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ORIGINAL INVESTIGATION

Gene-based analysis suggests association of the nicotinic acetylcholine receptor β1 subunit (*CHRNB1*) and M1 muscarinic acetylcholine receptor (*CHRM1*) with vulnerability for nicotine dependence

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Abstract Based on our previously identified linkage regions for nicotine dependence (ND), we selected six and five single nucleotide polymorphisms (SNPs) in the muscarinic cholinergic receptor subtype M1 (*CHRM1*) and nicotinic cholinergic receptor β1 (*CHRNB1*), respectively, to determine the association of the two genes with ND in a total of 2,037 subjects from 602 nuclear families of either African-American (AA) or European-American (EA) origin. Individual SNP- and/or haplotype-based analyses indicated that the *CHRNB1* was significantly associated with ND, which was assessed by smoking quantity (SQ), the Heaviness of Smoking Index (HSI), and the Fagerström Test for ND (FTND), in both ethnic samples. The association of rs2302763 in the *CHRNB1* was significant with adjusted SQ in the EA sample after correction for multiple testing ($P = 0.013$). Haplotype A-T-A formed by SNPs rs2302765, rs2302762, and rs9217 in the *CHRNB1* was significantly associated with the high risk allele for all the three ND measures (minimum $P = 0.009$, 0.006, and 0.008 for SQ, HSI and FTND, respectively) in the AA sample while haplotype A-T-A formed by rs2302765, rs2302763, and rs9217 was significantly positively associated with ND (minimum $P = 0.005$, 0.016, and 0.016 for SQ, HSI and FTND, respectively) in the EA sample. The *CHRM1* exhibited significant protective associations of haplotype C-C-A-T-G-G formed by all six SNPs of this gene with at least one ND measure in the AA sample after Bonferroni correction (minimum $P = 0.008$, 0.013, and 0.009 for SQ, HSI and FTND, respectively), but no significant association was found in the EA sample. The significant associations, together with their location of linked region to ND, suggest that the *CHRNB1* and *CHRM1* are likely candidates for further investigation.

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Introduction

Cigarette smoking, which is highly prevalent in the US and throughout the world, is associated with considerable morbidity, mortality and public health costs (USDHHS 2000; WHO 2002; Mokdad et al. 2004). Numerous studies have demonstrated that nicotine is the addictive ingredient in tobacco, and nicotine dependence (ND) is the primary factor responsible for continued smoking (USDHHS 1988; Rose 1996; Henningfield and Fant 1999). The likelihood of progression from smoking initiation to ND involves many genetic and environmental factors. Genetic differences that affect nicotine absorption, metabolism, and other functions underlie the expression of smoking-related phenotypes, and influence characteristics such as sensitivity and responsiveness to nicotine intake, whether administered via cigarette smoking or therapeutic treatments (Berrettini and Lerman 2005; Munafo et al. 2005). Epidemiological studies have pointed to a genetic contribution of at least 50% in the liability for ND (Sullivan and Kendler 1999; Niu et al. 2000; Li

et al. 2003a). Identification of susceptibility genes for ND would have a significant impact in characterizing the genetic architecture and understanding tobacco use behavior, and ultimately facilitate our efforts in tobacco prevention and cessation, via the refinement of tailored treatment approaches.

Positive (e.g. stimulatory, relaxing) and negative (avoidance of nicotine-withdrawal symptoms) reinforcement serve to establish and maintain tobacco use and addiction (Henningfield and Jude 1999). By acting on a family of nicotinic acetylcholine receptors (nAChRs), nicotine modulates release of various neurotransmitters, which produce those pleasant and rewarding psychopharmacologic effects (Rose 1996; Swan 1999; Picciotto et al. 2000; Watkins et al. 2000). Examples include stimulating the release and increasing the turnover of dopamine, serotonin, acetylcholine, glutamate, gamma-aminobutyric acid (GABA), opioid peptides, catecholamine, and norepinephrine. Further, chronic exposure to nicotine causes alterations in brain structure and cerebral metabolism, such as receptor desensitization and inactivation, and reduction in capacity to synthesize 5-hydroxytryptamine. Withdrawal from nicotine may elicit an abstinence syndrome (Henningfield and Jude 1999; Watkins et al. 2000).

