Published OnlineFirst August 25, 2009; DOI: 10.1158/0008-5472.CAN-09-0786

Epidemiology

The *CHRNA5-CHRNA3-CHRNB4* Nicotinic Receptor Subunit Gene Cluster Affects Risk for Nicotine Dependence in African-Americans and in European-Americans

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Abstract

Genetic association studies have shown the importance of variants in the *CHRNA5-CHRNA3-CHRNB4* cholinergic nicotinic receptor subunit gene cluster on chromosome 15q24-25.1 for the risk of nicotine dependence, smoking, and lung cancer in populations of European descent. We have carried out a detailed study of this region using dense genotyping in both European-Americans and African-Americans. We genotyped 75 known single nucleotide polymorphisms (SNPs) and one sequencing-discovered SNP in an African-American sample (N = 710) and in a European-American sample (N = 2,062). Cases were nicotine-dependent and controls were nondependent smokers. The nonsynonymous *CHRNA5* SNP rs16969968 is the most significant SNP associated with nicotine dependence in the full sample of 2,772 subjects [$P = 4.49 \times 10^{-8}$; odds ratio (OR), 1.42; 95% confidence interval (CI), 1.25–1.61] as well as in African-Americans only (P = 0.015; OR, 2.04; 1.15–3.62) and in European-Americans only ($P = 4.14 \times 10^{-7}$; OR, 1.40; 1.23–1.59). Other SNPs that have been shown to affect the mRNA levels of *CHRNA5* in European-Americans are associated with nicotine dependence in African-Americans but not in European-Americans. The *CHRNA3* SNP rs578776, which has a low correlation with rs16969968, is associated with nicotine dependence in European-Americans but not in African-Americans. Less common SNPs (frequency ≤ 5%) are also associated with nicotine dependence. In summary, multiple variants in this gene cluster contribute to nicotine dependence risk, and some are also associated with functional effects on *CHRNA5*. The nonsynonymous SNP rs16969968, a known risk variant in populations of European-descent, is also significantly associated with risk in African-Americans. Additional SNPs contribute to risk in distinct ways in these two populations. [Cancer Res 2009;69(17):6848–56]

Introduction

Cigarette smoking is a major public health problem and is the single largest cause of preventable death in the world, contributing to >5 million deaths a year (1). In the United States, smoking causes ~443,000 deaths each year including an estimated 82% of lung cancer deaths (2).

Cigarette smoking is common in European-Americans and African-Americans, with a similar prevalence of current smoking among adults: 21.4% [95% confidence interval (CI), 20.4–22.4] in European-Americans and 19.8% (19.2–21.4) in African-Americans (3). In past-month smokers, the rates of current nicotine dependence, defined by the Fagerström Test for Nicotine Dependence (FTND; ref. 4), were also similar although slightly lower in African-Americans: 57.3% (55.1–59.5) versus 61.7% (61.0–62.4) in European-Americans (5). Despite these similarities, African-Americans have a higher incidence of lung cancer than European-Americans (82.7 per 100,000 versus 64.3 per 100,000) and higher lung cancer mortality (64.1 per 100,000 versus 54.1 per 100,000; ref. 6). Many issues could contribute to these differences in lung cancer, including discrepancies in quit rates and disparities in medical care (7, 8). Genetic factors such as differences in allele frequencies and linkage disequilibrium (LD) might also play a role.

We previously carried out a high-density whole-genome and candidate gene association study of nicotine dependence in European-descent samples (9, 10). Among the top association signals were chromosome 15q24-25.1 variants, including a biologically compelling finding at rs16969968, a nonsynonymous single nucleotide polymorphism (SNP) in the α5 cholinergic nicotinic receptor subunit gene (*CHRNA5*; ref. 10). The risk allele at rs16969968 causes an amino acid change (D398N, aspartate to asparagine) in the α5 subunit and functional changes in the receptor *in vitro* (11). Independent replication of this association with nicotine dependence or smoking has now been confirmed in multiple populations of European descent, using rs16969968 or SNPs highly correlated ($r^2 \geq 0.8$) with rs16969968 (11–19). Fine-mapping in our original sample continued to highlight rs16969968 and other highly correlated SNPs, which extend across *CHRNA5-CHRNA3-CHRNB4* and into neighboring genes *IREB2, PSMA4*, and *LOC123688* (20).

Also in this region, a locus tagged by rs578776 and rs3743078 is significantly associated with nicotine dependence and smoking in European-descent samples (10, 12, 14, 16). This locus has been described as protective because the minor allele is more frequent in controls than in cases. There is evidence that this locus is

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-09-0786

Published OnlineFirst August 25, 2009; DOI: 10.1158/0008-5472.CAN-09-0786

statistically distinct from rs16969968: in two independent European-ancestry samples, the correlation between rs578776 and rs16969968 is low ($r^2 = 0.2$), with $D' = -1.0$ indicating repulsion phase (the minor allele at one locus co-occurs with the major allele at the other), and both SNPs are significant in joint analyses (14, 20).

Genome-wide studies of lung cancer report strong association with rs16969968 and its r^2 correlates (16, 21–23). The locus tagged by rs578776 is also associated with lung cancer. Thus, there has been a convergence of genetic findings for nicotine dependence and for lung cancer.

The association studies cited above were carried out in European-descent samples. Studying this region in individuals of African descent provides an opportunity to confirm its role in an important and understudied population. Also, the contrasting genetic architecture in Africans and Europeans can be leveraged to narrow down the potential functional source of the disease associations (24). In the European ancestry population, many highly correlated SNPs could often detect the same association signal, and determining which variant(s) are "causal" is not straightforward. LD and allele frequencies can differ between European and African populations. For instance, in HapMap, rs16969968 is nonpolymorphic in the YRI (Yoruba in Ibadan, Nigeria) sample; for rs3743078, the minor allele in YRI ("C", 33%) is the major allele (77%) in CEU (CEPH, Utah residents with ancestry from northern and western Europe; ref. 25).

Here, we present an association study of nicotine dependence across the *CHRNA5-CHRNA3-CHRNB4* region in a new African-American sample and an expanded European-American sample. We also analyze novel variants discovered from DNA resequencing of European-Americans in a 26 kb region between *CHRNA5* and *CHRNA3*. Our goal is to determine whether any variants consistently affect nicotine dependence risk in both European-Americans and African-Americans. Additionally, we are interested in evidence of variants that affect risk in the African-American sample alone.

