Original Investigation

Examination of the Nicotine Dependence (NICSNP) Consortium findings in the Iowa adoption studies population

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Abstract

Introduction: Nicotine dependence results from a complex interplay of genetic and environmental factors. Over the past several years, a large number of studies have been performed to identify distinct gene loci containing genetic vulnerability to nicotine dependence. Two of the most prominent studies were conducted by the Collaborative Study of the Genetics of Nicotine Dependence (NICSNP) Consortium using both candidate gene and high-density association approaches.

Methods: We attempted to confirm and extend the most significant findings from the high-density association study and the candidate gene study using the behavioral and genetic resources of the Iowa Adoption Studies, the largest case–control adoption study of substance use in the United States.

Results: We found evidence that genetic variation at *CHRNA1*, *CHRNA2*, *CHRNA7*, and *CHRNA1* alters susceptibility to nicotine dependence, but we did not replicate any of the most significant single nucleotide polymorphism associations from the NICSNP high-density association study.

Discussion: Further examination of the NICSNP findings in other population samples is indicated.

Introduction

Approximately 27% of Americans use nicotine regularly, with approximately 24% meeting *Diagnostics and Statistical Manual of Mental Disorders, fourth edition (DSM-IV)* criteria for nicotine dependence at some point in their lives (Breslau, Johnson, Hiripi, & Kessler, 2001). Decades of twin and family studies have

demonstrated consistently that approximately 50% of total vulnerability to nicotine dependence results from heritable factors (True et al., 1999; Tsuang, Bar, Harley, & Lyons, 2001). Since smoking results in 400,000 deaths and \$157 billion in economic expenditures annually in the United States, the identification of the genetic architecture that initiates and maintains nicotine dependence is a high public health priority (Centers for Disease Control and Prevention, 2002).

In response to this challenge, a large number of genetic studies have attempted to identify gene loci containing variability that affects vulnerability to nicotine dependence. Before 2005, these reports consisted largely of candidate gene and linkage analyses (Li, 2006). Many of these studies were pivotal in advancing our understanding of the role of genetic variation in critical gene pathways, such as the cholinergic neurotransmission system, in altering vulnerability to nicotine dependence. However, these linkage and candidate gene studies were limited by either sample size or scale of genotyping.

In an attempt to transcend these problems, the National Institute on Drug Abuse funded a pair of large-scale association studies by a group of investigators in collaboration with Perlegen Sciences (Mountain View, CA) referred to as the Collaborative Study of the Genetics of Nicotine Dependence (NICSNP) consortium. This consortium first performed a high-density association case–control analysis of 482 nicotine dependence cases and 466 controls using a 2.4 million single nucleotide polymorphism (SNP) platform and a DNA pooling technique (Bierut et al., 2007). This was then followed by individual genotyping of the 39,213 SNPs showing the strongest evidence of association in a sample of 1,050 cases and 879 controls. In the second study, the same team conducted an association analysis of 348 of the leading candidate genes using the same population (Saccone

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doi: 10.1093/ntr/ntn034

Advance Access publication on March 23, 2009

Received March 13, 2008; accepted September 16, 2008

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et al., 2007). The former study reported 35 SNPs with suggestive evidence of association with nicotine dependence ($p < .0001$), found strong evidence for small effects being played by a number of cholinergic genes, and nominated *Neurexin 1* and *CHRNA3* as genes having a potentially critical role in nicotine dependence. The latter study identified more than 20 genes with significant evidence of association with a substantial portion of those genes coding for nicotinic receptors.

We attempted to confirm and extend the most significant of these findings into an epidemiologically sound population using the Iowa Adoption Studies, the largest case-control adoption study of substance abuse in the United States (Cadoret, Troughton, O'Gorman, & Heywood, 1986; Cadoret, Yates, Troughton, Woodworth, & Stewart, 1995; Yates, Cadoret, & Troughton, 1998), for the most significantly associated SNPs from the NICSNP Consortium's high-density association study and for every SNP from their genotyping of nicotinic receptors that were analyzed in the candidate gene analysis.

Methods

The methods and procedures of the Iowa Adoption Studies have been described extensively elsewhere (Yates et al., 1998). All procedures described here were approved by the University of Iowa Institutional Review Board for Human Subjects.

