

Significant associations of *CHRNA2* and *CHRNA6* with nicotine dependence in European American and African American populations

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Abstract The direct physiological effects that promote nicotine dependence (ND) are mediated by nicotinic acetylcholine receptors (nAChRs). In line with the genetic and pharmacological basis of addiction, many previous studies have revealed significant associations between variants in the nAChR subunit genes and various measures of ND in different ethnic samples. In this study, we first examined the association of variants in nAChR subunits $\alpha 2$ (*CHRNA2*) and $\alpha 6$ (*CHRNA6*) genes on chromosome 8 with ND using a family sample consisting of 1,730 European Americans (EAs) from 495 families and 1,892 African Americans (AAs) from 424 families (defined as the discovery family sample). ND was assessed by two standard quantitative measures: smoking quantity (SQ) and the Fagerström Test for ND (FTND). We found nominal associations for all seven tested SNPs of the genes with at least one ND measure in the EA sample and for two SNPs in *CHRNA2* in the AA sample. Of these, associations of SNPs rs3735757 with FTND ($P = 0.0068$) and rs2472553 with

both ND measures (with a P value of 0.0043 and 0.00086 for SQ and FTND, respectively) continued to be significant in the EA sample even after correction for multiple tests. Further, we found several haplotypes that were significantly associated with ND in the EA sample in *CHRNA6* and in the both EA and AA samples in *CHRNA2*. To confirm the associations of the two genes with ND, we conducted a replication study with an independent case–control sample from the SAGE study, which showed a significant association of the two genes with ND, although the significantly associated SNPs were not always the same in the two samples. Together, these findings indicate that both *CHRNA2* and *CHRNA6* play a significant role in the etiology of ND in AA and EA smokers. Further replication in additional independent samples is warranted.

Introduction

Tobacco use continues to be an important worldwide health concern. According to World Health Organization, there were 1.3 billion tobacco users world wide in 2004 (World Health Organization 2012). In the USA, 46.0 million adults were cigarette smokers in 2008, and the number of deaths annually from smoking-related illnesses accounts for 30 % of deaths from cancer and nearly 80 % of deaths from chronic obstructive pulmonary disease (CDC 2008; Mokdad et al. 2004). The annual economic burden of smoking is also substantial, with a staggering \$193 billion in medical costs and productivity losses (CDC 2008; Mokdad et al. 2004).

Cigarette smoking is a complex behavior, with both genetic and environmental components (Al Koudsi and Tyndale 2005; Sullivan and Kendler 1999). Many family, adoption, and twin studies of smoking addiction have

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indicated a heritability of 11–78 %, with an average heritability of 59 % for both male and female smokers (Kendler et al. 1999; Li et al. 2003; Maes et al. 2004; Vink et al. 2005).

Nicotine, the primary psychoactive ingredient in cigarette smoke, exerts its effects by readily crossing the blood–brain barrier and binding to nicotinic acetylcholine receptors (nAChRs) in various brain structures (Wonnacott 1997). Activation of nAChRs on dopaminergic terminals induces dopamine release in the mesolimbic brain reward system (Kleijn et al. 2011; Wonnacott et al. 2000). To date, 17 nAChR subunits have been identified, which are divided into muscle and neuronal types (Kalamida et al. 2007). Neuronal nAChRs are widely expressed in the nervous system in peripheral ganglia and certain areas of the brain, as well as in nonexcitable cells, such as epithelium and cells of the immune system (Cui and Li 2010). Of the neuronal nAChRs, genes for nine α ($\alpha 2$ – $\alpha 10$) and three β ($\beta 2$ – $\beta 4$) subunits have been cloned. The $\alpha 7$ – $\alpha 10$ subunits are found either as homopentamers (of five $\alpha 7$, $\alpha 8$, or $\alpha 9$ subunits) or as heteropentamers (of $\alpha 7/\alpha 8$ and $\alpha 9/\alpha 10$) (Plazas et al. 2005). By contrast, the $\alpha 2$ – $\alpha 6$ and $\beta 2$ – $\beta 4$ subunits form heteropentameric receptors, usually with a $(\alpha_x)_2(\beta_y)_3$ stoichiometry.

Whereas several human neuronal nAChR subunit genes have been investigated for association with ND and other smoking-related behaviors in human subjects [for reviews, see (Berrettini and Doyle 2012; Greenbaum and Lerer 2009; Li and Burmeister 2009)], *CHRNA2* has received less attention. Early linkage analysis of the Collaborative Studies on Genetics of Alcoholism (COGA) data showed modest evidence of linkage to 8p22–23, near *CHRNA2*, using two smoking phenotypes (ever-smoked and average number of packs per year) (Bergen et al. 1999). The association of *CHRNA2* with smoking was reported in the schizophrenia families through linkage analysis and the candidate gene approach (Faraone et al. 2004). Although there is a reported association of *CHRNA2* with ND, measured by DSM-IV and FTND score, in the Iowa Adoption Studies, the results were not corrected for multiple comparisons (Philibert et al. 2009; Yates et al. 1998). In a smoking cessation trial, SNP rs2565065 in *CHRNA2* appeared to have pharmacogenetic relevance (Heitjan et al. 2008). In contrast, several other studies have failed to reveal a significant association of this gene with ND or other smoking-related phenotypes (Keskitalo-Vuokko et al. 2011).

