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Variants in the Nicotinic Receptors Alter the Risk for Nicotine Dependence

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Abstract

Objective—A recent study provisionally identified numerous genetic variants as risk factors for the transition from smoking to the development of nicotine dependence, including an amino acid change in the α5 nicotinic cholinergic receptor *(CHRNA5)*. The purpose of this study is to replicate these findings in an independent dataset and more thoroughly investigate the role of genetic variation in the cluster of physically linked nicotinic receptors, *CHRNA5-CHRNA3-CHRNB4*, and the risk of smoking.

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Methods—Individuals from 219 European American families (N=2,284) were genotyped across this gene cluster to test the genetic association with smoking. The frequency of the amino acid variant (rs16969968) was studied in 995 individuals from diverse ethnic populations. *In vitro* studies were performed to directly test whether the amino acid variant in the *CHRNA5* influenced receptor function.

Results—A genetic variant marking an amino acid change showed association with the smoking phenotype (p=0.007). This variant is within a highly conserved region across non-human species, but its frequency varied across human populations (0% in African populations to 37% in European populations). Furthermore, functional studies demonstrated that the risk allele decreased response to a nicotine agonist. A second independent finding was seen at rs578776 (p=0.003), and the functional significance of this association remains unknown.

Conclusions—This study confirms that at least two independent variants in this nicotinic receptor gene cluster contribute to the development of habitual smoking in some populations, and it underscores the importance of multiple genetic variants contributing to the development of common diseases in various populations.

INTRODUCTION

Cigarette smoking is a major public health problem that contributes to nearly 5 million deaths every year worldwide (1). Despite knowledge of the adverse health effects, 45 million adults in the U.S. continue to smoke, and about half of these individuals are dependent on nicotine (2,3). Nicotine is the component in cigarettes that is responsible for the maintenance of smoking, and the physiological effects of this drug are mediated largely through the neuronal nicotinic acetylcholine receptors (nAChRs) (4).

Our group recently completed a genome-wide association and candidate gene study of nicotine dependence based on the contrast between nicotine dependent smokers and smokers who used at least 100 cigarettes in their lifetime, but never developed any symptoms of dependence (5, 6). These genetic studies focused on the transition from smoking to the development of nicotine dependence. Intriguing findings for further follow-up included the identification of strong association of nicotine dependence with genetic polymorphisms in the nicotinic receptor gene cluster, α5-α3-β4, on chromosome 15, which included a variant that results in an amino acid change (aspartic acid (D) to asparagine (N)) in the α5 neuronal nicotinic acetylcholine receptor subunit (*CHRNA5*). There was also evidence of a second independent finding in this cluster marked by rs578776 (pairwise $r^2 < 0.2$ with rs16969968) in the α5-α3-β4 gene cluster.

Neuronal nicotinic receptors are pentameric ligand-gated cation channels that are expressed in the central and peripheral nervous systems and are comprised of different combinations of primarily α and β subunits (see Gotti et al., 2006 for review) (7). The α5 subunit participates in multiple nicotinic receptor subtypes (8-14), including an α4β2α5 subtype that contributes to nicotine-stimulated dopamine release in the striatum, a region involved in the reward pathway and crucial to the development of substance dependence (15,16). The α3 subunit has limited distribution in the brain and forms α3β2 and α3β4 receptors (7).

The purpose of the present study was to further define the genetic contribution of these findings to smoking in families affected with alcoholism. This involved testing whether the original observations could be replicated and fine mapped in an independent dataset, examining the frequency of this variant in diverse populations, and performing a functional study to determine whether the amino acid substitution changed receptor function.

METHOD

Collaborative Study on the Genetics of Alcoholism (COGA) dataset

The Collaborative Study on the Genetics of Alcoholism (COGA), a genetic study of alcohol dependence, had high rates of smoking and allowed for the genetic study of habitual smoking and light smoking contrast groups (17). The institutional review boards of all participating institutions approved the study. After providing a complete description of the study to subjects, written informed consent was obtained.

