional scaling (MDS) algorithm (Li and Yu, 2008) implemented in PLINK (Purcell *et al*, 2007) using ancestry informative markers; all 472 participants fall into one strong cluster suggesting a homogeneous study population (Supplementary Figure S1). This method identifies both clustered and continuous patterns of genetic variation and corrects for potential confounding effects by adjusting each subject's positions along identified axes of genomic variation and his/her memberships in detected clusters simultaneously. We analyzed up to 10 dimensions of variation in our MDS analyses.

Initial SNP analysis. Individual SNP associations with cessation were assessed using two-sided χ^2 tests or Fisher's exact tests. Both the one degree of freedom genotypic trend test (analogous to the Cochran-Armitage test) and the two degree of freedom tests of independence were performed. As there is no *a priori* knowledge of the underlying biological mechanism for these intronic SNPs, an additive model was considered most informative in both of the cohorts (Foulkes, 2009). Logistic regression models provided adjusted odds ratios and 95% confidence intervals

adjusting for age, sex, and nicotine dependence score. Odds ratios present the increase in risk associated with each additional copy of the minor allele. Associations between individual SNPs and nicotine dependence were modeled using linear regression. The distribution of FTND was found to approximate a Gaussian distribution and hence no transformation of this outcome was applied. As the primary objective of the discovery cohort was to find potential target genes of interest, *p*-values in this cohort were not corrected for multiple testing. Analyses were conducted using PLINK (Purcell *et al*, 2007) and SAS Genetics Version 9.2 (SAS Institute, Cary, NC).

Gene selection for further analysis. Gene selection criteria were (a) ≥ 5 SNPs with *p*-values ≤ 0.10 with at least three of these clustered within a 10-kb DNA sequence, or (b) any gene with a SNP association at a level of $p < 0.001$. Only the *ChAT* gene met this first criterion, and none met the second criterion. Therefore, subsequent analysis of nicotine dependence and haplotype analysis focused only on the *ChAT* SNPs, as did analyses in the replication cohort.

Haplotype analysis. Pair-wise LD between all SNP markers in *ChAT* was computed using Haploview (Barrett *et al*, 2005) and determination of haplotype blocks was based on criteria recommended by Gabriel *et al* (2002) (Figure 1). The single-SNP analysis informed which haplotype blocks would be examined (specifically haplotype block 6 in *ChAT*). We used the EM algorithm (Excoffier and Slatkin, 1995) to estimate haplotype frequencies, and haplotype-specific associations with cessation were tested using generalized linear models (GLM) (Schaid *et al*, 2002). This approach allowed us to assess the global significance between all haplotypes and outcome. For ease of interpretation, we also conducted haplotype-specific tests and

estimated haplotype-specific odds ratios and confidence intervals using the common haplotype as the reference haplotype. We chose this method because when comparisons are made between each haplotype to all others, the reference haplotype does not remain the same, and hence makes interpretation of the results more difficult. Testing used the haplo.stat program (haplo.glm, haplo.score, haplo.em; R version 2.7.2, <http://www.R-project.org>). As the score statistic distribution (to test for overall haplotype association) may not be normal in our data, *p*-values were calculated from empirical null distributions based on 1000 simulations.

The power analysis was conducted in SAS using a program based on the Schlesselman formulas (Schlesselman, 1982, 1987). Analyses of cessation in the discovery cohort were powered to detect an odds ratio of 1.8 or greater for allelic frequencies of 0.2 or greater with 80% power and type I error rate (α) of 5%. For a variant allele frequency of 0.1, our study provided 80% power to detect an odds ratio > 2.0 at $\alpha = 5\%$. This level of effect would be considered clinically significant, and is comparable to effects in previous pharmacogenetic trials of smoking cessation (David *et al*, 2007; Johnstone *et al*, 2007; Lerman *et al*, 2006).

