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A Novel Permutation Testing Method Implicates Sixteen Nicotinic Acetylcholine Receptor Genes as Risk Factors for Smoking in Schizophrenia Families

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Key Words
Acetylcholine · Linkage · Nicotinic receptor · Linkage · Genetics · Permutation testing · Candidate genes

Abstract
Smoking is a common correlate of schizophrenia, which leads to medical morbidity. Although twin and adoption studies have consistently implicated genes in the etiology of both smoking and schizophrenia, finding genes has been difficult. Several authors have suggested that clinical or neurobiological features associated with schizophrenia, such as smoking, might improve the ability to detect schizophrenia susceptibility genes by identifying genes related to the etiology of that feature. The objective of this study is to assess evidence for linkage of sixteen nicotinic acetylcholine receptor genes and smoking in schizophrenia families, using data from the NIMH Genetics Initiative for schizophrenia. Sixteen nicotinic acetylcholine receptor genes were selected prior to analysis. We used a multipoint sibling pair linkage analysis program, SIBPAL2, with a smoking trait in schizophrenia families. The significance of the group of candidate genes, in addition to each individual candidate gene, was assessed using permutation testing, which adjusted for multiple comparisons. The group of genes showed significant linkage to the smoking trait after adjusting for multiple comparisons through permutation testing ($p = 0.039$). In addition, two of the individual candidate genes were significant (CHRNA2, $p = 0.044$) and (CHRNB2, $p = 0.015$) and two genes were marginally significant (CHRNA7, $p = 0.095$; CHRNA1, $p = 0.076$). The significance of the complex hypothesis, involving sixteen genes, implicates the nicotinic system in smoking for schizophrenic families. Individual gene analysis suggests that CHRNA2 and CHRNB2 may play a particular role in this involvement. Such findings help prioritize genes for future case control studies. In addition, we provide a novel permutation method that is useful in future analyses involving a single hypothesis, with multiple candidate genes.

Introduction

Twin and adoption studies have consistently implicated genes in the etiology of schizophrenia, and molecular genetic studies have found some support for linkage to ten regions of the genome: 1q21.3, 5q11.2-q13.3, 6q13-

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q26, 6p23, 8p21, 11q14-q21, 13q32, 15q14, 18p, 22q11-q13 [Tsuang et al., 1999]. Despite those encouraging results, none of the molecular genetic findings have been consistently replicated, and none have led to gene discovery. For that reason, novel approaches to molecular genetic studies of complex disorders have received increasingly widespread attention [Ott, 1990; Risch, 1990; Faraone et al., 1999; Tsuang and Faraone, 2000].

In this paper, we present a novel approach to the genetics of schizophrenia. This approach incorporates three features: (a) the use of a refined phenotype that has the potential for improving the power to detect linkage to schizophrenia using a correlated phenotype; (b) the a priori specification of a complex hypothesis based on a system of candidate genes believed to be relevant to the phenotype, and (c) the use of a permutation method that corrects for the multiple comparisons inherent in the complex hypothesis, allowing a test of the aggregate set of genes as well as gene-specific significance.

As we have discussed elsewhere [Tsuang et al., 1993; Faraone et al., 1995, 1999], many authors have attempted to redefine the schizophrenia phenotype to make it suitable for genetic studies. For the present work, we chose the phenotype of smoking intensity in schizophrenia families to identify a genetic etiology in schizophrenics related to smoking intensity. We did this because the literature, as described below, suggests a meaningful complex hypothesis for a linkage study of smoking in schizophrenic families. By 'meaningful complex hypothesis' we mean a hypothesis that specifies several candidate genes known to be biologically related to one another and plausibly implicated in the disease or trait being studied.