Materials and Methods

Study design and sample. All individuals ($N = 2,772$) were recruited by the Collaborative Genetic Study of Nicotine Dependence (COGEND), a multi-site project from the United States. All cases and controls reported smoking ≥100 cigarettes lifetime, the threshold classically used to define a smoker (26). In the recruitment of new subjects, cases were nicotine-dependent, defined by current FTND ≥4, and controls were required to have a lifetime FTND of 0 or 1. The African-American genetic sample in COGEND consists of 461 cases and 249 controls. The European-American genetic sample of 1,063 cases and 999 controls consists of 454 new subjects (266 cases and 188 controls) together with 1,608 original COGEND European-American individuals previously analyzed in refs. [9, 10; 797 cases (FTND ≥4) and 811 controls (FTND = 0)].

The study complies with the Code of Ethics of the World Medical Association and obtained informed consent from participants and approval from the appropriate institutional review boards.

Genotyping and quality control. Thirty-three SNPs covering the *CHRNA5-CHRNA3-CHRNB4* region were genotyped in the additional subjects by the Genome Center at Washington University.[8] These SNPs were genotyped using Illumina GoldenGate assay as part of a set of 1,536 SNPs that included SNPs for checking population stratification and SNPs to follow-up several genomic regions. Cleaning was performed using all 1,536 SNPs. Four DNA samples had poor call rates (≤63% of the 1,536 SNPs) and were dropped. All remaining DNA samples had call rates above 90% and were retained; 99.5% of DNA samples had call rates ≥95%. Twenty samples were included in duplicate. SNPs with more than one nonconcordant duplicate sample genotype were dropped; the 33 SNPs reported here were 100% concordant. SNPs were also required to pass a call rate threshold of 98%. Self-reported race was verified using EIGENSTRAT (27). An additional 42 SNPs were genotyped using Sequenom MassArray iPLEX technology.[9] These SNPs passed a call rate threshold of 95%. Map positions were obtained from the National Center for Biotechnology Information Human Reference Build 36.2 and dbSNP Build 129.

These 75 SNPs were selected to include the same SNPs used to cover this region in our previous publications (9, 10, 20). Forty-nine of the genotyped SNPs lie between 76642961 bp and 76722642 bp, encompassing *CHRNA5-CHRNA3-CHRNB4* and the 2 kb flanking either side (5′ of *CHRNA5* and 5′ of *CHRNB4*), matching the dbSNP definition of in or near a gene. These SNPs provide good coverage of the 72 SNPs in this region that are common [minor allele frequency (MAF) ≥0.05] in either the HapMap CEU or YRI samples. Of the 61 SNPs that have MAF ≥5% in CEU, 54 (93%) are tagged by at least one genotyped SNP at an $r^2 \geq 0.8$ according to CEU LD data; all 61 are tagged at an $r^2 \geq 0.5$. Of the 65 SNPs in this region that have MAF ≥5% in YRI, 46 (71%) are tagged at an $r^2 \geq 0.8$ in the YRI LD data; 60 (92%) are tagged at an $r^2 \geq 0.5$ (HapMap release 23a). Twenty-six additional SNPs flanking the cluster or in *IREB2, LOC123688*, and *PSMA4* were included because of the high r^2 extending from *CHRNA5-CHRNA3-CHRNB4*.

Genotype data were then combined for this new sample and the previously genotyped sample of 1,608 European-Americans. Allele correspondence was checked. All association analyses are reported using the major allele in the European-American sample as the reference allele.

DNA resequencing in the *CHRNA5* and *CHRNA3* intergenic region. Human *CHRNA3* and *CHRNA5* are physically linked and partially overlap tail-to-tail (28). We targeted a 26-kb region spanning exon 5 of *CHRNA5* to exon 4 of *CHRNA3* and sequenced 10 cases and 12 controls of European descent. The goal was to identify novel variants, genotype them in the larger sample, and test them for association with nicotine dependence.

Sequencing was performed at the Genome Center at Washington University using standard protocols.[10] We used Sequencher (Gene Codes) to assemble and scan sequence traces for variation from the reference sequence. Novel variants were genotyped using Sequenom MassArray iPLEX technology.

Linkage disequilibrium. LD was calculated in European-Americans and African-Americans using PLINK (29) and Haploview (30). LD (r^2) plots were generated using WGAviewer (31).[11]

Genetic association analyses. Association analysis of case-control status was performed on the entire sample using logistic regression with gender and race as covariates. This analysis highlights SNPs that have a similar effect in both European-Americans and African-Americans. The full genetic model is

$$\ln\left(\frac{P}{1-P}\right) = \alpha + \beta_1 g + \beta_2 s + \beta_3 G, \qquad (A)$$

where P is the probability of being a case, g is gender (0 = male, 1 = female), s is race (0 = European-American, 1 = African-American), and G is genotype, coded as the number of copies of the minor allele in the European-American sample, corresponding to a log additive (multiplicative) model. The term $\beta_3 G$ is tested for significance by the standard likelihood χ^2 with 1 *df*. We then analyzed African-Americans and European-Americans separately with gender as a covariate. Association tests were carried out using PLINK (29) and SAS.

Cross-population heterogeneity analysis. Heterogeneity of the odds ratios (OR) in European-Americans and African-Americans was tested by adding the interaction term $\beta_4 Gs$ to Eq. A (24). This test is of interest for SNPs that are associated with disease in at least one population. If

[8] http://genome.wustl.edu/services/microarray.cgi

[9] www.sequenom.com

[10] http://genome.wustl.edu/activity/med_seq/protocols.cgi

[11] http://people.genome.duke.edu/~dg48/WGAViewer/

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Table 1. Selected genotyped SNPs and allele frequencies, sorted by position

