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Report

Single- and Multilocus Allelic Variants within the $GABA_B$ Receptor Subunit 2 (*GABAB2*) Gene Are Significantly Associated with Nicotine Dependence

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Twelve single-nucleotide polymorphisms (SNPs) in the human γ-aminobutyric acid type B ($GABA_B$) receptor subunit 2 gene (*GABAB2*) were tested for association with nicotine dependence (ND) in an extensively phenotyped cohort of 1,276 smokers and nonsmokers, representing ~404 nuclear families of African American (AA) or European American (EA) origin. The *GABAB2* gene encodes a subunit of the $GABA_B$ receptor for GABA, an inhibitory neurotransmitter involved in the regulation of many physiological and psychological processes in the brain. The gene is located within a region of chromosome 9q22 that showed a "suggestive" linkage to ND. Individual SNP analysis performed using the PBAT-GEE program indicated that two SNPs in the AAs and four SNPs in the EAs were significantly associated with ND. Haplotype analysis using the Family-Based Association Test revealed that, even after Bonferroni correction, the haplotype C-C-G of *rs2491397-rs2184026-rs3750344* had a significant positive association with ND in both the pooled and the AA samples. In the EAs, we identified two haplotypes, C-A-C-A and T-A-T-A, formed by SNPs *rs1435252-rs378042-rs2779562-rs3750344,* that showed a highly significant negative and positive association with ND, respectively. In summary, our findings provide evidence of a significant association of *GABAB2* variants with ND, implying that this gene plays an important role in the etiology of this drug addiction.

Tobacco use is a major worldwide health problem, and nearly one-third of the global adult population smokes tobacco products (United States Department of Health and Human Services [USDHHS] 2000). Nicotine is the primary reward component in tobacco that maintains continued use and results in addiction (USDHHS 2000). Nicotine dependence (ND [MIM 188890]), like many other substance dependencies, is a complex trait determined by strong genetic and environmental factors (Sullivan and Kendler 1999; Li et al. 2003*a*). Our previous meta-analysis of the genetic parameter for ND, which was based on 17 reported twin studies, indicated an average weighted mean polygenic heritability of 0.56 for adult smokers (Li et al. 2003*a*). On the basis of the results from genomewide linkage analyses for smoking behavior, several chromosomal regions appear to harbor susceptibility loci for ND (for recent reviews, see the studies by Uhl et al. [2002] and Li et al. [2004]). However, no susceptibility genes for ND have yet been identified within these positive regions. In addition, although numerous population-based association studies have been conducted to examine the effects of functional candidate genes on ND, only a limited number of variants have been confirmed in multiple cross-sectional studies (Li et al. 2004).

We previously identified an ~13-cM interval on chromosome 9q22 that showed "suggestive" linkage with ND (measured as the number of cigarettes smoked per day, called "smoking quantity") in the Framingham Heart Study (FHS) samples (Li et al. 2003*b*). Furthermore, three additional independent studies reported linkage—at a nominally significant level—of smoking to a region on chromosome 9 overlapping with our linked region (Ber-

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Table 1

Clinical Characteristics of the AA, EA, and Pooled Sample Groups

Characteristic	AA	EA	Pooled
No. of families	253	151	404
Average no. of members/family (mean ± SD)	3.1 ± .8	3.2 ± .7	3.2 ± .8
No. of subjects	793	483	1,276
% Female	64.6	68.7	66.1
Age (years) (mean ± SD)	40.0 ± 14.5	41.1 ± 15.2	40.4 ± 14.8
No. of smokers	622	468	990
Age at smoking onset (years) (mean ± SD)	17.2 ± 4.7	15.5 ± 4.4	16.6 ± 4.7
Years smoked (mean ± SD)	21.0 ± 12.7	24.1 ± 12.8	22.1 ± 12.8
SQ/day (mean ± SD)	17.9 ± 12.5	18.0 ± 13.7	18.0 ± 12.9
HSI score (mean ± SD)	3.7 ± 1.4	3.9 ± 1.4	3.8 ± 1.4
FTND score (mean ± SD)	6.3 ± 2.1	6.2 ± 2.2	6.2 ± 2.2