A complex hypothesis for the relationship between smoking and schizophrenic families derives from the work of Freedman et al. [1997]. In these families, they found linkage between markers on 15q14 and a measure of sensory gating known to be abnormal among schizophrenic patients and their non-schizophrenic relatives [Waldo et al., 1991]. Freedman et al.'s strongest evidence for linkage (LOD = 5.3, $p < 0.001$) was for marker D15S1360, which is physically close to the α-7 nicotinic acetylcholine receptor (nAChR) subunit gene (CHRNA7). Additional studies using 15q14 markers have implicated CHRNA7 in schizophrenia using linkage [Coon et al., 1994; Riley et al., 1997; Kaufmann et al., 1998; Leonard et al., 1998; Stober et al., 2000; Gejman et al., 2001; Liu et al., 2001; Tsuang et al., 2001] and linkage disequilibrium methods [Riley et al., 2000; Stassen et al., 2000; Freedman et al., 2001; Xu et al., 2001]. In contrast, several studies have not confirmed these results [Moises et al., 1995; Pulver et al., 1995; Faraone et al., 1998; Levinson et al., 1998; Neves-Pereira et al., 1998; Curtis et al., 1999; Hovatta et al., 1999; Ekelund et al., 2000; Gurling et al., 2001].

Additional evidence suggesting that nAChR dysfunction may mediate susceptibility to smoking in schizophrenia is as follows: (a) schizophrenic patients have a very high prevalence of nicotine dependence [Leonard et al., 1996; Adler et al., 1998]; (b) nicotine normalizes sensory gating deficits in schizophrenic patients [Adler et al., 1993] and their non-schizophrenic relatives [Adler et al., 1992]; (c) schizophrenic patients have decreased numbers of hippocampal nicotinic receptors [Freedman et al., 1995]; (d) smoking transiently decreases negative symptoms [Smith et al., 2002]; (e) nicotine nasal spray modestly improve some aspects of cognitive function in schizophrenia [Smith et al., 2002], and (f) nicotine improves continuous performance task and smooth pursuit eye movements, both of which are known to be impaired among schizophrenic patients and their relatives [Depatie et al., 2002; Sherr et al., 2002].

The idea that nAChR dysfunction is involved in the etiology of smoking in schizophrenic families suggests a complex candidate gene hypothesis: that a system of genes relevant to nAChR functioning predisposes to smoking in schizophrenia. To see if this was a testable hypothesis, we searched internet databases for all genes expressed in brain that coded for proteins relevant to nAChRs. As table 1 shows, most of these code for additional nAChR subunits. Each nAChR is constituted from five subunits that create a ligand-gated ion channel [Paterson and Nordberg, 2000]. The various subunits combine in groups of five to create many subtypes of nAChRs. Of the sixteen subunits known to be expressed in brain, fourteen have known chromosomal locations (table 1).

Our search identified two additional genes relevant to nAChR functioning. TMPO codes for Thymopoietin, a polypeptide linked to immune function, which inhibits nAChRs [Quik et al., 1989, 1991; Quik, 1992]; and RAPSN, that codes for receptor-associated protein of the synapse (which helps anchor nAChRs to the postsynaptic cytoskeleton [Quik et al., 1989]. If variants of nAChRs predispose schizophrenic families to smoking, then a sensible complex hypothesis follows, i.e., that the set of sixteen genes in table 1 should provide evidence for linkage to a smoking trait in families with schizophrenia.

The plausibility of linkage between the set of genes in table 1 and smoking in schizophrenia families is demonstrated in the literature. In addition to CHRNA7, two of the sixteen genes in table 1 are located in regions showing

Table 1. Genes relevant to the neuronal acetylcholine nicotinic receptor

Gene	Protein	Position	Previous schizophrenia findings	Previous smoking findings
CHRNB2	Beta 2 subunit	1q21.3	1q21-22	
CHRNA1	Alpha polypeptide 1	2q24-q32		
CHRND	Delta polypeptide	2q33-q34		
CHRNG	Gamma polypeptide	2q33-q34		
CHRNB3	Beta 3 subunit	8p11.2	8p	
CHRNA2	Alpha 2 subunit	8p21	8p21, 8p	
CHRNA6	Alpha 6 subunit	8p22	8p22, 8p	8p22, 8p23.1-23.2
RAPSN	Receptor-associated protein	11p11.2-p11.1		
CHRNA10	Alpha polypeptide 10	11p15.5		
TMPO	Thymopoietin	12q22		
CHRNA7	Alpha 7 subunit	15q14	15q11-14	15q13.1-14
CHRNA3	Alpha 3 subunit	15q24		15q26.2-26.3
CHRNA5	Alpha 5 subunit	15q24		15q26.2–26.3
CHRNB4	Beta 4 subunit	15q24		15q26.2–26.3
CHRNB1	Beta polypeptide 1	17p12-p11		
CHRNA4	Alpha 4 subunit	20q13.2-q13.3		

