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Multiple cholinergic nicotinic receptor genes affect nicotine dependence risk in African and European Americans

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Abstract

Several independent studies show that the chromosome 15q25.1 region, which contains the *CHRNA5-CHRNA3-CHRNB4* gene cluster, harbors variants strongly associated with nicotine dependence, other smoking behaviors, lung cancer, and chronic obstructive pulmonary disease.

We investigated whether variants in other cholinergic nicotinic receptor subunit (*CHRN*) genes affect risk for nicotine dependence in a new sample of African-Americans (N = 710). We also analyzed this African-American sample together with a European-American sample (N=2062, 1608 of which have been previously studied), allowing for differing effects in the two populations. Cases are current nicotine-dependent smokers and controls are non-dependent smokers.

Variants in or near *CHRND-CHRNG*, *CHRNA7*, and *CHRNA10* show modest association with nicotine dependence risk in the African-American sample. In addition, *CHRNA4*, *CHRNB3-CHRNA6*, and *CHRNB1* show association in at least one population. *CHRNG* and *CHRNA4* harbor SNPs that have opposite directions of effect in the two populations. In each of the population samples, these loci substantially increase the trait variation explained, although no loci meet Bonferroni-corrected significance in the African-American sample alone. The trait variation explained by three key associated SNPs in *CHRNA5-CHRNA3-CHRNB4* is 1.9% in European-Americans and also 1.9% in African-Americans; this increases to 4.5% in EAs and 7.3% in AAs when we add six variants representing associations at other *CHRN* genes.

Multiple nicotinic receptor subunit genes outside of chromosome 15q25 are likely to be important in the biological processes and development of nicotine dependence, and some of these risks may be shared across diverse populations.

Keywords

genetic association; smoking; cholinergic nicotinic receptors; nicotinic acetylcholine receptors

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Conflict of Interest disclosure statement: Drs. Bierut, Goate, Rice and Wang are listed as inventors on a patent, “Markers of Addiction,” covering the use of certain SNPs in diagnosing, prognosing, and treating addiction. Dr. Saccone is the spouse of Dr. S. Saccone, who is also listed as an inventor on the above patent. Dr. Bierut has served as a consultant to Pfizer in 2008. D. Hatsukami has a pending grant from NabiBiopharmaceuticals to conduct a clinical trial with a nicotine vaccine.

INTRODUCTION

Multiple large-scale, independent association studies conclusively demonstrate that variants in the *CHRNA5-CHRNA3-CHRNB4* cluster of nicotinic receptor subunit genes on chromosome 15q25.1 are associated with nicotine dependence, smoking behavior, lung cancer, and chronic obstructive pulmonary disease in European-Americans (Amos *et al*., 2008, Berrettini *et al*., 2008, Bierut *et al*., 2008, Broderick *et al*., 2009, Caporaso *et al*., 2009, Chen *et al*., 2009, Freathy *et al*., 2009, Grucza *et al*., 2008, Hung *et al*., 2008, Le Marchand *et al*., 2008, Lips *et al*., 2009, Liu *et al*., 2008, Pillai *et al*., 2009, Saccone *et al*., 2007, Sherva *et al*., 2008, Spitz *et al*., 2008, Stevens *et al*., 2008, Thorgeirsson *et al*., 2008, Wang *et al*., 2009, Weiss *et al*., 2008, Young *et al*., 2008). Recent work reveals that some of these variants also affect nicotine dependence and lung cancer risk in African-Americans (Li *et al*., 2010, Saccone *et al*., 2009b, Schwartz *et al*., 2009) and in Asians (Shiraishi *et al*., 2009, Wu *et al*., 2009). These strong, consistent findings in *CHRNA5-CHRNA3-CHRNB4* raise the question of whether additional cholinergic nicotinic receptor subunit (*CHRN*) genes also play an important role in risk for these diseases.

We previously carried out a high-density association study covering the complete family of 16 *CHRN* genes in European-ancestry subjects from the United States and Australia (Saccone *et al*., 2009a). Evidence for association was observed not only for *CHRNA5-CHRNA3-CHRNB4*, but also for the *CHRNB3-CHRNA6*, *CHRND-CHRNG*, *CHRNA4* and *CHRNB1* genes. This evidence nominates additional *CHRN* genes in the development of nicotine dependence.

An important question is whether *CHRN* genes are also associated with nicotine dependence in African-ancestry populations. We recently examined the *CHRNA5-CHRNA3-CHRNB4* region on chromosome 15q25 using an expanded United States sample (N=2772) (Saccone *et al*., 2009b). The expanded sample included a new African-American (AA) sample (N=710) and a new European-American (EA) sample (N=454), in addition to 1608 European-Americans previously studied together with 319 European-descent Australians (Saccone *et al*., 2009a, Saccone *et al*., 2007). The analysis of chromosome 15q25.1 in the expanded U.S. sample demonstrated that the non-synonymous *CHRNA5* SNP rs16969968 is significantly associated with nicotine dependence in AAs as well as in EAs (Saccone *et al*., 2009b). Additional, distinct variants in that region appear associated in at least one of these populations.

