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Report

A Common Haplotype of the Nicotine Acetylcholine Receptor α4 Subunit Gene Is Associated with Vulnerability to Nicotine Addiction in Men

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Nicotine is the major addictive substance in cigarettes, and genes involved in sensing nicotine are logical candidates for vulnerability to nicotine addiction. We studied six single-nucleotide polymorphisms (SNPs) in the *CHRNA4* gene and four SNPs in the *CHRNB2* gene with respect to nicotine dependence in a collection of 901 subjects (815 siblings and 86 parents) from 222 nuclear families with multiple nicotine-addicted siblings. The subjects were assessed for addiction by both the Fagerstrom Test for Nicotine Dependence (FTND) and the Revised Tolerance Questionnaire (RTQ). Because only 5.8% of female offspring were smokers, only male subjects were included in the final analyses (621 men from 206 families). Univariate (single-marker) family-based association tests (FBATs) demonstrated that variant alleles at two SNPs, rs1044396 and rs1044397, in exon 5 of the *CHRNA4* gene were significantly associated with a protective effect against nicotine addiction as either a dichotomized trait or a quantitative phenotype (i.e., age-adjusted FTND and RTQ scores), which was consistent with the results of the global haplotype FBAT. Furthermore, the haplotype-specific FBAT showed a common (22.5%) *CHRNA4* haplotype, GCTATA, which was significantly associated with both a protective effect against nicotine addiction as a dichotomized trait ($Z = -3.04$, $P < .005$) and significant decreases of age-adjusted FTND ($Z = -3.31$, $P < .005$) or RTQ scores ($Z = -2.73$, $P = .006$). Our findings provide strong evidence suggesting a common *CHRNA4* haplotype might be protective against vulnerability to nicotine addiction in men.

Nearly one-third of adults worldwide are smokers, and the majority started the habit as adolescents (World Health Organization 1997). In 2000, smoking caused ~2.43 million deaths in industrialized countries (~19% of total adult mortality) and ~2.41 million deaths in developing countries (~9% of total adult mortality) (Ezzati and Lopez 2003).

Smoking cigarettes is both psychologically and physiologically addictive (Nair and Brandt 2000). A vast body of literature indicates that nicotine is the component of tobacco smoke that leads to addiction (Stolerman and Jarvis 1995). Nicotine addiction (i.e., tobacco addiction [MIM #188890]), like many other drug dependencies, is believed to be a complex, multifactorial behavior with both genetic and environmental determinants. Twin and adoption studies have shown that heritabilities of vulnerabilities for both smoking initiation (SI) and smoking persistence (SP) are at least 50% (Li 2003). A familial aggregation study among siblings of nuclear families (Niu et al. 2000) also suggests that genetic influences may be an important determinant in vulnerability to nicotine addiction. Furthermore, studies using animal models found that genetic factors play a critical role in both behavioral and physiological effects of nicotine (Batra et al. 2003). However, the identification of nicotine-addiction genes has lagged far behind environmental risk-factor classification (Straub et al. 1999; Batra et al. 2003).

Nicotine functions by binding to nicotinic acetylcholine receptors (nAChRs), which, in turn, modulate the release of dopamine in the mesolimbic system (Pido-

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plichko et al. 1997). To date, nine α (α2–α10) and three β (β2–β4) subunit genes have been identified (Mansvelder and McGehee 2002). Among them, α4 and β2 subunits are the most widely and concurrently expressed high-affinity nAChR subunits in the brain (Mathieu-Kia et al. 2002) and are both upregulated under chronic nicotine exposure (Whiteaker et al. 1998). The gene that encodes the human nAChR α4 subunit (*CHRNA4* [MIM 118504] [National Center for Biotechnology Information {NCBI} locus ID 1137]) was mapped by FISH to 20q13.2-13.3 (Steinlein et al. 1994). The gene is ~17 kb in size and comprises six exons (Steinlein et al. 1996). The nAChR β2 subunit gene (*CHRNB2* [MIM 118507] [NCBI locus ID 1141]) was first mapped by FISH to chromosome 1q21 (Rempel et al. 1998) and was further narrowed to chromosome 1q21.3 (Lueders et al. 1999). The genomic sequence of the *CHRNB2* gene is ~12 kb and comprises six exons (Lueders et al. 2002). Genetic polymorphisms in the *CHRNA4* gene have been reported to be associated with autosomal dominant nocturnal frontal lobe epilepsy (Hirose et al. 1999; Chioza et al. 2000; Steinlein et al. 2000; Rozycka et al. 2003), attention-deficit/hyperactivity disorder (ADHD) (Kent et al. 2001; Todd et al. 2003), Alzheimer disease (Kawamata and Shimohama 2002), and febrile convulsions (Chou et al. 2003), but there has been no prior study of the association of this gene with addictive behaviors. Studies on *CHRNB2* gene variants are also quite limited, with two previous studies showing no associations with nicotine dependence (Silverman et al. 2000; Lueders et al. 2002).