gen et al. 1999; Bierut et al. 2004; Gelernter et al. 2004). The gene encoding the human γ-aminobutyric acid (GABA) type B receptor subunit 2 (*GABAB2,* also called "G-protein–coupled receptor 51" [MIM 607340]) has been mapped to the 9q22.1 region, spans ~420,000,000 bp, and consists of a 3.1-kb coding region that is distributed over 19 exons (Martin et al. 2001). GABA is a major neurotransmitter in the mammalian brain and controls neuronal excitability. The $GABA_B$ receptor inhibits neuronal activity through G-protein–coupled second-messenger systems, which regulate the release of neurotransmitters and the activity of ion channels and adenylyl cyclase (Kaupmann et al. 1998). Two types of $GABA_B$ receptors ($GABA_{B1}$ and $GABA_{B2}$) have been identified (Jones et al. 1998; Kaupmann et al. 1998). Although no unique pharmacological or physiological roles have been determined for either subtype of the $GABA_B$ receptor to date (Schuler et al. 2001), studies in both humans and animals have implicated GABAergic mechanisms in drug dependence, including ND (Dewey et al. 1999; Corrigall et al. 2000). Moreover, baclofen, a widely used $GABA_B$ agonist, is able to antagonize nicotine-rewarding effects in mice and rats (Fattore et al. 2002). Although there are lines of evidence suggesting biological links between *GABAB2* and smoking behavior (Cousins et al. 2001; Fattore et al. 2002), few genetic studies have examined the role of *GABAB2* in the etiology of ND, and no polymorphism in the *GABAB2* gene has been reported to be associated with any addictive behavior. On the other hand, association of the α2 subunit of the $GABA_A$ receptor with alcohol dependence has been reported (Edenberg et al. 2004). The linkage results in the chromosomal 9q22 region, together with a likely biological function of *GABAB2* in drug addiction, make this gene a plausible candidate for association with ND.

The subjects used in this study were of either African American (AA) or European American (EA) origin and were recruited primarily from the mid-South states in the United States from 1999 to 2004. The symptoms of ND of probands and other smoker participants were assessed with the Fagerstrom Test for Nicotine Dependence (FTND) scale (Heatherton et al. 1991). Once a proband was recruited, additional siblings and parents were recruited whenever possible, regardless of smoking status. Extensive clinical data were collected on all participants, including demographics (e.g., sex, age, race, biological relationships, weight, height, years of education, and marital status), medical history, smoking history, smoking experience, ND, and personality characteristics, as assessed by various questionnaires (National Institute on Drug Abuse, Center for Genetic Studies). Of the families recruited, 75.7% (EAs: 74.2%; AAs: 76.7%) had at least two siblings whose FTND score was 5 or above. All subjects agreed to participate in this study and signed the consent form, which was approved by each institutional review board.

In the present study, the ND of each smoker was ascertained by the three most commonly used measures in the literature: smoking quantity (SQ) (also used in our previous linkage study for ND in the FHS samples [Li et al. 2003*b*]); 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 score (0–10 scale). Given the presence of overlap in the contents of the three ND measures, there exist fairly robust correlations among them ($r = 0.88$–0.94). The primary reason for running similar analyses for each of the three ND measures was for comparison with previous findings. Demographic and clinical characteristics are presented in table 1. DNA from the participants was obtained using a blood extraction kit from Qiagen. Selection of the SNPs for association analysis was based on (1) a preference for SNPs located in coding or regulatory regions of the gene, (2) high heterozygosity (minor-allele frequency ≥ 0.15), and (3) a uniform coverage of the gene. The 12 SNPs used were selected from the dbSNP, JSNP, and Celera Genomics databases. SNPs *rs3750344* and *rs2304389* are synonymous variants located in exons 2 and 15, respectively, of *GABAB2* (*rs3750344* has an A/G transition at the third

Table 2

***P* Values of PBAT-GEE Statistic for Significant Association ($P < .05$) for Individual *GABAB2* SNPs with Three ND Measures**