Acetylcholine receptors (AChRs), found extensively throughout the central and peripheral nervous systems, respond not only to acetylcholine, but other molecules as well. Based on their relative affinities and sensitivities, AChRs are classified into two broad categories: nicotinic and muscarinic. nAChRs are ionotropic receptors, a group of ligand-gated ion channels that are opened in response to binding of acetylcholine or nicotine through an allosteric mechanism (Karlin 2002). nAChRs are assembled as pentamers of diverse subunits, of which there are 16 varieties in human, α1-10, β1-4, δ and either γ, or ε (Le Novere et al. 2002; Gotti and Clementi 2004). The numerous resulting receptor subtypes with variable kinetic, pharmacological and biophysical properties are present in many brain regions throughout the nervous system, potentially mediating the various behavioral and pharmacologic effects of nicotine on the central nervous system (Lukas et al. 1996). On the other hand, muscarinic AChRs (mAChRs) are monomeric and belong to a class of metabotropic receptors, which use G-protein as their signalling mechanism, and activate other ionic channels via a second messenger cascade. Five subtypes have been determined, namely M1–M5. Because the conditioning actions of nicotine are mediated largely by AChRs, the genes encoding different subunits of such receptors are usually considered as plausible candidates in association analysis for ND (Sullivan et al. 2001, 2004; Li et al. 2005).

Previously, we identified several chromosomal regions that are likely to harbor susceptibility loci for ND in the Framingham Heart Study (FHS) sample, including one region on chromosome 17 and one on chromosome 11 (Li et al. 2004; Wang et al. 2005), where the two genes of interest in this study, nicotinic cholinergic receptor β1 (*CHRNB1*) and muscarinic cholinergic receptor subtype M1 (*CHRM1*) are located, respectively. Further, the region on chromosome 11 or its vicinity has also been linked to habitual smoking (Bierut et al. 2004) and the region on chromosome 17 linked to smoking behavior (Duggirala et al. 1999) and Attention-Deficit/Hyperactivity Disorder (Arcos-Burgos et al. 2004). The *CHRNB1*, that encodes a 501 amino acid (aa) polypeptide, is mapped to ~17p13, is about 12.5 kb long, and contains 11 exons. Expression analysis indicated that the *CHRNB1* is abundantly expressed in the neuromuscular junctions of muscles and components of muscular subtypes that cause excitatory effects on muscle leading to contraction; but a low RNA level of the *CHRNB1* was also found in the brain (Su et al. 2002). Also, the *CHRNB1* was included in a list of genes possibly relevant to ND derived from a bioinformatic study (Sullivan et al. 2004). The *CHRM1* encoding 460 aa subtype M1 of mAChRs is mapped to ~11q13, is about 12.9 kb long, and contains only one intron and two exons. The *CHRM1* is present predominantly in cerebral hippocampus and cortex as well as the parasympathetic ganglia and is involved in many processes, such as modulation of excitatory aa neurotransmission in cortical and limbic areas (Levey 1996). The *CHRM1* has been implied to play a significant role in Alzheimer's disease and possibly substance abuse (Levey 1993; Hamilton et al. 1998, 2001; Felder et al. 2001). Although these two genes are potentially important and biological relevant, no genetic study has been reported on what roles they play in the etiology of ND. Thus, the primary objective of this study was to determine whether a significant association exists between the variants of the *CHRM1* or *CHRNB1* genes and ND in 602 nuclear families of either African-American (AA) or European-American (EA) origin.

Materials and methods

Study subjects and measurement

The participants of this study have been previously identified as the Mid-South Tobacco Family (MSTF)

sample, recruited primarily from Tennessee, Mississippi and Arkansas in the US during 1999–2004. The primary determination of ND for probands and other smoking participants was assessed using the Fagerstrőm Test for Nicotine Dependence (FTND) scale (Heatherton et al. 1991). Once a proband and a full-sib who was also nicotine dependent (for majority of our families) as assessed by FTND scale were recruited, we recruited additional siblings and parents into the study whenever possible regardless of their smoking status. A total of 2,037 subjects from 402 AA and 200 EA families (or sibships) were included in the study. The families varied in size from two to nine and the average size ± standard deviation (SD) was 3.14 ± 0.75 for AAs and 3.17 ± 0.69 for EAs, respectively. Extensive questionnaire data were collected on each participant, including demographics (e.g. sex, age, race, biological relationships, weight, height, years of education, and marital status), medical history, smoking history and current smoking behavior, ND, and personality traits. Average age ± SD was 39.4 ± 14.4 years for the AA sample and 40.5 ± 15.5 years for the EA sample, respectively. Detailed demographic and clinical characteristics are presented in Table 1. All participants provided informed consent and the study was approved by all participating Institutional Review Boards.