The *CHRNA6* and *CHRNA3* genes are located contiguously in a tail-to-tail configuration on chromosome 8. Both $\alpha 6$ - and $\beta 3$ -nAChRs are found in various brain regions, including the substantia nigra, ventral tegmental area (VTA), striatum, and locus coeruleus (Gotti et al.

2006a, b), which have significant roles in dopaminergic neurotransmission, thus contributing to reward and reinforcement of behavior (Cui et al. 2003). The $\alpha 6\beta 3$ - as well as $\alpha 6\alpha 4\beta 2\beta 3$ -containing receptors in the striatum mediate α -conotoxin MII-sensitive dopamine release. In contrast, $\alpha 6\beta 2$ -containing receptors in the superior colliculus seem to be involved in GABA release (Champtiaux et al. 2003; Gotti et al. 2006a, b; Salminen et al. 2004). A recent meta-GWAS study indicated that rs2304297 in *CHRNA6* is significantly associated with ND in the European sample, but the finding did not reach genome-wide significance (Thorgeirsson et al. 2010). Candidate gene-based association studies indicated that SNPs rs2304297 in the 3'-UTR of *CHRNA6* was associated with ND in the European sample (Hoft et al. 2009b; Saccone et al. 2007), as was rs1072003 in intron 2 of *CHRNA6* with ND in an Israeli female sample (Greenbaum et al. 2006).

Considering that nearly all subjects used in these GWAS or candidate gene-based association studies were of European origin, it would be interesting to know whether *CHRNA6* and *CHRNA2* genes are also associated with ND in smokers of other ethnicities. Thus, the primary objective of this study was to determine whether significant association of variants in *CHRNA6* and *CHRNA2* with ND can be detected in independent samples, especially in African American smokers.

Materials and methods

Subjects and ND measures

Discovery family sample

Subjects of this sample include persons of both AA and EA origin who were recruited primarily from the states of Tennessee, Mississippi, Arkansas, and Michigan. Proband smokers were required to be at least 21 years old, to have smoked for at least the last 5 years, and to have smoked at least 20 cigarettes per day during the last 12 months. Once proband smokers were identified, their biological parents and siblings were invited to participate whenever possible. Table 1 provides the detailed characteristics of the two ethnic groups. All participants provided written informed consent, and the Institutional Review Boards of each participating institution approved the study.

The ND of each smoker was assessed with the two commonly used measures of smoking quantity (SQ; the number of cigarettes smoked per day) and the Fagerström test for ND (FTND; 0–10 scale) (Fagerstrom 1978). Because of the overlap of the contents of the two measures, a fairly

Table 1 Description of discovery and replication samples

Ethnicity	Discovery family samples		Replication case–control samples	
	AA	EA	AA	EA
No. of nuclear families	424	495	–	–
Avg. members/family (SD)	4.46 (0.88)	3.49 (0.80)	–	–
No. of subjects	1,892	1,730	1,136	2,428
Female (%)	57	57	52	56
Age [years (SD)]	40.32 (14.60)	45.39 (15.77)	39.68 (6.71)	38.37 (9.65)
No. of smokers	1,013	1,088	626	1,048
CPD (SD)	21.55 (12.07)	22.07 (12.60)	24.33 (17.46)	26.16 (19.71)
FTND score (SD)	5.49 (3.60)	4.62 (3.55)	3.90 (2.88)	2.96 (3.27)

robust correlation exists among them in both populations ($r = 0.88$ for AAs and 0.89 for EAs).

Replication case–control sample

All subjects included in this sample were participants in the Study of Addiction: Genetics and Environment (SAGE) (Bierut et al. 2010) through the NCBI dbGaP database (dbGaP study accession phs000092.v1.p1). Quantitative measurements of severity of addiction to various substances, including nicotine, are provided in this dataset. For a detailed description, please see http://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000092.v1.p1.

Genotyping and imputation

For the discovery family samples, genomic DNA was either extracted from cells in the peripheral venous blood of each participant using a Maxi kit (Qiagen Inc, Valencia, CA, USA) or obtained from the NIDA Genetics Repository at Rutgers University. Seven SNPs in *CHRNA2* (rs2292976, rs3735757, rs891398, and rs2472553) and *CHRNA6* (rs9298628, rs892413, and rs2217732) were selected based on results reported by other researchers (Heitjan et al. 2008; Hoft et al. 2009b; Philibert et al. 2009), the location of each SNP and a uniform coverage of the gene of interest, and allele frequency in samples with European and African origins from NCBI SNP database. All SNPs were genotyped using *TaqMan* assays in the 384-well microplate format (Applied Biosystems Inc., Foster City, CA, USA) as reported previously (Beuten et al. 2005; Li et al. 2005; Ma et al. 2005). Briefly, 15 ng of DNA was amplified in a total volume of 7 μ l containing an MGB probe and 2.5 μ l of *TaqMan* universal PCR master mix. Allelic discrimination analyses were performed on the ABI Prism 7900HT. To ensure the quality of genotyping, four no-template negative controls and four positive controls were added to each 384-well plate.