	P Value(s) for Sample Group and ND Measure								
	Pooled			AA			EA		
SNP ID	SQ	HSI	FTND	SQ	HSI	FTND	SQ	HSI	FTND
rs2304389	***.01***[a,b]	***.01***[a,b]	***.03***[a,b]	.53	.74	.81	.07	.06	.14
rs1435252	***.03***[a,b]	.10	.13	.07	.09	.09	***.003***[a,b]	***.009***[a,b]	***.004***[a], ***.005***[b]
rs3780422	.35	.28	.21	.43	.56	.49	.09	.08	***.04***[a,b]
rs1537959	.09	***.03***[a,b]	.07	***.02***[a,b]	***.007***[a,b]	***.02***[a,b]	.49	.40	.38
rs2491397	***.03***[a,b]	***.03***[a,b]	.07	***.02***[a,b]	***.04***[a,b]	.11	.17	.19	.12
rs2779562	.40	.29	.38	.78	.71	.44	.06	***.01***[a,b,c]	***.008***[c]
rs3750344	.14	.30	.35	.33	.56	.74	***.004***[c], ***.003***[a,b]	***.03***[c], ***.02***[a,b]	***.01***[c], ***.007***[a], ***.006***[b]

NOTE.—Significant *P* values are shown in bold italics. The adjusted *P* value after correction for multiple testing at the .05 significance level is .0043. For the pooled samples, the three ND measures were adjusted for age, sex, and ethnicity; for each ethnic-specific sample group, only age and sex were used as covariates for the ND measures.

[a] Dominant model.

[b] Recessive model

[c] Additive model.

position of the 120th codon for alanine; *rs2304389* has a G/A transition at the third position of the 684th codon for proline). The remaining 10 SNPs are located within intronic regions of *GABAB2*. Data for these SNPs, including their locations within the gene, chromosomal position, allele frequency, and primer/probe sequences, are provided in table A1 (online only). SNPs were genotyped using the TaqMan assay in a 384-well microplate format (Applied Biosystems). Briefly, 15 ng of DNA was amplified in a total volume of 7 μl containing a minor groove binder (MGB) probe and 2.5 μl of TaqMan 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 9700 Sequence Detection System. We applied the PedCheck program (O'Connell and Weeks 1998) to identify any inconsistent Mendelian inheritance, nonpaternity, or other typing errors. To avoid bias, 135 inconsistencies (88 in the AA samples and 47 in the EA samples) among ~18,000 reactions for 12 SNPs were excluded from further statistical analyses.

The pairwise $|D'|$ values for the 12 selected SNPs within *GABAB2* were calculated using the GOLD program (Abecasis and Cookson 2000) and showed a $|D'|$ value of >0.72 for pairwise combinations of *rs1547272, rs1930139,* and *rs509747* in the AA, EA, and pooled sample groups (see fig. A1 [online only]). In the AA samples, an additional SNP pair (*rs1537959* and *rs3780422*) showed a $|D'|$ value of >0.72. These findings suggest that the SNPs *rs1547272-rs1930139-rs509747* and SNPs *rs1537959-rs3780422* are part of a single haplotype block. We noticed differences for several SNPs, compared with the allele frequencies presented on various SNP Web sites, when we calculated the allele frequency by directly counting the numbers of each allele from the progenitors of our samples, especially in the AA samples (see table A2 [online only]), suggesting an ethnicity-specific allele distribution.

Association between a single SNP and three ND measures was determined by the Pedigree-Based Association Test (PBAT) program with the use of generalized estimating equations (Lange et al. 2003). Association between each ND measure and a haplotype was examined using the Family-Based Association Test (FBAT) program, with the option of computing *P* values of the *Z* statistic with the use of Monte Carlo sampling under the null distribution of no linkage and no association (Horvath et al. 2004). Three genetic models (additive, dominant, and recessive) were tested, with correction for sex and age in the AA or EA samples and for sex, age, and ethnicity in the pooled samples.