Alcohol dependent probands were identified through inpatient or outpatient chemical dependency treatment programs. Probands and their relatives were administered a poly-diagnostic instrument, the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA) interview. Though a diagnostic assessment of nicotine dependence was not administered to all subjects, smoking history was collected and proxies for nicotine dependent and non-dependent status were developed. Case status was classified by habitual smoking, defined as ever smoking at least one pack (20 cigarettes) daily for 6 months or more. Unaffected subjects were smokers who 1) smoked at least 100 cigarettes in their lifetime; or 2) smoked daily for a month or more, but never smoked more than 10 cigarettes daily (18). In a set of subjects also assessed with the Fagerström Test for Nicotine Dependence (FTND) (19), 78% of habitual smokers were nicotine dependent using an FTND score of 4 or more, and 73% of light smokers were not nicotine dependent (see Supplemental Figure 1 in the data supplement that accompanies the online version of this article). Those who never smoked or did not meet the affected or unaffected status were considered ”unknown” phenotypically in the genetic analyses.

The families that participated in the genetic phase of this study included an alcohol dependent proband and at least two first-degree relatives with alcohol dependence. A total of 262 families including 2309 individuals, were selected for the genetic study (20,21). The 219 pedigrees of European descent with *2,284* genotyped individuals (955 habitual smoking subjects and 281 light smoking individuals) were analyzed.

Human Diversity Cell Line Panel (HGDP-CEPH)

The Human Diversity Cell Line Panel (HGDP-CEPH) is a resource of DNA from individuals from different world populations that is banked at the Foundation Jean Dausset (CEPH) in Paris (22). These samples were collected as part of the Human Genome Diversity Project to provide DNA for studies of sequence diversity and history of human populations. Information includes the population and geographic origin of the samples. Genetic structure analysis on these human populations identified six main genetic clusters, five of which correspond to major geographic regions, and subclusters that often correspond to individual populations (23). Informed consent was obtained from all subjects.

SNP assays

dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) was used to identify single nucleotide polymorphisms (SNPs) within and flanking each of the nAChRs. Sequenom MassArray technology (http://www.sequenom.com), homogenous MassEXTEND (hME) or iPLEX assays were used for genotyping. A detailed protocol was described previously (24). The primer sequences used for genotyping are listed in Supplemental Table 1. For the 22-bp insertion/deletion polymorphism (rs3841324), PCR primers (forward primer 5′-AAAAGGAACAAGGCGAGGATTG-3′; reverse primer 5′-GAGTGTGAGTCGTGAGACAAAACG-3′) were selected using the MacVector 6.5.3 program (Accelrys) to yield a 166-bp or 188-bp genomic fragment containing the SNP, rs3841324. The amino acid change coding SNP in exon 5 of the CHRNA5 gene, rs16969968

was genotyped using an RFLP assay. PCR products were generated with forward primer 5′-CGCCTTTGGTCCGCAAGATA-3′ and reverse primer 5′-TGCTGATGGGGGAAGTGGAG-3′ and then digested with Taq1 restriction enzyme. Genotypes of rs3841324 and rs16969968 were detected by electrophoresis on 2% agarose gel.

Statistical analyses

Linkage disequilibrium (LD) between markers was computed using the program Haploview (25). The family-based association test (FBAT) was used to examine association between genetic variants and habitual smoking (26). The significant covariates, sex and age were incorporated in the model. There was no correction for multiple testing.

Functional Studies of CHRNA5 Genetic Variant

Cell culture—HEK293T cells were maintained at 37°C in a humidified, 5% CO_2 environment in Dulbecco's modified Eagle's medium (high glucose, no pyruvate) (DMEM), 10% heat-inactivated fetal bovine serum and antibiotic/antimycotic (100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B). Culture reagents were purchased from either Biowhittaker (East Rutherford, NJ, USA) or Invitrogen (Carlsbad, CA, USA).