To identify families affected by nicotine addiction, we screened a population of ~25,000 siblings, aged 20–60 years, from November 2000 to July 2001, by use of the Fagerstrom Test for Nicotine Dependence (FTND) (Heatherton et al. 1991; Niu et al. 2000). The siblings resided in a relatively remote region (Huoqiu County) of Anhui Province, China. A total of 901 subjects (815 siblings [age 47.0 years ± 10.1 years, men 78.8%]; 86 parents) from 222 qualified nuclear families (22 [9.9%] families had both parents, 42 [18.9%] families had one single parent, and 158 [71.2%] families had no available parents) were finally recruited. The criteria used for subject selection were as follows: (1) at least two siblings, aged 20–60 years, with FTND scores $\geq$8 and (2) at least one parent or one additional sibling. In addition to the FTND, the Revised Tolerance Questionnaire (RTQ) (Tate and Schmitz 1993) was also administered to eligible individuals. All subjects gave their written informed consent, and the study protocol was approved by the institutional review boards of the Harvard School of Public Health and the Anhui Medical University. Because only 10 women offspring were smokers (5.8%), all women and nonsmoking male offspring were excluded in the final analyses. A total of 621 male subjects from 206 nuclear families were finally used in the analysis. Among the 577 smoking male offspring, FTND scores (8.1 ± 2.1) and RTQ scores (35.1 ± 7.0) were found to be moderately but significantly correlated (Pearson correlation coefficient $r = 0.52$, $P < .0001$), which was in accord with results of other independent studies (Tate and Schmitz 1993; Niu et al. 2000).

A SNP set of 30 *CHRNA4* SNPs and 24 *CHRNB2* SNPs from NCBI dbSNP (dbSNP Home Page) or published literatures was evaluated by the PCR-RFLP method in a panel of 45 previously established B-lymphoblastoid cell line DNA samples from the same geographical region. Among the 54 SNPs evaluated, 18 (33.3%) were validated to be polymorphic (*CHRNA4,* 14 SNPs; *CHRNB2,* 4 SNPs). From the 14 *CHRNA4* SNPs, we excluded 1 low-heterozygosity (HET) SNP (i.e., HET $\leq$18%; minor allele frequency [MAF] $\leq$10%), as well as an additional 7 SNPs that were in perfect linkage disequilibrium (LD) with at least one of the remaining six *CHRNA4* SNPs (the extent of LD was measured by use of $|D'|$, which was calculated according to Lewontin [1964]). Finally, we handpicked six *CHRNA4* SNPs and four *CHRNB2* SNPs on the basis of both HET (HET > 18%, which implies MAF > 10%) and pairwise LD ($0.5 < |D'| \leq 1.0$) criteria. Large-scale genotyping for these 10 SNPs in the 901 DNA samples was performed by use of either PCR-RFLP or PCR-OLA (oligonucleotide ligation assay). The PCR primer sequences (designed by use of the Primer3 or SNPkit [Hao et al. 2002]), as well as genotyping methods, are summarized in table A1 (online only).

The distributions of the six *CHRNA4* SNPs and the four *CHRNB2* SNPs are depicted in fig. 1*A* and 1*b*, respectively. Among the six SNPs residing in the *CHRNA4* gene, rs1044396 (C→T transition; Ser→Ser) and rs1044397 (G→A transition; Ala→Ala) are synonymous changes located in exon 5, whereas all others are located either in an intronic region (rs2273504 [G→A transition], rs2273502 [C→T transition], rs3827020 [T→C transition]) or in the 3′ UTR region (rs2236196 [A→G transition]). Among the four SNPs residing in the *CHRNB2* gene, rs2072658 (G→A transition) is located in the 5′ UTR region, whereas A10160C is located at intron 5, and rs2072660 (C→T transition) and rs2072661 (G→A transition) are located in the 3′ UTR. In the DNA panel of 45 cell line samples, all 10 SNPs had a MAF > 10% and all were in Hardy-Weinberg equilibrium ($P > .05$ in respective χ^2 tests).