In the present study, ND was ascertained by the three measures most commonly used in the literature: Smoking Quantity (SQ; defined as the number of cigarettes smoked per day), the Heaviness of Smoking Index (HSI; 0–6 scale), which includes SQ and smoking urgency (i.e. how soon after waking up does the subject smoke the first cigarette?), and the FTND on a 0–10 scale (Heatherton et al. 1991). Given the content overlap of these measures, there exists a fairly robust correlation among them ($r = 0.88$–0.94). The average FTND score ± SD for smokers was 6.26 ± 2.15 for AAs and 6.33 ± 2.22 for EAs (see Table 1). The average number of cigarettes smoked per day ± SD was 19.4 ± 13.3 for AA smokers and 19.5 ± 13.4 for EA smokers, respectively.

Table 1 Clinical characteristics (Mean ± SD) for pooled, AA, and EA samples

Characteristic	AA	EA	Pooled
Number of nuclear families	402	200	602
Family size	3.14 ± 0.75	3.17 ± 0.69	3.15 ± 0.73
Number of subjects	1,366	671	2,037
Gender (% female)	66.1	69.5	67.2
Age (years)	39.4 ± 14.4	40.5 ± 15.5	39.7 ± 14.8
Number of smokers	1,053	515	1,568
Age of smoking onset (years)	17.3 ± 4.7	15.5 ± 4.4	16.7 ± 4.7
Years smoked	20.4 ± 12.5	23.2 ± 13.5	21.3 ± 12.9
Smoking quantity/day	19.4 ± 13.3	19.5 ± 13.4	19.5 ± 13.3
HSI score	3.7 ± 1.4	3.9 ± 1.4	3.8 ± 1.4
FTND score	6.26 ± 2.15	6.33 ± 2.22	6.29 ± 2.17

SNP genotyping

DNA was extracted from peripheral blood samples of each participant using a kit from Qiagen Inc (Valencia, CA, USA). Selection of the SNPs for association analysis was based on (1) high heterozygosity (minor allele frequency ≥ 0.15 as reported by the NCBI SNP database), and (2) coverage of the whole gene that was as uniform as possible across the *CHRM1* and *CHRNB1* genes. Based on the size and availability of polymorphisms of each gene, we selected six SNPs for the *CHRM1* and five SNPs for the *CHRNB1* using the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/). The final SNP density is ~6 kb for the *CHRM1* and 4 kb for the *CHRNB1*, respectively. Detailed information on SNP locations, chromosomal positions, allelic variants, minor allele frequency, and primer/probe sequences is summarized in Table 2.

All SNPs were genotyped using the *Taq*Man assay in a 384-well microplate format (Applied Biosystems Inc., Foster City, CA, USA). Briefly, 15 ng of DNA was amplified in a total volume of 7 µl containing an MGB probe and 2.5 µl of *Taq*Man universal PCR master mix. Amplification conditions were 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 95°C for 25 s and 60°C for 1 min. Allelic discrimination analysis was performed on the ABI Prism 7900HT Sequence Detection System (ABI, Foster City, CA, USA). To ensure the quality of the genotyping, at least five SNP-specific control samples were added to each 384-well plate.

Data analysis

The PedCheck program (O'Connell and Weeks 1998) was used to identify any inconsistent Mendelian inheritance, nonpaternity, or other typing errors. To avoid bias, a total of 35 and 38 inconsistencies in the AA sample and 26 and 11 in the EA sample, for the *CHRM1* and *CHRNB1* genes, respectively, i.e., 0.49% of the ~23,000 assays, were treated as missing in further statistical analysis. To verify our genotyping quality, we also checked the SNP data for any significant departure from Hardy–Weinberg equilibrium (HWE). Pair-wise linkage disequilibrium (LD) between all SNP markers was assessed using the Haploview program (Barrett et al. 2005), with the option of determining haplotype blocks using the block definitions proposed by Gabriel and colleagues (Gabriel et al. 2002).