From: http://www3.ncbi.nlm.nih.gov/; http://bioinfo.weizmann.ac.il/cards/

some prior evidence for linkage to schizophrenia: CHRNA2 is located on 8p21–22, a region several linkage studies have implicated in schizophrenia [Gurling et al., 2001; Badner and Gershon, 2002]. But arguing against this idea is the work of Blaveri et al. [2001] who found no association between CHRNA2 alleles and schizophrenia in a case-control study. CHRNB2 is located at 1q21.3, in a region Brzustowicz et al. [2000] implicated in schizophrenia with a peak LOD score of 6.5. Hovatta et al. [1997] reported linkage to the same region, and schizophrenia has been associated with a balanced translocation between chromosomes 1 and 11, t(1;11)(q42.1;q14.3) [St. Clair et al., 1990; Millar et al., 2000].

Our complex hypothesis has also been informed by studies of smoking, which is well known to be elevated among schizophrenic patients [Stassen et al., 2000; de Leon et al., 2002] and shown by twin studies to be a heritable trait [Lundman, 1966; Crumpacker et al., 1979; Kaprio et al., 1982; Hopper et al., 1992; Heath and Martin, 1993; Edwards et al., 1995; Heath et al., 1995; True et al., 1997; Kendler et al., 1999]. In a study of twins discordant for schizophrenia, Lyons et al. [2002] showed that the unaffected co-twins of schizophrenic patients had higher rates of smoking than controls, which suggests smoking is influenced by familial vulnerability to schizophrenia, not just clinical schizophrenia per se.

These data suggest that identifying genes for smoking intensity in schizophrenic families may increase the understanding of the etiology that underlies schizophrenia, as some genes may influence both smoking and schizophrenia. Some evidence for this idea can be gleaned from molecular genetic studies of smoking. Table 1 shows five of the 16 nAChR-related genes are in regions that might contain susceptibility genes for smoking. Duggirala et al. [1999] reported a genome scan of smoking intensity (average cigarettes per day for one year) using the Collaborative Study on the Genetics of Alcoholism (COGA) data set. They found modest evidence for linkage at 15q26.2–26.3, which is near CHRNA5 and CHRNB4.

Another analysis of the COGA data set using two phenotypes (ever smoked and average number of packs per year) implicated additional loci. Both phenotypes showed modest evidence of linkage to 8p22–23, near CHRNA2 [Bergen et al., 1999]. Using a questionnaire-based measure of smoking intensity, Straub et al. [1999] found their strongest evidence for linkage to smoking at 2q14.1, but also reported that chromosomes 4, 10, 16, 17, and 18 deserved further study. Their study did not implicate any genes relevant to nAChRs.

These prior studies suggest that several genes relevant to nAChR functioning may be involved in smoking intensity in schizophrenic families. We tested this hypothesis

Table 2. Description of families

	European-American	African-American	Total
Number of families	27	18	45
Total genotyped	57	41	98
Age at interview, mean (SD)	44.7 (15.4)	39.7 (10.4)	42.6 (13.7)
Males	36	20	56
Females	21	21	42
Affected siblings			
2	24	14	38
3	3	3	6
4	0	1	1

with a genome scan of cigarette smoking using families from the NIMH Genetics Initiative Collection, originally ascertained for studies of schizophrenia [Cloninger et al., 1998; Faraone et al., 1998; Kaufmann et al., 1998]. We hypothesized that the genes in table 1 would show significant linkage to smoking in schizophrenia pedigrees.

Methods

Subjects

Probands affected with either DSM-III-R schizophrenia or schizoaffective disorder, depressed type, were identified by systematic screening of patients in psychiatric hospitals and clinics at Washington University, Harvard University, and Columbia University. If a case had at least one living first-degree relative with either disorder, the family was retained for further examination. Families were excluded if both parents were schizophrenic. We included all available first-degree relatives of the probands and then all first-degree relatives who had schizophrenia, schizoaffective disorder, depressed, delusional disorder, manic subtype of schizoaffective disorder, brief reactive psychosis, schizophreniform disorder, psychosis not otherwise specified and schizotypal personality disorder. The diagnostic distribution has been described by Cloninger et al. [1998]. Table 2 gives the number of families, number of family members, and demographics for the families used in the analysis. There are a total of 80 schizophrenic individuals, 14 schizoaffective individuals, and 4 individuals with other psychiatric diagnoses.