Replication Cohort

Sample. Participants were recruited from the Mid-South states of Tennessee, Mississippi, and Arkansas from 1999 to 2004. Proband smokers were 18 years of age and older and reported smoking at least 20 cigarettes per day for the last 12 months. Siblings and parents of the smoking probands were recruited whenever possible. The 629 participants of European ancestry represented 200 families; 69.5% were female, with a mean age of 39.4 years (SD = 14.4) and mean nuclear family size of 3.17

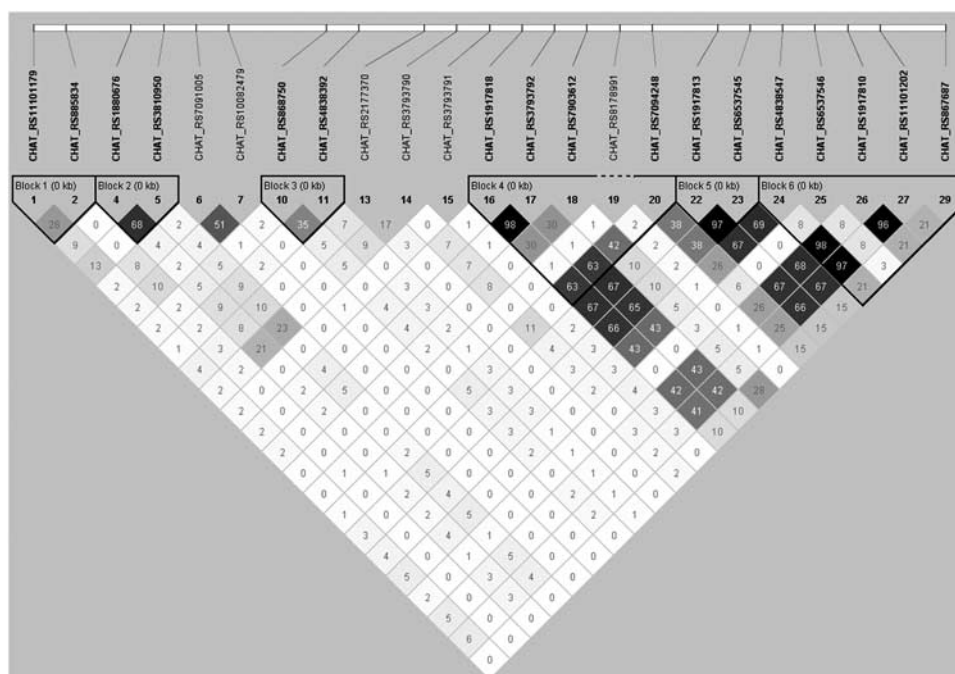


Figure 1 LD plot for SNPs on *ChAT* gene in the discovery cohort.

(SD = 0.69). The mean cigarettes per day was 19.5 (SD = 13.4) and median cigarettes per day was 25. The mean heaviness of smoking index (HSI) was 3.9 (SD = 1.4) with a median of 4; the mean FTND score was 6.33 (SD = 2.22) with a median of 7.

Procedures. Current nicotine dependence was ascertained by: smoking quantity (SQ: defined as the number of cigarettes smoked per day), the HSI (0–6 scale), which includes SQ and smoking urgency (ie, how soon after waking up does the subject smoke the first cigarette?), and the previously described FTND (Heatherton *et al*, 1991). The correlations among these measures ranged from 0.88 to 0.94, suggesting that these measures assess common and unique aspects of dependence (Li *et al*, 2008). All of the three measures showed a normal distribution.

The replication cohort analysis focused on seven SNPs in *ChAT* haplotype blocks 2 and 6, based on the presence of SNPs with $p < 0.05$ associations with either cessation or nicotine dependence in the discovery cohort, as well as to capture SNPs in both the 3' and 5' end regulatory regions of the gene. These included two SNPs in haplotype block 2 (rs1880676 and rs3810950) and five SNPs in haplotype block 6 (rs4838547, rs6537546, rs1917810, rs11101202, and rs867687). Genotyping used the *TaqMan* SNP Genotyping Assay in a 384-well microplate format (Applied Biosystems, Foster, CA). Allelic discrimination analysis was performed on the ABI Prism 7900HT Sequence Detection System, and SNP-specific control samples were added to each 384-well plate. Detailed procedure and conditions for genotyping in the replication cohort were described previously (Beuten *et al*, 2005; Li *et al*, 2005).