The SAGE samples were genotyped on commercially available platforms, including Illumina (San Diego, CA,

USA). Quality control was performed in each group separately, with the goal of excluding those samples with sex or chromosomal anomalies, a low call rate, or first- or second-degree relatedness. Imputations of non-genotyped SNPs in the 1000 Genome CEU v2 (2010–11 release) and the HapMap Phase II CHB + JPT were carried out for the SAGE data using MaCH (Li et al. 2009, 2010) and IMPUTE v2 (Howie et al. 2009; Marchini et al. 2007), respectively.

Statistical analysis

To test for genotyping quality in the discovery family sample, we assessed Mendelian inconsistencies and departure from Hardy–Weinberg equilibrium (HWE) using Haploview (v. 4.0) software (Barrett et al. 2005). Subjects with any inconsistent SNP data for a given genetic variant were excluded from analysis.

Individual and haplotype-based association analysis for the discovery family sample

Associations between the seven SNPs in *CHRNA2* and *CHRNA6* and the two ND measures were determined using the pedigree-based association test (PBAT v. 3.5) based on the generalized estimating equation approach (Lange et al. 2004). Pair-wise linkage disequilibrium (LD) and haplotype blocks for the four SNPs in *CHRNA2* and three SNPs in *CHRNA6* were assessed by Haploview (v. 4.0) software (Barrett et al. 2005; Gabriel et al. 2002). Association analysis for haplotypes located in each LD block with the two ND measures was performed using the family-based association test (FBAT v.1.7.3) (Horvath et al. 2004). Three genetic models (additive, dominant, and recessive) were tested for all association analyses, with sex and age as covariates in the AA and EA samples. Statistically significant results ($P < 0.05$) for individual SNPs and major haplotypes (frequency $\geq 5\%$) were adjusted for multiple testing using Bonferroni correction.

Table 2 ND-associated *P* values under the three genetic models for the first given allele of each SNP in *CHRNA2* and *CHRNA6* in the discovery family sample

Gene	dbSNP ID (Gene Location)	Alleles	African American			European American			
			Allele freq.	SQ	FTND	Allele freq.	SQ	FTND	
CHRNA2	rs2292976 (Exon 8)	A/G	0.13/0.87	0.120 ^a	0.0866 ^a	0.14/0.86	−0.902 ^a	−0.664 ^a	
				0.0578 ^d	0.0461 ^d		0.502 ^d	0.631 ^d	
				−0.444 ^r	−0.700 ^r		−0.0378 ^r	−0.0221 ^r	
	rs3735757 (Intron 5)	G/C	0.22/0.78	−0.378 ^a	−0.613 ^a	0.14/0.86	−0.589 ^a	−0.291 ^a	
				−0.635 ^d	−0.810 ^d		0.750 ^d	−0.891 ^d	
				−0.239 ^r	−0.464 ^r		−0.00782 ^r	−0.00675 ^r	
	rs891398 (Exon 5)	T/C (T125A)	0.25/0.75	0.0201 ^a	0.00790 ^a	0.52/0.48	−0.511 ^a	−0.450 ^a	
				0.026 ^d	0.0287 ^d		0.228 ^d	0.294 ^d	
				0.302 ^r	0.0814 ^r		−0.0357 ^r	−0.0338 ^r	
	rs2472553 (Exon 2)	T/C (T22I)	0.16/0.84	0.481 ^a	0.534 ^a	0.13/0.87	−0.595 ^a	−0.192 ^a	
				0.324 ^d	0.387 ^d		0.776 ^d	−0.724 ^d	
				−0.538 ^r	−0.649 ^r		−0.00429 ^r	−0.000863 ^r	
	CHRNA6	rs9298628 (3′-flanking)	C/T	0.25/0.75	−0.422 ^a	−0.375 ^a	0.81/0.19	−0.0455 ^a	−0.216 ^a
					−0.266 ^d	−0.232 ^d		−0.336 ^d	−0.547 ^d
					−0.913 ^r	−0.996 ^r		−0.0625 ^r	−0.251 ^r
rs892413 (Intron 2)		C/A	0.25/0.75	−0.612 ^a	−0.397 ^a	0.80/0.20	−0.00769 ^a	−0.152 ^a	
				−0.566 ^d	−0.340 ^d		−0.117 ^d	−0.189 ^d	
				−0.704 ^r	−0.732 ^r		−0.0195 ^r	−0.304 ^r	
rs2217732 (Intron 2)		A/G	0.26/0.74	−0.423 ^a	−0.405 ^a	0.81/0.19	−0.0195 ^a	−0.126 ^a	
				−0.247 ^d	−0.215 ^d		−0.335 ^d	−0.433 ^d	
				−0.972 ^r	0.864 ^r		−0.0235 ^r	−0.163 ^r	

