Original Investigation

Analysis of Detailed Phenotype Profiles Reveals *CHRNA5-CHRNA3-CHRNB4* Gene Cluster Association With Several Nicotine Dependence Traits

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Abstract

Introduction: The role of the nicotinic acetylcholine receptor gene cluster on chromosome 15q24-25 in the etiology of nicotine dependence (ND) is still being defined. In this study, we included all 15 tagging single nucleotide polymorphisms (SNPs) within the *CHRNA5-CHRNA3-CHRNB4* cluster and tested associations with 30 smoking-related phenotypes.

Methods: The study sample was ascertained from the Finnish Twin Cohort study. Twin pairs born 1938–1957 and concordant for a history of cigarette smoking were recruited along with their family members (mainly siblings), as part of the Nicotine Addiction Genetics consortium. The study sample consisted of 1,428 individuals (59% males) from 735 families, with mean age 55.6 years.

Results: We detected multiple novel associations for ND. *DSM-IV* ND symptoms associated significantly with the proxy SNP Locus 1 (rs2036527, $p = .000009$) and Locus 2 (rs578776, $p = .0001$) and tolerance factor of the Nicotine Dependence Syndrome Scale (NDSS) showed suggestive association to rs11636753 ($p = .0059$), rs11634351 ($p = .0069$), and rs1948 ($p = .0071$) in *CHRNB4*. Furthermore, we report significant association with *DSM-IV* ND diagnosis (rs2036527, $p = .0003$) for the first time in a Caucasian population. Several SNPs indicated suggestive association for traits related to ages at smoking initiation. Also, rs11636753 in *CHRNB4* showed suggestive association with regular drinking ($p = .0029$) and the comorbidity of depression and ND ($p = .0034$).

Conclusions: We demonstrate novel associations of *DSM-IV* ND symptoms and the NDSS tolerance subscale. Our results confirm and extend association findings for other ND measures. We show pleiotropic effects of this gene cluster on multiple measures of ND and also regular drinking and the comorbidity of ND and depression.

Introduction

Genes are known to be involved in the etiology of nicotine dependence (ND) with heritability estimates ranging from 40% to 75% (Rose, Broms, Korhonen, Dick, & Kaprio, 2009). Similarly, moderate to high heritability estimates have been reported for smoking initiation (32%–78%; Rose et al., 2009), first smoking experiences (37%; Haberstick, Ehringer, Lessem, Hopfer, & Hewitt, 2011), cigarettes smoked per day (CPD; 40%–56%; Rose et al., 2009), and smoking cessation (11%–86%; Rose et al., 2009). However, studies on smoking and ND based on linkage scans and candidate gene approach has yielded somewhat inconsistent results (Han, Gelernter, Luo, & Yang, 2010), even though multiple pathways and neurotransmitter systems have been implicated (Wang & Li, 2010).

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The most established finding involves the *CHRNA5-CHRNA3-CHRNB4* nicotinic acetylcholine receptor (nAChR) gene cluster on chromosome 15q24-25. A number of studies have implicated the consistent role of this gene cluster in a wide range of smoking-related phenotypes: ever smoking (smoked 100 cigarettes or more in lifetime; Erlich et al., 2010), CPD (Berrettini et al., 2008; Caporaso et al., 2009; Erlich et al., 2010; Keskitalo et al., 2009; Li et al., 2010; Liu et al., 2010; Saccone et al., 2010; Spitz, Amos, Dong, Lin, & Wu, 2008; Stevens et al., 2008; Thorgeirsson et al., 2008, 2010; Tobacco and Genetics Consortium, 2010), persistent smoking (Bierut et al., 2008), heavy smoking (Stevens et al., 2008), number of quitting attempts (Erlich et al., 2010), age at smoking initiation (Grucza et al., 2010; Schlaepfer et al., 2008; Weiss et al., 2008), pleasurable early smoking experience (Sherva et al., 2008), physical effects reported after smoking first experimental cigarette (Hoft, Stitzel, Hutchison, & Ehringer, 2011), as well as serum cotinine levels (Keskitalo et al., 2009). The *CHRNA5-CHRNA3-CHRNB4* gene cluster has been associated with ND defined by the Fagerström Test for Nicotine Dependence (FTND; Heatherton, Kozlowski, Frecker, & Fagerström, 1991) (Bierut et al., 2007; L. S. Chen et al., 2009; X. Chen et al., 2009; Erlich et al., 2010; Johnson et al., 2010; Kim et al., 2011; Li et al., 2010; Saccone et al., 2009; Saccone, Hinrichs, et al., 2007a; Spitz et al., 2008; Weiss et al., 2008; Wessel et al., 2010), as well as the Heaviness of Smoking Index (HSI) consisting of two key items of FTND (Li et al., 2010; Marques-Vidal et al., 2011). In addition, association has been found with the *DSM-IV* ND diagnosis (American Psychiatric Association, 1994) in a Black sample (Sherva et al., 2010) and in an Islandic sample defining ND as a score of 4 or more on the FTND or endorsement of 3 or more *DSM-IV* ND criteria (Thorgeirsson et al., 2008). Furthermore, haplotypes within the gene cluster were associated with the multidimensional ND scale, the Wisconsin Inventory of Smoking Dependence Motives, (WISDM-68) among individuals with early smoking initiation (Baker et al., 2009).

The *CHRNA5-CHRNA3-CHRNB4* gene cluster codes for the α5, α3, and β4 nAChR subunits. Nicotinic acetylcholine receptors are the primary targets for nicotine and initiate the brain and peripheral responses to smoking. It is thus biologically plausible that genetic variants in the genes coding for the nAChR subunits influence smoking intensity and ND. Supporting this hypothesis, functional studies have shown that a common nonsynonymous variant (rs16969968) in *CHRNA5* affects receptor function (Bierut et al., 2008; Kuryatov, Berrettini, & Lindstrom, 2011). Furthermore, rs588765 and correlates are associated with *CHRNA5* mRNA levels in brain tissue (Wang et al., 2009). In addition, *Chrna5* knockout mice have shown reduced sensitivity to nicotine-induced hypolocomotion and seizures (Salas et al., 2003).