Here we extend our study of this U.S.-based sample to the 13 *CHRN* genes outside the chromosome 15q25 region. Our goals are to determine whether variants in other nicotinic cholinergic receptor subunit genes are associated in African-Americans, and also to study the effects of *CHRN* variants with the increased power of the full U.S. sample, which was recruited with a uniform ascertainment scheme.

MATERIALS AND METHODS

Study design and sample

All individuals were recruited by the Collaborative Genetic Study of Nicotine Dependence (COGEND), a United States multi-site project. Cases and controls reported smoking at least 100 cigarettes lifetime, the threshold classically used to define a smoker (Centers for Disease Control and Prevention, 2006). Cases are nicotine dependent according to the Fagerström Test for Nicotine Dependence (FTND) (Heatherton *et al*., 1991, Heatherton *et al*., 1989), with an FTND score of 4 or more. Controls were never nicotine dependent and had an FTND of 0 or 1 even when smoking the most. Genome-wide and candidate gene

genetic data on 1608 European-American COGEND subjects (797 cases (FTND ≥ 4), 811 controls (FTND=0) were previously reported together with an Australian sample (N= 319) (Bierut *et al*., 2007, Saccone *et al*., 2009a, Saccone *et al*., 2007). With additional recruitment, we have extended our U.S.-based sample and now report on 710 African-Americans (AAs) (461 cases, 249 controls) and 454 European Americans (EAs) (140 cases, 126 controls) for a total of 1164 new subjects. These subjects have been combined with the 1608 original EA subjects recruited from COGEND. The Australian subjects are not retained because there are significant differences in recruitment, ages and gender distributions in that cohort. The entire U.S.-based COGEND sample size is 2772 subjects and Table 1 gives demographics (cases/controls, sex, age) for the sample. When reporting results for the EA groupings, we use “original” and “new” to specify those two subsamples, and “all EAs” or “EAs” to indicate the complete EA sample. The full sample of all EAs and AAs combined (N=2772) is denoted by “full sample.” This full U.S. sample was recently analyzed for SNPs in the *CHRNA5-CHRNA3-CHRNB4* region (Saccone *et al*., 2009b); here, we extend the study of this sample to SNPs in other nicotinic receptors.

The study obtained informed consent from all participants and approval from institutional review boards. DNA was derived from whole blood maintained by the Rutgers University Cell and DNA Repository (www.rucdr.org).

Genotyping and quality control

We analyzed 144 SNPs covering the 13 *CHRN* genes outside chromosome 15q25; SNPs were selected to match those covered in the previously reported subjects (Bierut *et al*., 2007, Saccone *et al*., 2009a, Saccone *et al*., 2007). SNPs were genotyped using Illumina Golden Gate and Sequenom iPlex technologies. All DNA samples had call rates above 90% across the genotyped SNPs and 99.5% of DNA samples had call rates ≥ 95%; Illumina-based SNPs (N=133) passed a call rate threshold of 98% and Sequenom-based SNPs (N=11) satisfied a call rate threshold of 95%. Self-reported race was verified with an EIGENSTRAT (Price *et al*., 2006) principal components analysis of 162 ancestry informative markers genotyped both in our samples and in the HapMap CEU, YRI and HCB-JPT samples which were included as anchors. Map positions and genomic annotations were obtained from the National Center for Biotechnology Information (NCBI) Human Reference Build 36.2 and dbSNP build 129.

Linkage disequilibrium

Linkage disequilibrium (LD) between SNPs was calculated for cases and controls in EAs and AAs using Haploview (Purcell *et al*., 2007). LD plots based on r^2 were generated with WGAviewer (Ge *et al*., 2008).

Genetic association analyses

For all association analyses, the genotype status at each SNP was consistently coded as the number of copies of the allele that is minor in the EA sample, so the major allele in EAs is the reference. Association tests were carried out using PLINK (Purcell *et al*., 2007) and SAS (Cary, NC).

A series of logistic regression analyses was undertaken. We first present the primary analysis of case-control status in African-Americans using logistic regression in a 1 degree of freedom (df) test of the SNP term, with gender and age as covariates.

A second main set of analyses analyzed the combined sample of AAs and EAs. In this full sample we included gender, age, population (0=EA, 1=AA), SNP, and SNP*population in the model, and examined the 2 degree of freedom test for significance of the SNP and SNP x

population terms together. This approach is sensitive both to SNPs that show consistent effects across populations and also to SNPs that show population-specific effects. This approach is therefore useful for the discovery phase in a diverse sample such as this one. A region harboring SNPs with population-specific effects is of interest, given the differing LD and population histories in EAs and AAs. Such a region can later be fine-mapped or resequenced to determine whether additional underlying genetic variation evidences more similar effects across populations, as would be consistent with a common biological mechanism (Saccone *et al*., 2008).

