Original Investigation

Chromosome 20 Shows Linkage With DSM-IV Nicotine Dependence in Finnish Adult Smokers

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Abstract

Introduction: Chromosome 20 has previously been associated with nicotine dependence (ND) and smoking cessation. Our aim was to replicate and extend these findings.

Methods: First, a total of 759 subjects belonging to 206 Finnish families were genotyped with 18 microsatellite markers residing on chromosome 20, in order to replicate previous linkage findings. Then, the replication data were combined to an existing whole-genome linkage data resulting in a total of 1,302 genotyped subjects from 357 families. ND diagnosed by *DSM-IV* criteria, the Fagerström Test for Nicotine Dependence (FTND) score, and the lifetime maximum number of cigarettes smoked within a 24-hr period (MaxCigs24) were used as phenotypes in the non-parametric linkage analyses.

Results: We replicated previously reported linkage to *DSM-IV* ND, with a maximum logarithm of odd (LOD) score of 3.8 on 20p11, with females contributing more (maximum LOD [MLOD] score 3.4 on 20q11) than males (MLOD score 2.6 on 20p11). With the combined sample, a suggestive LOD score of 2.3 was observed for *DSM-IV* ND on 20p11. Sex-specific analyses revealed that the signal was driven by females with a maximum LOD score of 3.3 (on 20q11) versus LOD score of 1.3 in males (on 20q13) in the combined sample. No significant linkage signals were obtained for FTND or MaxCigs24.

Conclusions: Our results provide further evidence that chromosome 20 harbors genetic variants influencing ND in adult smokers.

Introduction

It has been clearly established that smoking behaviors are genetically influenced (Rose, Broms, Korhonen, Dick, and Kaprio,

2009). Despite several gene-mapping studies, the genes underlying liability to nicotine dependence (ND) remain largely unknown. Recently, Han, Gelernter, Luo, and Yang (2010) performed a meta-analysis of 15 genome-wide linkage scans of smoking behavior. Linkage signals were observed on chromosomal regions 17q24.3–q25.3, 5q33.1–q35.2, 20q13.12–32, and 22q12.3–13.32. The relevance of the chromosome 20 finding is highlighted by the fact that *CHRNA4* encoding the nicotinic acetylcholine receptor (nAChR) subunit $\alpha 4$ resides on 20q13.2–13.33. This subunit is crucial to form a functional $\alpha 4$ - $\beta 2$ receptor which is the most widely expressed nAChR subtype in the human brain and plays a central role in the mediation of physiological effects of nicotine (Collins, Salminen, Marks, Whiteaker, and Grady, 2009).

Finnish twin sample has yielded linkage signals on chromosome 20 for maximum number of cigarettes smoked within a 24-hr period (MaxCigs24; 20q13, logarithm of odds [LOD] score = 4.22; Saccone et al., 2007) and *DSM-IV* ND (20p13, LOD score = 2.36; Loukola et al., 2008). In genetic association studies, single nucleotide polymorphisms (SNPs) residing at *CHRNA4* have shown association with ND (Breitling et al., 2009; Saccone et al., 2009), salivary cotinine levels (Etter et al., 2009), sensitivity to the effects of nicotine (Hutchison et al., 2007), and with the success of smoking cessation in a clinical trial (King et al., 2009). In addition, SNPs residing in *CHRNA4* have shown gender- and ethnicity-specific association with vulnerability to ND (Feng et al., 2004; Li et al., 2005). However, no genome-wide association study or meta-analysis of smoking-related traits so far has found an association in chromosome 20 (The Tobacco and Genetics Consortium, 2010).

Our aim was to replicate the linkage signal between chromosome 20 markers and ND (Study 1) and to delineate these findings in an extended Finnish family sample (Study 2) in order to study (a) the sex specificity of the signal and (b) whether the genomic area influences the persistence to smoke.

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Methods

Subjects

Sample was drawn from the Finnish Twin Cohort comprising of Finnish adult twins born between 1938 and 1957 (Kaprio and Koskenvuo, 2002). Based on earlier health questionnaires, twin pairs concordant for ever-smoking were identified and recruited along with their family members (mainly siblings) for the Nicotine Addiction Genetics (NAG) study which is a consortium among Finland, Australia, and United States (Broms et al., 2007; Loukola et al., 2008; Saccone et al., 2007). At the time of the data collection, the mean age of the sample was 57 years (range 31–93, *SD* 9.5). The study has been approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa in 2001.