Diagnostic and Smoking Assessment

The structured interview was the Diagnostic Interview for Genetic Studies (DIGS) [Nurnberger et al., 1994; Faraone et al., 1996]. Test-retest reliabilities of DIGS-based diagnoses were shown to be excellent within sites and across sites [Nurnberger et al., 1994; Faraone et al., 1996]. We supplemented structured interview data by medical records and a semi-structured itemized assessment of psychopathology in family members, called the Family Instrument for Genetic Studies (FIGS). Best estimate diagnoses were made by two experienced psychiatrists or psychologists based on all available information. If there was any disagreement between the two independent clinicians, a third diagnostician was used as a tiebreaker.

The DIGS quantified smoking with the question, 'have you ever smoked cigarettes on a daily basis?' Those who responded affirmatively were next asked to estimate the average number of packs per day they smoked and the number of years they had smoked. Subsequently, we computed the number of 'pack years' as follows: the number of packs smoked per day was multiplied by the number of years of smoking and that quantity was divided by age. Pack years is a commonly used method of retrospectively assessing the cumulative intensity of smoking [e.g. Giles et al., 2001; Hernan et al., 2001; Hittelman, 2001]. Bernaards et al. [2001] found a high Cohen's kappa coefficient (0.79) between prospectively and retrospectively assessed pack years. We transformed pack years to be normal by taking its square root.

People who never smoked have ambiguous phenotypes: Because they never engaged in smoking, they did not have the opportunity to become addicted. For that reason, it would not make sense to include them in an analysis of smoking intensity. Because the DIGS does not ask the question 'Have you ever smoked?', we used negative responses to the question 'Have you ever smoked cigarettes on a daily basis' to exclude individuals. Although some information might have been lost regarding probands who were occasional smokers, it provided a conservative approach to the analysis of these data.

Due to the limited number of unaffected individuals, there was no significant difference in the smoking trait between those affected with schizophrenia, schizoaffective disorder, or neither one of these conditions ($p > 0.99$).

Genotyping and Genetic Map

Details of the genotyping and genetic map creation are described in detail elsewhere [Cloninger et al., 1998]. The genome scan was conducted by Millennium Pharmaceuticals using 459 markers spaced at an average of 10 c*M* intervals. The markers were selected from the CHLC-6 set and supplemental markers were added from the Genethon map. The markers were di-, tri-, and tetra-nucleotide repeats that can be reliably scored using automated methods. The polymerase chain reactions (PCR) were set up with 5.0 microliter genomic DNA (4 ng/µl), 5.05 ul primer cocktail, and 4.95 µl Taq cocktail. The PCR cycling was 95 °C for 5 min, (95 °C for 30 s, 55 °C 30 s, 72 °C 60 s) for 30 cycles, 72 °C for 10 min. The gels were run on Applied Biosystems (ABI) 377 DNA sequencers using ABI Prism* 377 data collection software. The data were analyzed with the ABI Prism* GeneScan* 2.0.2 with Genotyper 1.1.1. The program CRIMAP was used to create the genetic map using the Kosambi mapping function.

Candidate Gene Selection

Prior to the linkage analysis, we identified all genes expressed in the brain that coded for proteins relevant to nAChRs. The specific genes included in this analysis are provided in table 1. Genes were identified through online searches of the gene card database in Genebank (http://bioinfo.weizmann.ac.il/cards/) and the Online Mendelian Inheritance in Man (OMIM) (http://www3.ncbi.nlm.nih.gov/). Keywords typed into these databases include 'nicotinic' 'receptor' 'cholinergic'. Of the identified genes, exclusion criteria included genes either (1) involved in 'muscarinic' or (2) genes not localized to a specific chromosomal region. Of the identified genes, four were related to 'muscarinic' and two did not have specific chromosome loca-

tions (cholinergic receptor, nicotinic, epsilon polypeptide and cholinergic receptor, neuronal nicotinic, alpha polypeptide 9).