Variation in genes coding for nAChRs has an established role in ND but may also play a role in alcohol dependence (Sherva et al., 2010; Wang et al., 2009), early alcohol use (Schlaepfer et al., 2008), cocaine dependence (Sherva et al., 2010), and opioid dependence (Erlich et al., 2010). The effects of different drugs share biological mechanisms, most importantly the increase of dopamine release from limbic brain areas. The α4α5β2 nAChR subtype is involved in nicotine-stimulated dopamine release (Salminen et al., 2004). Recent work highlights the role of the medial habenula, with high density of α4α5β2 receptors, in responses to nicotine (Fowler, Lu, Johnson, Marks, & Kenny, 2011; Salas, Sturm, Boulter, & De Biasi, 2009).

The *CHRNA5-CHRNA3-CHRNB4* gene cluster exhibits extensive linkage disequilibrium (LD). *CHRNA5* and *CHRNA3* are oriented in opposite directions and share part of their 3′UTR; thus, coordinated expression of these two genes may occur (Solda et al., 2005). Previous studies support the existence of multiple distinct smoking behavior loci within the *CHRNA5-CHRNA3-CHRNB4* region (Liu et al., 2010; Saccone et al., 2010; Thorgeirsson et al., 2010; Tobacco and Genetics Consortium, 2010). The large meta-analysis on CPD (Saccone et al., 2010) identified three statistically distinct loci: Locus 1 tagged by rs16969968, Locus 2 tagged by rs578776, and Locus 3 tagged by rs588765. For each loci, a list of so-called proxy single nucleotide polymorphisms (SNPs; Saccone et al., 2010), that is, adjacent SNPs in high LD with these variants, has been provided to ease future study comparisons and meta-analyses.

Both depression and alcohol use are known to co-occur with smoking and ND (Dani & Harris, 2005; Durazzo & Meyerhoff, 2007; Korhonen et al., 2007). Twin and family studies show that significant genetic correlations underlie this co-occurrence (Rose et al., 2009), suggesting shared genetic predisposition. As nAChRs have an important role in mediating the effect of nicotine on the dopaminergic pathway (Benowitz, 2010), it is reasonable to consider that variation in nicotine receptor genes may have pleiotropic effects and potentially associates not exclusively to ND but also to alcohol use and depression. Furthermore, the various ND measurements are suggested to capture slightly different aspects of ND, and by including several measures, we attempt to comprehensively portray the dimensions of ND. Here, the study sample utilized was specifically enriched for smoking and ND, with detailed phenotype profiles including not only assessment of CPD and ND but also age at smoking initiation, diagnoses and symptoms of depression, as well as of alcohol use, abuse, and dependence. The aim of this study was to utilize detailed phenotype information to more comprehensively clarify the involvement of the *CHRNA5-CHRNA3-CHRNB4* gene cluster in the etiology of ND and other smoking related traits as well as co-occurring phenotypes.

Methods

Study Sample

The sample collection has been previously described in detail (Broms et al., 2007; Loukola et al., 2008; Saccone, Pergadia, et al., 2007b). Briefly, the study sample was ascertained from the Finnish Twin Cohort study consisting of adult twins born between 1938 and 1957. Based on earlier health questionnaires, the twin pairs concordant for ever smoking were identified and recruited along with their family members (mainly siblings) for the Nicotine Addiction Genetics Finland study (N = 2,265), as part of the consortium including Finland, Australia, and United States. Data collection took place between 2001 and 2005. Because of the relatively old age of the siblings, very few parents were available for the study. The study sample consisted of 1,428 individuals (59% males) from 735 families, with mean age 55.6 years, who smoked on average 19.7 (SD = 9.9) CPD. Ninety-

Table 1. Basic Characteristics of the Study Sample

	Families	Individuals	MZ twins	DZ twins	Regular smoker	CPD ≥ 20	*DSM-IV* ND[a]	FTND ≥ 4[a]	*M* age (years)
Total	735	1,428	140	970	1,347	649	735	687	55.6
Males		845 (59%)	98	589	818	456	440	437	55.2
Females		583 (41%)	42	381	529	193	295	250	56.1

Notes. CPD = cigarettes per day; *DSM-IV* = Diagnostic and Statistical Manual of Mental Disorders, 4th edition (American Psychiatric Association, 1994); DZ = dizygotic; FTND = Fagerström Test for Nicotine Dependence (Heatherton et al., 1991); MZ = monozygotic; ND = nicotine dependence.

[a]Conditioned for regular smoking.

four percent had smoked 100 or more cigarettes over lifetime. Sample characteristics are presented in Table 1. The study was approved by the Ethics committee of the Hospital District of Helsinki and Uusimaa, Finland and by the IRB of Washington University, St. Louis, Missouri, USA.

In order to gain further information on the LD structure, we utilized a total of 679 genome-wide association (GWA) samples ascertained from the population-based FinnTwin12 study (unselected for smoking; Kaprio, 2006).

Phenotypes

Participants were telephone interviewed using the diagnostic Semi-Structured Assessment for the Genetics of Alcoholism (Bucholz et al., 1994) protocol, including an additional section on smoking behavior and ND adapted from the Composite International Diagnostic Interview (Cottler et al., 1991). The customized computer-assisted telephone interviews included more than 100 questions on smoking behavior. All participants provided written informed consent forms by mail. All phenotypes used in the analyses are based on the interview data (survey for the Nicotine Dependence Syndrome Scale [NDSS]; Shiffman, Waters, & Hickcox, 2004). Phenotype definitions are presented in Table 2. The examined binary, continuous, and categorical smoking-related phenotypes are divided into five groups: (a) amount smoked, (b) smoking initiation, (c) ND, (d) *DSM-IV*–based lifetime major depression, and (e) alcohol use, abuse, and dependence.

Prevalences and correlations of phenotypes were calculated with Stata 11.1 statistical software (StataCorp, 2009). Phenotype correlations are presented in Supplementary Table 1.

Genotyping

Participants were mailed a blood sample kit, which they took to the nearest health center laboratory for phlebotomy, and the venous blood samples were returned to the National Public Health Institute by mail. DNA was extracted by standard methods.