Superscripts indicate genetic model used for analysis: *a* additive, *d* dominant, *r* recessive. For each ethnic-specific sample, age and sex were used as covariates. Negative signs indicate protective effect with the model specified in superscript letters

Individual and haplotype-based association analysis for the replication case–control SAGE sample

The association analysis was performed using a linear regression model by regressing two ND measures in PLINK (Purcell et al. 2007) on age, sex, SNP allele dosage, and other drug dependences (alcohol, cocaine, marijuana, opiates, and other drugs) covariates. Non-smokers were excluded.

Meta-analysis of individual SNP association for both the discovery and replication samples

Prior to conducting meta-analysis, we measured the heterogeneity for the SAGE case–control AA and EA samples using the program METAL (Willer et al. 2010). To combine the association analysis results from the discovery family sample and replication case–control sample, we conducted our meta-analysis of each SNP under the same genetic model used for analyzing each individual sample using Fisher's combining *P* value method (Fisher 1932). Considering that the PBAT approach used in the discovery family sample provides only *Z* score and *P* value, we used an equal weight for each studied sample.

Results

Individual SNP-based association analysis for discovery and replication samples

Results from the individual SNP-based association analyses of the discovery family sample are shown in Table 2. Of the *CHRNA2* polymorphisms, SNP rs891398 showed strong associations with FTND in the AAs (*P* = 0.0079) under an additive model and weak association in the EAs (*P* = 0.0338) under a dominant model. Also, rs373575 (*P* = 0.00782 and 0.00675) and rs2472553 (*P* = 0.00429 and 0.000863) showed strong association with SQ and FTND in the EAs under a dominant model. For the *CHRNA6* polymorphisms, the only variant significantly associated with SQ in the EAs was rs892413, with a *P* value of 0.00769 under the additive model. All these associations remained significant after Bonferroni correction for multiple testing.

The results from the replication case–control SAGE sample showed that SNP rs2292976 in *CHRNA2* had a significant association with FTND in the AAs under both an additive (*P* = 0.00533) and a dominant (*P* = 0.0079) model

Table 3 ND-associated *P* values under the three genetic models for the first given allele of each SNP in *CHRNA2* and *CHRNA6* with ND in the replication case–control sample

Gene	dbSNP ID (gene location)	Alleles	African American			European American		
			Allele freq.	SQ	FTND	Allele freq.	SQ	FTND
<i>CHRNA2</i>	rs2292976 (Exon 8)	A/G	0.10/0.90	−0.041 ^a	−0.00533 ^a	0.13/0.87	0.684 ^a	0.0487 ^a
				−0.058 ^d	−0.00790 ^d		0.619 ^d	0.0767 ^d
				−0.209 ^r	−0.147 ^r		−0.952 ^r	0.157 ^r
	rs3735757 (Intron 5)	G/C	0.20/0.80	−0.152 ^a	−0.267 ^a	0.13/0.87	0.982 ^a	0.114 ^a
				−0.209 ^d	−0.380 ^d		0.975 ^d	0.167 ^d
				−0.275 ^r	−0.290 ^r		−0.876 ^r	0.217 ^r
	rs891398 (Exon 5)	T/C (T125A)	0.25/0.75	−0.463 ^a	0.343 ^a	0.51/0.49	−0.172 ^a	−0.297 ^a
				−0.462 ^d	0.398 ^d		0.043 ^d	−0.124 ^d
				−0.724 ^r	0.506 ^r		−0.825 ^r	−0.864 ^r
	rs2472553 (Exon 2)	T/C (T22I)	0.16/0.84	0.185 ^a	0.102 ^a	0.13/0.87	−0.942 ^a	−0.0785 ^a
				0.192 ^d	0.122 ^d		0.812 ^d	−0.0953 ^d
				0.518 ^r	0.340 ^r		−0.635 ^r	−0.326 ^r
<i>CHRNA6</i>	rs9298628 (3′-flanking)	C/T	0.29/0.71	0.0323 ^a	0.056 ^a	0.79/0.21	0.104 ^a	0.000889 ^a
				0.0502 ^d	0.013 ^d		0.589 ^d	0.680 ^d
				0.143 ^r	0.941 ^r		0.036 ^r	0.000218 ^r
	rs892413 (Intron 2)	C/A	0.31/0.69	−0.119 ^a	0.208 ^a	0.80/0.20	−0.189 ^a	0.00153 ^a
				−0.0799 ^d	−0.486 ^d		−0.083 ^d	0.688 ^d
				−0.586 ^r	0.035 ^r		−0.515 ^r	0.000530 ^r
	rs2217732 (Intron 2)	A/G	0.29/0.71	0.105 ^a	0.098 ^a	0.80/0.20	0.080 ^a	0.000865 ^a
				0.125 ^d	0.026 ^d		0.828 ^d	0.513 ^d
				0.304 ^r	−0.938 ^r		0.034 ^r	0.000282 ^r