In Study 1, a total of 759 samples belonging to 206 Finnish families with a mean age of 56.7 years were included to form a replication material for our earlier findings. Altogether 44% (188 males, 149 females) fulfilled the criterion for lifetime *DSM-IV* ND, and 41.5% (190 males, 125 females) fulfilled the criterion for lifetime ND by Fagerström Test for Nicotine Dependence (FTND).

In Study 2, a total of 1,302 samples including 759 samples from Study 1 and 543 samples previously genotyped were combined. These samples with a mean age of 57.4 years were included. Altogether 42.1% (313 males, 235 females) fulfilled the criterion for lifetime *DSM-IV* ND, and 40.1% (316 males, 206 females) fulfilled the criterion of lifetime ND by FTND. Affected pairs consisted of 344 sib pairs (122 males, 81 females, 141 opposite sex), 4 half-sib pairs, and 13 parent–child pairs. The sample set included 1,106 regular smokers, who had smoked during the heaviest period of smoking, on average, 18.7 cigarettes/day (*SD* 10.4). Female smokers ($N = 489$) had a mean CPD of 18.6 which is at the same level with male smokers ($N = 617$) whose mean CPD was 18.7. The sample included 508 current smokers (260 males, 248 females) and 594 former smokers (355 males, 239 females). Data on smoking status (current/former) were missing for four regular smokers.

Phenotyping

The participants were telephone interviewed using trained interviewers during 2001–2005. Diagnostic *DSM-IV* ND criteria (American Psychiatric Association, 1994) were measured by Semi-Structured Assessment for the Genetics of Alcoholism (Bucholz et al., 1994), modified for use in Australian and Finnish populations, with the section of nicotine use and dependence based on the Composite International Diagnostic Interview (Cottler et al., 1991). The ND diagnosis requires the presence of at least three out of seven criteria during a 12-month period. In addition, the FTND (Heatherton Kozlowski, Frecker, and Fagerström, et al., 1991) was administered (scored 0–10), and the largest number of cigarettes ever-smoked during a 24-hr period was asked. The cutpoint of ≥ 4 was used as the criterion for the lifetime ND by FTND similarly as in the case–control studies by Bierut et al. (2007) and Berrettini et al. (2008). All of the smoking- and ND-related questions were presented to regular smokers (defined as having smoked ≥ 100 cigarettes during lifetime and for at least once a week for a minimum period of 2 months in a row), and after fulfilling the criteria for regular smoking, no stem structures were used in the telephone interview

of smoking behavior (i.e., all regular smokers responded to all the questions regarding smoking behavior).

Genotyping

Genotyping of chromosome 20 microsatellite markers was performed in two phases. First, in 2005, a whole-genome scan with 380 markers (11 residing on chromosome 20 between 2.90 and 100.63 cM, with an average distance between markers of 9 cM) was performed using MegaBASE (Amersham Biosciences) and ABI (Applied Biosystems) platforms. In 2009, four markers included in the genome-wide scan and giving the strongest evidence for linkage for MaxCigs24 (Saccone et al., 2007) were genotyped in an additional sample. Furthermore, for fine-mapping purposes, 14 additional microsatellite markers residing around and between these four markers (at 39.56–83.19 cM) were genotyped in the additional sample as well as in the original whole-genome scan sample. After the fine mapping, the average distances between all 25 markers and 18 markers within the fine-mapped region were 4 and 2.4 cM, respectively. The genotyping in 2009 was performed using ABI platform.

Data Analysis

The data genotyped in 2009 were checked for genotyping success ($>85\%$) by sample and by marker. After removing eight families yielding more than three Mendelian inconsistencies, no errors in the pedigrees were detected by program PedCheck (O'Connell and Weeks, 1998). The unlikely but Mendelian consistent genotypes were identified by the error-detection algorithm of program MERLIN (Abecasis, Cherny, Cookson, and Cardon, 2002) and were erased from the data using the program Pedwipe.