A total of 303 individuals were genotyped using Sequenom's homogeneous Mass Extend (hME) and iPLEX Gold technology (Sequenom, San Diego, CA, USA). Tagging SNPs were selected based on the HapMap Project (http://www.hapmap.org) and NCBI (http://www.ncbi.nlm.nih.gov) databases. The selected variants were biallelic and had a minor allele frequency (MAF) for more than 10% in the Caucasian population. The ability to amplify the flanking regions of each SNP was determined by using the applications SNPper (http://www.snpper.chip.org) and RealSNP (http://www.realsnp.com), which define, respectively, the most reliable regions for designing primers and the quality of the amplicons. All tagging SNPs failing during the procedure were replaced by newly generated tagging SNPs proposed by Haploview (Barrett, Fry, Maller, & Daly, 2005). The polymerase chain reaction (PCR) and extension primers were designed using Sequenom's MassARRAY Assay Design software (version 2.0). SNPs were genotyped in 384-well plates according to manufacturer's instructions. For quality controls, each plate contained at least eight water controls and 22 duplicate samples. PCR reactions were performed in a total reaction volume of 5 µl using 20 ng of genomic DNA. The alleles were automatically called by Sequenom's MassARRAY Typer Analyzer software and verified by two independent persons. Further, marker-specific quality controls included a call rate more than 80% and a Hardy–Weinberg equilibrium (HWE) p value > .01 (estimated using unrelated individuals). Mendelian errors were excluded using PedCheck (O'Connell & Weeks, 1998).

For 1,125 individuals, genotypes for the same 15 SNPs were derived from GWA data. Genotyping was performed at the Welcome Trust Sanger Institute (Hinxton, UK) on the Human670-QuadCustom Illumina BeadChip (Illumina, Inc., San Diego, CA, USA). The data were checked for minor allele frequency (>1%), genotyping success rate per SNP and per individual (>95%), HWE ($p > 1 \times 10^{-6}$), gender, and heterozygosity. In addition, to check whether any individuals were unexpectedly related to each other, an multidimensional scaling plot (using a pairwise-IBS matrix) with only one member of each known family was created. After the pedigree was reassured to be correctly formulated, the basic filters (MAF, genotyping success, HWE) were reapplied to the data. Seven of the markers were genotyped and eight were imputed using the software IMPUTE v2.1.0 (Howie et al., 2009). The reference panel used in the imputation was HapMap rel#24 CEU—NCBI Build 36 (dbSNP b126), which is available on the IMPUTE website (https://mathgen.stats.ox.ac.uk/impute/impute_v1.html#Using_IMPUTE_with_the_HapMap_Data). The posterior probability threshold for "best-guess" imputed genotype was .9. Genotypes below the threshold were set to missing.

Marker quality controls are presented in Supplementary Table 2.

Statistical Analyses

The LD between SNPs was estimated among nonrelated individuals (one per family) by using Haploview 4.2 (Barrett et al., 2005). The pairwise comparisons of markers more than 500 kb

Table 2. Phenotypes Used in the Study

Phenotype	Definition	Continuous/categorical: min–max (*M*), binary: prevalence (%)
I Amount smoked		
(1) Regular smoker	Smoked at least 100 cigarettes in lifetime and at least once a week for at least two consecutive months.	94.7%
(2) CPD	Number of cigarettes smoked per day during month of heaviest smoking (eight categories: 1–2, 3–5, 6–10, 11–15, 16–19, 20–25, 26–39, ≥40)[a]	1–2 to ≥40 (19.7)[b]
(3) Maximum CPD	Maximum number of cigarettes ever smoked during one day (24-hr period).	2–98 (29.8)
(4) Heavy smoker	Smoked 20 cigarettes or more daily during heaviest period of smoking or, 40 or more in a single day.	51.1%
II Smoking initiation		
(5) Age at first puff	Age (years) at first puff of cigarette ("How old were you the very first time you smoked even a puff of a cigarette?").	3–54 (14.1)
(6) Age at first cigarette	Age (years) when first whole cigarette smoked ("How old were you the first time you smoked a whole cigarette?").	4–54 (15.8)
(7) Immediacy	Tried smoking again on same or next day ("After you tried smoking a cigarette for the first time, how soon did you try smoking again?").	18.3%
(8) Second cigarette	Second cigarette smoked same or next day after first one ("After you first smoked a whole cigarette, how long was it before you smoked your second whole cigarette?").	51.6%
(9) Age of onset of weekly smoking	Age (years) when started to smoke weekly ("How old were you when you first smoked a cigarette at least once a week for at least two months in a row?").	6–54 (17.5)
(10) Age of onset of daily smoking	Age (years) when one started to smoke daily ("How old were you when you first smoked cigarette every day or nearly every day for at least two months in a row?").	7–54 (18.4)
(11) First time sensation	Sensation felt after smoking the first cigarette or first puffs ("While smoking your very first cigarettes, did you (1) like the taste or smell of the cigarette, (2) cough, (3) feel dizzy or light headed, (4) feel more relaxed, (5) get a headache, (6) feel a pleasurable rush or buzz, (7) feel your heart racing, (8) feel nauseated, like vomiting, (9) feel your muscles tremble or become jittery, (10) feel burning in your throat"). Sum score of 10 questions (items #1, #4, and #6 were reverse scored before summation): 0 points if answered "No," 1 = "A little bit," 2 = "Some," 3 = "Quite a bit," and 4 = "A great deal." Cronbach's alpha = .70.	3.6–15.8 (10.1)
III Nicotine dependence		
(12) *DSM-IV* ND	ND by *DSM-IV* diagnosis (three symptoms or more of seven occurring within a year).	51.5%
(13) *DSM-IV* ND symptoms	Number of *DSM-IV* ND symptoms from 0 to 7.	0–7 (3.0)
(14) FTND score	FTND score: 0–10 points.	0–10 (3.7)
(15) FTND (≥4)	Nicotine dependent if 4 or more of 10 points in FTND.	48.1%
(16) FTND TTF	TTF in the morning (one item of the FTND scale). Five categories: 0–5 min, 6–15 min[c], 16–30 min[c], 31–60 min, and >60 min.	1–5 (3.1)[b]
(17) NDSS sum score	NDSS[d] sum score as a continuous variable from 0 to 56.	0–56 (21.0)
(18) NDSS drive/priority factor[e]	Drive reflects craving, withdrawal, and smoking compulsions; priority reflects preference for smoking over other reinforces.	−2.0–3.3(−0.1)
(19) NDSS stereotypy/continuity factor[e]	Continuity reflects the regularity of smoking rate; stereotypy reflects the invariance of smoking.	−2.6–2.3(−0.3)
(20) NDSS tolerance factor[e]	Tolerance reflects reduced sensitivity to the effects of smoking.	−2.5–2.7(−0.1)
(21) *DSM-IV* ND and HSI	Co-occurrence of *DSM-IV* ND diagnosis and HSI (Heatherton et al. 1989; HSI ≥ 4 of 6 points).	17.9%
IV Depression		
(22) *DSM-IV* depression	Lifetime Major Depressive Disorder *DSM-IV* diagnosis (five symptoms or more of nine, including insufficiency).	17.2%
(23) *DSM-IV* depression symptoms	Number of *DSM-IV* defined depression symptoms ranging from 0 to 9.	0–9 (1.6)
(24) Comorbidity of depression and ND	Fulfilling the criteria of depression and nicotine dependence (either *DSM-IV* ND criteria or FTND ≥ 4).	13.8%