To clarify comparisons between previously published data and new data, we also present separate association results in the new EA sample only (N=454), the original EA sample only (N = 1608), and all EAs (N=2062), using a 1-df test of the SNP term with gender and age covariates included.

Because of possible concerns about population stratification in the AA sample, which includes individuals of admixed ancestry, in AAs we compared the above results with those obtained using covariates for gender, age and the first two principal components from the EIGENSTRAT analysis.

Multiple test correction

We are examining 144 SNPs. A conservative Bonferroni correction would result in an uncorrected p-value threshold of $\alpha' = 3.47 \times 10^{-4}$ for an α of 0.05 for our primary experiment in AAs only. Even after accounting for correlation between SNPs (Li & Ji, 2005, Nyholt, 2004), the low correlations in AAs afford only a marginal reduction to 118 tests ($\alpha'=4.24 \times 10^{-4}$).

RESULTS

Genetic association analyses

Supplementary Table 1 shows the allele frequencies, in the EA and AA samples, for all 144 genotyped SNPs across the 13 *CHRN* genes. The last two columns of Supplementary table 1 show that almost all of the SNPs have significantly different allele frequencies between the EA and AA samples by Fisher's exact test (120/144 (83%) at $\alpha = 0.001$).

Table 2 shows the results for the top associated SNPs in the AA-only sample ($p \leq 0.05$ in the 1-df test of SNP). Table 3 shows the results for the top associated SNPs in the full sample ($p \leq 0.01$ in the 2-df test of SNP and SNP*population). Results for all 144 SNPs are available in Supplementary Table 2.

Analysis of the African-American sample

In the AA-only sample, none of the SNPs pass multiple-test correction for significance (Table 2). However, the top SNPs suggest some interesting findings. First, rs1881492 in *CHRNG* is the most significant SNP in AAs (OR = 1.57 (1.17-2.11), p = 0.00245), and is also associated in EAs, but the odds ratio is in the opposite direction (OR = 0.77 (0.66-0.90), p = 0.0012). Therefore, the 2-df test of SNP and SNP × population, which allows effects to differ between populations, yields a p-value of 4.25×10^{-5}.

The second interesting result from Table 2 is that in AAs, there is nominal evidence for association at multiple SNPs in *CHRNA7*. For *CHRNA7*, the most significant SNP in the AAs is rs6494212 (OR = 1.44 (1.14-1.83), p = 0.0027), but in all the EAs, OR = 1.1 (0.96-1.26) and p = 0.16. However, rs904951 shows some consistent evidence in both populations (OR = 1.30 (1.02-1.65), p = 0.03 in AAs and OR = 1.12 (0.99-1.27), p = 0.069

in EAs). Other *CHRNA7* SNPs such as rs4779565 and rs10438287 have $p < 0.05$ in EAs and consistent odds ratios in both populations (Supplementary Table 2).

Finally, Table 2 shows nominal evidence in AAs for two SNPs in the *CHRNA10* region on chromosome 11. For this region, we genotyped SNPs flanking and tagging the gene. While rs2231532 is the most significant in AAs, rs16925377 has modest evidence for consistent association in both AAs and EAs (OR = 1.43 (1.00-2.03), p = 0.049 in AAs and OR = 1.21 (0.99-1.48), p = 0.069 in EAs) (Table 2).

Because of possible admixture concerns in the AA sample, we re-analyzed the AA sample using the first two principal components from the EIGENSTRAT analysis as added covariates. Comparing these results to those in Table 2, the 3 top-ranked SNPs in the AA sample (one each from *CHRNG*, *CHRNA7* and *CHRNA10*) were essentially unchanged (p = 0.0017, 0.0025 and 0.014 respectively). We conclude that these signals are unlikely to be population stratification artifacts.

Analysis of the full sample

Table 3 highlights all SNPs for which the 2-df test of SNP and SNP*population has $p \leq 0.01$. Three additional regions of interest, besides those already seen in Table 2, are highlighted: *CHRNA4*, *CHRNB3-CHRNA6*, and *CHRNB1*.

Rs2236196 in *CHRNA4* is the most significant not only in the 2-df full sample test, but also in the EA sample. Only one *CHRNA4* SNP, rs2229959, shows any hint of association in AAs with an odds ratio of 0.82 (0.66-1.02) and p = 0.08; thus it has an odds ratio opposite that in all the EAs (OR = 1.33 (1.09-1.62), p = 0.004).