Superscripts indicate genetic model used for analysis: *a* additive, *d* dominant, *r* recessive. For each ethnic-specific sample, age, sex, and other non-nicotine drug dependences were used as covariates. Negative signs indicate protective effect with the model specified in superscript letters

(Table 3). Of the *CHRNA6* polymorphisms, rs9298628, rs892416, and rs2217732 showed a significant association with FTND in the EAs, with *P* values of 0.000889, 0.00153, and 0.000865, respectively, under the additive model and 0.000218, 0.00053, and 0.000282 under the recessive model. Again, all these associations remained significant after Bonferroni correction.

Meta-analysis associations for both discovery and replication samples

Meta-analysis was performed for the seven SNPs by combining the results from the discovery and replication samples, which included the AA sample only, EA sample only, and the AA and EA samples together (Table 4). The reason to perform meta-analysis on the AA and EA samples together was that the heterogeneity test for SAGE AA and EA case–control samples revealed no heterogeneity between the two ethnic samples on these SNPs. Of the meta-analyzed SNPs, rs2292976 in *CHRNA2* (*P* = 0.0053) and rs892413 in *CHRNA6* (*P* = 0.00311) showed the strongest association with FTND.

Haplotype-based association analysis

According to the haplotype block criteria defined by Gabriel et al. (2002), only one block was identified within each ethnic sample in the *CHRNA2* as well as in *CHRNA6* (Fig. 1). We employed the FBAT program to perform haplotype-based association analysis for all major (defined as >5 %) haplotypes in each of the above-mentioned LD blocks with the two ND measures in *CHRNA2* (Table 5) and *CHRNA6* (Table 6) from the discovery family sample.

In *CHRNA2*, significant haplotypes in the AAs were: (1) G-C-T, formed by SNPs rs2292976, rs3735757, and rs891398 (Fig. 1), with a frequency of 22.3 %, which was associated significantly with FTND (*Z* = 2.54, *P* = 0.011); and (2) G-G-C, formed by the same SNPs, with a frequency of 9.6 %, which was associated significantly with FTND (*Z* = −2.74; *P* = 0.0063) under a dominant model. The identified haplotypes in the EAs were: (1) C-T-T, formed by SNPs rs3735757, rs891398, and rs2472553, with a frequency of 51.9 %, which was significantly associated with FTND (*Z* = −2.60; *P* = 0.0093); and (2) C-C-T, formed by SNPs rs3735757, rs891398, and rs2472553, with a

Table 4 Meta-analysis results of SNPs in *CHRNA2* and *CHRNA6* with ND in both the discovery and replication samples

Gene	dbSNP ID	Allele	African American	European American	AA + EA samples	P^2
<i>CHRNA2</i>	rs2292976	A	0.00325 ^d	0.195 ^d	0.00530 ^d	0
	rs3735757	G	0.404 ^r	0.011 ^r	0.0252 ^r	0
	rs891398	T	0.0187 ^a	0.402 ^a	0.0443 ^a	0
	rs2472553	T	0.554 ^r	0.00258 ^r	0.0108 ^r	0
<i>CHRNA6</i>	rs9298628	C	0.998 ^r	0.000592 ^r	0.00498 ^r	0
	rs892413	C	0.120 ^r	0.00290 ^r	0.00311 ^r	0
	rs2217732	A	0.981 ^r	0.000505 ^r	0.00426 ^r	0

For each SNP, meta-analysis was performed on only one genetic model, which was selected on the basis of the association analysis result for both the discovery and the replication samples; P^2 was calculated only for the replication case–control samples with the METAL program