The pairwise $|D'|$ values for the six *CHRNA4* SNPs were found to be quite high (range 0.84–1.00, median 1.00) (fig. 2*A*), which indicates that these six SNPs can be considered to be one haplotype block. Similarly, the four *CHRNB2* SNPs were also found in almost complete LD with respect to each other (range 0.86–1.00, median 1.00) and, therefore, could also be grouped in one block (fig. 2*B*). Thus, we either analyzed one SNP at a time

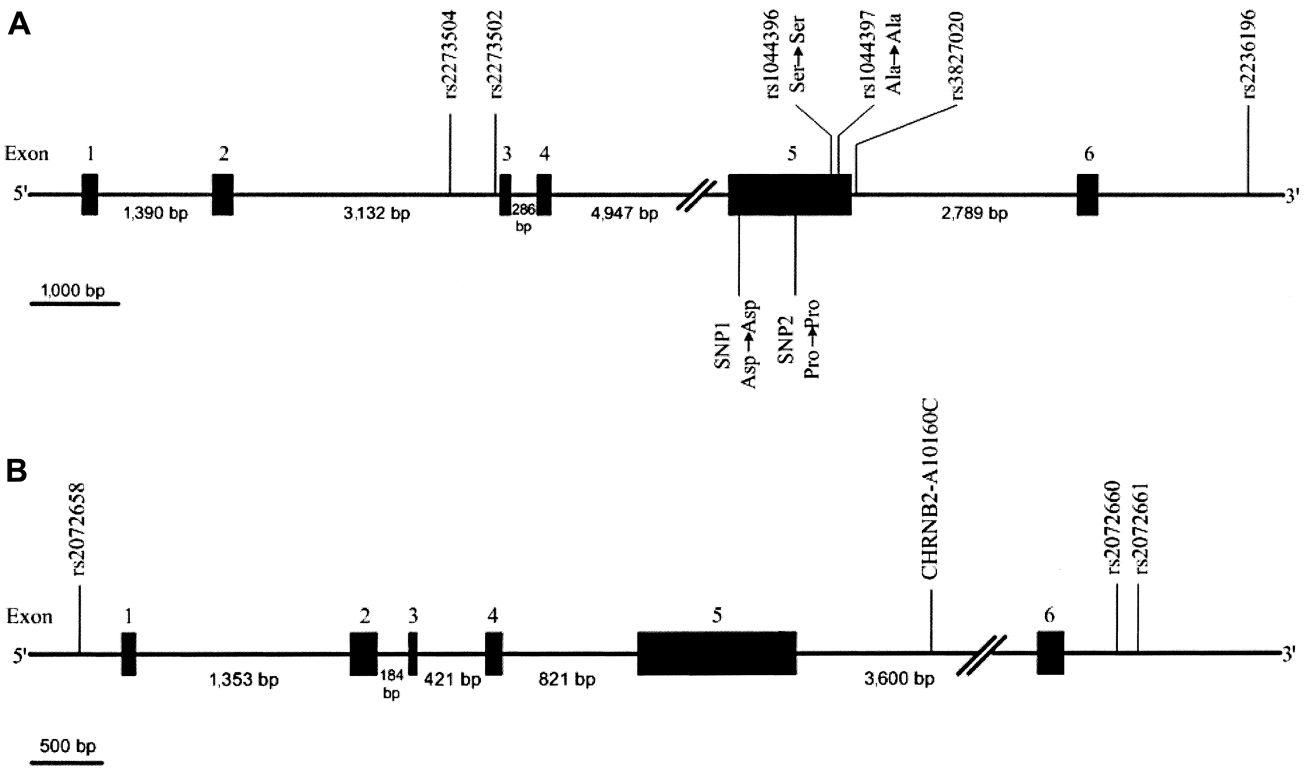

Figure 1 *A,* Schematic representation of the six SNPs located on the *CHRNA4* gene, showing their locations according to the information from the chromosome 20 genomic contig (GenBank accession number NT_011333). SNP1 and SNP2 in exon 5 are synonymous SNPs revealed by direct DNA sequencing. *B,* Schematic representation of the four SNPs located on the *CHRNB2* gene, showing their locations according to the information from the chromosome 1 genomic contig (GenBank accession number NT_004668).