Table 2. Continued

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Phenotype	Definition	Continuous/categorical: min–max (*M*), binary: prevalence (%)
V Alcohol use		
(25) Regular drinker	Drinks at least one alcoholic drink at least once a week.	67.2%
(26) Heavy drinker	Drinks at least five or more alcoholic drinks once a week.	50.8%
(27) *DSM-IV* alcohol dependence	*DSM-IV* alcohol dependence (three or more of seven symptoms occurring within a year).	25.6%
(28) Binge drinker	Drinks at least five alcoholic drinks at least three days per year.	79.4%
(29) Comorbidity of binge drinking and ND	Comorbidity of binge drinking (five or more alcoholic drinks three or more days per year) and FTND (≥4 points).	41.5%
(30) Maximum drinks	Maximum number of alcoholic drinks ever drunk during one day (24-hr period).	1–72 (15.4)

Notes. The 30 examined binary, continuous, and categorical phenotypes are presented in five groups: (I) amount smoked, (II) smoking initiation, (III) ND, (IV) *DSM-IV*–based lifetime major depression, and (V) alcohol use, abuse, and dependence. CPD = cigarettes per day; *DSM-IV* = Diagnostic and Statistical Manual of Mental Disorders, 4th edition; FTND = Fagerström Test for Nicotine Dependence; HSI = Heaviness of Smoking Index; ND = nicotine dependence; TTF = time to first cigarette.

[a]In the statistical analyses of the CPD variable, original categorical observations (1–8) were replaced with class means of CPD (1.5, 3.5, 8, 13, 17.5, 22.5, 32.5, and 45 CPD, respectively). Regression coefficients can therefore be interpreted as the average change in number of cigarettes smoked per day when the number of minor allele is increased by one.

[b]Weighted arithmetic mean, where weights determined by class frequencies.

[c]Categorization differs from original four categories (Heatherton et al., 1991), that is, 6–30 min is split into 6–15 min and 16–30 min. In our dataset, 46% of smokers belong to the group of 6–30 min, and from the smoking behavior point of view, there is a significant difference whether one smokes the first cigarette within 6 min or 30 min from waking up. In this dataset, 22% of smokers belong to the 6–15 min and 24% to the 16–30 min group.

[d]NDSS = Nicotine Dependence Syndrome Scale (Shiffman et al., 2004).

[e]Factor structure of the NDSS scale with 31 items conditioned for current smoking and CPD more than 10 generated a three-factor solution (as reported also earlier in Broms et al., 2007). Factors accounted for 92% of the common variance among items: 1. drive/priority factor, 11 items (Cronbach's alpha (α) = .88, accounted for 33%); 2. stereotypy/continuity factor, 9 items (α = .89, accounted for 33%); and 3. tolerance factor, 5 items (α = .82, accounted for 26%).

apart were excluded. Haplotype blocks were defined according to the "solid spine of LD" algorithm by using the default threshold values for block estimation (Figure 1).

The associations between discrete phenotypes and candidate SNPs (qualitative association) were estimated with pseudomarker (Goring & Terwilliger, 2000), which performs separate and joint linkage and LD analyses, testing each marker locus against a phenotype-based "pseudomarker" locus. This likelihood-based estimation method is numerically equivalent to model-free analysis and efficiently uses data on all family types. Both recessive and dominant models (default parameters) were fitted. We report uncorrected *p* values minimized over "LD given linkage," "LD given no linkage," and "LD and linkage" (joint test) as well as dominant and recessive models.

The associations between continuous phenotypes and candidate SNPs (quantitative association) were performed with quantitative transmission disequilibrium test (QTDT; Abecasis, Cardon, & Cookson, 2000). In the analysis, the proportion of alleles shared identically by descent (IBD) was estimated by multipoint computation of MERLIN (Abecasis, Cherny, Cookson, & Cardon, 2002) to extract maximal inheritance information from the pedigrees. Prior to analysis, we estimated our sample not being stratified with the appropriate "population stratification" model of QTDT, which compares the between and within family components of association (Abecasis et al., 2000). The total association model was used, allowing powerful analysis of the sample, including incomplete families. In the analysis, the variance components "polygenic," "nonshared environment" (environmental effects unique to each family member), "common environment" (environmental effects shared by all related individuals), "nuclear family environment" (environmental effects shared by all members of a nuclear family), and "twin environment" (environmental effects shared only by twins) were used to model the phenotypic similarities between the pedigree members. In the analysis of traits related to age of initiation (age at first puff, age at first cigarette, age of onset of weekly smoking, age of onset of daily smoking), sex was included as a covariate, whereas both sex and age at recruitment were used as covariates for all other continuous traits.