The next group of SNPs in Table 3 all lie upstream of *CHRNB3-CHRNA6* (rs13277254, rs10958726, rs1955186, rs13277524). They all have p-values ≤ 0.003 and their association evidence is improved when analyzing all EAs compared to the original EA sample (e.g. for rs13277254, OR = 0.76 (0.66-0.89) p = 0.00063 in all EAs; OR = 0.78 (0.64-0.91), p = 0.0031 in the original EAs). However, there is no evidence for association of these SNPs in AAs, with point estimates for the ORs ranging from 0.9 to 1.01 and p-values from 0.33-0.94. A different *CHRNB3* SNP, rs4952, though less common (minor allele frequency < 5%), is of interest because it has a strong, similar odds ratio in EAs and in AAs (0.42 (0.65 (0.47-0.91), p = 0.01 in EAs, 0.16-1.08), p = 0.07) in AAs).

CHRNB1 is represented in Table 3 by rs7210231 and rs2302761, which are highly correlated in our EA sample ($r^2 = 0.99$) but somewhat less so in AAs ($r^2 = 0.61$). Rs7210231 shows greater consistency across populations (in EAs only, OR=0.80 (0.69-0.94), p=0.0052; in AAs only, OR = 0.79 (0.62-1.02), p = 0.065)

Linkage Disequilibrium

Supplementary figures 1-6 display LD (r^2) plots, in EAs and AAs separately, across associated genes. As expected, strong pairwise r^2 in EAs is often reduced in AAs.

Explaining phenotypic variation

To examine the impact of these associated *CHRN* variants, we calculated Nagelkerke's adjusted R2 from logistic regression of case-control status (Nagelkerke, 1991), comparing the base model with intercept, gender, and age to the model with selected SNPs added, in AAs and EAs separately. This quantity represents the proportion of trait variation explained by the SNPs, in the given sample; it is scaled so that an appropriate maximum of 1 is achieved in the case where the sample is 50% cases and 50% controls, and case-control

status is predicted perfectly by the variables. We first calculated R^2 for three SNPs (rs16969968, rs578776, and rs588765) on chromosome 15q25 that demonstrate association with nicotine dependence (Saccone *et al*., 2009b). We then added SNPs representing the regions highlighted by our current results: *CHRND-CHRNG* (rs1881492), *CHRNA4* (rs2236196), *CHRNB3-CHRNA6* (rs13277254), *CHRNB1* (rs7210231), *CHRNA7* (rs6494212), and *CHRNA10* (rs2231532). Each of these regions contains at least one SNP with $p \leq 0.05$ in the full COGEND sample (2-df association test), and for each region we chose the SNP with the lowest p-value.

Table 4 shows the results for individual SNPs as well as for all SNPs included together in the model. In EAs, the three SNPs on chromosome 15q25 together give an R^2 of 1.87%; in AAs, the three SNPs give a very similar R^2 of 1.85%. In EAs, rs16969968 has the highest individual R^2 of the SNPs considered (1.4%). In AAs, rs16969968 has a similar individual R^2 of 1.3%, but there are two other SNPs with higher R^2 in AAs: rs6494212 in *CHRNA7* (R^2 = 2.2%) and rs1881492 in *CHRNG* (R^2 = 1.6%). Together, the 9 selected SNPs explain 4.9% of the phenotypic variation in the EA sample and 7.3% of the phenotypic variation in the AA sample.

DISCUSSION AND CONCLUSIONS

This study highlights variants in several nicotinic receptor subunit genes, besides *CHRNA5-CHRNA3-CHRNB4* on chromosome 15q25, that affect the risk of developing nicotine dependence in our diverse sample of African-Americans and European-Americans. These include SNPs in or near *CHRNG*, *CHRNA7*, *CHRNA10*, *CHRNA4*, *CHRNB1*, and in the putative promoter region of *CHRNB3-CHRNA6*. In *CHRNG* and *CHRNA4*, the associated SNPs show differing effects in African-Americans and European Americans, while the other genes harbor SNPs that show some evidence for consistent effects in the two populations. In both populations, these additional nicotinic receptor loci substantially increase the explained trait variation. In the new African-American sample, no SNPs surpass Bonferroni-corrected significance for the 144 SNPs tested. However, in the full sample, combining African-Americans and both new and previously-reported European-Americans, the *CHRNG* SNP rs1881492 is significant with a 2-df test p-value of 4.25×10^{-5} and opposite directions of effect in the two populations. Strengths of the current study include the careful phenotyping of stringently defined nicotine-dependent cases and non-dependent smoking controls, and the inclusion of both European-ancestry and African-ancestry subjects.