by use of univariate (single-marker) family-based association tests (FBATs) or considered SNPs that were on the same gene as a single haplotype block and tested the associations by use of the haplotype FBAT for various nicotine-addiction phenotypes (both qualitative and quantitative). As mentioned above, all analyses were performed in men only. In the univariate FBAT, a significant association of *CHRNA4* rs1044396 (the variant T allele is the protective allele) (MAF = 0.286, polymorphism information content [PIC]= 0.325, calculated by use of the POLYMORPHISM software [Niu et al. 2001]) was consistently shown across all addiction-related phenotypes: nicotine addiction (addicted and nonaddicted patients with FTND scores of ≥ 8 and ≤ 2, respectively) ($P < .01$), age-adjusted FTND score ($P < .01$), and age-adjusted RTQ score ($P < .01$) before Bonferroni correction. Similarly, rs1044397 (MAF = 0.416, PIC = 0.368), which is in almost complete LD with rs1044396 ($|D'| = 1.00$) (the variant A allele is the protective allele), was observed to be significantly associated with nicotine addiction ($P < .01$) before Bonferroni correction and with age-adjusted FTND and RTQ scores ($P < .001$ for both) after Bonferroni correction for multiple comparisons (for 10 SNPs being tested individually, the global significance level needs to be adjusted to 0.05/10 = 0.005) (table 1). For *CHRNB2,* no SNP was found to be significantly associated with any nicotine-addiction phenotype (table 1).

Because haplotype-based analysis is arguably more powerful than single-marker analysis (Niu et al. 2002), we performed the global haplotype FBAT as the main haplotype test. In all FBAT analyses, we used both the age-adjusted FTND score and the age-adjusted RTQ score as the continuous phenotypes. For *CHRNA4,* the global test of the haplotype FBAT demonstrated a significant association of the haplotypes formed by rs2273504, rs2273502, rs1044396, rs1044397, rs3827020, and rs2236196 (in the direction 5′→3′) with both nicotine addiction ($\chi^2 = 19.19$, 4 df, $P < .001$) and the age-adjusted FTND score ($\chi^2 = 17.96$, 5 df, $P < .005$), even after Bonferroni correction (for two genes, the significance level is adjusted to be 0.05/2 = 0.025). In addition, a significant association was observed with regard to the age-adjusted RTQ score ($\chi^2 = 10.43$, 4 df, $P < .05$) before Bonferroni correction (table 2). For *CHRNB2,* the global test shows that the haplotypes formed by rs2072658, A10160C,

Table 1

Univariate FBAT for Association of *CHRNA4* and *CHRNB2* SNPs with Nicotine-Addiction Phenotypes

Gene and SNP	Variant	MAF	Result for Phenotype: Nicotine Addiction[a] Z	Nicotine Addiction[a] P	FTND Score Z	FTND Score P	RTQ Score Z	RTQ Score P
CHRNA4:								
rs2273504	A	.441	.753	.452	1.284	.199	.845	.398
rs2273502	T	.179	2.13	.033[b]	2.497	.013[b]	1.704	.088
rs1044396	***T***	***.286***	***−2.673***	***.008***[b]	***−2.832***	***.005***[b]	***−2.724***	***.006***[b]
rs1044397	***A***	***.416***	***−2.699***	***.007***[b]	***−3.781***	***<.001***[c]	***−3.505***	***<.001***[c]
rs3827020	C	.399	.237	.813	.602	.547	−.265	.791
rs2236196	G	.185	1.834	.067	1.828	.067	1.169	.242
CHRNB2:								
rs2072658	A	.19	−.628	.530	−1.692	.091	−.821	.412
A10160C	C	.2	−1.143	.253	−1.686	.092	−.912	.362
rs2072660	T	.278	1.646	.100	1.515	.130	1.052	.293
rs2072661	A	.283	1.36	.174	1.152	.249	.972	.331

Note.—The univariate FBAT was performed by use of the additive model. The minor allele is also referred to as the "variant allele" in the text. MAF = minor (i.e., variant) allele frequency. SNPs are listed in order (5′→3′) for each gene. Both rs1044396 and rs1044397 are shown in bold italics because they were consistently significant before Bonferroni correction for nicotine addiction, as well as FTND and RTQ scores. Both FTND and RTQ scores were age-adjusted.

[a] When nicotine addiction was considered as a dichotomized trait, the addicted and nonaddicted subjects were defined as having scores of ⩾8 and ⩽2, respectively (Fagerstrom et al. 1990).

[b] Significant only before Bonferroni correction (i.e., $P < .05$).