Table 2 Biological information on the 11 SNPs used in this study

dbSNP ID	Gene/domain	Physical position	Alleles[a]	Reported MAF[b]	Forward (F) and reverse (R) primer and probe (P) sequences (5′→3′)
rs2507821	CHRM1/3′-flanking	62423182	A/**C**	0.398	F: TCTGACGAGACTCCCTTGTTAGAAA R: GCAGCAGCAGAAATAAAAAGCAGAT P: TTACGATGCA[A/C]AAAAG
rs4963323	CHRM1/ 3′-flanking	62431179	C/**G**	0.143	F: GGGCCCTCTTAGGAACTTGAG R: GGAAGGATCCAAACCCCATATCC P: TCACAACCC[C/G]GTAAGC
rs544978	CHRM1/ Intron	62441806	A/**C**	0.204	F: CCTCCTGATGAAGGCTGTTAGAAG R: CAGCCAGTAAATACCAGTTACTATAATATGAGTAATT P: TAAGGAGTATTAGTGATA[T/G]TATTA
rs542269	CHRM1/ Intron	62442113	**C**/T	0.182	F: CTCCGAGACCAACCTCTTGAAC R: CAAGGAGGCTGGCAAACC P: CCCTCAAG[G/A]GCAGGAG
rs2075748	CHRM1/ Intron	62444845	**A**/G	0.168	F: GCATCCTGTGGGAGGTTGAC R: AGGGAGGACAGGAGGTTCTG P: ACTGGCA[C/T]CCCCATC
rs1938677	CHRM1/ 5′-flanking	62453527	**A**/G	0.471	F: GCCCAGTGTTCCTCAGTCTATTTTG R: TTCCCATTTCATCACAGAGGACTTTT P: TGGAAGTTAGTGG[A/G]TGTTA
rs3829603	CHRNB1/ 5′-flanking	7287766	**A**/C	0.379	F: CAGCCTTTTCTCTGGATCTGTCA R: GCCCAAATCTAGCCTCTTTCTACTT P: CCTCTGTC[A/C]TTGACTC
rs2302765	CHRNB1/ Intron 6	7291699	A/**G**	0.176	F: GGAAATGATAGAGCCCATGGATGTA R: GGCATCAGGAAATCCACATTCATG P: CCATGCCT[A/G]CTCACCA
rs2302762	CHRNB1/ Intron 9	7299585	**C**/T	0.443	F: CCAGAAGAGTGTGCGGAAGAAG R: CAACTCCCATGATGCTCAACCA P: TCTTCCG[G/A]GAGCATG
rs2302763	CHRNB1/ Intron 10	7300001	**C**/T	0.168	F: CCACGATGCGGTATGTCCAA R: GGCCAAGGTCGCCTAGAC P: CCTTGTTCC[G/A]CCCCCG
rs9217	CHRNB1/3′-flanking	7303812	A/**G**	0.314	F: TTTTTTGGGAGAATTGAGATTGTAGACATTTTTT R: GAACCACCACATTCTGGAAGCTAT P: TTTCCTTGATCATA[T/C]TTAAG

[a,b] The bold-faced nucleotide in allele column is a minor allele, which is based on the allele frequency presented in the NCBI dbSNP database

Associations between individual SNPs and the three ND measures were determined by the PBAT program using generalized estimating equations (Lange et al. 2003). Associations between each ND measure and haplotypes from multiple SNP combinations were examined using the FBAT program under the null distribution of no linkage and no association (Horvath et al. 2004). Three genetic models (additive, dominant, and recessive) were tested, using gender and age as covariates. Relationships with the three ND measures were analyzed individually. All associations found to be significant were corrected for multiple testing according to the SNP spectral decomposition (SNPSpD) approach (Nyholt 2004; Li and Ji 2005) for individual SNP analysis, and using Bonferroni correction for haplotype-based association analysis by dividing the significance level by the number of major haplotypes (with > 5% frequency).

Results

To determine if population stratification and admixture exist within our sample, we performed heterogeneity tests and found significant differences in the allele frequencies for the following SNPs between two ethnic samples: rs2507821, rs4963323, rs544978, rs542269, rs2075748, and rs1938677 in the *CHRM1*, and rs2302762 in the *CHRNB1* (Table 3). This suggests the presence of distinct allele distributions between two samples. The tests for HWE indicated that with the sole exception of SNP rs2075748 of the *CHRM1* in the EA sample ($P = 0.0014$), no other SNPs deviated significantly from HWE in either ethnic sample, confirming the high quality of our genotyping data. As the FBAT is generally considered immune to population stratification, admixture, and Hardy–Weinberg disequilibrium, we choose to report the result for SNP

Table 3 Minor allele frequencies and *P*-values for heterogeneity test and association of individual SNPs of *CHRM1* and *CHRNB1* with three ND measures in the AA and EA samples