SNP	Chr	Position (bp)	Gene	Reference allele*	Minor allele		Reference allele frequency	
					European-American sample	African-American sample	European-American sample	African-American sample
rs17483548	15	76517368	*IREB2*	G	A	A	0.662	0.941 †
rs17405217	15	76518204	*IREB2*	C	T	T	0.662	0.944 †
rs2656052	15	76527987	*IREB2*	T	G	G	0.653	0.565 †
rs17484235	15	76548469	*IREB2*	C	G	G	0.662	0.944 †
rs7164594	15	76590112	*LOC123688*	C	T	T	0.785	0.584 †
rs8034191	15	76593078	*LOC123688*	T	C	C	0.648	0.840 †
rs10519203	15	76601101	*LOC123688*	A	G	G	0.643	0.697 †
rs2036534	15	76614003	*LOC123688*	T	C	C	0.789	0.778
rs12916483	15	76619452	*PSMA4*	G	A	A	0.575	0.835 †
rs3813570	15	76619887	*PSMA4*	A	G	G	0.789	0.739 †
rs11858230	15	76622607	*PSMA4*	G	A	A	0.574	0.771 †
rs2036527	15	76638670		C	T	T	0.645	0.778 †
rs667282	15	76650527	*CHRNA5*	T	C	C	0.779	0.704 †
rs588765	15	76652480	*CHRNA5*	C	T	T	0.576	0.705 †
rs6495306	15	76652948	*CHRNA5*	A	G	G	0.576	0.702 †
rs17486278	15	76654537	*CHRNA5*	A	C	C	0.648	0.710 †
rs601079	15	76656634	*CHRNA5*	T	A	A	0.574	0.578
rs680244	15	76658343	*CHRNA5*	G	A	A	0.573	0.576
rs637137	15	76661031	*CHRNA5*	T	A	A	0.779	0.701 †
rs951266	15	76665596	*CHRNA5*	C	T	T	0.649	0.888 †
rs555018	15	76666297	*CHRNA5*	T	C	C	0.576	0.699 †
rs647041	15	76667536	*CHRNA5*	C	T	T	0.578	0.704 †
rs16969968	15	76669980	*CHRNA5*	G	A	A	0.650	0.948 †
rs660652	15	76674887	*CHRNA3*	G	A	A	0.632	0.717 †
rs578776	15	76675455	*CHRNA3*	C	T	C ‡	0.726	0.451 †
rs6495307	15	76677376	*CHRNA3*	C	T	T	0.580	0.595
rs1051730	15	76681394	*CHRNA3*	C	T	T	0.651	0.882 †
rs3743078	15	76681814	*CHRNA3*	C	G	C ‡	0.774	0.416 †
rs3743077	15	76681951	*CHRNA3*	G	A	A	0.579	0.847 †
rs1317286	15	76683184	*CHRNA3*	A	G	G	0.645	0.753 †
rs938682	15	76683602	*CHRNA3*	T	C	C	0.778	0.711 †
rs11637630	15	76686774	*CHRNA3*	A	G	G	0.778	0.697 †
GSC3_26	15	76693692	*CHRNA3*	A	G	G	0.938	0.986 †
rs7177514	15	76694461	*CHRNA3*	C	G	G	0.777	0.698 †
rs6495308	15	76694711	*CHRNA3*	T	C	C	0.777	0.702 †
rs8042059	15	76694914	*CHRNA3*	A	C	C	0.777	0.702 †
rs8042374	15	76695087	*CHRNA3*	A	G	G	0.780	0.750
rs4887069	15	76696125	*CHRNA3*	A	G	G	0.772	0.698 †
rs8192475	15	76698285	*CHRNA3*	G	A	A	0.950	0.991 †
rs6495309	15	76702300	*CHRNB4*	C	T	T	0.794	0.748 †
rs12914008	15	76710560	*CHRNB4*	G	A	A	0.955	0.994 †
rs17487223	15	76711042	*CHRNB4*	C	T	T	0.629	0.897 †
rs1996371	15	76743861		A	G	G	0.587	0.929 †

*The reference allele is the major allele in the European-American sample.
† Allele frequencies differ significantly between European-Americans and African-Americans by Fisher's exact test ($P < 10^{-3}$).
‡ Minor allele differs in European-Americans and African-Americans.

the interaction term is significant (e.g., $P \leq 0.05$, unadjusted for multiple tests), we conclude that the genetic effect is different in the two population samples.

A significant difference in ORs may occur because the genetic effect is present in only one of the two populations. In this case, one interpretation is that this SNP is not biologically causal, although in the associated population it may tag the functional SNP. This interpretation is based on the hypothesis that the underlying biological processes leading to disease risk are shared in common across human populations (32).

Results

Genetic Association Analyses

Supplementary Table S1 shows the allele frequencies of the 75 known SNPs, and one novel SNP from our SNP discovery project, in the European-American and African-American samples; selected SNPs, including those most associated with nicotine dependence in each sample, are in Table 1. Sixty-three SNPs show significant allele frequency differences between European-Americans and

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African-Americans. Nevertheless, for most SNPs, the minor allele is the same in both populations; notable exceptions are rs578776 and rs3743078, which are known to be associated with nicotine dependence in European-Americans.

Table 2 shows association results for the Table 1 SNPs; Supplementary Table S2 shows all 76 SNPs. The most significant SNP in the combined sample is the nonsynonymous *CHRNA5* SNP rs16969968 ($P = 4.49 \times 10^{-8}$; OR, 1.42; 95% CI, 1.25–1.61). Furthermore, rs16969968 is the most significant of the 76 SNPs in the African-Americans alone ($P = 0.0147$; OR, 2.04; 1.15–3.62) and in the European-Americans alone ($P = 4.14 \times 10^{-7}$; OR, 1.40; 1.23–1.59). The same allele (A) is elevated in European-American and African-American cases. Additional SNPs correlated with rs16969968 in European-Americans and African-Americans are also significant.

In European-descent samples, a locus distinct from rs16969968, tagged by rs578776, has been consistently associated with nicotine dependence (10, 14). In the full sample, rs578776 is significantly associated ($P = 7.09 \times 10^{-5}$; OR, 0.79; 0.70–0.88) and is the most strongly associated of the correlated SNPs representing this

Table 2. Chromosome 15 association results for selected SNPs, sorted by chromosomal position