[c] Significant after Bonferroni correction (i.e., $P < .005$).

rs2072660, and rs2072661 (in the direction 5′→3′) yielded marginally significant association with the age-adjusted FTND score ($\chi^2 = 8.84$, 4 df, $P = .065$) before Bonferroni correction but demonstrated no association with nicotine addiction as a dichotomized trait ($\chi^2 = 7.05$, 4 df, $P > .10$) or with the age-adjusted RTQ score ($\chi^2 = 3.23$, 4 df, $P > .10$) (table 2). The association with age-adjusted FTND score was no longer statistically significant after Bonferroni correction. By use of the expectation-maximization algorithm implemented in the haplotype FBAT, four major haplotypes (i.e., frequency ⩾0.05) were revealed for both *CHRNA4* and *CHRNB2*. Haplotype-specific tests (haplotype FBATs) were used to analyze the effects on nicotine addiction, age-adjusted FTND score, and age-adjusted RTQ score for each haplotype, compared with all other haplotypes grouped together on each gene. The GCTATA haplotype of the *CHRNA4* gene, bearing the variant alleles at both rs1044396 and rs1044397 (underscored), was found to be significantly associated with a protective effect against nicotine addiction ($Z = -3.04$, $P = .002$), as well as significant decreases of age-adjusted FTND score ($Z = -3.31$, $P = .001$) and age-adjusted RTQ score ($Z = -2.73$, $P = .006$) after Bonferroni correction. These results were highly consistent with the univariate FBAT results showing that the variant alleles at both of these two exonic SNPs had significant protective effects. For *CHRNB2,* we found that the haplotype GCCG was associated with a significant protective effect against nicotine addiction ($Z = -2.13$, $P = .033$), as well as a significant decrease of age-adjusted FTND score ($Z = -2.13$, $P = .033$) before Bonferroni correction, but such associations were no longer statistically significant after Bonferroni correction (table 3).

Because both rs1044396 and rs1044397 were located on exon 5 of the *CHRNA4* gene, we sequenced exon 5 and its flanking regions (50 bp upstream and 50 bp downstream) in a selected sample of 36 subjects (7 parents and 29 offspring) from 25 nuclear families with extreme *Z* values. Among the 29 offspring, 22 subjects had FTND scores ⩾8 and 7 subjects had FTND scores ⩽2. Sequencing results confirmed the three genotyped SNPs in this region—rs1044396, rs1044397, rs3827020—and revealed two new synonymous polymorphisms in exon 5, which have been indicated in figure 1. The MAFs of SNP1 (Asp→Asp) and SNP2 (Pro→Pro) were 0.237 and 0.257, respectively, in the sequenced subjects. Both SNP1 and SNP2 have been recently reported in dbSNP (dbSNP Home Page), corresponding to rs1044393 and rs2229959, respectively.

Our study is the first to demonstrate significant associations, not only of individual *CHRNA4* SNPs but also of a multilocus *CHRNA4* haplotype, with vulnerability to nicotine addiction in men. To our knowledge, there

have been no prior studies regarding the role of molecular variants of the human *CHRNA4* gene in addictive behavior, although two groups have linked this gene to attention problems such as ADHD (Kent et al. 2001; Todd et al. 2003). A biological link between *CHRNA4* or *CHRNB2* and the nicotine-addiction phenotype has been well established by multiple studies in animal models. By constructing α4 nAChR subunit knockout mice, Ross et al. (2000) confirmed that the α4 nAChR subunit plays a pivotal role in anxiety in mice. Actually, several in vitro studies showed that a number of nAChR agonists that bind to α4β2 receptor complexes have anxiolytic-like effects (Pomerleau 1986; Gilbert et al. 1989; Brioni et al. 1993), and nicotine is shown to reduce anxiety in chronic smokers (Pomerleau 1986; Gilbert et al. 1989), allowing them to relax, focus, and work more efficiently. Furthermore, knock-in mice with a Leu→Ser mutation (a point mutation in the M2 transmembrane domain) in the α4 nAChR subunit demonstrated increased anxiety and showed a reduced nigrostriatal dopaminergic function (Labarca et al. 2001). With regard to *CHRNB2*, nicotine-evoked dopamine release is abolished (Zhou et al. 2001) and nicotine self-administration is attenuated (Picciotto et al. 1998) in β2 nAChR subunit knockout mice.