Measurement of intracellular calcium—Agonist (epibatidine)-evoked changes in intracellular calcium were performed using an aequorin-based luminescence assay as previously described (27). HEK293T cells were seeded onto six-well plates (1.5×10^6 cells/well) and were transfected the following day with plasmids (0.25 μg/well for each plasmid) containing a human codon-optimized aequorin cDNA (28), the mouse α4 and β2 cDNAs, and either the wild-type mouse α5 cDNA (D398) or a mouse α5 cDNA in which D398 was mutated to N398. Transfection was performed using either the LipofectAmine Plus Reagent (Invitrogen) or Fugene HD transfection reagent (Roche, Indianapolis, IN, USA) as recommended by the manufacturers. Approximately 48 h following transfection, culture media was replaced with DMEM + 0.1% fetal bovine serum and 2.5 μM coelenterazine-hcp (Invitrogen) and the cells were incubated for 3 h at 37°C in a humidified 5% CO_2 incubator. Following the coelenterazine incubation, cells were gently aspirated from the culture dishes and transferred to 2 ml tubes. The cells were then pelleted by centrifugation at 4°C for 5 min at 800 *g*, the supernatant was discarded, and the cells were resuspended in 1× assay buffer (Hank's Balanced Salt Solution (Cambrex, East Rutherford, NJ, USA) supplemented to 10 mM $CaCl_2$) and incubated for 1 h at 4°C prior to initiating the assay. Sample size was n = 12 for each nAChR variant (12 separate transfections per variant from 3 independent experiments).

For the epibatidine concentration–response curves, 50 μL of cells were added to each well of a 96-well opaque white plate and placed in a Victor3V plate reader (Perkin Elmer). Following a 1 second baseline read, 50 μl epibatidine was injected onto each sample and luminescence (in Lux) was recorded at 0.2 s intervals for 20 s immediately following the addition of agonist. At the completion of the agonist stimulation, 100 μL of a solution containing 0.1% Triton X-100 and 100 mM $CaCl_2$ was injected into each well, and luminescence was recorded for 5 s at 0.1 s intervals. In order to control for differences in cell number per well as well as variation in transfection efficiency and coelenterazine loading, agonist responses were normalized by dividing the maximal peak value for the agonist-stimulated luminescence (L) by the total peak luminescence value (L_{max}) (maximal peak agonist-stimulated luminescence + maximal peak luminescence resulting from cell lysis in the presence of high calcium).

The EC_{50} and maximal response values for the concentration response curves were calculated using a four parameter logistic equation in Graphpad Prism 3.0 software (San Diego, CA, USA). Concentration response curves for the two nAChR populations were evaluated using 2-

way ANOVA for epibatidine concentration and receptor variant. Maximal response and EC_{50} values between the α4β2α5D398 and α4β2α5N398 were compared using Student's t-test (two-tailed). Verification that the α5 subunit was being incorporated into functional α4β2α5 nAChRs was established using the reporter mutation approach as described by others (27, 29-32) (data not shown).

[^{125}I]-epibatidine binding—Membrane fractions were prepared from samples as previously described (33), with the exception that a 15 minute incubation at 37°C with 50 μg/mL DNAse was performed prior to the first centrifugation. The binding of [^{125}I]-epibatidine to the membrane fractions was performed essentially as described previously (33) in a 30 μL reaction that included binding buffer (118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl2, 1.2 mM Mg2SO4 and 20 mM HEPES pH 7.5) and 200 pM [^{125}I]-epibatidine. Non-specific binding was determined by the inclusion of 10 μM cytisine in the reaction. Ligand binding was performed with an amount of homogenate that did not produce ligand depletion. Homogenate protein levels were determined by the method of Lowry (34). Differences in binding were assessed using Student's t-test (two-tailed).

Western blot immunochemistry—Unused samples from the aequorin assay were lysed in Tris buffered saline (25 mM Tris pH = 7.4, 150 mM NaCl) , 1% triton X-100, 1 mM EDTA and protease inhibitor cocktail (Sigma, St. Louis, MO). Equal volumes of sample were denatured in Laemmli buffer (Pierce, Rockford, IL), run on an SDS PAGE gel and transferred to a PVDF membrane as described previously (35). The membrane was incubated with mAb 268 (a generous gift of Dr. Jon Lindstrom) at a concentration of 1 μg/mL followed by an HRP-conjugated goat-anti rat antibody (Pierce) at a dilution of 1:5000. The membrane subsequently was incubated with ECL Advance HRP substrate (GE Healthcare, Piscataway, NJ) and the resulting image was captured using an Image Station 2000R (Kodak, New Haven, CT). Intensity levels for the α5 subunit were determined using Imagequant software (Kodak). Differences in protein levels were assessed using Student's t-test (two-tailed).