SNP ID	African-American sample				European-American sample				Heterogeneity test
	MAF	SQ	HIS	FTND	MAF	SQ	HSI	FTND	
CHRM1									
rs2507821	0.399	0.210[r]	0.175[d]	0.191[d]	0.465	0.177[r]	0.038[d,r]	0.057[r]	0.019
rs4963323	0.034	0.429[r]	0.368[a]	0.454[r]	0.126	0.712[d]	0.686[d]	0.593[d]	0.000
rs544978	0.150	0.244[a]	0.240[d]	0.184[d]	0.299	0.186[a]	0.157[a]	0.136[a]	0.000
rs542269	0.063	0.478[d]	0.705[d]	0.172[d]	0.284	0.691[r]	0.346[a]	0.307[a]	0.000
rs2075748	0.071	0.579[a]	0.936[a]	0.956[a]	0.216	0.223[d]	0.147[a]	0.187[a]	0.000
rs1938677	0.520	0.660[a]	0.573[a]	0.444[a]	0.438	0.502[a]	0.144[a]	0.048[a]	0.007
CHRNB1									
rs3829603	0.278	0.637[a]	0.752[d]	0.744[d]	0.293	0.359[a]	0.150[a]	0.043[a]	0.515
rs2302765	0.168	0.247[d]	0.245[d]	0.287[d]	0.144	0.037[d], 0.036[r]	0.129[d]	0.345[d]	0.166
rs2302762	0.406	0.077[r]	0.067[r]	0.046[r]	0.707	0.361[a]	0.149[a]	0.047[a]	0.000
rs2302763	0.167	0.757[a]	0.461[d]	0.579[a]	0.147	0.013[d,r]	0.028[d], 0.027[r]	0.102[r]	0.248
rs9217	0.326	0.237[r]	0.178[r]	0.132[r]	0.346	0.024[d] 0.026[r]	0.047[d,r]	0.039[d] 0.037[r]	0.423

Corrected *P*-value at 0.05 significance level is 0.009 and 0.013 for *CHRM1*, and 0.013 and 0.013 for *CHRNB1* in the AA and EA samples, respectively, which was calculated from the website: http://genepi.qimr.edu.au/general/daleN/SNPSpD/

Superscripts indicate the genetic models used for analysis: *a* additive, *d* dominant, and *r* recessive

MAF: the frequency of the allele in the AA or EA sample, which is reported as the minor allele in the NCBI dbSNP database

rs2075748 in Table 3, given the lack of evidence that the deviation was due to genotyping error.

Individual SNP analysis using the PBAT program documented a significant association for three of the five SNPs in the *CHRNB1* gene, i.e., rs2302765, rs2302763, and rs9217, with at least one age- and gender-adjusted ND measure in the EA sample (Table 3). The association of rs2302763 remained significant with adjusted SQ in the EA sample after correction for multiple testing based on the SNPSpD approach (Nyholt 2004; Li and Ji 2005). SNP rs2302762 in the *CHRNB1* gene was significantly associated with the FTND in both the AA ($P = 0.046$) and EA ($P = 0.047$) samples, but not after correction for multiple testing. For the *CHRM1* gene, SNP rs2507821 was significantly associated with HSI ($P = 0.038$), and rs1938677 with the FTND ($P = 0.048$) in the EA sample; both findings became non-significant after correction for multiple testing. No significant associations were detected for the *CHRM1* SNPs in the AA sample.

The pair-wise normalized LD (D′) values for the six SNPs within the *CHRM1* and 5 SNPs within the *CHRNB1* were calculated using Haploview (Barrett et al. 2005) (see Fig. 1). Overall, there is a good agreement with the ethnic evolutionary history in which the EA sample shows a stronger LD than the AA sample, with few exceptions. For example, the following D′ values were larger in the AA sample: (1) rs2507821–rs4963323 in the *CHRM1*, and (2) rs3829603–rs2302763 and rs2302762–rs2302763 in the *CHRNB1*. This implies that the LD patterns differed between the two ethnic samples. Two tightly contiguous SNP-pairs, rs544978 and 542269 (0.3 kb) of the *CHRM1* and rs2302762 and rs2302763 (0.4 kb) of the *CHRNB1*, did not yield a high LD as expected (e.g. all D′ values were less than 0.90, that of rs544978–rs542269 pair was 0.66 in the AA sample and that of rs2302762–rs2302763 was 0.56 in the EA sample), suggesting the LD is nonlinear with respect to their physical distance, and there might exist strong genetic shuffling even within a short genomic region. There were high (range from 0.97 to 1.00) D′ values for the SNP pairs between rs3829603 and rs2302765 in the AA sample and among rs3829603, rs2302765, and rs2302762 (ca. 11 kb) in the EA sample, and one haplotype block was defined by Haploview program according to the criteria of Gabriel et al. (2002). No haplotype block was defined for the SNPs in the *CHRM1*. The difference shown in Fig. 1, together with different haplotype frequencies (partially shown in Tables 4 and 5) between the EA and AA samples revealed a different LD structure and haplotype profile across two ethnic samples.