To account for multiple testing we used a modified Bonferroni correction to set *p* value thresholds for significant and suggestive association signals. Since neither analyzed markers nor traits are independent, we utilized an established methodology to evaluate the numbers of corresponding independent markers and traits with the programs SNPSpD and matSpD, respectively (Cheverud, 2001; Li & Ji, 2005; Nyholt, 2004), and their MeffLi

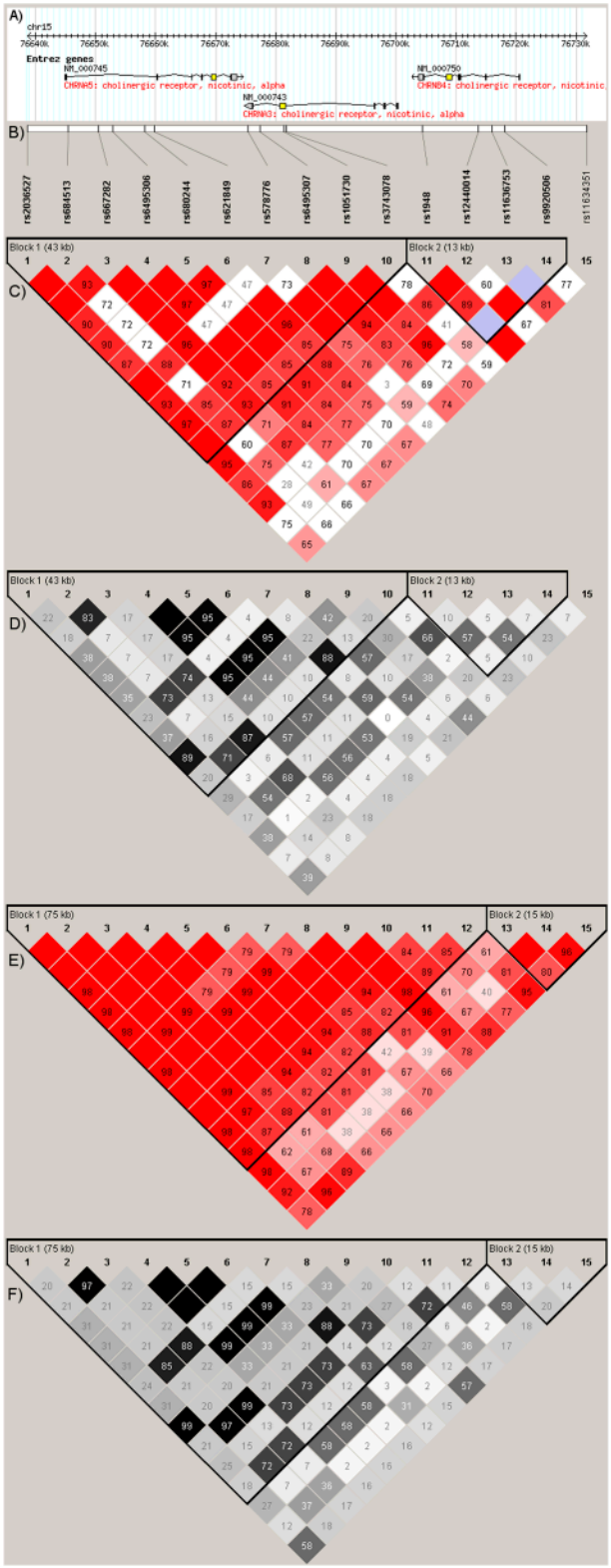

Figure 1. (A) Gene structures in the *CHRNA5-CHRNA3-CHRNB4* region, (B) genotyped single nucleotide polymorphisms, (C) D′ in the HapMap CEPH data (NCBI Build 36), (D) r2 in the HapMap CEPH data, (E) D′ in the study sample (nonrelated individuals; one per family), (F) r2 in the study sample.

and VeffLi estimates (Li & Ji, 2005) were used as they were smaller than Meff and Veff, respectively, as recommended by the author (http://gump.qimr.edu.au/general/daleN/SNPSpD/). The trait "regular smoker" was not accounted for when estimating the number of independent traits, as it is the ascertainment criteria for our families. In our dataset, the number of independent markers was 6.0022 and the number of independent traits was 16.956. A p value threshold of .0005 for significant association was achieved by dividing $p = .05$ by the product of the number of independent markers and the number of independent traits. A p value threshold of .0083 for suggestive association was achieved by dividing $p = .05$ by the number of independent markers.

Nonnormally distributed continuous variables (kurtosis and/or skewness >1 or <−1) were transformed with square root or 10-base logarithm, whichever provided most normal-like distribution. Trait values above 30 years considered as outliers were removed from the analysis for phenotypes measuring the age at first puff (seven individuals), age at first cigarette (nine individuals), age of onset of weekly smoking (20 individuals), and age of onset of daily smoking (28 individuals).

To estimate effect sizes for all SNPs showing significant or suggestive p values in the genetic association analyses, we performed regression analyses with Stata 11.1 statistical software (StataCorp, 2009) by using an additive model. Odds ratios (*OR*) and beta coefficients (β) with 95% *CI*s were reported for binary and quantitative traits, respectively. Regression analyses were adjusted for sex and age. Furthermore, individuals were clustered based on the possible lack of statistical independence of observations of subjects who came from the same family (Williams, 2000).

Results

In the study sample, the LD blocks were similar to those in the HapMap CEPH data (Figure 1) and the somewhat stronger LD between markers is in agreement with previous findings from the Finnish population (Service et al., 2006). In the HapMap CEPH data, *CHRNB4* SNPs lie outside the *CHRNA5–CHRNA3* LD block, whereas in the study sample, the LD block extends to rs1948 and rs12440014 in *CHRNB4* (Figure 1). The LD blocks were almost identical between the study sample and the FinnTwin12 sample (data not shown).

We detected significant association with a variety of traits measuring ND, the strongest signal emerging for *DSM-IV* ND symptoms in Locus 1 (rs2036527 $p = .000009$, rs1051730 $p = .00002$) and Locus 2 (rs578776 $p = .0001$, rs667282 $p = .0002$). A significant association was observed with *DSM-IV* ND diagnosis in Locus 1 (rs2036527 $p = .0003$, rs1051730 $p = .0004$). Altogether we detected significant or suggestive p values for six traits measuring ND (*DSM-IV* ND, *DSM-IV* ND symptoms, FTND score, NDSS tolerance factor, Time to first cigarette (TTF), and *DSM-IV* ND + HSI; Table 3).