The behavioral data for this study were derived from interviews conducted during the past two waves of the study (1999–2004 and 2004–current) using an adapted version of the Semi-Structured Assessment for the Genetics of Alcoholism, Version II (Bucholz et al., 1994), a robust, widely used instrument that allows assessment of *DSM-IV* substance use dependence and a variety of other common behavioral illnesses. Using these data, we derived symptom counts for nicotine dependence (maximum score of 7) in this population using criteria from *DSM-IV* (American Psychiatric Association, 1994). Similarly, total scores for the Fagerström Test for Nicotine Dependence (FTND) Scale (maximum score of 10; Heatherton, Kozlowski, Frecker, & Fagerström, 1991) were determined using these data.

The first set of SNPs (see Supplementary Table 1) contained 33 of the 35 most significantly associated SNP variants from the 2006 high-density association analysis (Bierut et al., 2007). The second set of SNPs (see Supplementary Table 1) contained 129 polymorphisms from 21 candidate genes for nicotine dependence, including 15 nicotinic genes, from the candidate gene (Saccone et al., 2007) and high-density association analyses.

Genotyping for the present study was performed by Sequenom Inc. (San Diego, CA) using DNA prepared in our laboratory from whole blood using cold protein precipitation (Lahiri & Nurnberger, 1991) or from lymphoblast DNA provided from the National Institutes of Health Rutgers Repository. The resulting genotypic data were successively inspected for informativeness, successfulness, and conformance with Hardy–Weinberg equilibrium. Loci were excluded if they were monoallelic ($n = 5$), had less than 95% genotyping success ($n = 22$), or had Hardy–Weinberg p values of less than .01 (χ^2 , $n = 5$). In total, only 22 of the 33 SNPs (67%) from the NICSNP high-density association study and 115 of the 137 SNPs (84%) from the candidate gene study provided usable genotyping information.

The surviving 137 SNPs were then analyzed using an ordinal regression analysis and an additive genetic model to identify SNPs significantly associated with nicotine dependence. To maintain internal consistency with our previous publications, our primary data analyses were conducted using *DSM-IV* nicotine dependence counts. However, to make these results more consistent with those conducted by the NICSNP Consortium and more useful to all investigators, where appropriate, we have provided parallel regression analyses using the FTND data. Both these symptom counts were treated as ordinal variables. Where appropriate, intermarker disequilibrium between SNPs at each candidate gene locus was calculated using Haploview (Stephens, Smith, & Donnelly, 2001). Haplotypes for genes with at least one significantly associated SNP were inferred using PHASE (Stephens et al., 2001), as described previously (Bradley, Dodelzon, Sandhu, & Philibert, 2005). Haplotypes with frequencies greater than 0.10 were then incorporated as additive factors in ordinal regression analyses that sometimes included sex and nicotine exposure data, as described in the text, using JMP Genomics, SAS version 9.1, and the chi-square test. All test results reported are two-tailed.

Results

The demographic and clinical characteristics of the sample population are described in Tables 1 and 2. The sample is largely White and predominantly female. Consistent with intentional loading of the sample cohorts for the genetic diatheses for substance use, there are high levels of nicotine use. Some 90% of subjects reported smoking at least once in their lifetime and 51% reported smoking at least 100 cigarettes in their lifetime.

As a first step in our analyses, we conducted ordinal regression analyses of nicotine dependence symptom counts with respect to genotype at each of the 137 SNPs that survived quality assurance assessment using *DSM-IV* nicotine dependence symptom counts and an additive model (Philibert, Ryu, et al., 2007; Philibert, Sandhu, et al., 2007). In total, 12 SNPs were nominally associated with nicotine dependence ($p < .05$ before correction for multiple comparisons); all these were from the candidate gene analysis (Table 3). Six of these SNPs were from *CHRNA2*, three were from *CHRNA7*, three were from *CHRNA1*, and one was from *CHRNA1*. In general, the correlation between results obtained using *DSM-IV* symptom counts and our