Haplotype-based association analysis indicated that haplotype A-T-A formed by SNPs rs2302765, rs2302762, and rs9217 in the *CHRNB1*, the most common haplotype with a frequency of 52.7%, was significantly and positively associated with all three adjusted ND measures in the AA sample. These associations remained significant after Bonferroni correction (Table 4), suggesting that AA individuals carrying this haplotype have a higher risk of developing ND. However, the haplotypic associations identified in the AA sample were not significant for the EA sample, whereas another common haplotype (64.4%), A-T-A

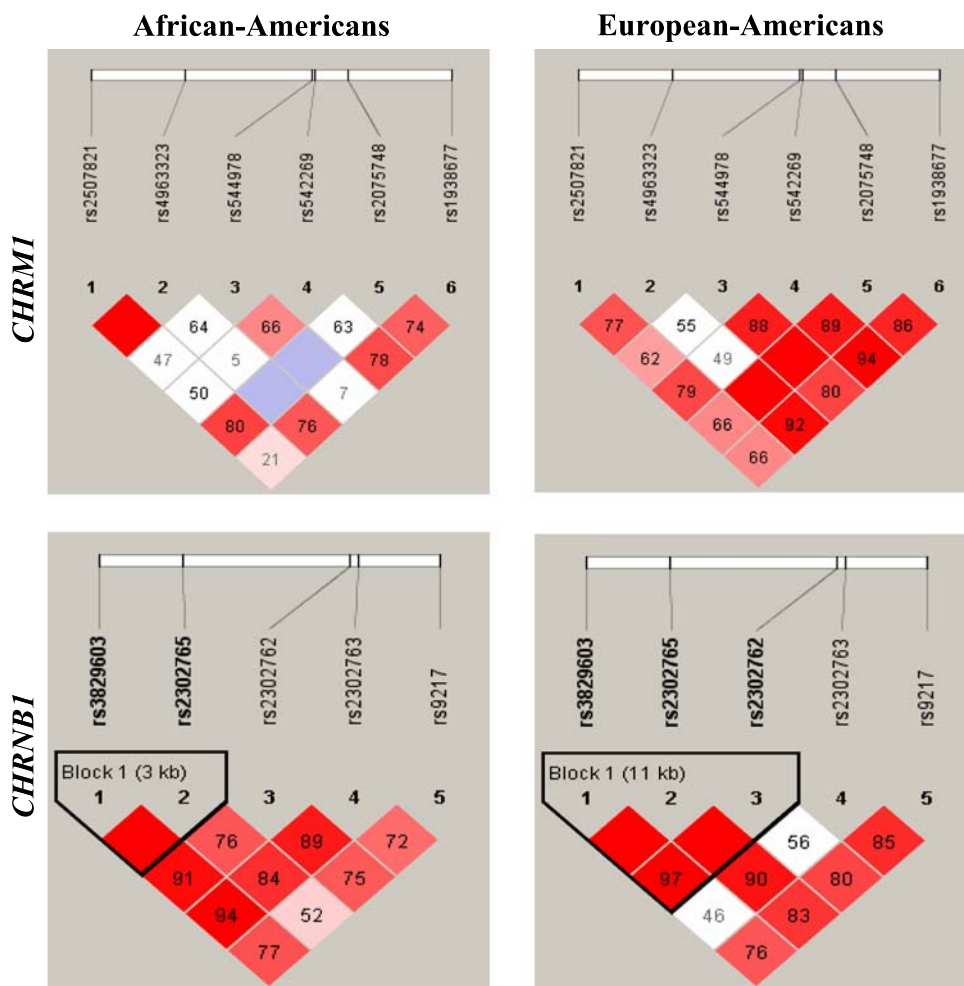

Fig. 1 Linkage disequilibrium structures of the six SNPs and the five SNPs within the *CHRM1* gene (*top row*) and the *CHRNB1* (*bottom row*) gene, respectively, in the AA (*left side*) and EA (*right side*) samples. The LD computation was performed with the Haploview program (Barrett et al. 2005) and haplotype block was defined based on the criteria of Grabiel et al. (2002)

Table 4 *Z*- and *P*-values for association of major haplotypes in the *CHRNB1* gene with three ND measures in AA and EA samples