RESULTS

After careful examination of the linkage disequilibrium pattern across the gene cluster, there was evidence of two independent findings of genetic association with habitual smoking versus light smoking in the *CHRNA5-CHRNA3-CHRNB4* gene cluster. The nonsynonymous coding SNP of the *CHRNA5* gene, rs16969968 (p=0.007) was associated with habitual smoking, and other SNPs that were highly correlated with rs16969968 (rs2036527, rs17486278, rs1051730, rs17487223, $r^2 > 0.79$; see Supplemental Figure 2 in the online data supplement), were also associated (p value range 0.020 – 0.086) (Table 1). This SNP cluster which spans the three genes *CHRNA5-CHRNA3-CHRNB4* most likely represents one group of correlated associated genetic variants.

There was a second finding of association in this gene cluster at rs578776. This result is independent given its low correlation with rs16969968 ($r^2 < 0.15$) (Table 1 and Supplemental Figure 2). Several SNPs which were moderately correlated with rs578776 (r^2 0.60-0.76) and which were significant in the previous study (6) were not associated in this family based analysis.

The finding of the amino acid change associated with nicotine dependence at rs16969968 was further examined in biological studies. Using sequence data from public databases, the protein sequences for *CHRNA5* homologues were aligned to determine conservation in the region surrounding codon 398 in divergent species (http://www.ncbi.nlm.nih.gov/entrez/). The aspartic acid residue at amino acid position 398 was completely conserved from human to chicken, suggesting it has functional importance, see Figure 1.

To assess the frequency of the minor allele (A) at *rs16969968* across multiple populations, this SNP was successfully typed in the HGDP-CEPH Human Genome Diversity Cell Line Panel, which included 995 individuals representing 39 different populations (22). In populations of European and Middle Eastern origin, the frequency of the A allele was 37-43%. The A nucleotide was not detected or was uncommon in African, East Asian, and Native American populations (Figure 2; Supplemental Table 2).

To establish whether the D398N polymorphism altered receptor function, nicotinic agonist-evoked changes in intracellular calcium were measured from HEK293T cells that heterologously expressed either α4β2α5D398 or α4β2α5N398 nAChRs. Receptor expression and α5 protein levels also were determined for each α5 variant. Two-way ANOVA indicated that the concentration response curves for the nicotinic agonist epibatidine were significantly different between the α4β2α5N398 and α4β2α5D398 nAChR variants ($p < 0.0001$) (Figure 3a). This difference in concentration-response curves and maximal response to agonist was not due to a shift in sensitivity to activation by epibatidine between the nAChR variants, as their EC_{50} values did not differ (α4β2α5D398 EC_{50} = 14.7 ± 2.4 nM; α4β2α5N398 EC_{50} = 22.0 ± 3.5 nM, $p = 0.09$). However, the maximal response to agonist was found to be over two-fold higher for the α4β2α5D398 nAChR variant relative to the α4β2α5N398 nAChR variant (0.044 ± 0.002 and 0.023 ± 0.002, respectively; $p < 0.0001$) (Figure 3b). In contrast, the two variant nAChRs did not differ in expression (Figure 3c) nor were the two isoforms of the α5 subunit differentially expressed (Figure 3d). The sum of these data indicates that the variant forms of the α5 subunit alter receptor function without affecting receptor expression.

DISCUSSION

This study validates the importance of genetic variants within the α5-α3-β4 nicotinic acetylcholine receptor gene cluster that contribute to the risk of a light smoker transitioning to heavy smoking and sheds light on a potential biological mechanism. There are two distinct genetic associations – one marked by rs16969968 which results in an amino acid change in the α5 nicotinic cholinergic receptor *(CHRNA5)* and a second marked by a SNP rs578776 in the 3' untranslated region of the α3 nicotinic cholinergic receptor (*CHRNA3). Because the* r^2 between these two variants is low (0.15), the statistical significance at both cannot be explained solely by the linkage disequilibrium between them. Therefore, these data imply that there are two independent loci in this region that alter the risk for nicotine dependence and habitual smoking. These findings confirm our previous results in a case-control series that examined nicotine dependence (6) and the recent work by Berrettini and colleagues that studied heavy smoking (36).