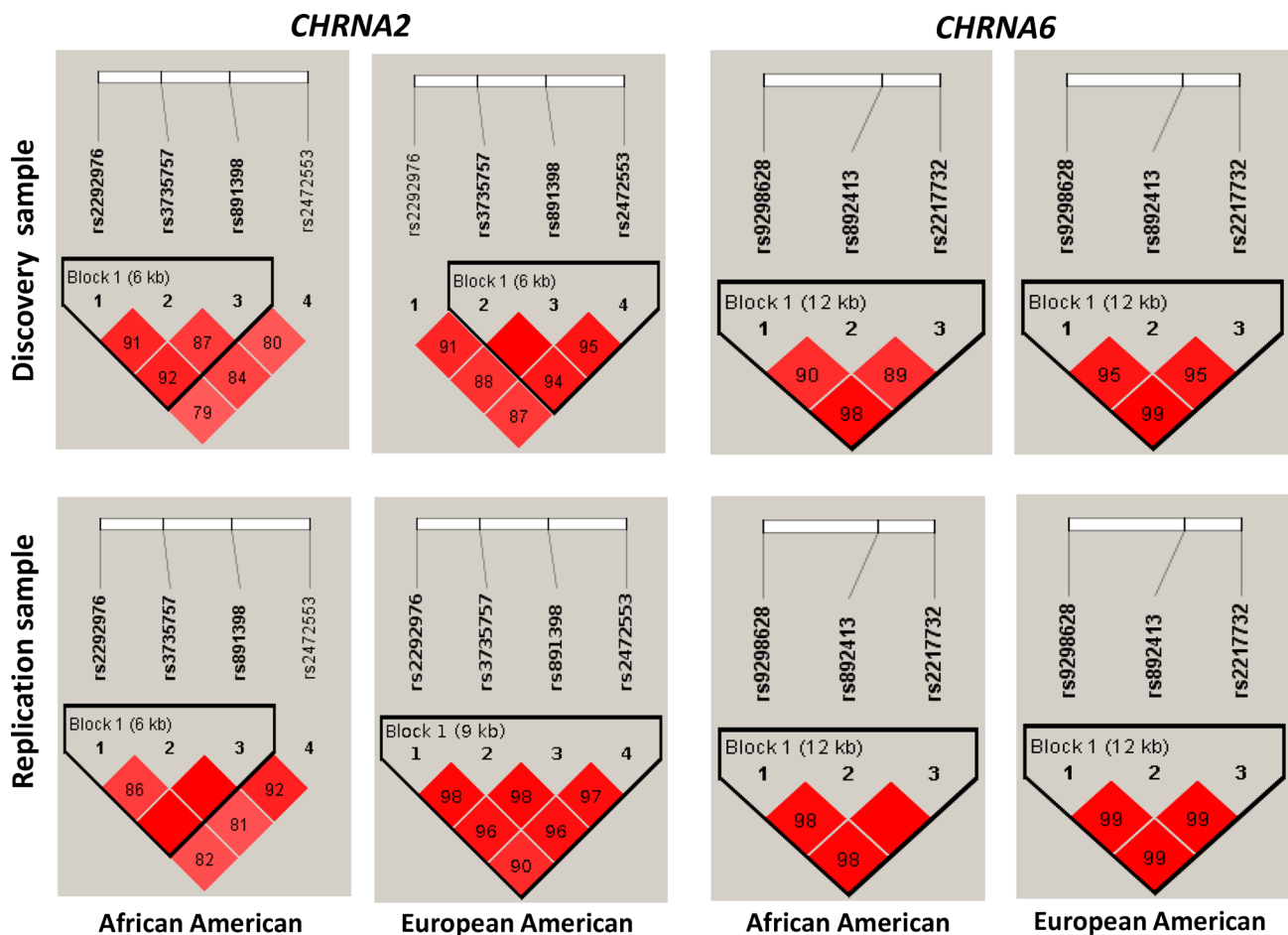


Fig. 1 LD structures for *CHRNA2* (left) and *CHRNA6* (right) SNPs in AAs and EAs from discovery and replication samples. Haploview (Barrett et al. 2005) was used to calculate all D' values, and haplotype

blocks were defined according to Gabriel et al. (2002). The number in each box represents the D' value for each SNP pair surrounding that box

frequency of 34.4 %, which was significantly associated with FTND ($Z = 2.85$; $P = 0.0043$).

In *CHRNA6*, for the AA sample, we found no haplotypes showing significant association with ND. In the EA sample (Table 5), we found one haplotype, C-C-A, formed by SNPs rs9298628, rs892413, and rs2217732, with a

frequency of 79.1 %, significantly associated with SQ ($Z = -2.71$; $P = 0.0067$). Several haplotype-based associations remained significant after Bonferroni correction for each LD block.

In the replication case–control sample, only one haplotype in *CHRNA2*, A-G-C, formed by SNPs rs2292976,

Table 5 Association analysis results for haplotypes in *CHRNA2* and *CHRNA6* with ND in the discovery family sample