It is important to study the genetics of nicotine dependence in diverse populations. Differences in allele frequencies and genetic architecture between populations can help narrow association signals to biologically causal variants. Also, there can be important phenotypic differences between populations. Current smoking prevalence is similar in European-Americans and African-Americans (Centers for Disease Control and Prevention, 2008). Nicotine dependence is common in both groups, with evidence of slightly lower levels of dependence in African-Americans by standard measures currently in use (Breslau *et al*., 2001, Substance Abuse and Mental Health Services Administration, 2007). Smoking cessation rates, however, are lower in African-Americans compared to European-Americans (Breslau *et al*., 2001, Covey *et al*., 2008). Furthermore there is evidence that African-Americans have a higher risk of dependence at lower cigarettes-per-day levels compared to European-Americans (Luo *et al*., 2008). Also important are the disparities in health consequences from smoking: African-Americans have higher lung cancer incidence and mortality than European-Americans (Haiman *et al*., 2006, Ries *et al*., 2008). An understanding of the genetic loci involved, and their effects and allele frequencies in diverse populations, can provide important clues to the risk of developing nicotine dependence across all populations.

The most significant SNP in the African-American sample, rs1881492 in *CHRNG*, is also modestly associated in European-Americans, but the odds ratios are in opposite directions (OR = 1.57 (1.17-2.11) in African-Americans; OR=0.77 (0.66-0.90) in European-Americans). For the other gene regions nominally associated in African-Americans – *CHRNA7* and *CHRNA10* – there is little evidence for association with nicotine dependence in European-Americans, even though the latter sample is larger. However, we note that in the *CHRNA10* region, rs2231532 (OR = 1.35 (1.07-1.70), p=0.0108 in AAs versus OR = 1.07 (0.94-1.22), p=0.285 in EAs) is also associated with "dizziness in response to tobacco" in the original European-American subset of this sample (Ehringer *et al*., 2010).

In *CHRNA4*, rs2236196 shows association in European-Americans (OR = 1.30 (1.12-1.50) p = 0.0004) and has previously been associated with smoking in independent European-descent samples (Breitling *et al*., 2009, Hutchison *et al*., 2007) and a sample of African-American women (Li *et al*., 2005). However, neither rs2236196 nor other tested *CHRNA4* SNPs show consistent evidence in both European-Americans and African-Americans (OR=1.04 (0.83-1.30), p = 0.73 in African-Americans). The one SNP with a trend towards association in AAs, rs2229959, has odds ratios in opposite directions in the two groups (OR = 0.82 (0.66-1.02) in AAs; OR = 1.33 (1.09-1.62) in EAs).

In *CHRNB3-CHRNA6*, evidence suggests that there may be at least two distinct loci associated with nicotine dependence. In this region, the SNP most strongly associated in the full sample is rs13277254, upstream of the gene cluster (2-df p = 0.0021), and this association is driven by the EAs. Some of the additional associated SNPs in this region constitute the same signal because they are very highly correlated with rs13277254. However, the synonymous coding SNP rs4952 in *CHRNB3* has the strongest odds ratio in this region (OR = 0.65 (0.47-0.91), p = 0.01 in EAs; OR = 0.42 (0.16-1.08), p = 0.07 in AAs). Rs4952 may tag a distinct involved locus in this gene cluster because it has only low correlation with rs13277254 (r^2 = 0.153 in EAs and 0.009 in AAs). The region upstream of the *CHRNB3-CHRNA6* cluster has been associated with nicotine dependence and smoking behavior in several European-ancestry samples (Bierut *et al*., 2007, Ehringer *et al*., In press, Hoft *et al*., 2009, Saccone *et al*., 2009a, Saccone *et al*., 2007, Zeiger *et al*., 2008), though the biological implications are still largely unclear. Interestingly, β3 knock-out mice show significantly reduced α6 expression in the brain resulting in deviated receptor targeting activity (Gotti *et al*., 2005). The α6 subunit is also known to occur in nicotinic receptor subtypes that modulate the dopamine reward pathway in mice (Klink *et al*., 2001).

In *CHRNB1*, rs7210231 is the most strongly associated in the full sample and shows modest evidence for consistent effects across populations (OR = 0.79(0.62-1.02) in AAs, OR = 0.77(0.66-0.9) in EAs). *CHRNB1* is abundantly expressed in the neuromuscular junctions of muscles as well as showing a low level of mRNA expression in the brain.

We chose a 2-df test of SNP and SNP × population to analyze the full sample. This approach allowed us to detect SNPs having population-specific effects as well as SNPs with similar effects in the two populations, at the expense of an extra degree of freedom. This same 2-df test has been used in other association studies of diverse samples (Sleiman *et al*., 2010).