Table 1. Subject demographics and characteristics

	Male	Female
Gender	231	285
Ethnicity		
Native American	0	1
White	216	271
Black	7	4
White, of Hispanic origin	6	5
Other or no answer	2	4
Mean age, years (<i>SD</i>)	47 (8)	45 (7)
Exposure		
Reported smoking at least once	208	254
Reported smoking ≥ 100 cigarettes	116	148

Table 2. *DSM-IV* nicotine dependence symptom counts and Fagerström Test for Nicotine Dependence (FTND) scores

	Nicotine dependence symptoms		FTND score	
	Male	Female	Male	Female
0	109	145	127	157
1	14	20	17	22
2	19	18	11	14
3	25	22	17	21
4	28	35	11	16
5	17	28	12	19
6	16	14	10	17
7	3	3	14	7
8			6	10
9			4	2
10			1	0

Note. DSM-IV, Diagnostic and Statistical Manual of Mental Disorders, fourth edition.

secondary analyses of FTND scores was quite good. Of the 12 nominally significant associations from the primary analysis (*DSM-IV* symptom count), 8 had at least a trend for an association when FTND scores were used. Conversely, only one SNP

Table 3. Gene loci examined in the study

Gene	Chr	Contig size (kb)	SNPs tested	SNPs failed	Source ^a
<i>ART1</i>	11	19	1	0	CG
<i>CHRNA1</i>	2	16.8	4	1	CG
<i>CHRNA2</i>	8	18.5	17	1	CG
<i>CHRNA3</i>	15	25.7	11	2	CG
<i>CHRNA4</i>	20	18.1	9	3	CG
<i>CHRNA5</i>	15	28.6	7	2	CG
<i>CHRNA6</i>	8	15.9	9	2	CG
<i>CHRNA7</i>	15	138.5	13	2	CG
<i>CHRNA9</i>	4	19.5	11	2	CG
<i>CHRNA1</i>	11	5.8	2	0	CG
<i>CHRNA1</i>	17	12.5	8	1	CG
<i>CHRNA2</i>	1	8.8	4	0	CG
<i>CHRNA3</i>	8	40.8	9	1	CG
<i>CHRNA4</i>	1	17	5	0	CG
<i>CHRNA5</i>	2	9.3	3	0	CG
<i>CHRNA6</i>	2	6.6	5	0	CG
<i>FGF11</i>	1	5.6	1	0	CG
<i>MINK</i>	17	58.5	5	0	CG
<i>NRXN</i>	2	1108.1	8	5	HDA
<i>NUP9</i>	11	122.7	1	0	CG
<i>ZBTB</i>	17	24.8	1	0	HDA

Note. Chr, chromosomal localization; contig size, the region of chromosome covered by the single nucleotide polymorphisms (SNPs). SNPs tested are the number of SNPs from each locus genotyped by Sequenom, Inc. SNPs failed denote the number of SNPs from that locus that failed quality control testing. Some of the 33 HDA SNPs also map to within 10 kb of the coding section of a gene. These localizations are noted in Supplementary Table 1.

^aCG, candidate gene study; HDA, high-density association study.

(rs1031006, $p < .03$) was significantly associated when using FTND criteria but not when using *DSM-IV* criteria. The complete results for each of these analyses for all the SNPs are given in Supplementary Table 1.

We then inferred haplotypes for each of the genes that contained at least one SNP that was nominally significantly associated with nicotine dependence. The major haplotypes, their frequencies, and a disequilibrium map for each of the four loci analyzed (*CHRNA2*, *CHRNA7*, *CHRNA1*, and *CHRNA1*) are given in Supplementary Figure 1.

To reduce the number of false positives occurring as a result of multiple testing, we analyzed the haplotype outputs and determined which haplotypes contained our “risk variants” (Table 4). These haplotypes were then termed “risk haplotypes.” This was easily accomplished for two of the genes. First, for *CHRNA7*, all three variants were found on a single haplotype whose total frequency was 0.529. Second, for *CHRN1*, both SNP risk variants were found on a single haplotype with a frequency of 0.131. However, this approach was more problematic for *CHRNA1* and *CHRNA2*. For *CHRNA1*, the risk variant was found on four different haplotypes whose totaled frequency was 0.86. Therefore, we regressed each of the three common haplotypes (frequency greater than 0.10) with respect to the symptom counts for the various substance use disorders. For *CHRNA2*, our approach was problematic because six “risk” SNPs were spread across two distinct haplotype blocks. However, because one of these SNPs was nearly monomorphic (rs1211756) and rs1346726 was not in tight linkage disequilibrium with either haplotype block, we only used the data from rs2292974, rs2292975, rs2565061, and rs2472553 to define a single risk haplotype block with a frequency of 0.113.