Linkage Analyses

After the candidate genes were identified, a linkage analysis was performed on the genetic data. Estimates of the marker allele frequencies were calculated for European-American and African-American families separately to account for locus heterogeneity that was found previously in this sample [Cloninger et al., 1998a]. The GENIBD program from the Statistical Algorithms for Genetic Epidemiology (SAGE), beta version 7.0 was then used to estimate the multipoint identity by descent (IBD) allele sharing. A combined linkage analysis using the respective IBD information would only be appropriate if the variance of the smoking quantitative trait was homogeneous across the two ethnic groups, which was the case for our data as indicated Bartlett's test ($\chi^2_{[1]} = 0.074$, $p = 0.79$). The variance estimate was 0.65 for the African-American sample and 0.71 for the European-American sample. When racial groups are analyzed in the combined analysis, each ethnic sample contributes proportionally to its sample size in the linkage analysis as well as subsequent analyses. The combined linkage analysis was performed on the smoking phenotype and concatenated allele sharing IBD matrix using SIBPAL2 from SAGE, beta version 7.0. This method implements an enhanced multipoint version of the Haseman-Elston linkage method. In SIBPAL2, the smoking trait is modeled using linear regression in full siblings as a function of IBD sharing based on multipoint IBD information [Elston et al., 2000]. The default trait regression option, mean corrected smoking trait cross products, was used as the dependent variable in the Haseman-Elston regression. This method allows separate marker allelic heterogeneity to exist between the two ethnicities, while increasing the power to find any common genetic effects. In addition, there were no gender differences in the smoking trait ($p = 0.66$), therefore it was not necessary to make adjustments to the sibling-pair correlations in the linkage analysis. Linkage analysis using SIBPAL2 was run on the European-American, African-American, and combined samples.

Permutation Test Conditional on Genome Scan Results

This permutation testing method was applied to the combined ethnic sample. The genome scan provided multipoint p values for a total of 2,202 consecutive 2 c*M* intervals throughout the genome. For each candidate gene we selected the p value from the 2 c*M* bin in which the gene was located. Because four of the candidate genes were located in the same c*M* bin, there were only 12 independent p values that could be derived from the 16 candidate genes. These 12 observed p values constitute our observed data. The goal of the permutation testing was to determine the probability of observing these p values by chance alone, correcting for the number of locations being examined. The four steps needed for implementing the method are as follows:

Step 1: All of the p values generated at 2 c*M* intervals by the genome scan formed the pool of p values for the permutation test. From this pool, 12 p values were randomly selected. This procedure was repeated 25,000 times, resulting in 25,000 sets of 12 p values.

Step 2: We selected the lowest p value from each of the 25,000 sets of 12 p values. We defined this group of 25,000 p values as the empirical distribution for the lowest observed p value. We then selected the second lowest p value from each of the 25,000 sets of 12 p values. We defined this group of 25,000 p values as the empirical distribution for the second lowest observed p value. In like manner, we defined 10 more distributions for the third lowest p value, fourth lowest p value and so forth.

Step 3: To assess the statistical significance of the lowest p value from the set of 12 p values associated with our candidate genes, we determined at what point that p value was located on our empirical distribution for the lowest observed p value. From that distribution, we calculated the probability of observing a p value of that magnitude or smaller. That probability was used as the empirical p value for the lowest p value. To assess the statistical significance of the second lowest p value from the set of 12 p values associated with our candidate genes, we determined at what point that p value was located on our empirical distribution for the second observed p value. From that distribution, we calculated the probability of observing a p value of that magnitude or smaller. That probability was used as the empirical p value for the second lowest p value. In like manner, we computed the empirical p values for the third lowest p value, the fourth lowest and so forth. If the empirical p value was less that 0.05, we concluded it was statistically significant.

The permutation results are conditional on the combined linkage analysis findings. After 12 locations throughout the genome were randomly selected for each iteration of the simulation, the observed p values at those locations were obtained from the combined genome scan. It is when the p values from the genome scan are selected that we condition on and genome scan results. Any linkage disequilibrium present in the linkage analysis is reflected in the obtained p value that is subsequently used in the permutation test. Therefore, through conditioning on the genome scan in the permutation test, existing chromosomal dependencies are accounted for in the permutation method.