A biological link between *CHRNA4* or *CHRNB2* and nicotine-addiction phenotypes has also been suggested by multiple studies in humans. Human α4β2 nAChRs are functionally upregulated by chronic nicotine exposure (Buisson and Bertrand 2001), and these receptors are more likely to enter desensitization states (Fenster et al. 1999) than without such exposure. Moreover, the increased numbers of α4β2 nAChRs in the human postmortem brain significantly correlated with intensity and duration of smoking history (Breese et al. 1997). When nicotine is avoided, the excess number of α4β2 nAChRs will recover from desensitization, resulting in hyperexcitability at cholinergic synapses that could contribute to the unrest and agitation that, in turn, motivate the smoker to smoke the next cigarette, to re-desensitize these excess nAChRs (Dani and De Biasi 2001). Todd et al. (2003) identified an intron 2 SNP in the *CHRNA4*

Table 2

Global (Multihaplotype) Tests of the Haplotype FBAT for Association of *CHRNA4* and *CHRNB2* SNPs with Nicotine-Addiction Phenotypes

Gene and Phenotype	χ^2	df	*P* Value
CHRNA4:			
Nicotine addiction	***19.19***	***4***	***.0007***[a]
FTND score	***17.96***	***5***	***.003***[a]
RTQ score	10.43	4	.034[b]
CHRNB2:			
Nicotine addiction	7.05	4	.133
FTND score	8.84	4	.065
RTQ score	3.23	4	.520

NOTE.—The haplotype FBAT was performed by use of the additive model. Globally significant results are shown in bold italics.

[a] Significant after Bonferroni correction (i.e., $P < .025$).

[b] Significant only before Bonferroni correction (i.e., $P < .05$).

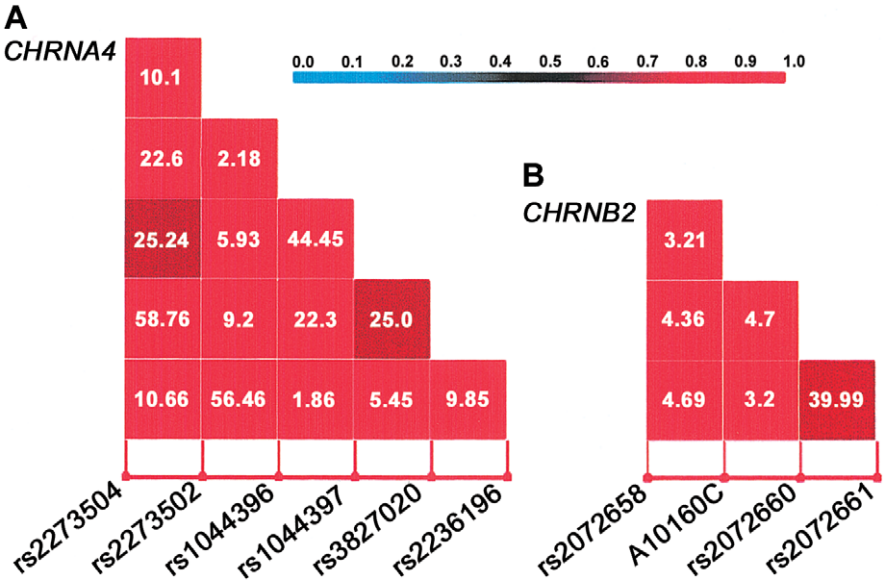

Figure 2 *A*, Pairwise LD for the 15 *CHRNA4* SNP pairs, evaluated by $|D'|$. *B*, Pairwise LD for the six *CHRNB2* SNP pairs, evaluated by $|D'|$. Because all SNP pairs have high $|D'|$ values, we presented χ^2 values inside their corresponding boxes.

Table 3

Haplotype-Specific Tests of the Haplotype FBAT for Association of *CHRNA4* and *CHRNB2* SNPs with Nicotine-Addiction Phenotypes

Gene and Haplotype	Frequency	Result for Phenotype: Nicotine Addiction Z	Nicotine Addiction P	FTND Score Z	FTND Score P	RTQ Score Z	RTQ Score P
CHRNA4:							
ACCGCA	.381	1.865	.062	2.432	.015	1.875	.061
GCTATA	***.225***	***−3.039***	***.002***[a]	***−3.312***	***.001***[a]	***−2.727***	***.006***[a]
GTCGTG	.153	2.297	.022[b]	2.424	.015[b]	1.713	.087
GCCATA	.129	.765	.444	−.310	.756	−.063	.950
CHRNB2:							
GACG	.418	.179	.858	1.529	.126	.805	.421
GATA	.225	1.979	.048[b]	1.828	.068	1.185	.236
GCCG	.163	−2.134	.033[b]	−2.128	.033[b]	−.848	.396
AACG	.135	−.17	.865	−1.406	.160	−1.297	.195

Note.—The haplotype-specific test of the haplotype FBAT was performed by use of the additive model. The nucleotides at rs1044396 and rs1044397 of *CHRNA4* and the nucleotides at rs2072658 and A10160C are underscored for comparisons with the single-marker FBAT results of the respective SNPs in table 1. Only haplotypes with frequencies ⩾.05 are presented. Globally significant results are shown in bold italics.