Analysis of individual SNPs indicated a significant association for 4 of the 12 SNPs with at least one age-, sex-, or ethnicity-adjusted ND measure in the pooled samples (table 2). Given the potential genetic differences in ND between ethnic groups, we analyzed all 12 SNPs in the two ethnic groups separately. Two SNPs in the AA samples and four SNPs in the EA samples showed significant or highly significant association with at least one ND measure under different genetic models. The association of SNPs *rs1435252* and *rs3750344* in the EA samples remained significant with the adjusted SQ and FTND after correction for multiple testing, on the basis of the SNP spectral decomposition approach (Nyholt 2004).

Haplotype analysis of SNPs *rs2491397, rs2184026,* and *rs3750344,* which cover ~135 kb of *GABAB2,* revealed four major haplotypes (≥5%) in the AA samples and five major haplotypes in the pooled and EA samples.

Table 3

***Z* Values and Permutation *P* Values for Highly Significant Association ($P < .01$) of Major Haplotypes Formed by a Three- or Four-SNP Combination within *GABAB2* with Three ND Measures**

No. of SNPs and Haplotype	Percentage and *Z* Value(s) (Permutation *P* Value[s]) for Sample Group and ND Measure											
	Pooled				AA				EA			
	%	SQ	HSI	FTND	%	SQ	HSI	FTND	%	SQ	HSI	FTND
Three:[a]												
C-C-G	6.2	***3.65 (.0002)***[b], ***3.73 (.0002)***[c]	***3.69 (.0002)***[b], ***3.74 (.0002)***[c]	***3.98 (.0001)***[b], ***4.02 (.0001)***[c]	7.6	***3.03 (.002)***[b], ***3.12 (.002)***[c]	***3.20 (.001)***[b], ***3.26 (.001)***[c]	***3.50 (.0003)***[b], ***3.55 (.0004)***[c]	3.9	1.98 (.05)	1.59 (.12)	1.73 (.10)
Four:[d]												
C-A-C-A	7.7	−1.05 (.33)	−1.19 (.24)	−1.07 (.29)	4.6	2.22 (.028)[b], 2.24 (.02)[c]	2.21 (.03)[b], 2.2 (.02)[c]	2.10 (.03)[b], 2.07 (.04)[c]	12.8	***−3.05 (.001)***[b], ***−3.28 (.0006)***[c]	***−3.22 (.0009)***[b], ***−3.40 (.0003)***[c]	***−3.03 (.001)***[b], ***−3.16 (.0008)***[c]
T-A-T-A	3.8	1.61 (.11)	1.30 (.18)	1.69 (.09)	2.6	−.11 (.90)	−.47 (.64)	−.24 (.81)	6.6	2.41 (.015)[b], 2.28 (.024)[c]	2.43 (.014)[b], 2.42 (.017)[c]	***2.73 (.006)***[b], ***2.69 (.007)***[c]

Note.—Significant *P* values are shown in bold italics. The adjusted *P* value at the .01 significance level after Bonferroni correction for five major haplotypes formed by the three SNPs in the pooled samples is .002, and, for the four major haplotypes in the AA samples, the adjusted *P* value is .0025. The adjusted *P* values for six major haplotypes formed by the four SNPs in the EA samples are .0083 and .0016 for the .05 and .01 significance levels, respectively. The ND measures used in the analysis were corrected for age, sex, and ethnicity in the pooled samples and for age and sex in each ethnic-specific sample group.

[a] *rs2491397-rs2184026-rs3750344.*

[b] Additive model.

[c] Dominant model.

[d] *rs1435252-rs3780422-rs2779562-rs3750344.*

In both the pooled and the AA samples, the C-C-G haplotype of the three SNPs, with a frequency of 6.2% and 7.6%, respectively, showed a highly significant positive association with the three adjusted ND measures (max $Z = 4.02$ and permutation $P = .00001$ for association with FTND in the pooled samples and max $Z = 3.55$ and permutation $P = .0004$ for association with FTND in the AA samples, with 76 and 58 families contributing to the associations, respectively) (see table 3). These results remained highly significant after Bonferroni correction for testing of the major haplotypes in each sample group. In the EA samples, we did not find a haplotype for these three SNPs that remained significantly associated with ND after Bonferroni correction.