Family-based association analysis accounted for family structure. We used the PedCheck program to determine genotyping consistency for Mendelian inheritance of all SNPs. Departure from HWE was assessed at each locus by the χ^2 test at a significance threshold of $p < 0.01$. The allele frequencies for each SNP were calculated using the FREQ program of SAGE (v. 5.0). Associations between individual SNPs and the dependence measures were determined by the PBAT program (version 3.6) using generalized estimating equations assuming an additive genetic model to be consistent with the discovery cohort (Lange *et al*, 2003).

An exploratory haplotype analysis in the replication cohort used a sliding window approach as performed in previous association studies of these nicotine dependence measures and other complex traits (Huang *et al*, 2009; Lin *et al*, 2004; Nussbaum *et al*, 2008). We used the FBAT program, with the computation of p -values for the Z -statistic based on the Monte Carlo sampling option under the null distribution of no linkage and no association (Horvath *et al*, 2004). Gender and age were included as covariates. Significant associations were corrected for multiple testing using the Bonferroni correction for haplotype analysis.

RESULTS

Discovery Cohort

Smoking cessation. Of the 472 participants, 150 were verified quitters and 322 had relapsed. The quit rates

observed in our study are comparable to our previous NRT study (Lerman *et al*, 2004) and meta-analyses of other NRT studies (Stead *et al*, 2008). Eight SNPs in *ChAT* showed nominal associations ($p < 0.10$) with smoking cessation (uncorrected for multiple testing) (Table 1). These include one SNP in haplotype block 2 (rs1880676), which is located in an alternatively spliced version of *ChAT* that produces a 74-kDa protein (*ChAT* isoform 3), three SNPs in haplotype block 4 (rs1917818, rs3793792, and rs7094248) and four SNPs in haplotype block 6 (rs4838547, rs6537546, rs1917810, and rs11101202). However, if we correct for multiple testing using a stringent Bonferroni correction for all the SNPs tested, none of the associations would remain statistically significant. In all logistic regression models of 8-week smoking cessation, FTND score was significantly associated with outcome ($p < 0.0001$); however, age and sex were not.

The most significant ($p \leq 0.005$) single SNP associations were observed in haplotype block 6 (formed by rs4838547, rs6537546, rs1917810, rs11101202, and rs867687). Therefore, we focused our haplotype analysis on this block. Common haplotypes accounted for 99% of all haplotypes, with the most common one being G–A–G–G–A with a frequency of 43%. Haplotype block 6 was significantly associated with cessation using a global test for haplotype association under an additive model ($p = 0.02$). When each haplotype was compared with the most common haplotype (G–A–G–G–A), haplotypes A–A–A–C–G and A–T–A–C–A were significantly associated with relapse (Table 2). The odds of cessation at the end of treatment was 0.65 (CI = 0.44–0.95, $p = 0.04$) among individuals with haplotype A–A–A–C–G, and 0.5 (CI = 0.29–0.88, $p = 0.02$) among individuals with haplotype A–T–A–C–A compared with the reference haplotype (Table 2).

Smoking cessation was not associated with SNPs in any of the nAChR genes examined (p -values ranged from 0.15 to 0.83) (Supplementary Table S1). Allele frequencies for the SNPs in the *CHRNA5/A3/B4* gene cluster previously associated with nicotine dependence (Saccone *et al*, 2007; Thorgeirsson *et al*, 2008) were not different in relapsers and abstainers. For example, frequencies for the major A allele of rs16969968 were 61% in the relapsed and 62% in the abstinent groups, respectively; for the G allele of rs1051730, allele frequencies were 61% and 63% respectively. Supplementary Table S3 includes the allele frequencies and p -values for selected SNPs in the nAChR genes that have been associated with smoking behavior in previous studies (Etter *et al*, 2009; Greenbaum *et al*, 2006; Hutchison *et al*, 2007; Li *et al*, 2005; Saccone *et al*, 2007, 2009).