In these three genetic studies that demonstrated the associations, there are important differences in the definition of phenotypes, recruitment procedures, and analytic methods. The COGA sample was recruited as a high-risk population for alcoholism, and a broader smoking phenotype was used: habitual smoking, defined as smoking 20 cigarettes a day for 6 months or more, and light smoking, defined as smoking 10 cigarettes or fewer per day. A family based analytic design for association was used which is less likely to be biased by population stratification. The previous genetic study sample by our group was recruited from the community, the phenotypic status was defined by the Fagerström Test for Nicotine Dependence, and a case control design of unrelated individuals was used (5,6,19). Similar SNPs were analyzed in these two studies. The recent publication by Berrettini was based on genetic studies of heart disease and other common illnesses in a population based sample of 15,000 people (36). Smoking status was collected and a quantitative phenotype defined by cigarettes per day was studied in a secondary genetic analysis. Though different genetic variants were tested in the Berrettini sample, the associated SNPs are highly correlated (in linkage disequilibrium) with the variants in our studies. The consistency of results across three different

studies shows that these genetic findings are robust across populations, phenotypic classification systems, and analytic methods.

Three additional research groups recently published strong evidence of genetic association of the α5-α3-β4 nicotinic acetylcholine receptor gene cluster on chromosome 15 with lung cancer (37-39). The findings highlighted in their work are highly correlated with the genetic variant that results in the amino acid change in the α5 nicotinic acetylcholine receptor gene. The groups differed in their interpretation of whether this genetic association with lung cancer acts through the indirect effect of smoking or whether this variant also directly increases the vulnerability to lung cancer.

When a genetic association is found, it represents not only association with the tested variants, but all genetic variants (tested and untested) that are highly correlated. Identifying the variant that causes functional changes requires biological investigations. We have focused on the amino acid change in the α5 nicotinic cholinergic receptor for further studies.

The aspartic acid at position 398 in *CHRNA5* in humans occurs at a residue that is otherwise invariant across vertebrate species. Frogs, chickens, rodents, cattle and non-human primates all possess an aspartic acid residue at this location. In humans, the amino acid may be either an aspartic acid, the predominant residue at this position, or asparagine. The α5 nicotinic subunit is not involved in receptor binding *in vivo* (7) , and this variant is located in the cytoplasmic loop between transmembrane domains.

Evidence that the amino acid change is functionally relevant is supported by the fact that *in vitro*, α4β2α5nicotinic receptors with the aspartic acid variant (D398) exhibited a greater maximal response to a nicotinic agonist than did α4β2α5 nicotinic receptors with the asparagine amino acid substitution (N398). Because the allele that codes for asparagine is associated with increased risk for developing nicotine dependence, and nicotinic receptors containing the α5 subunit with this amino acid (N398) exhibit reduced function *in vitro*, reduced function of α4β2α5 nicotinic receptors may lead to an elevated risk for developing nicotine dependence. The observation that decreased nAChR function is associated with increased risk for nicotine dependence is consistent with the observation that individuals who are extensive metabolizers of nicotine (reduced receptor activation per cigarette) are at increased risk for nicotine dependence (40,41). We believe that this combined evidence of high conservation across species and biological change in receptor function supports the amino acid variant in the α5 nicotinic receptor as a causative biological factor that alters the risk of nicotine dependence, though we cannot definitively rule out the other correlated SNPs across the three gene cluster.

The α4β2α5-containing nicotinic receptors are expressed on dopaminergic neurons in the striatum (16) where they modulate nicotine-stimulated dopamine release (15). In addition, α4β2α5 nicotinic receptors also are found on GABAergic neurons in the striatum and ventral tegmental area (42).This region of the brain is associated with the reward pathway, and the neurotransmitter dopamine plays a crucial role in the development of dependence. Individuals with reduced α4β2α5 cholinergic receptor activity may require greater amounts of nicotine to achieve the same activation of the dopaminergic pathway. Alternatively, reduced activity of the receptor complex on GABAergic neurons may lead to increased dopaminergic activity in response to nicotine. How the altered receptor activity caused by the *CHRNA5* amino acid change modifies liability to nicotine dependence via the reward system in response to nicotine requires further study.