(A) <i>CHRNA2</i>												
rs2292976	rs3735757	rs891398	rs2472553	Freq.	SQ			FTND				
					<i>P</i> haplotype	Z score	<i>P</i> global	Family #	<i>P</i> haplotype	Z score	<i>P</i> global	Family #
African American	G	C	C	54.9	0.36 ^d	−0.92		130	0.097 ^a	−1.66		221
	G	C	T	22.3	0.058 ^a	1.90	0.13	175	0.011 ^a	2.54	0.023	175
	A	G	C	10.6	0.44 ^d	0.77		105	0.28 ^d	1.09		109
	G	G	C	9.6	0.0099 ^d	−2.58		101	0.0063 ^d	−2.74		102
European American		C	C	34.4	0.020 ^d	2.32		193	0.0043 ^d	2.85		193
		C	T	51.9	0.014 ^d	−2.46	0.012	152	0.0093 ^d	−2.60	0.0039	152
		G	C	11.7	0.049 ^d	−1.97		10	0.015 ^d	−2.42		10
	rs9298628	rs892413	rs2217732	Freq.	SQ			FTND				
					<i>P</i> haplotype	Z score	<i>P</i> global	Family #	<i>P</i> haplotype	Z score	<i>P</i> global	Family #
African American	C	C	A	22.1	0.66 ^a	−0.44	0.54	176	0.66 ^d	−0.44	0.32	157
	T	A	G	72.9	0.26 ^d	1.12		163	0.13 ^d	1.50		163
European American	C	C	A	79.1	0.0067 ^a	−2.71	0.014	176	0.12 ^a	−1.54	0.18	179
	T	A	G	18.3	0.011 ^a	2.55		171	0.094 ^a	1.67		170

Table 6 Association analysis results for SNPs in *CHRNA2* and *CHRNA6* with ND in the replication case–control sample

		rs2292976	rs3735757	rs891398	rs2472553	Freq.	SQ		FTND	
							<i>P</i> haplotype	<i>P</i> global	<i>P</i> haplotype	<i>P</i> global
(A) <i>CHRNA2</i>										
African American	G	C	C			54.0	0.0713 ^a		0.744 ^a	
	G	C	T			24.8	0.139 ^a	0.220	0.379 ^a	0.099
	G	G	C			10.9	0.823 ^a		0.324 ^a	
	A	G	C			9.16	0.0275 ^a		0.00649 ^a	
European American	A	G	C	T		11.4	0.769 ^a		0.0686 ^a	
	G	C	C	C		35.7	0.178 ^a	0.533	0.826 ^a	0.408
	G	C	T	C		50.1	0.158 ^a		0.240 ^a	
		rs9298628	rs892413	rs2217732		Freq.	SQ		FTND	
							<i>P</i> haplotype	<i>P</i> global	<i>P</i> haplotype	<i>P</i> global
(B) <i>CHRNA6</i>										
African American	T	A	G			68.4	0.0584 ^a	0.238	0.145 ^a	0.243
	C	C	A			28.9	0.105 ^a		0.0885 ^a	
European American	T	A	G			20.0	0.178 ^a	0.228	0.000947 ^a	0.00792
	C	C	A			79.2	0.122 ^a		0.00121 ^a	

Superscripts indicate genetic model used for analysis: *a* additive; Corrected *P* value at 0.05 is 0.0125 in AAs and 0.0167 in EAs for *CHRNA2* and 0.025 in both EAs and AAs for *CHRNA6*

rs3735757, and rs891398, was significantly associated with FTND in the AA sample ($P = 0.00649$) (Table 6). Two haplotypes formed by SNPs rs9298628, rs892413, and rs2217732 in *CHRNA6* (Fig. 1) showed significant associations in the EA sample: (1) T-A-G, with a frequency of 20.0 %, was significantly associated with FTND ($P = 0.000947$); and (2) C-C-A, with a frequency of 79.2 %, was significantly associated with FTND ($P = 0.00121$).

Discussion

Nicotinic acetylcholine receptors $\alpha 2$ and $\alpha 6$ play vital roles in the nervous system. To test for their association with ND, seven SNPs in *CHRNA2* and *CHRNA6* were investigated in two independent samples of either African or European origin. Association analysis revealed that multiple SNPs and haplotypes are significantly associated with ND in the both discovery and replication samples. In the discovery sample, individual SNP analysis for *CHRNA2* revealed a significant association of two SNPs in the EA sample and one SNP in the AA sample with FTND and/or SQ. In particular, we found that associations of SNPs rs3735757 and rs2472553 in *CHRNA2* with SQ and FTND remained significant after correction for multiple testing. However, such associations were not exactly the same at the SNP level in the replication sample, where only SNP rs2292976 showed significant association with FTND in the AA population. Although SNP rs892413 showed significant association with SQ in

the EA discovery samples, all three *CHRNA6* SNPs exhibited significant association with FTND in the EA replication sample, even after correction for multiple testing. Further, we found a significant association between several haplotypes of *CHRNA2/CHRNA6* and ND in both the discovery and the replication samples, although haplotypes formed by particular SNPs were sometimes different in the two samples. Meta-analysis of the discovery and the replication samples added further support for the association of *CHRNA2* and *CHRNA6* with ND.