Step 4: The goal of this step was provide a test of the joint significance of the 12 observed p values. For each of the 25,000 sets of 12 p values generated in step 2, we formed the product of the 12 p values. For each of the 25,000 sets, the product of these 12 p values can be interpreted as the joint probability of observing the 12 p values in the set. The result is one distribution of 25,000 joint probabilities. We then calculated the product of the set of 12 p values associated with our candidate genes. This product can be interpreted as the joint probability of observing the 12 p values not corrected for the number of p values used to form the product. To assess the statistical significance of the observed joint probability, we determined at what point that probability was located on our empirical distribution of 25,000 joint probabilities. From that distribution, we calculated the probability of observing a joint probability of that magnitude or smaller. That probability was used as the empirical p value for the joint probability of observing the 12 p values associated with our candidate genes. If it was less that 0.05, we concluded it was statistically significant.

In developing a global hypothesis test, low type I error and high power are important with respect to some alternative statistical tests. The type I error of the global hypothesis test is sufficient due to the nature of developing empirical distributions using permutation tests. To maximize power, the product of the p values is an ideal choice. When developing a global hypothesis, it is likely that not all candidate genes in the tested set will actually be susceptibility loci. That is, it is likely that we are under an alternative hypothesis for a subset of the nicotinic receptors while some of the genes are not genetically important. A test based on a multiplicative rather than an additive approach would be more likely to retain some power in this situation.

Results

The empirical p values, along with the physical and genetic locations for the 16 candidate genes are given in table 3. After using permutation testing to account for multiple comparisons, we found the group of sixteen nicotinic acetylcholine receptor genes to be significantly linked to smoking in schizophrenia families (p = 0.039). Two genes in the list of candidate genes were individually significant: CHRNA2 (p = 0.044) and CHRNB2 (p = 0.015). In addition, CHRNA7 and CHRNA1 were marginally significant (p = 0.095, p = 0.076 respectively).

Table 4 shows findings from the European-American, African-American, and combined genome scans having a significance level of 0.01 or less. These p values are not corrected for multiple testing and are presented only to show all regions showing potential linkage to smoking in our schizophrenia families. We included the results of the genome scan for purposes of completeness only. The genome scan and its significance as far as the peak LOD score are not part of the candidate gene hypothesis of this paper. These findings should not impact any of the statements and conclusions made with regard to the permutation test.

Table 3. Tests of genes relevant to the neuronal acetylcholine nicotinic receptor

Gene	Position	c*M*	Nominal p values	Empirical p values
CHRNA2	8p21	45	0.004	(1) 0.044
CHRNA7	15q14	14	0.027	(2) 0.095
CHRNB2	1q21.3	165	0.048	(3) 0.015
TMPO	12q22	128	0.132	(4) 0.646
CHRNB1	17p11-p12	23	0.335	(5) 0.217
CHRNA4	20q13.2-20q13.3	110	0.365	(6) 0.251
CHRNB3	8p11.2	65	0.411	(7) 0.219
CHRNA6	8p11.2	65	0.411	(7) 0.219
CHRNA10	11p15.5	4	0.506	(8) 0.198
CHRND	2q33-q34	247	0.566	(9) 0.166
CHRNG	2q33-q34	247	0.566	(9) 0.166
CHRNA1	2q24-q32	177	0.589	(10) 0.076
RAPSN	11p11.1-11p11.2	70	0.866	(11) 0.549
CHRNA3	15q24	80	0.990	(12) 0.961
CHRNA5	15q24	80	0.990	(12) 0.961
CHRNB4	15q24	80	0.990	(12) 0.961

Note: All p values are empirically derived as described in the Methods. Thus, they are corrected for multiple comparisons. Numbers in parentheses indicate the rank of each gene's p value from the SIBPAL2 analyses.