We then conducted regression analyses with respect to nicotine dependence symptom counts using sex and exposure data as covariates (Table 5). In addition, based on our prior findings

Table 4. SNPs with significant associations

SNP	Gene	Risk allele	Ancestral allele	Frequency ¹	ND ^{all}	FTND ^{all}
rs1376866	<i>CHRNA1</i>	T	C	<.75	<.03	<.09
rs2565061	<i>CHRNA2</i>	A	G	<.13	<.02	<.02
rs2472553	<i>CHRNA2</i>	T	C	<.12	<.04	<.05
rs12114756	<i>CHRNA2</i>	G	G	<.01	<.05	<.18
rs2292974	<i>CHRNA2</i>	C	C	<.51	<.03	<.001
rs2292975	<i>CHRNA2</i>	C	C	<.52	<.05	<.004
rs1346726	<i>CHRNA2</i>	G	T	<.03	<.05	<.17
rs904952	<i>CHRNA7</i>	C	T	<.44	<.02	<.03
rs10438287	<i>CHRNA7</i>	A	A	<.77	<.03	<.07
rs12915265	<i>CHRNA7</i>	T	T	<.78	<.03	<.10
rs3855924	<i>CHRNB1</i>	C	T	<.13	<.05	<.27
rs4796418	<i>CHRNB1</i>	G	C	<.13	<.05	<.28

Note. SNP, single nucleotide polymorphism; FTND, Fagerström Test for Nicotine Dependence; ND, nicotine dependence. ¹Frequency refers to the frequency of the risk allele; ND^{adj} and FTND^{adj} values are the *p* values from the uncorrected single-point association analysis that uses data from all 515 subjects and nicotine dependence as assessed by DSM-IV or the FTND criteria, respectively.

Table 5. Ordinal logistic regression of haplotype and symptom count data

Locus	Haplotype frequency		Model		
<i>CHRNA1</i>			ND ^{all}	ND ¹	ND ¹⁰⁰
	H1 0.457	Male	<.93	<.78	<.69
		Female	<.51	<.80	<.90
		Combined	<.57	<.68	<.41
	H2 0.374	Male	<.90	<.73	<.43
		Female	<.15	<.09	<1.00
		Combined	<.36	<.33	<.55
	H3 0.106	Male	<.83	<.49	<.70
		Female	<.02 ^a	<.002 ^a	<.05 ^a
Combined		<.04 ^a	<.003 ^a	<.07	
<i>CHRNA2</i>	Block 1				
	H4 0.113	Male	<.71	<.51	<.28
		Female	<.03 ^a	<.01 ^a	<.36
Combined		<.05 ^a	<.07	<.94	
<i>CHRNA7</i>	H1 0.529	Male	<.01 ^a	<.005 ^a	<.55
		Female	<.53	<.48	<.71
		Combined	<.02 ^{a*}	<.01 ^a	<.80
<i>CHRNB1</i>	H4 0.131	Male	<.19	<.23	<.35
		Female	<.24	<.28	<.10
		Combined	<.08	<.11	<.05 ^a

Note. Model refers to the exposure analytic model as described in the Methods and the Results. H1, H2, H3, and H4 refer to the haplotypes for each gene which are given in Supplementary Figure 1.

^aNominally significant findings.

at *GABRA2*, we used three different sets of exposure criteria (Philibert et al., in press). The first, referred to as ND^{all}, used data from all subjects including those who reported that they had never smoked a cigarette. The second, ND¹, was determined by the answer to the question "Have you ever smoked?" The criterion for the third, most stringent model, referred to as ND¹⁰⁰, was the question "Have you smoked at least 100 cigarettes in your lifetime?"