SNP	African-American sample		European-American sample		Full sample		Cross-population heterogeneity
	OR (95% CI)	*P*	OR (95% CI)	*P*	OR (95% CI)	*P*	*P*
rs17483548	1.58 (0.95–2.61)	7.98E−2	1.36 (1.19–1.54)	5.95E−6	1.36 (1.20–1.54)	1.88E−6	6.17E−01
rs17405217	1.78 (1.04–3.04)	3.45E−2	1.35 (1.18–1.54)	7.50E−6	1.37 (1.20–1.55)	1.55E−6	3.56E−01
rs2656052	0.97 (0.78–1.20)	7.90E−1	1.27 (1.11–1.44)	3.84E−4	1.18 (1.06–1.32)	3.05E−3	5.38E−02
rs17484235	1.79 (1.05–3.05)	3.36E−2	1.36 (1.19–1.54)	5.25E−6	1.37 (1.21–1.55)	1.06E−6	3.62E−01
rs7164594	1.00 (0.80–1.25)	9.79E−1	0.74 (0.64–0.86)	1.10E−4	0.81 (0.71–0.91)	1.04E−3	3.35E−02
rs8034191	1.32 (0.96–1.80)	8.84E−2	1.37 (1.20–1.56)	2.30E−6	1.36 (1.21–1.53)	5.59E−7	8.47E−01
rs10519203	1.13 (0.88–1.44)	3.45E−1	1.33 (1.17–1.51)	1.62E−5	1.29 (1.15–1.44)	1.65E−5	2.98E−01
rs2036534	0.89 (0.69–1.15)	3.88E−1	0.75 (0.64–0.87)	2.77E−4	0.79 (0.69–0.89)	3.40E−4	2.56E−01
rs12916483	0.86 (0.64–1.15)	3.16E−1	0.94 (0.83–1.06)	3.57E−1	0.93 (0.83–1.04)	2.11E−1	5.15E−01
rs3813570	0.82 (0.64–1.04)	1.09E−1	0.75 (0.64–0.87)	2.35E−4	0.77 (0.68–0.87)	7.39E−5	5.36E−01
rs11858230	0.86 (0.67–1.10)	2.37E−1	0.93 (0.82–1.05)	2.65E−1	0.92 (0.82–1.02)	1.30E−1	5.37E−01
rs2036527	1.36 (1.04–1.78)	2.37E−2	1.37 (1.21–1.56)	1.81E−6	1.37 (1.22–1.53)	1.41E−7	9.57E−01
rs667282	0.90 (0.71–1.14)	3.99E−1	0.75 (0.64–0.87)	1.68E−4	0.79 (0.69–0.89)	2.03E−4	2.28E−01
rs588765	**0.80 (0.63–1.00)**	**5.48E−2**	**0.91 (0.80–1.02)**	**1.18E−1**	**0.88 (0.79–0.98)**	**2.56E−2**	**3.56E−01**
rs6495306	0.80 (0.63–1.00)	5.29E−2	0.90 (0.80–1.02)	1.16E−1	0.88 (0.79–0.98)	2.44E−2	3.45E−01
rs17486278	1.28 (1.01–1.63)	4.43E−2	1.40 (1.23–1.59)	5.74E−7	1.37 (1.22–1.54)	6.77E−8	6.12E−01
rs601079	0.86 (0.69–1.06)	1.61E−1	0.90 (0.79–1.02)	1.03E−1	0.89 (0.80–0.99)	3.56E−2	6.65E−01
rs680244	0.87 (0.70–1.08)	2.08E−1	0.90 (0.79–1.01)	8.68E−2	0.89 (0.80–0.99)	3.66E−2	8.03E−01
rs637137	0.91 (0.71–1.15)	4.31E−1	0.74 (0.64–0.86)	1.03E−4	0.78 (0.69–0.88)	1.64E−4	1.76E−01
rs951266	1.45 (1.01–2.06)	4.15E−2	1.39 (1.22–1.57)	9.83E−7	1.39 (1.23–1.57)	1.25E−7	8.29E−01
rs555018	**0.79 (0.63–0.99)**	**4.50E−2**	**0.91 (0.80–1.02)**	**1.22E−1**	**0.88 (0.79–0.98)**	**2.43E−2**	**3.30E−01**
rs647041	0.80 (0.64–1.01)	6.74E−2	0.91 (0.80–1.02)	1.27E−1	0.89 (0.79–0.98)	3.15E−2	4.04E−01
rs16969968	**2.04 (1.15–3.62)**	**1.47E−2**	**1.40 (1.23–1.59)**	**4.14E−7**	**1.42 (1.25–1.61)**	**4.49E−8**	**2.37E−01**
rs660652	0.80 (0.63–1.00)	5.85E−2	0.95 (0.83–1.08)	4.49E−1	0.92 (0.82–1.02)	1.37E−1	2.27E−01
rs578776	**1.01 (0.80–1.26)**	**9.58E−1**	**0.72 (0.62–0.82)**	**3.21E−6**	**0.79 (0.70–0.88)**	**7.09E−5**	**1.26E−02**
rs6495307	0.86 (0.69–1.08)	2.06E−1	0.91 (0.81–1.03)	1.62E−1	0.90 (0.81–1.00)	7.22E−2	6.86E−01
rs1051730	1.38 (0.98–1.95)	6.76E−2	1.40 (1.23–1.59)	5.88E−7	1.39 (1.23–1.57)	1.16E−7	9.39E−01
rs3743078	**1.05 (0.84–1.30)**	**6.82E−1**	**0.76 (0.65–0.87)**	**2.68E−4**	**0.84 (0.74–0.94)**	**4.48E−3**	**1.82E−02**
rs3743077	0.80 (0.59–1.06)	1.28E−1	0.91 (0.80–1.02)	1.20E−1	0.89 (0.79–0.99)	3.97E−2	3.42E−01
rs1317286	1.27 (0.98–1.63)	6.56E−2	1.36 (1.19–1.54)	4.44E−6	1.33 (1.19–1.49)	1.10E−6	5.91E−01
rs938682	0.90 (0.71–1.15)	4.07E−1	0.76 (0.65–0.87)	2.87E−4	0.79 (0.70–0.89)	3.20E−4	2.59E−01
rs11637630	0.91 (0.71–1.15)	4.22E−1	0.75 (0.65–0.87)	2.10E−4	0.79 (0.70–0.89)	2.90E−4	2.12E−01
GSC3_26	**0.73 (0.29–1.84)**	**5.07E−1**	**0.77 (0.59–0.99)**	**4.69E−2**	**0.76 (0.59–0.97)**	**3.17E−2**	**7.94E−01**
rs7177514	0.97 (0.76–1.23)	7.87E−1	0.76 (0.65–0.88)	3.10E−4	0.81 (0.71–0.92)	1.21E−3	9.29E−02
rs6495308	0.98 (0.77–1.25)	8.82E−1	0.76 (0.65–0.87)	2.64E−4	0.81 (0.71–0.92)	1.34E−3	7.01E−02
rs8042059	1.00 (0.78–1.28)	9.94E−1	0.76 (0.65–0.88)	3.23E−4	0.82 (0.72–0.92)	2.01E−3	5.70E−02
rs8042374	1.01 (0.79–1.29)	9.15E−1	0.77 (0.66–0.89)	5.21E−4	0.83 (0.73–0.93)	3.33E−3	5.55E−02
rs4887069	1.01 (0.79–1.29)	9.31E−1	0.76 (0.65–0.87)	2.40E−4	0.82 (0.72–0.92)	1.83E−3	4.36E−02
rs8192475	**0.74 (0.23–2.35)**	**6.08E−1**	**0.77 (0.58–1.02)**	**6.80E−2**	**0.76 (0.58–0.99)**	**4.77E−2**	**7.68E−01**
rs6495309	0.94 (0.72–1.21)	6.13E−1	0.75 (0.64–0.87)	2.67E−4	0.79 (0.70–0.90)	5.41E−4	1.67E−01
rs12914008	**0.53 (0.13–2.12)**	**3.67E−1**	**0.73 (0.54–0.97)**	**3.53E−2**	**0.71 (0.53–0.95)**	**2.21E−2**	**5.39E−01**
rs17487223	1.59 (1.07–2.36)	2.07E−2	1.35 (1.19–1.54)	3.92E−6	1.37 (1.21–1.54)	3.58E−7	4.66E−01
rs1996371	1.10 (0.72–1.69)	6.55E−1	1.15 (1.01–1.30)	3.46E−2	1.14 (1.01–1.28)	3.95E−2	6.81E−01