Nicotine dependence. Three *ChAT* SNPs (rs1880676, rs3810950, and rs868750) were also significantly associated with level of nicotine dependence (allele p -values were 0.01, 0.02, and 0.04, respectively). The 2-SNP haplotype (rs1880676 and rs3810950 in block 2) showed borderline statistical significance for association with nicotine dependence under an additive model after adjustment for age and sex ($p = 0.06$). However, these reported p -values are unadjusted for multiple-testing in the discovery cohort.

Table 1 Allele Frequencies for *ChAT* SNPs and Association with Smoking Cessation

| dbSNP ID (alleles) ^a | Hap block | Position | SNP location | MAF | MAF abstinent | MAF relapsed | OR ^b (95% CI) | p-Value (per allele) |
|----------------------------------|-----------|----------|--------------|-------------|---------------|--------------|--------------------------|----------------------|
| rs11101179 (G /A) | 1 | 50480897 | 5' near gene | 0.29 | 0.3 | 0.29 | 1.05 (0.77, 1.43) | 0.75 |
| rs885834 (G /A) | 1 | 50485518 | 5' near gene | 0.41 | 0.44 | 0.39 | 1.25 (0.94, 1.43) | 0.12 |
| rs1880676 (A /G) | 2 | 50494123 | Intron | 0.34 | 0.39 | 0.33 | 1.34 (0.96, 1.87) | 0.08 |
| rs3810950 (A /G) | 2 | 50494625 | Exon | 0.26 | 0.28 | 0.25 | 1.19 (0.86, 1.66) | 0.29 |
| rs7091005 (A /G) | | 50496169 | Intron | <0.1 | 0.08 | 0.07 | 1.23 (0.7, 2.14) | 0.47 |
| rs10082479 (A /T) | | 50499225 | Intron | 0.1 | 0.09 | 0.11 | 0.96 (0.65, 1.41) | 0.61 |
| rs868750 (A /G) | 3 | 50503845 | Intron | 0.17 | 0.17 | 0.17 | 0.96 (0.65, 1.41) | 0.83 |
| rs4838392 (G /A) | 3 | 50504984 | Intron | 0.37 | 0.4 | 0.36 | 1.2 (0.88, 1.63) | 0.25 |
| rs2177370 (G /A) | | 50508880 | Intron | 0.39 | 0.38 | 0.4 | 0.92 (0.68, 1.23) | 0.56 |
| rs3793790 (G /A) | | 50510742 | Intron | 0.33 | 0.35 | 0.33 | 1.11 (0.82, 1.51) | 0.49 |
| rs3793791 (G /A) | | 50511710 | Intron | <0.1 | 0.06 | 0.08 | 0.68 (0.4, 1.17) | 0.16 |
| rs1917818 (C /A) | 4 | 50519348 | Intron | 0.39 | 0.43 | 0.37 | 1.3 (0.98, 1.7) | 0.072 |
| rs3793792 (A /G) | 4 | 50520169 | Intron | 0.39 | 0.43 | 0.38 | 1.29 (0.97, 1.7) | 0.08 |
| rs7903612 (G /C) | 4 | 50522533 | Intron | 0.32 | 0.3 | 0.32 | 0.9 (0.65, 1.23) | 0.49 |
| rs8178991 (A /G) | 4 | 50524643 | Exon | 0.03 | 0.03 | 0.02 | 1.3 (0.53, 3.15) | 0.57 |
| rs7094248 (C /G) | 4 | 50525374 | Intron | 0.50 | 0.54 | 0.48 | 1.32 (0.99, 1.75) | 0.06 |
| rs1917813 (G /A) | 5 | 50527841 | Intron | 0.48 | 0.52 | 0.46 | 1.27 (0.95, 1.68) | 0.1 |
| rs6537545 (G /A) | 5 | 50529255 | Intron | 0.47 | 0.51 | 0.46 | 1.23 (0.93, 1.64) | 0.15 |
| rs4838547 (G /A) | 6 | 50532410 | Intron | 0.44 | 0.5 | 0.42 | 1.53 (1.14, 2.04) | 0.004 |
| rs6537546 (T /A) | 6 | 50534049 | Intron | 0.09 | 0.06 | 0.11 | 0.60 (0.35, 1.03) | 0.06 |
| rs1917810 (G /A) | 6 | 50540077 | Intron | 0.44 | 0.5 | 0.42 | 1.53 (1.14, 2.04) | 0.004 |
| rs11101202 (G /C) | 6 | 50542408 | Intron | 0.43 | 0.5 | 0.41 | 1.51 (1.13, 2.02) | 0.005 |
| rs867687 (G /A) | 6 | 50547271 | 3'flanking | 0.22 | 0.19 | 0.23 | 0.76 (0.52, 1.1) | 0.14 |