The risk haplotype for *CHRNA2*, H4, was significantly associated with nicotine dependence risk in the female subjects using the ND^{all} and ND¹ models. However, the H4 haplotype was not associated with risk for the males under any condition and was significant for the combined sample when the ND^{all} model was used. A trend for association was observed when the ND¹ model was used.

In contrast, the risk haplotype for *CHRNA7*, H1, was significant for the males and the combined sample of subjects when either the ND^{all} or the ND¹ model was applied. However, we found no significant effects in the female subjects under any conditions.

The risk haplotype for *CHRNA1* was associated with nicotine dependence only when the most stringent model (ND¹⁰⁰) was used in the analysis; a trend for association was found when no exposure data were considered using the entire sample. In gender-specific analyses, none of the comparisons were significant.

Because of the complexity noted for at the *CHRNA1* locus, we analyzed all three major haplotypes (frequency > 0.10) at

this locus. The H3 haplotype, which contains the ancestral "C" variant as opposed to the risk "T" variant, was significantly associated with nicotine dependence under the two lower exposure model analyses (i.e., ND^{all} and ND¹). These associations resulted from the relative lack of nicotine dependence symptoms in those subjects with the H3 allele (the C variant). Exploratory analyses of the three minor haplotypes at this locus, whose combined frequencies totaled 0.06, were unremarkable (data not shown).

Discussion

In summary, we found evidence suggesting that nicotine receptor variation plays a role in vulnerability to nicotine dependence; this differential vulnerability may be gender specific. However, we did not replicate any of the most significant findings from the NICSNP Consortium's high-density association or candidate gene study.

Before we discuss these results, we note some potential limitations of the present study. First, the Iowa Adoption Studies are based on a largely White, high-risk population; approximately one-half of the subjects have at least one biological parent with significant psychopathology (Yates et al., 1998). Caution should be used when generalizing the findings to other populations. Second, as compared with the NICSNP Consortium population, the Iowa Adoption Studies population is relatively small. Therefore, failure to replicate findings may simply reflect a lack of power. Third, not all the most promising SNPs from either NICSNP study were successfully genotyped; a disproportionate share of the SNPs that failed was derived from the high-density association study. Hence, careful inspection of the supplementary material (Supplementary Table 1) accompanying this manuscript should be made to ensure that the SNP of interest was successfully genotyped before concluding that a given finding was not replicated. Finally, the designs of the present study and those conducted by the NICSNP Consortium in the initial analyses have important differences. The cases and the controls from the NICSNP Consortium must have smoked at least 100 cigarettes, and the NICSNP study design is a case-control analysis. In contrast, the Iowa Adoption Studies is a longitudinal study in which not all subjects report a history of smoking at least one cigarette. These differences could affect the impact of some of the findings. Finally, none of the present findings are corrected for multiple comparisons.

To our knowledge, this is the first study to specifically examine the findings of the NICSNP Consortium. These single-point (i.e., SNP) and haplotype analyses tended to confirm the suggestion by the NICSNP Consortium and others that nicotinic receptor variation affects vulnerability to nicotine dependence (Hutchison et al., 2007; Li et al., 2005; Saccone et al., 2007; Zeiger et al., 2008). However, we did not find any significant single-point associations at the candidate genes; most significant SNP signals came from the NICSNP candidate gene study. As noted earlier, this finding may be secondary to methodological or power issues. Using logistic regression analysis, our sample size of 515, and a significance level of .05, we estimated that the present study had 80% power to detect an association accounting for 1.6% of the overall trait variance and 60% power to detect an association accounting for 1.0% of the variance

(Gauderman & Morrison, 2006). Therefore, the present study was inadequately powered to detect risk SNPs of low frequency or low relative risk. In contrast, the NICSNP studies were case-control analyses that included only subjects who had smoked 100 cigarettes and compared subjects with FTND scores of 0 with those who had scores of at least 4. In addition, secondary to the complex method by which Saccone et al. (2007) corrected for multiple comparisons in their study and the hypothesized a priori likelihood of association, otherwise meritorious findings with respect to nicotine dependence may have been ignored. Hence, some of the differences between our two studies may not be as stark as they seem.