NOTE: Values in boldface denote SNPs discussed in the text. ORs are for each copy of the nonreference allele.

Published OnlineFirst August 25, 2009; DOI: 10.1158/0008-5472.CAN-09-0786

Table 3. Results for novel, sequencing-discovered genetic variants in the *CHRNA5-CHRNA3* intergenic region

Novel variant	Rs number (if available)	Position (bp)	Minor allele	Major allele	COGEND European-American original (N = 1,591)			
					MAF	P	OR (95% CI)	Maximum r^2 with previously genotyped SNPs
GSC3_2		76671157	TAAG	DEL	0.0100	0.6773	1.16 (0.57–2.36)	<0.2
GSC3_4		76671923	A	T	0.0028	0.351	0.51 (0.13–2.09)	0.46
GSC3_15	rs62010327	76679839	A	G	0.3666	0.8474	1.02 (0.87–1.18)	0.94
GSC3_16		76679938	DEL	TACTC	0.0493	0.03726	0.71 (0.51–0.98)	0.95
GSC3_17	rs55958820	76681412	T	G	0.0098	0.1722	1.70 (0.79–3.61)	<0.2
GSC3_18	rs57708953	76683263	T	C	0.0039	0.6057	0.74 (0.23–2.36)	0.35
GSC3_20		76684921	T	DEL	0.3719	0.9045	1.01 (0.87–1.17)	0.97
GSC3_26		**76693692**	**G**	**A**	**0.0648**	**0.006386**	**0.67 (0.50–0.89)**	**0.25**
GSC3_27		76694121	T	G	0.0060	0.7098	1.19 (0.48–2.97)	<0.2
GSC3_28		76695823	T	C	0.0063	0.6532	0.81 (0.33–2.00)	<0.2
GSC3_29		76696413	G	C	0.0028	0.3521	0.51 (0.13–2.09)	0.46

NOTE: Logistic regression, additive model.

"protective" locus. However, this association is driven by the European-American sample ($P = 3.21 \times 10^{-6}$; OR, 0.72; 0.62–0.82); in the African-American sample, rs578776 is not associated (P = 0.96; OR, 1.01; 0.80–1.26). This contrasts with the agreement between European-American and African-American association results for rs16969968.

In the African-American sample, the most significant SNP that confers a protective effect is rs555018 in *CHRNA5* (P = 0.045; OR, 0.79; 0.63–0.99). This SNP is not correlated with rs16969968 in African-Americans (r^2 = 0.02), although $|D'|$ = 1. It is highly correlated with rs588765 (r^2 = 0.99 in our African-American samples and 0.999 in the European-American samples), a SNP that has been shown to affect the expression of CHRNA5 in European-Americans (33, 34). Because of this high correlation, the evidence for association between rs588765 and nicotine dependence in African-Americans is similar (P = 0.055; OR, 0.80; 0.63–1.00) to that found for rs555018. In contrast, neither rs555018 nor rs588765 is associated with nicotine dependence in the European-Americans.

Finally, a nonsynonymous variant rs12914008 in *CHRNB4*, although rare in European-Americans (4.5%) and virtually nonpolymorphic in African-Americans (0.6%), is nominally associated with nicotine dependence in European-Americans (P = 0.035; OR, 0.73; 95% CI, 0.54–0.97). Although this SNP is too rare for a meaningful test in African-Americans alone, the OR is consistent in the two groups, and combining African-Americans and European-Americans improves the association evidence slightly (P = 0.022; OR, 0.71; 0.53–0.95).

DNA Resequencing in the *CHRNA5* and *CHRNA3* Intergenic Region

We detected 29 novel variants. Seven of these have also been reported by other groups while we were validating our sequencing results. Although many of these were rare (MAF ≤5%), 10 had MAF ≥10% based on the 44 chromosomes. We genotyped these novel variants in the original 1,608 European-Americans. Eleven were polymorphic in this larger sample and passed quality controls (per SNP call rate ≥0.96 and Hardy-Weinberg $P \geq 0.05$).

Table 3 shows association results for these 11 SNPs. Three SNPs were highly correlated with already genotyped SNPs ($r^2 > 0.9$) and did not provide new information. The most significant novel SNP, GSC3-26, had a relatively strong OR of 0.67 (0.50–0.89) and an $r^2 \leq 0.25$ with previously genotyped SNPs, although $|D'|$ was often high (e.g., r^2 = 0.23; $|D'|$ = 1.0 with rs3743078).

We therefore genotyped GSC3_26 in the remaining European-Americans and African-Americans (Tables 1 and 2). GSC3_26 is relatively uncommon (MAF of 0.062 in European-Americans and 0.014 in African-Americans). The combined sample with race and gender as covariates has P = 0.032 (OR, 0.76; 0.59–0.97).

LD and Cross-Population Heterogeneity

Figure 1 displays European-American and African-American results and r^2 for all 76 SNPs. The LD results confirm that the most significantly associated SNPs in the full sample are highly correlated with rs16969968 in European-Americans (r^2 ranging from 0.68 to 1.00). These SNPs all have OR > 1. In African-Americans, the LD is reduced (r^2 from 0.11 to 0.73). The next group of significantly associated SNPs, which have OR < 1, are highly correlated with rs578776 in European-Americans.