The “at risk” allele differs dramatically across human populations. It is predominantly seen in populations of European and Middle Eastern descent and is uncommon or non-existent in populations of African, Asian, or American origin. Interestingly, African Americans have a

lower prevalence of nicotine dependence than European Americans (43,44), and this may be explained in part by the low prevalence of this risk allele in the populations of African descent.

There is less information available regarding the second independent finding marked by the genetic variant rs578776 in this gene cluster. This SNP, rs578776, is located in the 3' untranslated region of the *CHRNA3* gene. The 3' untranslated regions contain regulatory sequences, and we can speculate that this SNP is a putative functional variant. It is important to also note that there are correlated SNPs with rs578776 in *CHRNA5* and *CHRNA3* and the localization of the functional alleles may be in either gene. Further experiments are needed to identify the potential functional variants and the biologic mechanisms.

In summary, there are at least two independent genetic variants in the *CHRNA5-CHRNA3-CHRNB4* gene cluster on chromosome 15 which are highly associated with smoking behaviors, and we have extended our work to identify a potential biological mechanism for one of the findings. This study provides strong evidence that an amino acid change in the α5 nicotinic receptor, which is highly conserved across species, results in a functional change that increases a smoker's risk of transitioning from non-dependence (light smoking) to dependence (habitual smoking) on nicotine. This variant is common in populations of European and Middle Eastern descent and increases the risk of developing nicotine dependence, but rare in populations of African, American and Asian descent. Intriguingly, three recent papers demonstrated that this genetic locus also contributes to the risk of developing lung cancer. A second independent finding in this gene cluster is also seen, though further study is needed to localize the potential functional allele and to determine whether the variant affects function of the CHRNA5 or CHRNA3 gene. These converging genetic associations and biological data support the importance of *CHRNA5* and potentially CHRNA3 in the development of nicotine dependence and highlight the pharmacogenetic response to nicotine which increases the susceptibility to dependence. Importantly, this finding may help predict response to pharmacologic therapies, such as varenicline and nicotine replacement, for those smokers who attempt to quit, and may shed important light onto the biological mechanisms that contribute to lung cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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D: Aspartic Acid (GAU or GAC)
N: Asparagine (AAU or AAC)

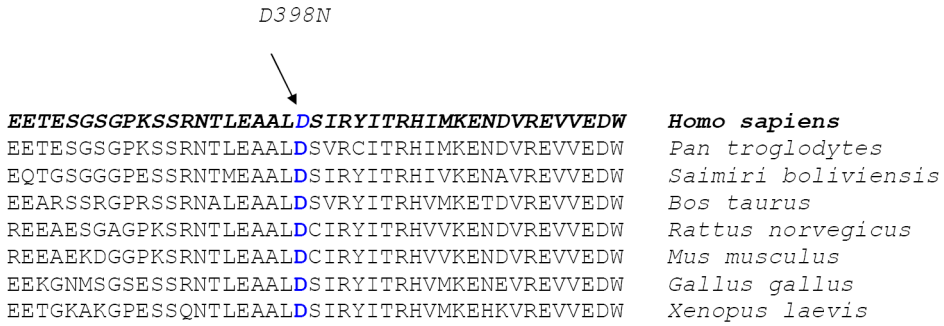

Figure 1.
Comparative sequence analysis surrounding aspartate D 398 of the α5 nicotinic receptor. The amino acid change to asparagine in the variant receptor (rs16969968).