Compared with other nAChR subunit genes such as *CHRNA5-A3-B4*, *CHRNA4*, *CHRNA2*, and *CHRNA3* (Bierut 2010; Cui et al. 2013; Li and Burmeister 2009; Saccone et al. 2009, 2010; Thorgeirsson et al. 2010; Wang et al. 2012), *CHRNA2* has not received much attention in nicotine research. Although *CHRNA2* was one of the first nAChRs investigated as an ND candidate gene in several GWAS and candidate studies, no significant associations have been reported after correction for multiple testing or replicated in independent studies. In the present study, we demonstrated that *CHRNA2* shows a strong association with FTND after correction for multiple testing. Importantly, the SNP rs2472553, which evinced the strongest association with ND, appears to encode a functional variant in the signal peptide, causing an amino acid change from threonine to isoleucine at the 22nd residue. Our functional study with oocyte electrophysiology indicates that this mutation changes the sensitivity of functional receptors (Dash et al. in preparation).

CHRNA2 plays a vital role in other neurologic disorders such as epilepsy, with an estimated prevalence in Europeans that ranges from 3 to 8 per 1,000 individuals (Forsgren et al. 2005). The mutation I279N in the $\alpha 2$ subunit (i.e., rs104894063) was the first identified functional variant associated with epilepsy in *CHRNA2*; electrophysiological investigation of the I279N mutation in HEK 293 cells indicates that the $\alpha 2^{I279N}/\beta 4$ receptor has a significantly higher sensitivity to the natural agonist than does the wild-type $\alpha 2/\beta 4$ receptor (Aridon et al. 2006). Another oocyte electrophysiology study found that $\alpha 2^{I279N}$, co-expressed with the $\beta 2$ subunit, causes a gain-of-function effect whose distinct biopharmacological profile includes reduced inhibition by carbamazepine and greater nicotine sensitivity (Hoda et al. 2009).

Several GWAS and candidate gene studies have revealed strong associations between *CHRNA6* and ND. However, most studies tested *CHRNA6*–*CHRNA6* associations as a cluster, in which most of the significant association was attributable to variants in *CHRNA6*; moreover, the association of *CHRNA6* SNPs typically did not survive correction for multiple testing (Hoft et al. 2009b; Saccone et al. 2009; Zeiger et al. 2008). In the present study, we demonstrated that *CHRNA6* is still significantly associated with FTND after correction for multiple testing. In a study by Hoft and colleagues (Hoft et al. 2009a), two SNPs from the *CHRNA6*–*CHRNA6* cluster were found to be associated with smoking quit attempts also: SNP rs2304297 ($P = 0.0044$) from *CHRNA6* and rs7004381 ($P = 0.0024$) from *CHRNA6*. Complementing our association study, animal self-administration studies suggest that $\beta 2^*nAChRs$ assembled with $\alpha 6$ subunits would be useful pharmacological targets for smoking cessation products (Brunzell 2012): nicotine self-administration is absent in $\alpha 6$ -knockout mice, and targeted re-expression of the $\alpha 6$ subunit in the VTA of $\alpha 6$ -KO mice promptly restores nicotine self-administration (Pons et al. 2008). Further, several in vitro electrophysiological, synaptosome-release assay, and cyclic-voltammetry studies have demonstrated that nicotine-mediated elevation of dopamine release is blocked following antagonism of $\alpha 6\beta 2^*nAChRs$ with α -CTX MII (Champtiaux et al. 2003; Drenan et al. 2008; Perez et al. 2009, 2010; Salminen et al. 2007; Zhao-Shea et al. 2011). Finally, an in vivo function study of $\alpha 6^*nAChRs$ in mesolimbic DA neurons has shown that the elevation of DA release caused by nicotine can be inhibited by intra-VTA infusion of α -CTX MII, implicating $\alpha 6\beta 2^*nAChRs$ in the regulation of this effect (Gotti et al. 2010). Collectively, the association and functional studies of *CHRNA6* suggest that $\alpha 6^*nAChRs$ are strong candidates for drug-development research on smoking cessation.

Recently, *CHRNA6* has been found to be associated, not only with ND, but also with alcohol dependence; three SNPs (rs1072003, $P = 0.015$; rs892413, $P = 0.0033$;

and rs2304297, $P = 0.012$) were associated with alcohol dependence in the National Youth Survey Family Study in a sample that was mostly EAs (Hoft et al. 2009a). Another study showed that two haplotypes of the *CHRNA6*, CCCC and TCGA, formed by SNPs rs10087172, rs10109429, rs2196129, and rs16891604, were associated with heavy alcohol consumption ($P = 0.004$ and $P = 0.035$, respectively) and with increased alcohol intake ($P = 0.004$) for the CCCC haplotype in a Spanish population (Landgren et al. 2009).

In sum, our results indicate that both *CHRNA2* and *CHRNA6* are significantly associated with ND. Such association with ND at both the individual SNP and haplotype level makes these genes good subjects for research on molecular mechanisms of dependence. A better understanding of the role of these genetic variants—especially the functional variants—may provide key insights for pharmacologic targeting to reduce or possibly eliminate some of the addictive properties of nicotine in susceptible individuals.

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Conflict of interest MDL has served as a consultant and board member of ADial Pharmaceuticals, LLC. All other authors declare that they have no conflicts of interest.

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