Three SNPs are of particular interest from the single-SNP association results: rs16969968 (associated with both European-Americans and African-Americans); rs555018 (associated with African-Americans only), and rs578776 (associated with European-Americans only). Among them, r^2 is low to moderate; $|D'|$ is higher. For rs16969968 and rs555018, r^2 is 0.39 and $|D'|$ = 0.99 in European-Americans, whereas r^2 = 0.024 and $|D'|$ = 1 in African-Americans. For rs16969968 and rs578776, r^2 = 0.20 and $|D'|$ = 1 in European-Americans; r^2 = 0.07 and $|D'|$ = 1 in African-Americans. For rs555018 and rs578776, r^2 = 0.075 and $|D'|$ = 0.52 in European-Americans; r^2 = 0.43 and $|D'|$ = 0.90 in African-Americans.

Fourteen SNPs show significantly different ORs in European-Americans versus African-Americans (Table 2; Supplementary Table S2, last column), including rs578776, which represents the "protective" finding in European-Americans. All other SNPs displaying significant heterogeneity are at least moderately correlated with rs578776 in European-Americans (r^2 ranges from

Published OnlineFirst August 25, 2009; DOI: 10.1158/0008-5472.CAN-09-0786

0.39 to 0.76) and are nominally associated ($P < 0.05$) in European-Americans with OR < 1. The correlation is sharply reduced in African-Americans (r^2 from 0.001 to 0.40). This difference in LD may explain the lack of association for these SNPs in African-Americans and suggests that the functional variant driving the signal is in LD with these SNPs in European-Americans. Notably, at rs16969968, the African-American and European-American ORs are not significantly different (heterogeneity $P = 0.24$).

Multilocus Analyses

rs555018 and rs16969968. The association between rs555018 and nicotine dependence in African-Americans is intriguing

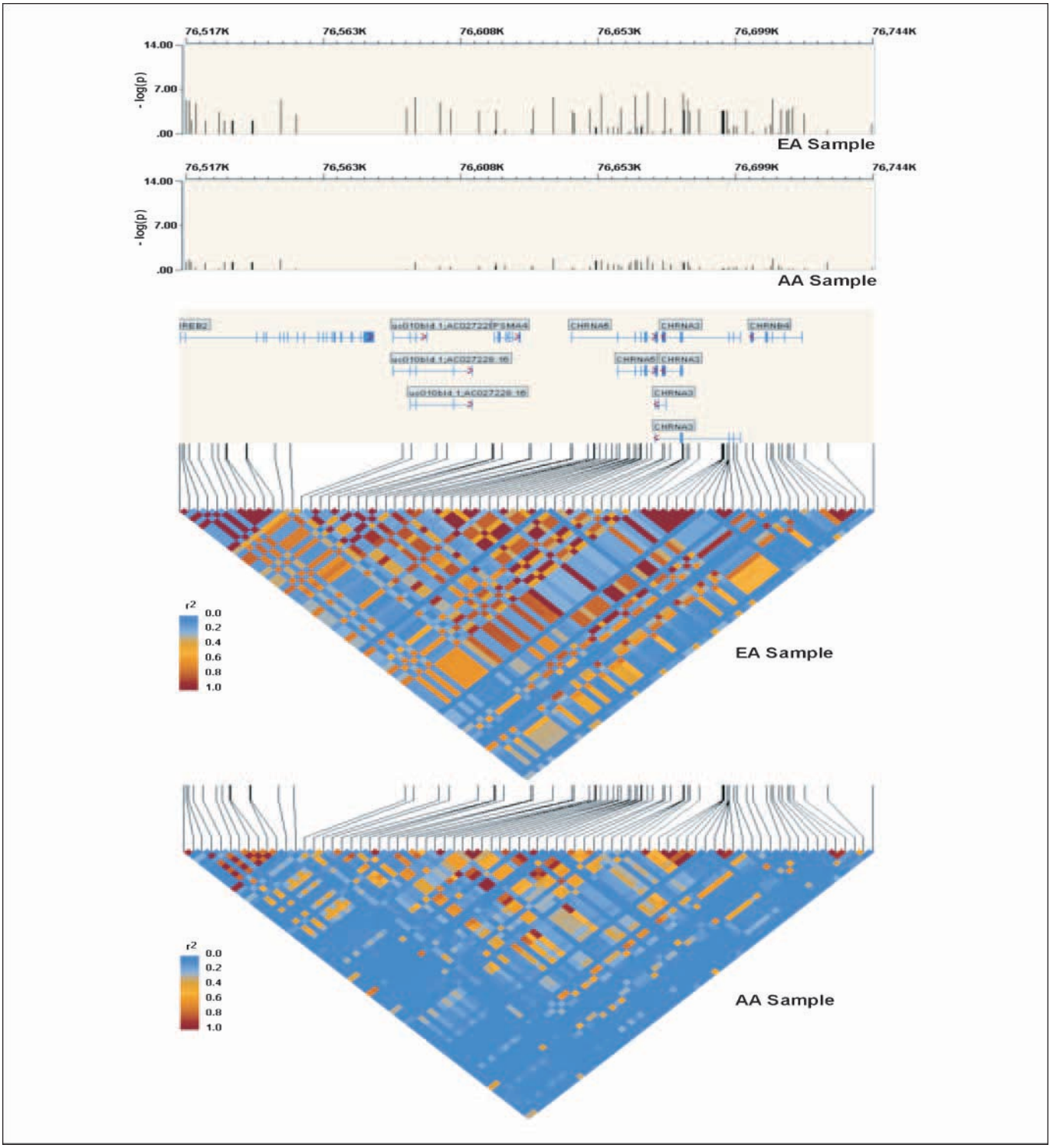

Figure 1. Association results and r^2 on chromosome 15q24-25.1 for the European-American and African-American samples.