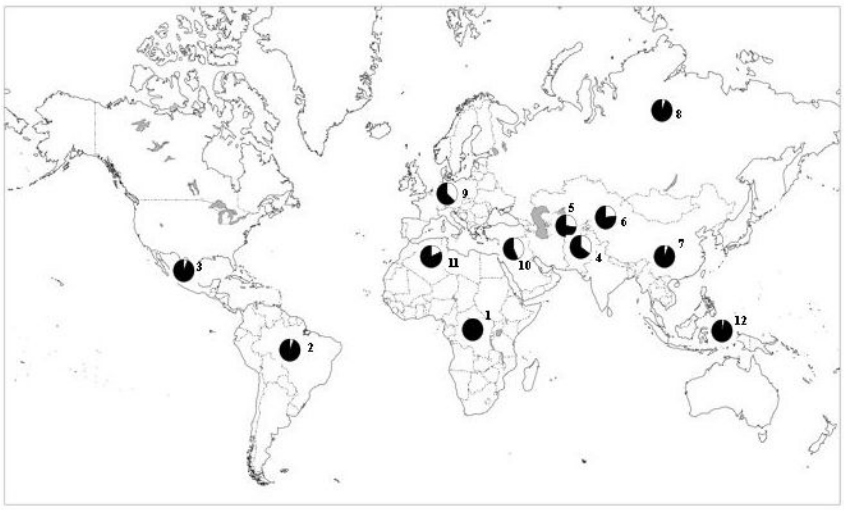

Figure 2.
Allele frequency distribution (%) of cSNP rs16969968 in different ethnic populations (A allele is white segment). Populations were grouped together based on their genetic structures reported by Rosenberg et al (23).

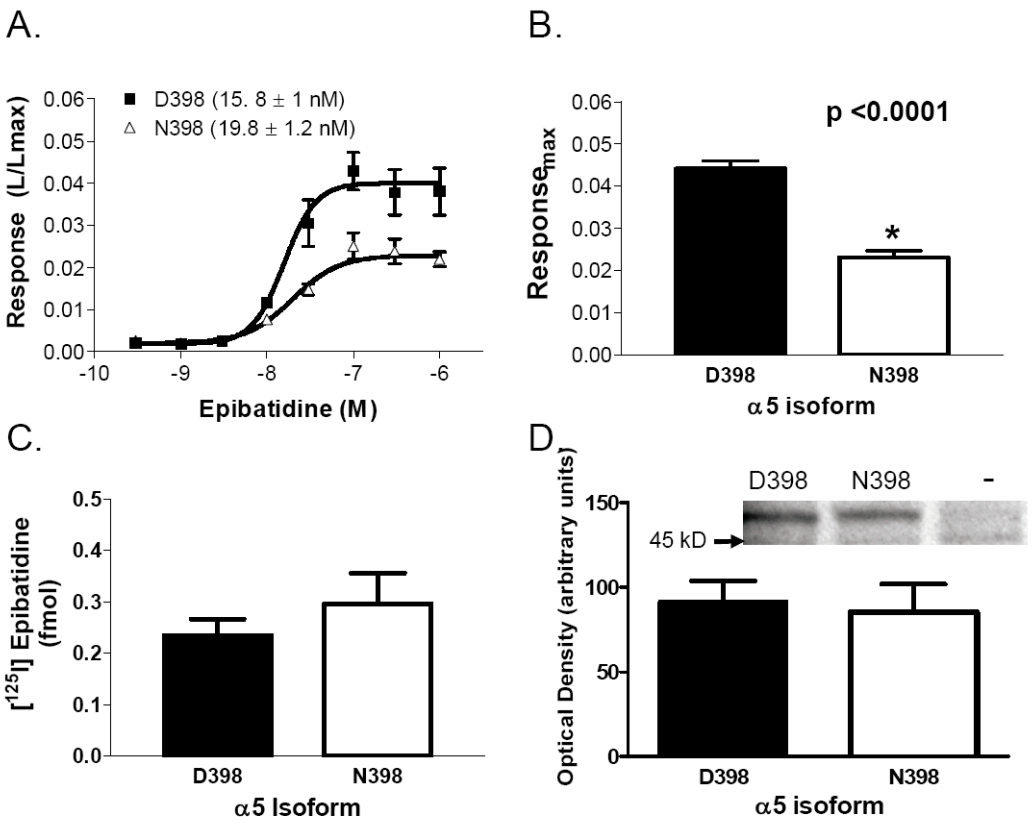

Figure 3.