Discussion

Results of Hypothesis Testing

From this study, conclusions can be made both from the complex hypothesis involving sixteen genes, and the individual hypotheses for each gene separately. The significance of the complex hypothesis suggests that this group of sixteen genes may influence cigarette smoking in schizophrenia families. This complex hypothesis does not involve a single gene, rather it identifies an unlikely pattern in a group of genes that are all major components of the nicotinic system, therefore implicating the system in

Table 4. Markers showing p values <0.01

Chromosome	Ethnicity	Closest marker to peak	Peak c*M*	Maximum LOD score	p value
6	EA	D6S470	8	1.53	0.008
8	Combined	D8S258	40	1.91	0.003
8	EA	D8S258	37	2.34	0.001
8	Combined	D8S557	166	1.58	0.007
12	AA	D12S84	154	1.75	0.005
13	Combined	D13S173	107	1.77	0.004
13	EA	D13S779	82	1.91	0.003
15	EA	D15S128	8	1.86	0.003
16	EA	D16S516	90	2.25	0.001
17	EA	D17S122	48	1.58	0.007

Note: The p values are as computed by SAGE. They are not empirical p values and are not corrected for multiple comparisons.

the smoking found in schizophrenic families. The individual gene analysis further targets specific genes that may have particular involvement, those being CHRNA2, CHRNB2, and possibly CHRNA7 and CHRNA1. Although more research is necessary, the results for CHRNA7 are compelling because this gene has been implicated in schizophrenia and sensory gating deficits by several prior studies [Coon et al., 1994; Freedman et al., 1997, 2001; Riley et al., 1997, 2000; Leonard et al., 1998; Stassen et al., 2000; Stober et al., 2000; Gejman et al., 2001; Liu et al., 2001; Tsuang et al., 2001; Xu et al., 2001]. Both the complex and individual analyses helps to further prioritize genes for case control studies relating to smoking in schizophrenics.

Results of Genome Scan

For completeness, we also presented the most significant results from the genome scan, regardless of their relationship to the candidate genes that are the primary interest of this study. Our highest peak LOD score (2.34) was found for the European-American sample on Chromosome 8 for marker D8S258. That marker is in the 8p21–22 region that contains CHRNA2. This region has also been linked to schizophrenia by several genome scans [Pulver et al., 1995, 2000; Kendler et al., 1996, 2000; Blouin et al., 1998; Kaufmann et al., 1998; Brzustowicz et al., 1999; Gurling et al., 2001], and one other genome scan of smoking implicated that region as well [Bergen et al., 1999].

In the combined sample, we found a peak LOD score of 1.77 at marker D13S173, which is near the 13q32 locus implicated in schizophrenia. Lin et al. [1995] studied families from the United Kingdom and Japan and reported a maximum LOD score of 1.62 for marker D13S119. In an independent sample from the UK, Lin et al. [1997] found a maximum LOD score of 1.72 at marker D13S128. Blouin et al.'s [1998] genome scan of 54 schizophrenia families reported a maximum LOD score of 4.18 at marker D13S174. Brzustowicz et al. [1999], in a study of 21 Canadian families, reported a LOD score of 4.42 at marker D13S793. In the 166 schizophrenia families collected by the Veterans Affairs Cooperative Linkage Study of Schizophrenia, Faraone et al. [2002] reported a peak LOD score of 1.43 between markers D13S1241 and D13S159. Although there are some negative reports [Coon et al., 1994; Faraone et al., 1998; Kaufmann et al., 1998; Riley et al., 1998; DeLisi et al., 2000; Levinson et al., 2000; Mowry et al., 2000; Schwab et al., 2000; Gurling et al., 2001], it is notable that the peak LOD scores reported by the positive studies cluster in a region ranging from 65 to 95 c*M*. For the present analyses, our peak LOD score was nearby at 107 c*M*.

Methodological Implications

In addition to the substantive findings, our work also provides a new methodological approach for addressing the genetics of complex diseases and traits. Our method requires three parts: (a) the use of a refined phenotype that has the potential for improving the power to detect linkage; (b) a meaningful complex hypothesis and (c) a method to adjust for multiple comparisons using permutation testing. By 'meaningful complex hypothesis' we mean a hypothesis that specifies several candidate genes biologically related to one another and plausibly implicated in the disease or trait being studies. As discussed in the Introduction, genes relevant to nAChR functioning work within the same neural systems and are plausible involved in both smoking and schizophrenia.