Several explanations would be consistent with differing results between populations, such as seen at rs1881492 in *CHRNG* and rs2229959 in *CHRNA4*. First, there may be an untyped variant that is causal and has similar effects across populations, but differing correlations with typed SNPs in the two populations lead to inconsistent association evidence. Alternatively, the variant may indeed have different effects in the two populations, perhaps because of differing history and genetic background, or because of interactions with other alleles or environmental factors that occur at different rates in the populations. In that case,

identifying the other background factors involved may reveal underlying common biological mechanisms. A third possibility is that the observed association may be a false positive. Potential reasons for "flip-flop" effects have been discussed by others (Lin *et al*., 2007, Zaykin & Shibata, 2008). Our interpretation of our "flipped" findings in *CHRNG* and *CHRNA4* is that these regions are likely to be involved in genetic risk contributing to nicotine dependence, but further genotyping or resequencing is necessary to refine these associations. Although SNPs in this study were selected to tag the common variation in European-Americans, the reduced LD in African-Americans means that more coverage is needed across this diverse sample. After more complete assessment of the genetic variation is obtained, analysis of a diverse sample can leverage LD differences and refine the associations to a smaller group of SNPs that show more consistent effects across populations (Saccone *et al*., 2008, Zaitlen *et al*., 2010).

For some of the *CHRN* genes highlighted here, other SNPs have been reported in independent studies of smoking or nicotine dependence. In *CHRNB1*, different SNPs have been reported (Lou *et al*., 2006, Philibert *et al*., 2009). Lou et al. did not genotype rs7210231, but reported rs2302763 as associated with smoking quantity in EAs; we genotyped the r^2 proxy rs3855924 ($r^2 = 1$ with rs2302763 in HapMap CEU) and did not find association with nicotine dependence in our sample. Philibert et al. did not report on rs7210231 but in their predominantly European-descent sample found evidence for rs3855924 and rs4796418, which are not associated in our samples. Our findings appear to highlight a different region in this gene. In *CHRNA7*, rs1909884 (Greenbaum *et al*., 2006) and rs904952, rs10438287 and rs12915265 (Philibert *et al*., 2009) have been reported; the latter two were tested in our sample but other SNPs are more strongly associated. For rs1909884 our closest proxy is rs904951 ($r^2 = 0.51$ in CEU but only 0.26 in YRI) which is highlighted in Table 2 and nominally associated in AAs. Rs904952 is completely correlated with rs904951 in both CEU and YRI ($r^2 = 1.0$). Thus our evidence for rs904951 supports the report in (Philibert *et al*., 2009) and extends the finding by showing association of rs904951 and other *CHRNA7* SNPs in a sizeable African-American sample.

The chromosome 15q25 region containing *CHRNA5-CHRNA3-CHRNB4* has been consistently associated with nicotine dependence and smoking in multiple studies (reviewed in (Greenbaum & Lerer, 2009)) and is clearly important. Evidence indicates there are multiple associated loci in this region that have low correlation with each other and may have distinct biological effects on risk. In European populations these distinct loci are represented by rs16969968 (a non-synonymous SNP in *CHRNA5*), rs578776 and rs588765. The association between rs16969968 and nicotine dependence has now been replicated in African-Americans. The results reported here now highlight additional nicotinic receptor genes as involved in nicotine dependence risk. However, none of these other *CHRN* genes show as much consistency in association across populations as rs16969968 and some of its correlates in *CHRNA5-CHRNA3-CHRNB4*. This suggests that these other *CHRN* genes need more investigation, and yet may be in a second "tier" of effects compared to the effects of variants on chromosome 15q25.

Given these multiple findings in *CHRN* genes, an important question is the proportion of phenotypic variance explained by these loci. Although large-scale association studies of complex diseases are reproducibly identifying common genetic "risk" variants, typically these variants have small effect sizes and account for only a small fraction of the heritability or phenotypic variance known to exist (Goldstein, 2009, Hirschhorn, 2009, Kraft & Hunter, 2009, Maher, 2008). In our sample, the variation explained by the chromosome 15q25 SNPs rs16969968, rs578776 and rs588765 is 1.9% in both EAs and AAs. With the addition of 6 SNPs representing the top associated *CHRN* genes in the full sample, the variation explained in each sample increases dramatically to 4.9% in EAs and 7.3% in AAs (Table 4). As with

many other complex diseases, these associated SNPs account for only a modest fraction of the trait variation. However, the important message is that we see a substantial additional contribution from variants in the other nicotinic receptor genes beyond *CHRNA5-CHRNA3-CHRNB4*. This is striking given that *CHRNA5-CHRNA3-CHRNB4* loci such as rs16969968 are genome-wide significant in multiple studies of European-descent subjects, while of the SNPs reported here, none even approach genome-wide significance.

In summary, this work provides further evidence that multiple cholinergic nicotinic receptor genes besides *CHRNA5-CHRNA3-CHRNB4* on chromosome 15q25 are involved in nicotine dependence risk not only in European-Americans but also in African-Americans. Future work to replicate these different findings in independent African-ancestry samples – and carry out meta-analysis – should improve our understanding and interpretation of these results. Larger sample sizes are needed to test the effect of these variants on smoking risk in other diverse human populations to help confirm and refine these findings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Demographics for the COGEND sample.