The CHRNA5 D398N polymorphism affects nAChR function but not expression of α4β2α5 nAChRs. **A**. Concentration-response curves for epibatidine-evoked changes in intracellular calcium were measured from HEK293T cells transfected with plasmids containing a calcium-sensing aequorin cDNA, α4 and β2 cDNAs and either α5D398 or α5N398 cDNA. Two-way ANOVA indicated that the response curves for each nAChR variant were significantly different (n = 12 per variant, $p < 0.0001$). However, EC_{50} values were not altered by the variant forms of α5 (n = 12, p = 0.098). **B**. nAChRs possessing the D398 variant of α5 also exhibited a significantly greater maximal response to epibatidine as compared to nAChRs containing the N398 variant (n = 12 per variant, $p < 0.0001$). **C**. [^{125}I] epibatidine binding on membranes prepared from cells used for the aequorin assay indicate that there is no difference in the expression of the receptors when either the D398 or N398 variant of α5 is co-transfected with α4 and β2 (n = 12, p = 0.375). **D**. Western analysis demonstrates that the variant forms of α5 do not differ in expression (n = 4 per variant, p = 0.78). Inset: sample western from α4β2α5D398 (D398) α4β2α5N398 (N398) and α4β2 (-) transfected cells. Data shown for all graphs are mean ± SEM.

Table 1

FBAT analysis of SNPs in the cluster of *CHRNA5/A3/B4* genes with habitual smoking in the COGA Caucasian subset (219 Families; 955 affected individuals; 281 unaffected individuals).
Age and gender adjusted. MAF = minor allele frequency;
Yellow and blue colors indicate two different correlated variant groups ($r^2 \geq 0.75$).

rs#	Gene	SNP	Minor Allele	MAF	P	P NICSNP *
rs1979906	Upstream of CHRNA5	A/G	G	0.44	0.508	--
rs880395		A/G	A	0.43	0.395	0.678
rs7164030		A/G	G	0.44	0.452	0.629
rs905739 *		C/T	C	0.22	0.410	0.005
rs2036527		C/T	T	0.33	0.086	--
rs3841324	Promoter of CHRNA5	Ins/Del	Del	0.44	0.624	na
rs503464		A/T	T	0.22	0.227	0.521
rs684513 *	CHRNA5	C/G	G	0.20	0.251	0.009
rs667282		C/T	C	0.23	0.378	-
rs17486278		A/C	C	0.32	0.028	--
rs601079		A/T	A	0.43	0.237	0.622
rs680244		A/G	A	0.44	0.780	--
rs621849		A/G	G	0.44	0.591	--
rs569207		A/G	A	0.23	0.139	--
rs692780		C/G	G	0.38	0.829	--
rs16969968		A/G	A	0.34	0.007	0.0006
rs514743		A/T	T	0.38	0.574	0.956
rs578776	3'UTR of CHRNA3	C/T	T	0.28	0.009	0.0003
rs6495307	CHRNA3	C/T	T	0.43	0.655	0.656
rs12910984		A/G	G	0.23	0.225	--
rs1051730		C/T	T	0.32	0.020	0.001
rs3743078		C/G	G	0.24	0.121	0.011
rs3743077		A/G	A	0.43	0.350	0.494
rs938682		C/T	T	0.23	0.163	--
rs11637630		A/G	G	0.23	0.224	--
rs7177514		C/G	G	0.24	0.332	--
rs6495308		C/T	C	0.24	0.224	--
rs8042059		A/C	C	0.23	0.339	--
rs8042374		A/G	G	0.23	0.349	--
rs3743075		A/G	A	0.39	0.641	0.966
rs3743073		A/C	C	0.39	0.629	0.886
rs1878399		C/G	G	0.43	0.468	--
rs17487223	CHRNB4	C/T	T	0.35	0.001	--
rs950776		C/T	C	0.35	0.100	--
rs11636605		A/G	A	0.22	0.493	--
rs9920506		A/G	A	0.19	0.120	--
rs3813567	Upstream of CHRNB4	C/T	C	0.22	0.592	0.012
rs17487514		C/T	T	0.30	0.211	--
rs1996371		A/G	G	0.39	0.374	--

* Primary p values from the NICSNP study (6)