Published OnlineFirst August 25, 2009; DOI: 10.1158/0008-5472.CAN-09-0786

Table 4. Joint analysis of rs16969968 and rs555018

European-American sample	rs555018		
rs16969968	TT	CT	CC
GG	42/63 1.00 (ref)	188/211 1.33 (0.86–2.07)	182/185 1.48 (0.95–2.29)
AG	157/161 1.46 (0.93–2.29)	317/280 1.70 (1.11–2.59)	3/0 —
AA	171/89 2.88 (1.81–4.60)	1/0 —	0/0 —

African-American sample	rs555018		
rs16969968	TT	CT	CC
GG	199/100 1.00 (ref)	155/101 0.77 (0.55–1.09)	37/30 0.62 (0.36–1.06)
AG	37/13 1.43 (0.73–2.81)	18/3 3.02 (0.87–10.48)	0/0 —
AA	1/0 —	0/0 —	0/0 —

NOTE: The first line of each cell indicates the number of cases and controls with the specific genotype combination; the second line indicates the OR and 95% CI when GG/TT for rs16969968/rs555018 is the reference genotype.

because this SNP is one of a group of highly correlated SNPs that are associated with *CHRNA5* mRNA levels in European-Americans (33, 34). However, in European-Americans, rs555018 and correlates such as rs588765 are not associated with nicotine dependence. To further investigate these differing results at these potentially functional SNPs, we carried out multilocus analyses to account for the established effects of rs16969968.

First, we tested rs555018 in European-Americans after controlling for rs16969968 (coded additively) as a covariate in the logistic regression. The goal was to determine if rs555018, which shows no main-effect association in European-Americans, might be associated after adjusting for rs16969968. After accounting for rs16969968, rs555018 has $P = 0.044$ (OR, 1.18; 1.01–1.39) and rs588765 has $P = 0.038$ (OR, 1.19; 1.01–1.40). The ORs in European-Americans at these the expression-associated SNPs have opposite direction to those in African-Americans.

This contrast in the direction of effect is clarified by joint analyses of rs16969968 with rs555018, using the genotype that is homozygous for both major alleles as the reference (Table 4). In the presence of "GG" at rs16969968, having one or two copies of the minor "C" allele at rs555018 increases risk in European-Americans, but confers protection in African-Americans (Table 4, *top row for each sample*). For rs16969968, we see a consistent direction of effect of the "A" risk allele in European-Americans and African-Americans, regardless of genotype at rs555018.

We also tested each SNP within the one available homozygous genotype group of the other. The test cannot be performed in the second homozygous group because there is essentially only one genotype present at the other locus ($|D'| > 0.99$; see also Table 4). In African-Americans, rs555018 remains significant ($P = 0.046$; OR, 0.78; 0.61–1.00) when tested within the 622 "GG" homozygotes at rs16969968; within the 350 rs555018 "TT" homozygotes, rs16969968 has $P = 0.22$ (OR, 1.51; 0.78–2.94). In European-Americans, rs555018 has $P = 0.12$ (OR, 1.17; 0.96–1.44) in the 871 rs16969968 "GGs", and rs16969968 has $P = 2.08 \times 10^{-6}$ (OR, 1.73; 1.38–2.17) in the 683 rs555018 "TTs".

rs578776 and rs16969968. Both rs16969968 and rs578776 show strong association with nicotine dependence in populations of European-descent. There is debate about whether these associations are independent or whether they reflect a single finding.

To explore this question in European-Americans, we tested for the association of rs578776 after controlling for rs16969968 (coded additively). Rs578776 remains significant ($P = 0.0055$; OR, 0.80; 0.68–0.94). We also tested for the effect of one locus in homozygotes of the other. Because $|D'|$ is high, this test can be carried out in only the "GG" genotype at rs16969968 or the "CC" genotype at rs578776. In both cases, the second SNP remains significant in European-Americans. Among "GGs" at rs16969968, rs578776 has $P = 0.018$ (OR, 0.79; 0.65–0.96); among "CCs" at rs578776, rs16969968 has $P = 0.0039$ (OR, 1.29; 1.09–1.54).

Discussion and Conclusions

This study contributes several new findings about genetic risk for nicotine dependence. The *CHRNA5-CHRNA3-CHRNB4* region clearly plays a role in nicotine dependence risk not only in European-Americans but also in African-Americans, and our evidence suggests that multiple variants are involved.

First, rs16969968, which causes an amino acid change in *CHRNA5* and is clearly implicated in risk for nicotine dependence and lung cancer in individuals of European descent, is also significantly associated with nicotine dependence in African-Americans. This SNP is the most important one to test first: it is

Published OnlineFirst August 25, 2009; DOI: 10.1158/0008-5472.CAN-09-0786

highly replicated, and *in vitro* studies show that α4β2α5 nicotinic receptors with the risk variant at rs16969968 exhibit lower maximal response to a nicotinic agonists than receptors with the other allele (11).

This replication, in African-Americans, of the rs16969968 association adds crucial information because of the different LD and population history for African-Americans, and because of the much lower allele frequency of rs16969968 in this population. The allele "A" at rs16969968 is rare in African-Americans, with a frequency of 5% (6% in cases and 3% in controls), compared with 35% in European-Americans (39% in cases and 31% in controls), and it is not present in the HapMap YRI. Nonetheless, it is a risk factor in our sample of African descent. The OR is 2.04 (1.15–3.62) in African-Americans and 1.40 (1.23–1.59) in European-Americans. These CIs overlap, and the ORs are not significantly different in the two populations (heterogeneity $P = 0.24$). Thus, the "A" allele at rs16969968 is a consistent risk factor in both populations. Furthermore, in each group, rs16969968 is the most significant and has the highest OR.

This chromosome 15q24-25.1 region also harbors SNPs that are correlated with each other and associated with brain mRNA levels of *CHRNA5* in European-descent samples (33, 34). Our second major finding is that in African-Americans, these expression-associated SNPs are associated with nicotine dependence. The evidence is nominally significant ($P = 0.045$; OR, 0.79; 0.63–0.99 for rs555018) in our modestly powered African-American sample. It is intriguing that in European-Americans, single-SNP association at rs555018 is not seen; however, when rs16969968 is included as a covariate, rs555018 does significantly alter risk. Joint genotype analysis clarifies that in the presence of "GG" at rs16969968, the major allele "T" at rs555018 seems to confer protection in European-Americans; however, in African-Americans, the minor allele "C" at rs555018 is more frequent in controls. These contrasting effects are difficult to interpret, but suggest that mRNA levels of *CHRNA5*, and thus nicotine dependence risk, may be influenced by additional variants in the region.

The two findings—one at rs16969968 and one at expression-associated SNPs such as rs588765—have the appealing property of being at least LD proxies for functional effects (11, 33, 34), if not the functional SNPs themselves. It is plausible that these SNPs are responsible for two biological mechanisms affecting nicotine dependence risk: a coding change at *CHRNA5* to asparagine (regardless of mRNA level) at rs16969968, and altered brain mRNA expression of the common (aspartate) form of *CHRNA5*, tagged by rs588765.