We detected suggestive association with the categorized variable of CPD in Locus 1 (rs1051730 $p = .0077$, rs2036527 $p = .0073$) and maximum CPD at rs12440014 ($p = .0074$) (Table 3). Additionally, four SNPs (rs578776 and rs667282 at Locus 2,

Table 3. Association Analyses Results for Traits Showing Significant ($p < .0005$, Bold and Underlined) or Suggestive ($.0005 < p < .008$, Bold) Association

		Amount smoked		Smoking initiation		Nicotine dependence						Depression	Alcohol use
Marker	Gene	CPD	Maximum CPD	Age of onset of weekly smoking	Age of onset of daily smoking	*DSM-IV* ND	*DSM-IV* ND symptoms	FTND score	FTND TTF	NDSS tolerance factor	*DSM-IV* ND and HSI	Comorbidity of depression and ND	Regular drinker
rs2036527 [L1]	*CHRNA5*	**0.0073**	0.0710	0.9188	0.2158	**0.0003**	**0.000009**	**0.0004**	**0.0061**	0.0844	**0.0047**	0.0676	0.3381
rs684513	*CHRNA5*	0.0336	0.0118	**0.0034**	**0.0065**	0.0609	**0.0005**	0.0447	0.0567	0.9696	0.0531	0.4674	0.2136
rs667282 [L2]	*CHRNA5*	0.0273	0.0120	**0.0070**	**0.0032**	0.0137	**0.0002**	0.0254	0.0848	0.9959	0.0370	0.4252	0.0189
rs6495306 [L3]	*CHRNA5*	0.7143	0.4308	0.0588	0.2134	0.4361	0.5403	0.2601	0.4519	0.0480	0.6088	0.0303	0.1686
rs680244 [L3]	*CHRNA5*	0.7607	0.4795	0.0460	0.1794	0.3855	0.5335	0.2753	0.4555	0.0468	0.5717	0.0203	0.0088
rs621849 [L3]	*CHRNA5*	0.7060	0.3919	0.0450	0.1254	0.4102	0.5538	0.2617	0.3998	0.0271	0.3757	0.0250	0.1685
rs578776 [L2]	*CHRNA3*	0.0125	0.0125	**0.0054**	**0.0077**	0.0217	**0.0001**	**0.0047**	0.0132	0.8518	0.0315	0.7507	0.0411
rs6495307 [L3]	*CHRNA3*	0.6800	0.4627	0.0581	0.1932	0.4648	0.5274	0.2621	0.3762	0.0544	0.5503	0.0228	0.1713
rs1051730 [L1]	*CHRNA3*	**0.0077**	0.0711	0.5518	0.1354	**0.0004**	**0.00002**	**0.0005**	0.0091	0.0138	0.0094	0.0726	0.3098
rs3743078	*CHRNA3*	0.0198	0.0084	**0.0062**	**0.0033**	0.0118	**0.0001**	0.0108	0.0545	0.7586	0.0632	0.3510	0.0619
rs1948	*CHRNB4*	0.8906	0.7394	0.0156	0.1698	0.1662	0.4901	0.4708	0.7041	**0.0071**	0.6646	0.0861	0.0615
rs12440014	*CHRNB4*	0.0722	**0.0074**	0.0957	0.0287	0.1264	**0.0040**	0.0411	0.1014	0.5114	0.1347	0.4426	0.3351
rs11636753	*CHRNB4*	0.0909	0.4171	0.4022	0.8948	0.3541	0.2331	0.0603	0.0830	**0.0059**	0.0403	**0.0034**	**0.0029**
rs9920506	*CHRNB4*	0.6322	0.9541	0.9561	0.4164	0.3091	0.1238	0.8613	0.8019	0.6695	0.5209	0.0672	0.3220
rs11634351	*CHRNB4*	0.1672	0.5042	0.4820	0.0902	0.0377	**0.0011**	0.0535	0.3443	**0.0069**	0.0939	0.0813	0.0168

Note. L1 = Locus 1 (tagged by rs16969968): proxy SNP identifier from Saccone et al. (2010) and by personal communication by S. M. Hartz. L2 = Locus 2 (tagged by rs578776): proxy SNP identifier from Saccone et al. (2010) and by personal communication by S. M. Hartz. L3 = Locus 3 (tagged by rs588765): proxy SNP identifier from Saccone et al. (2010) and by personal communication by S. M. Hartz. CPD = cigarettes per day; *DSM-IV* = Diagnostic and Statistical Manual of Mental Disorders, 4th edition; FTND = Fagerström Test for Nicotine Dependence; HSI = Heaviness of Smoking Index; ND = nicotine dependence; NDSS = Nicotine Dependence Syndrome Scale; TTF = time to first cigarette.

rs684513, rs3743078) indicated suggestive association for the age of onset of both weekly and daily smoking (Table 3).

Furthermore, rs11636753 in *CHRNB4* showed suggestive association with both regular drinking (p = .0029) and the comorbidity of depression and ND (p = .0034) (Table 3).

Association analysis results for all phenotypes are presented in Supplementary Table 3.

For those SNPs showing significant associations, notable effect sizes were detected (Supplementary Table 4). In Locus 1, *DSM-IV* ND diagnosis showed an *OR* of 1.25 (p = .0085), and corresponding effect size for *DSM-IV* ND symptoms was a beta coefficient of 0.30 (p < .0001). Similarly, Locus 1 showed a beta coefficient of 0.33 (p = .0017) for the FTND score. Locus 2 showed plausible protective effect for *DSM-IV* ND symptoms ($\beta = -0.28$, p = .0001). For SNPs showing suggestive association, modest effect sizes were detected. As an exception, a notable protective effect (*OR* = 0.76, p = .0239) was detected for rs11636753 in the comorbidity of depression and ND. The effect size for the regular drinker phenotype failed to meet statistical significance (p > .05; Supplementary Table 4).

Discussion

Multiple distinct loci at the *CHRNA5-CHRNA3-CHRNB4* locus on 15q24-25 appear to affect smoking behavior (Liu et al., 2010; Saccone et al., 2010; Thorgeirsson et al., 2010; Tobacco and Genetics Consortium, 2010). In a previous meta-analysis including the current study sample (Saccone et al., 2010), three SNPs in the *CHRNA5-CHRNA3-CHRNB4* gene cluster (rs16969968, rs578776, and rs588765) showed association with CPD (heavy i.e., CPD > 20 vs. light i.e., CPD ≤ 10). Presuming the previous evidence as an "a priori" hypothesis, we set out to extend and deepen the analyses of these three loci by utilizing previously established proxy SNPs (Saccone et al., 2010; S. M. Hartz [personal communication, May 30, 2011]) as well as all detected tagging SNPs within this gene cluster. We performed association analyses in a Finnish family sample of twins and their siblings ascertained specifically for smoking and ND using a broad spectrum of phenotypes covering aspects of smoking behavior, ND, and depression, as well as alcohol use, abuse, and dependence.