[a] Significant after Bonferroni correction (i.e., $P < .00625$).

[b] Significant only before Bonferroni correction (i.e., $P < .05$).

gene that was associated with a severe inattention problem. Since nicotine and nicotine agonists enhance attention among ADHD patients in clinical trials, some smokers with inattention problems may be addicted to nicotine because such addictive smokers can enhance attention through persistent self-administration of nicotine.

The pathway to substance dependence is complex and involves multiple genetic and environmental risk factors (Kendler et al. 1999; Swan 1999). By use of both univariate and haplotype FBATs, the present study found significant associations of two *CHRNA4*-coding SNPs, rs1044396 and rs1044397, with protective effects against nicotine addiction. Although these two SNPs do not result in amino acid changes, rs1044397 was located in a highly conserved protein motif, TKAP (see the boxed area of fig. A1 [online only]), across humans, chimpanzees, rhesus monkeys, rats, and mice. The most frequent haplotype in our study population contains the C allele at rs1044396 and the G allele at rs1044397 (i.e., ACCGCA population frequency 38.1%), and, therefore, the protective haplotype revealed here, GCTATA, carried the variant alleles at both SNP sites (table 1).

Our haplotype-analysis results provided some suggestive evidence of a tentative association of *CHRNB2* SNPs with vulnerability to nicotine addiction, but such results were uniformly insignificant after Bonferroni correction and therefore should be considered as principally negative. In agreement with our results, Lueders et al. (2002) undertook both single-marker and haplotype approaches and did not find any association of *CHRNB2* variants with nicotine dependence as either a continuous or a dichotomous variable. Silverman et al. (2000) analyzed four SNPs on *CHRNB2* in 872 subjects and also found no significant association with either SI or progression to nicotine dependence.

Our study focused exclusively on men. Perkins et al. (1999) discussed the emerging evidence that men and women differ in their responses to smoking, presumably because of both genetic and environmental factors. The relative importance of "non-nicotine" factors has been shown to be greater for women than for men, including factors such as pleasure from social reinforcements for smoking and comforts gained from having something to manipulate in social activities. Faraday et al. (1999) also pointed out that women are more inclined to view smoking as a useful tool to cope with social stress or unpleasant situations. Probably because of these reasons, women have been less successful than men in using nicotine-replacement therapy for quitting smoking. Thus, genetic factors underlying nicotine addiction probably play a different role in female and male smokers. In a meta-analysis in male and female twins, Li et al. (2003) found that genetic factors play a more significant role for SI but a less significant role for SP in female adults compared with male adults. Significant sex difference was also detected in shared environmental factors for SI and SP. However, no significant sex difference was detected for nonshared environmental effects for either phenotype.

Despite limitations, including an exclusively male sample and a single ethnic group, our study has several unique strengths. First, we applied both univariate and haplotype FBATs (they both allow for the analyses of dichotomized

or continuous traits), which are state-of-the-art tests of association in the presence of linkage (Lake et al. 2000; Horvath et al. 2004) on the basis of the Rabinowitz-Laird algorithm (Rabinowitz and Laird 2000). The FBAT is a generalized version of the classic transmission/disequilibrium test, which can be applied to any type of nuclear family study (Laird et al. 2000), thus avoiding the thorny problem introduced by population admixture that is commonly seen in case-control study designs involving multiple ethnic groups. Moreover, the FBAT results (both single-marker and haplotype-based) without age adjustments were quite similar to the results with adjustments for age (data not shown).

Second, in addition to using the dichotomized nicotine-addiction phenotype, we used both the age-adjusted FTND and the RTQ scores as quantitative measures of the degree of nicotine dependence. The FTND and the RTQ are both based on the Fagerstrom Tolerance Questionnaire (FTQ) (Fagerstrom 1978), and both have improved the internal consistency, the unitary-factor structure, and the psychometric properties of the FTQ (Heatherton et al. 1991; Tate and Schmitz 1993). In addition to covering all six items of the FTND, the RTQ has included items on inhalation patterns and the proportion of the physical lengths of the cigarettes smoked (Tate and Schmitz 1993). Thus, RTQ and FTND scores represent two moderately different perspectives in quantifying the degree of nicotine dependence. The RTQ appears to be more composite than the FTND, and this may explain the slight differences we detected by use of these two closely related continuous phenotypes ($r = 0.52$, $P < .0001$).