Our third important result involves rs578776 and its correlates. These SNPs represent an established association in populations of European-descent that has only a low correlation with rs16969968 ($r^2 < 0.25$), and the minor allele of which is elevated in controls. In our sample, rs578776 is associated with nicotine dependence in European-Americans, as expected, but is not associated in African-Americans. Furthermore, we detect significant heterogeneity in the ORs for rs578776 and some correlates ($P \sim 0.01$). These results suggest that those SNPs are not the biologically causal variants explaining the statistical association. Recently, rs6495309 and other SNPs correlated with rs578776 in HapMap CEU, but not in HapMap JPT and CHB (35), were associated with lung cancer and smoking in a Chinese population (36).

Our next key observation is that less common SNPs seem to be associated with nicotine dependence, suggesting that rarer variants in this gene cluster may have strong effects on susceptibility. In European-Americans, rs12914008 (MAF = 5%) is associated with nicotine dependence ($P = 0.035$; OR, 0.73; 0.54–0.97). In the African-American sample, this SNP is very rare (0.6%) and affords little power to detect association, although in the combined analysis, the evidence improves ($P = 0.022$; OR, 0.71; 0.53–0.95). This SNP causes a missense change in *CHRNB4* (threonine to isoleucine, T91I) and is also in LD ($r^2 = 0.8$ in CEU) with rs8192475, a nonsynonymous SNP in *CHRNA3* (arginine to histidine, R37H). We also identified a novel SNP that is nominally associated with nicotine dependence in the full sample and has a frequency of 6% in European-Americans and 1% in African-Americans. These rarer variants are intriguing. Although we cannot conclude that these SNPs represent additional independent risk factors, they hint that this region may play a more complex biological role in risk for nicotine dependence than will be explained by common variants.

A major question is whether the multiple SNP associations between *CHRNA5-CHRNA3-CHRNB4* and nicotine dependence represent the effect of a single causal locus, or whether multiple, statistically distinct loci are involved. Our data lead us to believe that there are at least two distinct, biologically causal loci in this region. In our analyses, the association at rs578776 in European-Americans is not explained by the association at rs16969968. Similarly, the association at rs555018 in African-Americans is not completely explained by rs16969968. The high $|D'|$ between these loci, however, does not allow us to determine the effect of each locus in the presence of all possible genotypes at other loci.

Rs16969968 is nonsynonymous, functional, and a strong candidate to be a causal allele. It remains unclear what biologically causal variant might explain a potential "second" distinct locus, and we are unable to fully exclude the possibility that a single causal variant underlies all observed signals. Interestingly, our evidence for multiple common nicotine dependence risk alleles in the same gene region is similar to other recent examples of multiple independent loci for other common diseases (37, 38).

In the majority of SNPs examined, allele frequencies differ significantly between European-Americans and African-Americans. Therefore, it is important to study associated variants that seem to be less common because of their potential for higher frequency in certain geographic or racial groups, and therefore, of higher impact on a population level.

Our results argue for further examination of this gene cluster, including DNA sequencing and variant discovery, especially in subjects of African descent. Such studies may reveal additional variants that affect nicotine dependence risk or *CHRNA5* mRNA expression, and thereby further explain some of the observed contrasts between the two populations.

In summary, rs16969968 represents a risk locus for nicotine dependence in African-Americans as well as in European-Americans. We have evidence of a potentially complex role for gene expression–associated SNPs such as rs555018 and rs588765 in the two populations. The "protective" association at rs578776 and its correlates is seen in European-Americans but not in African-Americans. Less common variants such as the novel SNP in the *CHRNA5-CHRNA3* overlapping region also are associated with risk. Overall, multiple variants in this gene cluster are contributing to nicotine dependence risk and therefore to risk for diseases for which smoking is a major contributing factor.

Published OnlineFirst August 25, 2009; DOI: 10.1158/0008-5472.CAN-09-0786

Disclosure of Potential Conflicts of Interest

L.J. Bierut, A.M. Goate, A.L. Hinrichs, J.P. Rice, S.F. Saccone, and J.C. Wang are listed as inventors on a patent "Markers of Addiction" held by Perlegen Sciences, Inc., covering the use of certain SNPs, including rs16969968, in diagnosing, prognosing, and treating addiction. N.L. Saccone is the spouse of S.F. Saccone, who is listed on the above patent. L.J. Bierut has served as a consultant to Pfizer. D. Hatsukami has a pending grant from NabiBiopharmaceuticals to conduct a clinical trial with a nicotine vaccine. The other authors disclosed no potential conflicts of interest.

Acknowledgments

Received 3/2/09; revised 5/15/09; accepted 6/12/09; published OnlineFirst 8/25/09.

Grant support: P01 CA89392 (L.J. Bierut) from the National Cancer Institute, R01DA013423 (L.J. Bierut), R01DA019963 (L.J. Bierut), K02DA021237 (L.J. Bierut), R03 DA023166 (R.C. Culverhouse), and K01DA024722 (S.F. Saccone) from the National Institute on Drug Abuse, K01 AA015572 (A.L. Hinrichs) from the National Institute on Alcohol Abuse and Alcoholism, K25 GM69590 (R.C. Culverhouse) from the National Institute of General Medical Sciences, and IRG-58-010-50 from the American Cancer Society (S.F. Saccone).

COGEND is a collaborative research group and part of the NIDA Genetics Consortium. Subject collection was supported by CA89392 (COGEND, PI—L.J. Bierut) from the National Cancer Institute. Genotyping of the original COGEND sample at Perlegen Sciences was performed under NIDA Contract HHSN271200477471C; additional genotyping was provided by the Center for Inherited Disease Research. The Center for Inherited Disease Research is fully funded through a contract from the NIH to the Johns Hopkins University (HHSN268200782096C). Genotyping of the expanded sample was carried out by the Microarray Core at the Washington University Genome Center and Dr. Alison Goate's laboratory at Washington University. Phenotypic and genotypic data are stored in the NIDA Center for Genetic Studies.[12] The authors thank Heidi Kromrei and Tracey Richmond for data collection assistance and Nanette Rochberg for data management assistance. We are grateful to Gary Chase for his contributions to COGEND. This work is in his memory, and in memory of Theodore Reich, founding Principal Investigator of COGEND.

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Cancer Res 2009;69:6848-6856. Published OnlineFirst August 25, 2009.