Abbreviation: MAF, minor allele frequency.

^aThe minor allele is bolded for each SNP.

^bOdds ratio for the minor allele adjusting for age, sex, and nicotine dependence score assuming an additive model (SNPs with $p < 0.05$ are in bold). The direction of association can be determined based on the MAF in the abstinent and relapsed groups.

Table 2 Haplotype Analyses of *ChAT* with Smoking Cessation in the Discovery Cohort (5 *ChAT* SNPs: formed by rs4838547, rs6537546, rs1917810, rs11101202, and rs867687 in Haplotype Block 6)

| Haplotype | Freq (%) | Haplotype frequency in abstinent | Haplotype frequency in relapsed | OR (95% CI) | p-Value ^a |
|----------------|----------|----------------------------------|---------------------------------|-------------------|----------------------|
| G-A-G-G-A | 43.1 | 49.6 | 40.1 | 1 | |
| A-A-A-C-A | 24.8 | 23.4 | 25.4 | 0.72 (0.51, 1.03) | 0.07 |
| A-A-A-C-G | 21.8 | 18.5 | 23.3 | 0.65 (0.44, 0.95) | 0.04 |
| A-T-A-C-A | 9.3 | 6.6 | 10.6 | 0.5 (0.29, 0.88) | 0.02 |
| Global p-value | | | | | 0.02 |

^aComputed using the haplotype score test using an additive model. Adjusted for age, sex, and nicotine dependence score.

Replication Cohort

Minor allele frequencies for the seven SNPs in the replication cohort were comparable to those in the primary discovery cohort. Three SNPs in haplotype block 6 showed $p \leq 0.05$ associations with at least one measure of nicotine dependence (Table 3a). Two SNPs located in haplotype block 2 were not significantly associated with any measure of nicotine dependence during individual SNP testing. Haplotype G-G-T-A-A (formed by rs1880676 and rs3810950 in block 2 and rs4838547, rs6537546, and rs1917810 in block 6; frequency of 34.7%) was significantly

associated with SQ, HSI, and FTND assuming a dominant model, yielding p -values of 0.018, 0.0085, and 0.0096, respectively (Table 3b). The haplotype A-A-C-A-G showed a trend toward association with SQ and HSI, with p -values of 0.058 and 0.056, respectively (Table 3b).

DISCUSSION

This study examined genetic variation in the nicotinic receptor system for associations with prospective smoking cessation and nicotine dependence. In the discovery cohort

of treatment-seeking smokers, we identified a cluster of SNPs in *ChAT* haplotype block 6 (formed by rs4838547, rs6537546, rs1917810, and rs11101202) showing nominal associations with smoking cessation in individual SNP-level as well as haplotype analysis. Specifically, three SNPs in haplotype block 6 (rs4838547, rs1917810, and rs11101202) were associated with smoking cessation at an individual SNP level ($p \leq 0.005$, uncorrected for multiple comparisons). Further, the 5-SNP haplotypes in block 6, A-A-A-C-G and A-T-A-C-A, were significantly associated with relapse as compared with the most common haplotype group. A closer examination of the haplotypes associated with greater relapse risk confirmed that the same SNPs that were significant in the single SNP analysis were also driving the haplotype results. Three *ChAT* SNPs were also associated with nicotine dependence in the discovery sample. In the replication sample of non-treatment-seeking smokers, three SNPs in haplotype block 6 (rs4838547, rs11101202, and rs867687) were also associated with nicotine dependence; rs4838547 and rs11101202 were also associated with cessation status in the discovery cohort with the same alleles predisposing to relapse and nicotine dependence phenotypes in the respective cohorts. Also in the replication cohort, a major haplotype (G-G-T-A-A) formed by 5 SNPs (rs1880676, rs3810950, rs4838547, rs6537546, and rs1917810) with a frequency of 34.7% was significantly associated with

all three measures of nicotine dependence. Although the overall effect sizes are modest, the convergent signals for haplotype blocks 2 and 6 across cohorts and phenotypes provide initial evidence that *ChAT* may contribute to nicotine dependence and smoking cessation.