	AA (N=710)	New EA (N=454)	Original EA[1] (N=1608)	Total (N=2772)
Cases, N	461	266	797	1524
Males	172	140	351	663
Females	289	126	446	861
Controls, N	249	188	811	1248
Males	89	57	250	396
Females	160	131	561	852
Age				
Mean ± SD	36.76 ± 5.86	36.61 ± 5.61	36.39 ± 5.44	36.52 ± 5.57
(Min-Max)	(25-47)	(23-44)	(25-45)	(23-47)

AA, African-American; EA, European-American; SD, standard deviation; Min, minimum; Max, maximum.

[1] Previously reported in Bierut *et al*., 2007, Saccone *et al*., 2007, and Saccone *et al*., 2009a.

Table 2

Association results for selected SNPs in *CHRN* genes: SNPs with p-value ≤ 0.05 in African-Americans.

					AA (N=710)		New EA (N=454)		Original EA (N=1608)		All EA (N=2062)		Full Sample (N=2772)
SNP	**Chr**	**Position**	**Gene**	**Role**	**OR (95%CI)**	**p**	**OR (95%CI)**	**p**	**OR (95%CI)**	**p**	**OR (95%CI)**	**p**	**2df *p***
rs1881492	2	233115242	*CHRNG*	Intron	1.57 (1.17-2.11)	2.45E-03	0.84 (0.59-1.20)	3.44E-01	0.76 (0.63-0.90)	2.09E-03	0.77 (0.66-0.90)	1.20E-03	4.25E-05
rs6494212	15	30172411	*CHRNA7*	Intron	1.44 (1.14-1.83)	2.70E-03	1.06 (0.79-1.43)	7.05E-01	1.10 (0.94-1.28)	2.21E-01	1.10 (0.96-1.26)	1.62E-01	4.37E-03
rs2231532	11	3649696	near *CHRNA10*	Nongenic	1.35 (1.07-1.70)	1.08E-02	1.15 (0.86-1.53)	3.41E-01	1.06 (0.92-1.22)	4.52E-01	1.07 (0.94-1.22)	2.85E-01	2.48E-02
rs904951	15	30205330	*CHRNA7*	Intron	1.30 (1.02-1.65)	3.14E-02	1.21 (0.92-1.59)	1.68E-01	1.10 (0.95-1.26)	2.05E-01	1.12 (0.99-1.27)	7.33E-02	2.12E-02
rs1913456	15	30133241	*CHRNA7*	Intron	1.28 (1.02-1.60)	3.29E-02	0.76 (0.53-1.09)	1.36E-01	1.03 (0.86-1.24)	7.41E-01	0.97 (0.83-1.14)	7.12E-01	1.47E-01
rs16925377	11	3633651	*ART1*	Intron	1.43 (1.00-2.03)	4.86E-02	1.45 (0.90-2.33)	1.23E-01	1.17 (0.93-1.47)	1.87E-01	1.21 (0.99-1.48)	6.89E-02	2.62E-02

AA, African-American; EA, European-American; OR, odds ratio; CI, confidence interval; df, degrees of freedom; p, p-value.

Table 3

Association results for selected SNPs in *CHRN* genes: SNPs with p-value ≤ 0.01 in full sample.