In the present study, our most significant associations were cholinergic genes. Each of these findings has prior support in the literature: *CHRNA2* (Faraone et al., 2004; Sullivan et al., 2004), *CHRNA7* (Faraone et al., 2004; Greenbaum et al., 2006), *CHRNA1* (Lou et al., 2006), and *CHRNA1* (Faraone et al., 2004). The present findings extend those earlier findings by specifying the haplotypes for each of the loci. This contribution should be of aid to others seeking to define more exactly the nature of the genetic variation at these loci affecting vulnerability. However, because we wished to follow closely the NICSNP Consortium's procedures and a number of our SNPs failed, our haplotypes do not completely cover all the genes in question. Furthermore, our haplotype analyses do not fully capture the effect of rare variation on risk. This rare variance, as epitomized by *CHRNA7* SNP rs12114756 (see Table 2), may make significant contributions to the overall effects contributed by these loci. Hence, because of these two methodological limitations of our haplotype analyses, the admonition "Absence of evidence does not mean evidence of absence" should be kept in mind regarding the loci at which we did not find evidence of association.

A significant contribution that longitudinal studies can make to our understanding of nicotine dependence is the importance of exposure data to the formation of nicotine dependence. For our analysis of nicotine dependence, we used three exposure models. The first exposure model, ND^{all}, is the lowest level of exposure and may capture some of the variance associated with exposure to passive smoking or with behaviors that result in individuals differentially segregating with respect to exposure environments. However, since the FTND is typically applied only to smokers, including nonsmokers in the analysis may weaken our ability to detect a finding when using this scale. The latter two levels of exposure, having smoked at least once or having smoked 100 times, are more conventional and allow us to directly compare our results with the results from the rest of the field. Overall, in this sampling of genes, the highest levels of statistical significance seem to be found when the ND¹ level of exposure is used. Biologically, this may mean that the genetic variation at these nicotinic loci comes into play even after smoking just one cigarette. If so, these findings suggest the public health importance of preventing that first cigarette. However, examination of this conclusion in other longitudinal studies is necessary.

As noted earlier, we did not correct any of our analyses for multiple comparisons. The reasons for this are straightforward. First, each of these genes was specifically nominated on the basis of prior studies. Second, in our stepwise analysis at each of these loci, the analyses were highly interrelated. We attempted to mit-

igate the number of false positives by the stepwise transition from risk SNP to risk haplotype, followed by a consideration of exposure data. It is reassuring to see that overall consideration of this exposure data sharpened the findings. However, some of these effects could be random effects. A number of researchers have suggested ways to address these problems (Nyholt, 2004; Risch, 2000). However, none of these solutions globally address the difficulties found in this type of candidate gene analysis. Therefore, we suggest *caveat emptor*, and we note that the *sine non qua* of validation is replication (Risch, 2000).

In contrast to the candidate gene analysis, we did not replicate any of the findings from the NICSNP high-density association study. In this regard, that only 22 of the 33 SNPs from the study's list of most highly significant findings could be genotyped successfully by our commercial collaborator, Sequenom Inc., using information from the public domain. Hence, the results from the high-density association study cannot be regarded as having been fully tested in our sample.

In summary, after examination of the most significant findings from the NICSNP Consortium, we confirm prior suggestions that sequence variation at nicotinic receptors may play an important role in nicotine dependence. We suggest that further confirmation and resequencing studies of the four loci listed here are in order.

Supplementary material

Supplementary Table 1 and Figure 1 can be found at *Nicotine and Tobacco Research* online (<http://www.ntr.oxfordjournals.org/>).

Funding

National Institutes of Health (DA015789) to Dr. Philibert.

Declaration of Interests

The authors do not have any conflicts with respect to this work. Dr. Philibert had full access to all data and calculations and takes full responsibility for the accuracy of the manuscript.

Acknowledgments

The authors thank Laura Bierut and Scott Saccone for their complete and prompt provision of loci for genotyping, Steve Orzack for his edits to an earlier version of the manuscript, and the Rutgers Repository staff for all their help in making our studies possible. Finally, the investigative team acknowledges a debt of gratitude to the late Remi Cadoret, the founder of the Iowa Adoption Studies who planned these studies jointly with Philibert, Todorov, Madden, and Heath.

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