In agreement with previous evidence from extensive studies with over 1,40,000 participants (Liu et al., 2010; Saccone et al., 2010; Thorgeirsson et al., 2008, 2010; Tobacco and Genetics Consortium, 2010), we detected some association with CPD. Larger samples may be needed to detect significant association with CPD, as shown by a study of over 13,000 Icelandic smokers (Thorgeirsson et al., 2008) in which variation in Locus 1 (rs1051730) accounted for only about 1% of the variance in CPD, the average effect per allele being about 1 CPD (Thorgeirsson et al., 2008). In agreement with this, an effect size of a beta coefficient 1.05 was detected in our sample, roughly corresponding to an increment of one cigarette per day for each allele of the locus.

The involvement of the *CHRNA5-CHRNA3-CHRNB4* gene cluster in FTND has been well documented in the literature, whereas studies reporting findings for *DSM-IV* ND are sparse. It is unclear whether this is due to lack of positive findings, publication bias, challenges in reliably creating *DSM-IV* ND diagnosis, or the predominance of FTND in measuring ND, possible due to ease of assessment. Here, we report a significant novel association with the number of *DSM-IV* ND symptoms, with Locus 1 conferring risk ($\beta = 0.30$) and Locus 2 showing plausible protective effect ($\beta = -0.28$), in agreement with a previous study reporting similar effects for CPD (Saccone et al., 2010). We also report the first significant association with *DSM-IV* ND diagnosis in a Caucasian sample, replicating previous findings in Blacks (Sherva et al., 2010). Further, we replicate association with the FTND as a continuous trait (X. Chen et al., 2009; Erlich et al., 2010; Kim et al., 2011; Wessel et al., 2010). These two widely used ND measures are suggested to extract partly different dimensions of ND (Moolchan et al., 2002; Piper, McCarthy, & Baker, 2006; Piper et al., 2008). *DSM-IV* predominantly measures loss of control in terms of smoking behavior, including cognitive, behavioral, and physiological symptoms, resulting in tolerance, withdrawal, and compulsive drug-taking behavior (American Psychiatric Association, 1994). FTND aims to measure the construct of physical dependence, associating with cessation outcome, predicting smoking relapse, and having the key component of difficulty to stand reduced nicotine levels (Haddock, Lando, Klesges, Talcott, & Renaud, 1999; Heatherton et al., 1991).

We demonstrate the first evidence of genetic association of the NDSS tolerance factor, TTF, as well as the combination of *DSM-IV* ND diagnosis and HSI (a combination of CPD and TTF) in the *CHRNA5-CHRNA3-CHRNB4* locus. Functional evidence for the involvement of the *CHRNA5-CHRNA3-CHRNB4* gene cluster in tolerance includes knockout mice showing association with alpha4beta2 nAChR subunits and the development of tolerance (McCallum, Collins, Paylor, & Marks, 2006; Tapper, McKinney, Marks, & Lester, 2007). Further, chronically treated mice lacking the beta4 subunit show an increased tolerance to an acute dose of nicotine (E. E. Meyers and M. J. Marks [personal communication, May 31, 2011]). Tolerance to nicotine is defined by the ability to smoke increased amounts of cigarettes without experiencing toxic effects (Piper et al., 2006). Repeated exposure to nicotine leads to tolerance (neuroadaptation), and as neuroadaptation develops, the number of binding sites on the nAChRs in the brain increases, probably in response to nicotine-mediated desensitization of receptors (Benowitz, 2010).

The gene cluster has previously been associated with the WISDM (Piper et al., 2004) tolerance factor among 886 early-onset smokers from the United States (Baker et al., 2009). NDSS and WISDM are measuring somewhat different aspects of ND and have a slightly different focus on the tolerance dimension as well (Piper et al., 2008). The use of multidimensional scales such as NDSS and WISDM potentially aids in deciphering the construct and nature of ND (Piper et al., 2006). Our results confirm the *CHRNA5-CHRNA3-CHRNB4* association to tolerance, one of the dimensions of ND, which supposedly also is embedded within the traditional unidimensional scales, *DSM-IV* and FTND.

Twin studies suggest that genetic influences on age at smoking initiation are correlated with the amount smoked (Broms,

Silventoinen, Madden, Heath, & Kaprio, 2006; Morley et al., 2007). Several SNPs within the *CHRNA5-CHRNA3-CHRNB4* locus associate with multiple phenotypes measuring age at onset of smoking, consistent with previous evidence (Schlaepfer et al., 2008). Previous results have been inconsistent, with findings supporting *CHRNA5-CHRNA3-CHRNB4* variation underlying ND in individuals with early age at smoking initiation (≤16 years; Weiss et al., 2008) and *CHRNA5* association with later age at smoking initiation (≥17 years; Grucza et al., 2010). Adolescence clearly is a crucial period of vulnerability for the development of substance use disorders (Chambers, Taylor, & Potenza, 2003; Crews, He, & Hodge, 2007), and also in the current study, we found that later starters were less dependent (see Supplementary Table 1) as previously reported (Breslau & Peterson, 1996; Broms, Silventoinen, Lahelma, Koskenvuo, & Kaprio, 2004; Chassin, Presson, Rose, & Sherman, 1996; J. Chen & Millar, 1998; Kandel, Hu, Griesler, & Schaffran, 2007; Lando et al., 1999). This study covered four stages of smoking initiation, allowing a comprehensive evaluation of the genetic factors contributing to this process.