Haplotype analysis of the *rs1435252-rs3780422-rs2779562-rs3750344* combination, which covers a region extending from exon 2 to intron 13 of *GABAB2* (~237 kb), revealed eight major haplotypes in the pooled and AA samples and six major haplotypes in the EA samples. In the EA samples, the haplotype consisting of alleles C-A-C-A at a frequency of 12.8% showed a highly significant inverse association, even after Bonferroni correction, with all three adjusted ND measures (max $Z = -3.40$ and permutation $P = .0003$ for HSI, with 33 families contributing to the analysis) (see table 3). Additionally, in this EA sample group, we found another haplotype (T-A-T-A) that was formed by the same four SNPs at a frequency of 6.6% and that showed a significant association with the three adjusted ND measures; however, this haplotype only remained significant for FTND after Bonferroni correction (table 3). These findings indicate haplotype specificities in the two ethnic groups and further suggest different effects of haplotypes within *GABAB2* on ND in AAs and EAs.

Our study has several advantages. We chose to use the single-marker PBAT program because it allows correction for covariates such as age, sex, and/or ethnicity. This represents an important feature for our analysis, as age, sex, and ethnic differences have been documented for ND (Heath et al. 1999; Madden et al. 1999; Li et al. 2003*a*; Tyndale 2003). We used a family-based approach for association analysis, which has an advantage over unconditional analyses (e.g., case-control analysis), in that it minimizes the potential for the confounding effects of population stratification. Furthermore, large samples of smokers and nonsmokers from two ethnic groups were analyzed, which allowed analysis of interethnic differences. Extensive phenotypic data on the individuals were collected, which can be used to investigate different aspects of ND in future studies. The three ND measures analyzed herein are commonly employed in the genetic research and clinical settings of tobacco smoking and thus would be readily available for cross-study replication. To our knowledge, this cohort represents one of the largest family-based genetic samples designed specifically for studies of tobacco smoking.

We here provide the first evidence of an association between the allelic variants of *GABAB2* and ND. We are aware that none of the *GABAB2* SNPs included in the current study represents an obvious causative variant. Only one significantly associated SNP (*rs3750344*) is located within a coding region of *GABAB2,* but the polymorphism does not result in an amino acid change. Thus, no coding differences are present in any of the *GABAB2* SNPs from the higher-risk or protective haplotypes. Because the associated SNPs are not necessarily exclusive to *GABAB2,* a functional allelic variant(s) close to or in strong linkage disequilibrium (LD) with these SNPs may be responsible for ND vulnerability. We therefore plan to analyze more SNPs within *GABAB2,* as well as adjacent genes in this 13-cM region on chromosome 9, not only because 9 of the 12 SNPs do not show significant LD but also because of the data of the haplotype analyses: the higher-risk haplotypes C-C-G and T-A-T-A were found in 7.6% of the AA smokers and in 6.6% of the EA smokers, respectively, and the protective haplotype C-A-C-A was found in 12.8% of the EA smokers. This suggests that these haplotypes play an important role in a relatively small proportion of smokers.

In summary, our association results provide evidence of a significant impact of *GABAB2* on ND—both for individual SNPs and for multilocus haplotypes. We also demonstrate that ethnic differences exist in the association between *GABAB2* and vulnerability to ND. This study not only confirms molecular and pharmacological data about the importance of the family of GABA receptor genes in addictive/behavioral traits but it also provides new information on the genetics of ND, showing the importance of the *GABAB2* gene as a biological candidate in future studies of ND.

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Electronic-Database Information

The URLs for data presented herein are as follows:

Celera Genomics, http://www.celera.com/
dbSNP Home Page, http://www.ncbi.nlm.nih.gov/SNP/
JSNP, http://snp.ims.u-tokyo.ac.jp/
National Institute on Drug Abuse, Center for Genetic Studies, http://zork.wustl.edu/nida/ (for questionnaires used in the study)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for ND and *GABRB2*)

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