These *ChAT* SNPs are likely to be surrogate markers for as yet undiscovered functional polymorphisms. Although our initial cohorts were not powered to test for associations with the known but rare non-synonymous coding SNPs included in the genotyping assay, a polymorphism (ie, rs1880676) previously unrecognized as affecting amino acid sequence was associated with nicotine dependence ($p = 0.01$) and smoking cessation ($p = 0.08$) in the discovery cohort. The SNP rs1880676 in haplotype block 2 is located in an alternatively spliced version of *ChAT* that encodes a rarely studied isoform (Ohno *et al*, 2001). The minor allele of *ChAT* rs1880676 alters amino acid 7 from aspartate to its uncharged amide asparagine in isoform 3, reducing the number of negatively charged residues from four to three in the 36 amino acid N-terminal extension (Figure 2). As this region also contains five basic (positively charged) residues, this polymorphism may affect regional electrostatic charge in this N-terminal domain. The development of assays to detect *ChAT* isoform 3 and determine its tissue and subcellular distribution, and to characterize the consequences of this coding polymorphism with respect to ACh levels, could be useful to determine whether this SNP may have a key role in neuronal function and possibly nicotine dependence. It is noteworthy that rs1880676 has also been associated with late-onset Alzheimer's disease (Harold *et al*, 2006), as well as schizophrenia and response to anti-psychotic treatment (Mancama *et al*, 2007); however, the molecular mechanism underlying its involvement in these disorders is largely undetermined.

In spite of the lack of knowledge regarding the functional significance of the SNPs in this report, there is a compelling biological rationale to support a potential contribution of *ChAT* to nicotine dependence and smoking persistence. *ChAT* is the key enzyme responsible for synthesis of endogenous ACh and is traditionally used as a marker for cholinergic terminals in the brain. Cholinergic projections from the posterior pendunculopontine tegmental nucleus (PPTg) to ventral striatum are thought to have a role in nicotine self-administration (Alderson *et al*, 2006; Corrigan *et al*, 2002). Nicotine administration causes release of ACh

Table 3a *p*-Values for Associations of Individual SNPs with Three Nicotine Dependence Measures under an Additive Model in the Replication Sample

| dbSNP ID | MAF | SQ | HSI | FTND |
|------------|------|-----------|------------------|------------|
| rs1880676 | 0.28 | 0.311 (G) | 0.276 (G) | 0.234 (G) |
| rs3810950 | 0.27 | 0.413 (G) | 0.339 (G) | 0.279 (G) |
| rs4838547 | 0.44 | 0.108 (A) | 0.038 (A) | 0.058 (A) |
| rs6537546 | 0.08 | 0.718 (A) | 0.621 (A) | 0.2589 (A) |
| rs1917810 | 0.45 | 0.106 (A) | 0.051 (A) | 0.077 (A) |
| rs11101202 | 0.45 | 0.095 (C) | 0.041 (C) | 0.066 (C) |
| rs867687 | 0.21 | 0.066 (G) | 0.050 (G) | 0.096 (G) |

Alleles in parentheses indicate the allele positively associated with the smoking phenotype (ie, the risk allele).

Bold entries indicate significant $p < 0.05$ associations.