Depression commonly co-occurs with smoking and ND (Chaiton, Cohen, O'Loughlin, & Rehm, 2009; Korhonen et al., 2007; Morrell & Cohen, 2006; Rose et al., 2009), recent evidence showing that ND predicts depression (Boden, Fergusson, & Horwood, 2010) but the causal nature of this association is unknown. Under certain conditions, nicotine can act as an antidepressant (Picciotto, Brunzell, & Caldarone, 2002), whereas chronic nicotine use can lead to neuroadaptation resulting in increase of depressed mood (Picciotto et al., 2002). On the other hand, depressed mood may promote continued nicotine intake to maintain desensitization of nAChRs (Mineur & Picciotto, 2009). So far, the effects of specific genes on the comorbidity of smoking and depression have been investigated in a limited number of studies with small sample sizes (Rose et al., 2009), mostly for dopaminergic genes (Audrain-McGovern, Lerman, Wileyto, Rodriguez, & Shields, 2004; Lerman & Niaura, 2002; Lerman et al., 1998). By stratifying on smoking status, it has been shown that genetic predisposition for depression actually run in families of smokers (Pergadia et al., 2011). The involvement of nAChRs in depression is a biologically plausible hypothesis. Preclinical studies show antidepressant-like effects in drugs targeting nAChRs for example, α4β2 nAChR modulators, such as varenicline (Philip, Carpenter, Tyrka, & Price, 2010). Interestingly, in our study, rs11636753 in *CHRNB4* showed suggestive association with the comorbidity of depression and ND, to our best knowledge a novel finding. It is noteworthy that the very same rs11636753 was associated also with the NDSS tolerance subscale. Such pleiotropic effect could be explained so that tolerance encompasses neuroadaptation, that is, quantitative and qualitative changes in nAChRs, being further associated with withdrawal symptoms, such as depressed mood (Benowitz, 2010).

Comorbidities for cigarette smoking and ND are alcohol use and dependence (Dani & Harris, 2005; Durazzo & Meyerhoff, 2007), shared genetic vulnerability being supported by twin studies (Madden & Heath, 2002; True et al., 1999). Genetic variation within the *CHRNA5-CHRNA3-CHRNB4* gene cluster has been associated with *DSM-IV* alcohol dependence (Sherva et al., 2010; Wang et al., 2009), *DSM-IV* alcohol symptoms (X. Chen et al., 2009), and early alcohol use onset (Schlaepfer et al., 2008). Interestingly, Locus 1 variants (rs16969968 and rs1051730) are associated with both ND and alcohol abuse, however, with opposite risk alleles (X. Chen et al., 2009), possibly reflecting the different actions of the two substances: alcohol being a depressant and nicotine a stimulant. In our sample ascertained for smoking, we detected suggestive association between rs11636753 and regular drinking. Heavy drinking or alcohol dependence did not show any association, although those traits likely are more genetically relevant extreme phenotypes. In the Finnish drinking culture, regular drinking defined as one or more drinks per week reflects a social drinking pattern, slightly surmising our association findings. Furthermore, the regular drinker phenotype showed statistically nonsignificant results ($p > .05$) in the effect size analysis (Supplementary Table 4).

Our study is based on a strong a priori hypothesis for the involvement of this locus in ND and smoking quantity. We set up to scrutinize a vast array of phenotypes to gain comprehensive information on the involvement of the 15q24-25 region and to study potential pleiotrophic effects in traits related to or co-occurring with ND. Although acknowledging the issue of increased number of tests, we chose to implement all relevant phenotypes within a single study rather than dividing them into separate entities. In order to account for multiple testing, we used a modified Bonferroni correction, utilizing estimated numbers of independent markers and traits, to set p value thresholds for significant and suggestive association signals. As the included markers and traits are correlated, standard procedures for the correction for multiple testing would certainly be overly conservative. The estimation of independent markers, based on LD matrixes, is rather straightforward. However, the number of independent traits is difficult to estimate and depends on what that information is used for. One criterion could be informativeness in predicting cessation or disease outcomes, which could be tested in a multivariate predictive model (logistic regression or survival models). In the current study, we used a statistical estimate based on the correlation/covariance matrix, resulting in a sample-based estimate that may fluctuate when applied to novel independent population samples. The use of estimated numbers of independent markers and traits in adjusting p value thresholds likely is quite conservative but nevertheless successful in reducing the type I error rate. We acknowledge that many of our findings are suggestive and need to be further confirmed in independent replication samples.

Although our study sample was of moderate size (n = 1,428), it harbors a number of advantages. Our sample of twins and their siblings was ascertained specifically for smoking and ND, being initially drawn from the population-based Finnish twin cohort with extensive phenotypic profiles. The population of Finland represents a well-established isolate with minuscule population admixture. In isolates, the genetic drift may lead to an overabundance of morbid alleles for particular disorders, and a high proportion of patients are likely to share these alleles IBD. Although the effect is strongest for rare disease alleles, isolates are also advantageous for genetic studies of common disorders (Peltonen, Palotie, & Lange, 2000).

The inclusion of other variants besides the well-established loci (tagged by rs16969968, rs578776, and rs588765; Saccone et al., 2010) proved beneficial. Although our strongest association signals emerged from Locus 1 (rs1051730 and rs2036527; in full LD with the functional and most well-known Locus 1 proxy SNP, rs16969968), we also detected association with SNPs not

included in the proxy lists. Although we aimed to study haplotype effects, the construction of haplotypes in our family sample with virtually all parental genotypes missing proved to be a challenging task. We tested several softwares including PHASE 2.1.1 (Stephens & Scheet, 2005; Stephens, Smith, & Donnelly, 2001; disregarding family structures) and FBAT 2.0.2c (Laird, Horvath, & Xu, 2000; including nuclear family information), but these led to either very inconsistent and unreliable haplotypes or the exclusion of virtually all pedigrees due to missing founder genotypes.

To conclude, we report the novel associations of *DSM-IV* ND symptoms, NDSS tolerance subscale, TTF, the combined phenotype of *DSM-IV* ND and HSI, as well as regular drinking and the comorbidity of ND and depression to the *CHRNA5-CHRNA3-CHRNB4* gene cluster. Our results confirm and extend the association findings for *DSM-IV* ND, FTND, CPD, and age at smoking initiation. Using detailed phenotype profiles, we show a broad involvement of this gene cluster on various traits measuring or co-occurring with ND.

Supplementary Material

Supplementary Tables 1–4 can be found online at http://www.ntr.oxfordjournals.org

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Declaration of Interests

Dr. Kaprio has served as a consultant to Pfizer in 2008 and 2011. Dr. Broms has served as a consultant to Pfizer in 2008. Dr. Korhonen has served as a consultant to Pfizer in 2011.

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