Haplotype	African-American sample				European-American sample			
	%	SQ	HSI	FTND	%	SQ	HIS	FTND
rs2302765–rs2302762–rs9217								
A-T-A	52.7	2.12 (0.034)^{a} 2.60 (0.009)^{r}	2.32 (0.020)^{a} 2.72 (0.006)^{r}	2.20 (0.028)^{a} 2.64 (0.008)^{r}	24.9	0.55 (0.584)^{d}	0.88 (0.379)^{a}	1.40 (0.161)^{a}
A-C-A	9.6	−1.27 (0.203)^{a}	−0.87 (0.386)^{a}	−0.87 (0.382)^{a}	39.5	0.59 (0.557)^{d}	−0.24 (0.813)^{r}	−0.62 (0.534)^{r}
A-C-G	17.2	0.83 (0.404)^{r}	−0.92 (0.360)^{d}	−0.91 (0.365)^{d}	19.4	−0.24 (0.808)^{r}	−0.28(0.778)^{a}	−0.39 (0.698)^{r}
G-C-G	10.9	−0.83(0.406)^{d}	−1.00 (0.318)^{d}	−1.22 (0.223)^{d}	12.1	−1.44(0.150)^{a}	−1.25 (0.211)^{a}	−1.01 (0.312)^{a}
Global *P*-value		0.287^{a} 0.361^{d} 0.020^{r}	0.292^{a} 0.289^{d} 0.014^{r}	0.384^{a} 0.312^{d} 0.021^{r}		0.641^{a} 0.893^{d} 0.983^{r}	0.630^{a} 0.951^{d} 0.941^{r}	0.561^{a} 0.872^{d} 0.762^{r}
rs2302765–rs2302763–rs9217								
A-T-A	61.1	1.74 (0.081)^{r}	2.30 (0.021)^{r}	2.18 (0.029)^{r}	64.4	2.80 (0.005)^{d}	2.41(0.016)^{d}	2.42 (0.016)^{d}
A-T-G	20.0	1.81 (0.070)^{r}	1.72 (0.086)^{r}	−1.55 (0.122)^{d}	19.1	−1.23 (0.219)^{a}	−1.04 (0.300)^{a}	−1.01(0.312)^{a}
G-C-G	10.9	−1.60 (0.110)^{a}	−1.54 (0.123)^{a}	−1.31(0.190)^{d}	11.6	−1.29(0.199)^{a}	−1.09 (0.276)^{a}	−0.74(0.460)^{a}
Global P-value		0.753^{a} 0.407^{d} 0.030^{r}	0.488^{a} 0.221^{d} 0.010^{r}	0.541^{a} 0.226^{d} 0.019^{r}		0.303^{a} 0.046^{d} 0.746^{r}	0.549^{a} 0.168^{d} 0.795^{r}	0.667^{a} 0.167^{d} 0.700^{r}

Superscripts indicate the genetic models used for analysis: *a* additive, *d* dominant, and *r* recessive

Table 5 *Z*- and *P*-values for association of major haplotypes formed by rs2507821–rs4963323–rs544978–rs542269–rs2075748–rs1938677 in the *CHRM1* gene with three ND measures in AA and EA samples

Haplotype	African-American sample				European-American sample			
	%	SQ	HSI	FTND	%	SQ	HIS	FTND
A-C-A-T-G-A	29.2	−1.21 (0.225)[d]	−1.01 (0.317)[d]	−1.14 (0.256)[d]	–	–	–	–
A-C-A-T-G-G	21.0	0.77 (0.443)[r]	1.15 (0.251)[r]	0.95 (0.343)[r]	12.1	−0.46 (0.646)[d]	−0.28 (0.779)[d]	−0.06 (0.956)[a]
C-C-A-T-G-G	13.1	−2.48 (0.013)[a] −2.67 (0.008)[d]	−2.48 (0.013)[a] −2.44 (0.015)[d]	−2.61 (0.009)[a] −2.55 (0.011)[d]	24.0	−1.59 (0.112)[r]	−1.33 (0.185)[r]	−1.64 (0.102)[r]
C-C-A-T-G-A	11.8	2.13 (0.033)[r]	1.56 (0.119)[r]	1.50 (0.135)[r]	–	–	–	–
A-C-C-C-G-A	–	–	–	–	21.5	−0.73 (0.468)[d]	−0.82 (0.413)[d]	−0.94 (0.347)[d]
C-C-A-T-A-G	–	–	–	–	11.4	1.36 (0.175)[a]	1.79 (0.074)[a]	1.93 (0.053)[a]
A-G-A-T-G-A	–	–	–	–	10.1	−0.63 (0.528)[d]	−0.95 (0.342)[d]	−1.38 (0.167)[a]
Global *P*-value		0.188[a] 0.261[r] 0.152[d]	0.266[a] 0.346[r] 0.289[d]	0.189[a] 0.334[r] 0.207[d]		0.649[a] 0.274[r] 0.808[d]	0.475[a] 0.389[r] 0.666[d]	0.252[a] 0.254[r] 0.461[d]

Superscripts indicate the genetic models used for analysis: *a* additive, *d* dominant, and *r* recessive model. "–" Indicates this haplotype could not be detected in the sample of interest

formed by SNPs rs2302765, rs2302763, and rs9217, was significantly and positively associated with the ND measures. The independent replication of association findings in both the AA and EA samples at the gene level (Neale and Sham 2004) indicates that the *CHRNB1* plays an important role in the etiology of ND, and that there is a common risk-conferring variant in AA and EA populations. As compared with individual SNP analysis, haplotype-based analysis appears to increase power in the AA sample, while supporting previous findings in the EA sample. This is consistent with the theoretical expectation that haplotype-based analysis can provide greater power in detecting associations as a result of capturing more information on the LD structure when the causative SNP(s) is not included in the set of measured SNPs.