The use of the continuously distributed phenotype in LD analysis has been shown to be a more powerful strategy in detecting genetic susceptibility to complex diseases than the simple classification of individuals into "addicted" and "nonaddicted" categories (Ebstein et al. 1996; Leckman et al. 2001; Rowe et al. 2001). By treating nicotine addiction as either a qualitative or a quantitative phenotype, we found highly consistent results across both univariate (table 1) and haplotype analyses (tables 2 and 3). In particular, the univariate FBAT found protective effects of the variant alleles at rs1044396 and rs1044397 against nicotine addiction, whereas the haplotype FBAT not only corroborated such findings at the global level but also revealed that the GCTATA haplotype (bearing the variant allele at these two loci) had a significant protective effect against nicotine addiction. Unobserved causal SNPs that are in and surrounding the *CHRNA4* gene and are in LD with the observed *CHRNA4* markers may explain the association we found. When we performed direct DNA resequencing for exon 5 of *CHRNA4* in a selected sample, two more synonymous SNPs were found (indicated as SNP1 and SNP2 in fig. 1), but no missense or splice-site mutations were detected. A recent promoter analysis in transgenic mice of the mouse orthologue of the human *CHRNA4* gene, *chrna4,* demonstrated that the regions either upstream of the transcription initiation site or in intron 1 may contain functional regulatory elements that are important for endogenous *chrna4* gene expression (Watanabe et al. 1998). Therefore, SNPs located in the 5′ upstream region and in intron 1 of the human *CHRNA4* gene may have functional effects on *CHRNA4* gene expression. The SNP rs6090387, located in the 5′ UTR of *CHRNA4,* deserves further investigation.

Third, our study population appeared to be both relatively stable and fairly homogeneous with respect to lifestyle variables, as well as social and cultural norms (Niu et al. 2000). Finally, none of the subjects in this study used nicotine-replacement therapies, such as nicotine gums, patches, or nasal sprays, or used tobacco products other than cigarettes (such as cigars or smokeless tobacco); thus, the potential for confounding by drug interventions or by an unknown cause of information bias was at least significantly minimized, if not virtually eliminated.

In conclusion, by use of a family-based design, we detected significant protective effects of variant alleles at the *CHRNA4* SNPs rs1044396 and rs1044397, not only at the individual SNP level but also at the global haplotype level (GCTATA being the protective haplotype), against vulnerability to nicotine addiction in men. Thus, this study provides important new information in the genetic research on addictive behaviors, pointing out that the *CHRNA4* gene should be more extensively studied as a critical biological candidate. Since nicotine addiction is a complex trait with significant genetic heterogeneity, candidate genes other than *CHRNA4* and *CHRNB2,* such as the genes encoding the D1, D2, and D4 dopamine receptors and the dopamine transporters (Bergen and Caporaso 1999), deserve further study. Because our study was limited to men, further investigations should also be performed to assess whether these *CHRNA4* and *CHRNB2* genes play similarly important roles in nicotine addiction in women from the same ethnic background. Also, our results need to be replicated in populations of ancestries other than Chinese.

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

dbSNP Home Page, http://www.ncbi.nlm.nih.gov/SNP/ (for *CHRNA4* polymorphisms [cluster IDs rs2273504, rs2273502, rs1044396, rs1044397, rs3827020, rs2236196, rs1044393, and rs2229959] and *CHRNB2* polymorphisms [cluster IDs rs2072658, rs2072660, and rs2072661])

FBAT, http://www.biostat.harvard.edu/~fbat/fbat.htm

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for chromosome 20 genomic contig [accession number NT_011333], chromosome 1 genomic contig [accession number NT_004668], human *CHRNA4* cDNA [accession number NM_000744], rhesus monkey *chrna4* cDNA [accession number AJ245973], rat *chrna4* cDNA [accession number NM_024354], and mouse *chrna4* cDNA [accession number NM_015730])

GSC BLAST Search, http://www.genome.wustl.edu/projects/chimp/blast/pan_client.pl (for chimpanzee genome sequence)

JavaScript DNA Translator 1.1, http://www.bioinformatics.vg/bioinformatics_tools/JVT.shtml

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for tobacco addiction, *CHRNA4*, and *CHRNB2*)

Primer3, http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi

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