					AA (N=710)		New EA (N=454)		Original EA (N=1608)		All EA (N=2062)		Full Sample (N=2772)
SNP	**Chr**	**Position**	**Gene**	**Role**	**OR (95%CI)**	**p**	**OR (95%CI)**	**p**	**OR (95%CI)**	**p**	**OR (95%CI)**	**p**	**2df *p***
rs1881492	2	233115242	*CHRNG*	Intron	1.57 (1.17-2.11)	2.45E-03	0.84 (0.59-1.20)	3.44E-01	0.76 (0.63-0.90)	2.09E-03	0.77 (0.66-0.90)	1.20E-03	4.25E-05
rs2236196	20	61448000	*CHRNA4*	mRNA-UTR	1.04 (0.83-1.30)	7.26E-01	1.15 (0.85-1.57)	3.65E-01	1.34 (1.14-1.58)	5.03E-04	1.30 (1.12-1.50)	4.36E-04	1.86E-03
rs13277254	8	42669139		Nongenic	0.95 (0.76-1.19)	6.36E-01	0.74 (0.53-1.02)	6.93E-02	0.77 (0.64-0.91)	3.13E-03	0.76 (0.66-0.89)	6.25E-04	2.12E-03
rs10958726	8	42655066		Nongenic	0.90(0.71-1.12)	3.35E-01	0.74 (0.54-1.03)	7.21E-02	0.78 (0.65-0.93)	5.67E-03	0.77 (0.66-0.90)	1.13E-03	2.50E-03
rs1955186	8	42668648		Nongenic	1.02 (0.80-1.29)	8.80E-01	0.72 (0.52-1.00)	5.22E-02	0.78 (0.65-0.93)	5.39E-03	0.77 (0.66-0.89)	7.38E-04	2.81E-03
rs13277524	8	42669214		Nongenic	1.01 (0.80-1.28)	9.35E-01	0.74 (0.54-1.03)	7.19E-02	0.77 (0.65-0.92)	3.78E-03	0.77 (0.66-0.90)	7.78E-04	3.00E-03
rs7210231	17	7298588	*CHRNB1*	Intron	0.79 (0.62-1.02)	6.49E-02	0.94 (0.68-1.29)	6.97E-01	0.75 (0.63-0.90)	1.64E-03	0.80 (0.69-0.94)	5.24E-03	3.47E-03
rs6474413	8	42670221	*CHRNB3*	Locus	1.02 (0.81-1.30)	8.55E-01	0.75 (0.54-1.04)	8.56E-02	0.77 (0.65-0.92)	4.07E-03	0.77 (0.66-0.90)	9.26E-04	3.49E-03
rs6494212	15	30172411	*CHRNA7*	Intron	1.44 (1.14-1.83)	2.70E-03	1.06 (0.79-1.43)	7.05E-01	1.10 (0.95-1.28)	2.21E-01	1.10 (0.96-1.26)	1.62E-01	4.37E-03
rs1955185	8	42668804		Nongenic	1.02 (0.80-1.29)	8.75E-01	0.75 (0.54-1.03)	7.99E-02	0.78 (0.65-0.93)	5.39E-03	0.78 (0.67-0.90)	1.17E-03	4.37E-03
rs3787138	20	61449668	*CHRNA4*	Intron	0.95 (0.76-1.17)	6.11E-01	1.17 (0.80-1.71)	4.22E-01	1.43 (1.15-1.79)	1.46E-03	1.38 (1.14-1.67)	1.02E-03	4.86E-03
rs6474412	8	42669655		Nongenic	0.97 (0.77-1.23)	8.13E-01	0.76 (0.55-1.05)	9.47E-02	0.78 (0.65-0.93)	5.55E-03	0.78 (0.67-0.91)	1.37E-03	4.91E-03
rs4950	8	42671790	*CHRNB3*	mRNA-UTR	1.01 (0.80-1.29)	9.08E-01	0.75 (0.55-1.04)	8.38E-02	0.78 (0.66-0.93)	6.21E-03	0.78 (0.67-0.91)	1.43E-03	5.30E-03
rs2229959	20	61451998	*CHRNA4*	Synonymous	0.82 (0.66-1.02)	7.75E-02	1.16 (0.79-1.71)	4.57E-01	1.37 (1.09-1.72)	6.60E-03	1.33 (1.09-1.62)	4.28E-03	5.98E-03
rs2302761	17	7299244	*CHRNB1*	Intron	0.82 (0.61-1.10)	1.88E-01	0.93 (0.68-1.28)	6.61E-01	0.74 (0.62-0.89)	1.09E-03	0.79 (0.68-0.93)	3.44E-03	6.19E-03
rs4952	8	42706222	*CHRNB3*	Synonymous	0.42 (0.16-1.08)	7.20E-02	0.75 (0.38-1.48)	4.12E-01	0.61 (0.42-0.89)	1.05E-02	0.65 (0.47-0.91)	1.08E-02	8.81E-03

AA, African-American; EA, European-American; OR, odds ratio; CI, confidence interval; df, degrees of freedom; p, p-value.

Table 4

Phenotypic variation explained by selected SNPs. R^2 difference" is Nagelkerke's adjusted R^2 from logistic regression, comparing the base model with intercept, gender, age and race to the model with the indicated SNP(s) added[1].

				EA		AA	
SNP[1]	**Chr**	**Gene region**	**ref allele**	**Ref allele freq**	**R^2 difference**	**Ref allele freq**	**R^2 difference**
rs16969968	15	*CHRNA5*	G	0.650	0.0142	0.948	0.0129
rs578776	15	*CHRNA3*	C	0.726	0.0131	0.451	0.0001
rs588765	15	*CHRNA5*	C	0.576	0.0013	0.705	0.0076
rs1881492	2	*CHRNG*	G	0.801	0.0066	0.803	0.0160
rs2236196	20	*CHRNA4*	A	0.740	0.0094	0.353	0.0000
rs13277254	8	*CHRNB3-CHRNA6*	A	0.791	0.0087	0.409	0.0008
rs7210231	17	*CHRNB1*	C	0.793	0.0050	0.772	0.0062
rs6494212	15	*CHRNA7*	C	0.683	0.0014	0.667	0.0220
rs2231532	11	*CHRNA10*	G	0.575	0.0005	0.333	0.0141
rs16969968/rs578776/rs588765					0.0187		0.0185
all 9 snps					0.0489		0.0733

[1]To ensure comparability, all models were run on a fixed sample of individuals with non-missing genotypes at all 9 SNPs (N = 1994 EAs, N = 667 AAs).