We have also showed how the use of permutation tests can compute statistical significance for a complex hypothesis through adjustments of multiple comparisons. When we limited our tests to plausible candidate genes, we were able to demonstrate statistical significance for the group of genes, as well as two individual genes (table 3). In contrast, if we had simply presented a genome scan (table 4), our results would have been much more equivocal, as none of the findings reached genome-wide statistical significance through linkage analysis.

Limitations and Conclusions

Several limitations to our study should be considered in assessing the present outcome. The Diagnostic Interview for Genetic Studies did not include a thorough assessment of smoking. Other measures may have given a better correspondence with prior genome scans of smoking. But our method of defining smoking is unlikely to have created false positive results because the assessment was blind to genotype status.

Gene locations were identified to the closest 2 c*M* interval. Therefore the genetic locations for the 16 genes were only approximations to their actual locations and the genes themselves were not tested. Furthermore, it is possible that there were errors in creating the genetic map, either in the order of the markers or the assumed distances between markers.

Genes that can be identified through databases, which are relevant to the nicotinic acetylcholine receptor pathway, change with time. Therefore, there are likely relevant genes yet to be identified which were not included in this analysis. One must also consider that the marginal effects

of several of the genes involved in this pathway may not be detectable due to the limited sample size in this analysis. It is also possible that the subset of most significant genes analyzed in this paper account for the entire group effect.

Our negative results should be viewed cautiously given that genes predisposing to schizophrenia and smoking are likely to have small incremental effects on our smoking phenotype. Thus, our power to detect any one of these genes was small. If our sample was bigger, the variance around estimated parameters would decrease, making it easier to identify true effects. Other limitations to consider are the likely heterogeneity in schizophrenia, including the multi-factorial nature of the disease and environmental risk factors for the disease [Tsuang and Faraone, 1995]. With the specified shortcomings of this study, no conclusions should be based on these findings alone.

Despite these reservations, present results suggest that the issues explored here deserve further scrutiny with larger samples and a more precise definition of the phenotype of smoking. The results for CHRNA2, CHRNB2, CHRNA7, CHRNA1 are particularly compelling for three reasons. Three of the four genes are in regions implicated by prior genome scans of schizophrenia at 8p21, 1q21 and 15q15, respectively. Other schizophrenia studies have already implicated CHRNA7 [Coon et al., 1994; Riley et al., 1997, 2000; Kaufmann et al., 1998; Leonard et al., 1998; Stassen et al., 2000; Stober et al., 2000; Freedman et al., 2001; Gejman et al., 2001; Liu et al., 2001; Tsuang et al., 2001; Xu et al., 2001]. And, because nicotinic acetylcholine neurons are known to modulate dopaminergic transmission in the frontal-subcortical pathways implicated in schizophrenia [Mereu et al., 1987; Izenwasser et al., 1991], it is reasonable to hypothesize that dysregulation of the nicotinic system is a risk factor for schizophrenia. Given our results, those of other groups and the biologic plausibility of this system's involvement in schizophrenia, future research in this area is warranted.

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The National Institute of Mental Health Genetics Initiative for schizophrenia is a multi-site study performed by three independent research teams in collaboration with staff from the National Institute of Mental Health (NIMH). The NIMH Collaborators include David Shore, M.D., Debra Wynne, M.S.W., Steven O. Moldin, Ph.D., Darrell G. Kirch, M.D. [1989–1994], Kate A. Berg, Ph.D. [1990–1994], Nancy E. Maestri, Ph.D. [1992–1994]; the NIMH Senior Scientific Consultant is Darrel A. Regier, M.D., M.P.H. The Principal Investigators and Co-Investigators from the three sites are: Harvard University, Boston, Massachusetts, U01 MH46318, Ming T. Tsuang, M.D., Ph.D., D.Sc., Stephen Faraone, Ph.D., and John Pepple, Ph.D.; Washington University, St. Louis, Missouri, U01 MH46276, C. Robert Cloninger, M.D., Theodore Reich, M.D., and Dragan Svrakic, M.D.; Columbia University, New York, New York U01MH46289, Charles Kaufmann, M.D., Dolores Malaspina, M.D., and Jill Harkavy Friedman, Ph.D. Blood samples are sent to the NIMH Cell Repository at the Coriell Institute for Medical Research. Clinical data is stored in the NIMH Data Management Center at SRA Technologies, Inc.

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