Table 3b Exploratory Analysis Examining the Association of Major Haplotypes Formed by rs1880676, rs3810950, rs4838547, rs6537546, and rs1917810 with Three Nicotine Dependence Measures in the Replication Sample

| Haplotype | Freq (%) | SQ | HSI | FTND |
|------------------------|----------|-----------------------------|------------------------------|------------------------------|
| G-G-T-A-A | 34.7 | 2.351 (0.0187 , 61) | 2.630 (0.00854 , 63) | 2.589 (0.00962 , 65) |
| G-G-G-A-G | 32.1 | -0.049 (0.961, 62) | -0.520 (0.603, 61) | -0.258 (0.797, 64) |
| G-G-A-T-A | 6.3 | 0.249 (0.803, 26) | 0.772 (0.440, 27) | 0.859 (0.390, 27) |
| A-A-G-A-G | 11.1 | -1.899 (0.0576, 43) | -1.913 (0.0557, 44) | -1.687 (0.0916, 45) |
| A-A-A-A-A | 13 | 0.186 (0.852, 49) | 0.301 (0.764, 49) | 0.108 (0.914, 52) |
| Global <i>p</i> -value | | 7.804 (0.167) | 9.940 (0.0770) | 9.350 (0.0959) |

Three numbers within each cell are Z score, *p*-value, and informative family size (last two are in parentheses), respectively. Results for the dominant model are reported.

```

atgtggccggaatgcaga (G/A) atgaagcactgagcacagtaggtccacacctctgcatccct
M W P E C R D/N E A L S T V G P H L C I P
gcaccaggactcaccaagacgcccacccatcctggaaaagggtcccccgtaatggcagcaaaa ...
A P G L T K T P I L E K V P R K M A A K ...

```

Figure 2 The N-terminal extension of 74-kDa ChAT.

in both *in vivo* and *in vitro* model systems (Rowell and Winkler, 1984; Tani *et al*, 1998), and repeated nicotine administration sensitizes ACh release (Arnold *et al*, 2003). Chronic nicotine administration to adult rats increases ChAT enzyme activity (Hernandez and Terry, 2005) and nicotine withdrawal in adolescent animals alters levels of ChAT enzyme activity in some brain regions (Slotkin *et al*, 2008). These studies support the biological plausibility of an association of smoking behavior with ChAT genetic variation.

The observed association of ChAT with smoking cessation is consistent with the results of a previous pharmacogenetic trial (Heitjan *et al*, 2008). ChAT SNP rs1917810 in haplotype block 6 was associated with response to bupropion *vs* placebo; consistent with the current findings, smokers with the minor allele had higher abstinence rates on placebo (Heitjan *et al*, 2008). Replication in additional clinical trials and community-based cohorts will be important to confirm whether variation in the ChAT gene is associated with nicotine dependence severity and cessation.

Some limitations of this study should be considered when interpreting the results. First, although SNPs chosen for the study provided adequate coverage of the ChAT gene, the functional consequences are unknown for the vast majority of these. Second, the sample sizes of these cohorts are not very large and most of the identified associations would not remain significant after correction for multiple comparisons. However, the studies were adequately powered to detect SNP associations at a level considered to be clinically significant. Third, the primary end of treatment (8-week) end-point might not be a sufficient duration to define someone as a quitter, given relapses that may occur after treatment. However, the vast majority of relapses occur within 5–10 days after a quit attempt (Piasecki, 2006), suggesting that we are probably able to capture most of the treatment success by our 8-week time point. Further, exploratory analysis of the ChAT SNP associations with quitting success at 12 months after the target quit date indicates that SNPs associated significantly with cessation at week 8 remain significant ($p = 0.01$) at 12 months (Supplementary Table S4).

In summary, this study provides novel convergent evidence for associations of ChAT with smoking cessation and nicotine dependence, suggesting that this gene warrants closer attention. Pre-clinical pharmacology studies that alter the activity of the ChAT enzyme and analyze effects on dependence phenotypes in rodents would be useful to increase our understanding of the role of the endogenous cholinergic system in nicotine dependence. Studies to identify functional variants in the ChAT gene that affect expression levels or enzymatic function of ChAT are needed. Pending further investigation, the current findings may have implications for medication development for nicotine dependence. Although molecules that alter ChAT activity have not been tested clinically, acetylcholinesterase

(AChE) inhibitors, such as galanamine, may decrease smoking behavior (Diehl *et al*, 2006).

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DISCLOSURE

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