The *CHRM1* exhibited significant associations of haplotype C-C-A-T-G-G, which has a frequency of 13.1% formed by all six SNPs in this gene, with at least one adjusted ND measure under the additive and/or dominant models in the AA sample (see Table 5). The association of the haplotype with SQ and FTND score remained significant after Bonferroni correction. Because these associations were negative, it suggests that a protective variant of the *CHRM1* is associated with this haplotype in the AA sample. No significant association was found for any haplotype formed by different combinations of the SNPs investigated in the EA sample.

Discussion

Nicotine is the primary psychoactive addictive agent responsible for maintaining and regulating tobacco use. It is well accepted that nicotine exerts its psychoactive and addictive effects through functionally diverse nAChRs coupling with both dopamine and non-dopamine neurochemical pathways (Picciotto et al. 2000). It is possible that gene variants of nAChRs play a role in receptor functioning in one or more of the systems. The rationale for this study concerns the fact that the *CHRM1* and *CHRNB1* are located in linkage regions identified in earlier studies by our research group (Li et al. 2003b; Wang et al. 2005) and other groups (Duggirala et al. 1999; Straub et al. 1999; Arcos-Burgos et al. 2004; Bierut et al. 2004; Stallings et al. 2005), as well as their plausible biological roles in ND. The *CHRNB1* has been suggested to mediate various nicotine effects on locomotion, endocrine, and cardiovascular systems, and its expression was reported to be modulated by chronic nicotine treatment in rat heart (Hu et al. 2002). Further, the *CHRNB1* is expressed in brain although the mechanism whereby *CHRNB1* is assembled into functionally nAChRs remains unknown (Su et al. 2002). These biological observations imply that the *CHRNB1* is of possible relevance to ND. Our replication of genetic association findings in two independent samples suggests a noteworthy role of the *CHRNB1* gene in the development of ND.

The *CHRM1* is a member of the G-protein-coupled superfamily and mediates various brain functions (Felder et al. 2001). Like its natural counterpart acetylcholine, nicotine can also bind to mAChRs, although with less affinity and sensitivity than muscarine (http://en.wikipedia.org/wiki/Muscarinic_acetylcholine_receptor). This provides a basis for implicating mAChRs in ND, although the underlying mechanism remains largely unknown. Our haplotype-based analyses indicated that *CHRM1* is associated with ND in the

AA sample, but not in the EA sample. One possible reason is that the association arises from a rare allele, and sampling error leads to an insufficient variant frequency, constraining our capacity to identify a detectable effect. To date, this has not been demonstrated, and requires confirmation in a follow-up study.

Our results suggest differences in allele frequency and LD structure across the two ethnic samples. As pointed out by Neale and Sham (2004), such differences may invalidate the assumption of holding the same allele frequencies and LD structure across diverse populations, behind the traditional concept considered at the single SNP and/or haplotype level, potentially leading to failed attempts to replicate association findings. This implies that it is helpful to scrutinize the replications among different populations under the gene-based concept proposed by Neale and Sham (2004). By shifting toward a gene-based approach, we replicated the significant association of the *CHRNB1* gene with ND in both AA and EA samples.

As pointed out by Clark (2004), haplotype-based association tests can improve power over the single SNP approach. In our study, only marginal significance was found for the *CHRNB1* in individual SNP-based association analysis within the AA sample (the minimum $P = 0.046$ for SNP rs2302762 with FTND). However, haplotype-based analysis indicated a highly significant association of *CHRNB1* with ND for both the ethnic samples. This supports the use of haplotype-based approach in examining associations with possible functional variant(s). Thus, with the haplotype-based approach, the cryptic component signals that were masked in association testing with individual SNPs may be resolved.

In summary, our individual SNP- and/or haplotype-based association analyses revealed that *CHRNB1* is significantly associated with ND in both AA and EA samples. Furthermore, we demonstrated haplotype-specificity within each ethnic sample. As for *CHRM1* gene, we only found a significant association with ND in the AA sample. These findings, along with the linkage results, imply these two genes are involved in the etiology of ND, although the mechanism underlying the observed association requires further exploration.

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