After the genotype quality check, the replication data (Study 1) consisted of 759 subjects with genotypes for 18 markers (4 whole-genome scan markers and 14 fine-mapping markers). The combined data (Study 2) included all 759 subjects from Study 1 and all 508 subjects from the existing whole-genome linkage scan (Loukola et al., 2008; Saccone et al., 2007) along with 35 additional family members, which had been genotyped after 2005. Overall, Study 2 sample consisted of 1,302 genotyped subjects including (a) 485 subjects with all 25 markers (11 whole-genome scan markers, 14 fine-mapping markers) genotyped, (b) 794 subjects with 18 markers (4 whole-genome scan markers, 14 fine-mapping markers) genotyped, and (c) 23 subjects with data for 11 genome-wide scan markers only (i.e., sample included in the genome-wide scan but for whom the fine-mapping was unsuccessful).

The multipoint and single-point linkage analyses were performed using program MERLIN (Abecasis et al., 2002). A nonparametric linkage analysis of *DSM-IV* ND affection status using Whittmore and Halpern (1994) NPL statistics to test for allele sharing among affected individuals was performed both within pairs (pairs) and arbitrary groups of individuals (all). The continuous traits were analyzed using MERLIN regression-based linkage analysis estimating the IBD at 2-cM intervals. When significant/suggestive linkage signals were obtained, sex differences were studied by analyzing males and females separately. In addition, in Study 2 the regular smokers of the sample were divided into groups of current and former smokers, and these groups were analyzed separately. The linkage analysis results are expressed as LOD scores, and the most

significant result of the trait, maximum LOD (MLOD) score, is presented.

Results

Study 1

The linkage analysis in the replication sample of 759 individuals yielded significant linkage with the *DSM-IV* phenotype on 20p11.21 at marker D20S871, with MLOD score of 3.8 (single-point, "pairs"; Figure 1). In sex-specific analyses (Figure 2), males provided suggestive evidence for linkage at marker D20S871 (20p11.21) with MLOD score of 2.6 (single-point, "all"), whereas a significant linkage with MLOD score of 3.4 (single-point, all) was observed in females at marker D20S884 (20q11.23). No significant linkage was observed with either FTND or the lifetime MaxCigs24.

Study 2

The linkage analyses of *DSM-IV* ND phenotype in the combined sample provided suggestive evidence for linkage on 20p11 peaking at marker D20S912 with MLOD score of 2.3 (multipoint, pairs; Figure 3). Sex-specific analyses revealed that this signal was driven by females; the MLOD score was 1.3 in males (single-point, all, D20S899, 20q13) and 3.3 in females (single-point, all, D20S884, 20q11; Figure 4). The ND measures correlated significantly with each other, and the tetrachoric

correlation between *DSM-IV* ND and FTND was 0.69 ($p < .001$, $SE\ 0.03$).

The analyses of the groups of current and former smokers (groups not further stratified by sex) did not yield any suggestive or significant linkage signals for *DSM-IV* ND (current smokers: multipoint, all, D20S912, MLOD score 0.7; former smokers: single-point, all, D20S861, MLOD score 0.9). The continuous trait analyses provided only nonsignificant linkage signals; thus, the sample was not stratified by smoking status. The highest LOD scores obtained were 1.5 for the continuous FTND score (at 44.9 cM) and 1.3 for the MaxCigs24 variable (at 70.9 cM).

To determine empirical significance of our linkage findings, we simulated 1,000 genome-wide scans of comparable structure using MERLIN and analyzed each simulated scan identically to the original data analysis. MERLIN performs gene-dropping simulation while retaining the genetic map, phenotype data, pedigree structure, and missing genotype data patterns, creating comparable data with random marker genotypes. Because the data are simulated under the hypothesis of no linkage, any linkage seen is due to chance alone, which therefore allows the evaluation of false-positive rate of the dataset analyzed. The empirical p value for a LOD score was defined as the proportion of simulated genomes where the LOD score in question was reached or exceeded. The highest LOD score (single-point 3.3 in females) produced an empirical p value of $<.001$ in the

Figure 1. Results of single-point and multipoint linkage analyses (testing for allele sharing within pairs of affected individuals) for the replication material (Study 1) on chromosome 20 for *DSM-IV* nicotine dependence diagnosis.

Figure 2. Result of single-point and multipoint linkage analyses (testing for allele sharing within arbitrary groups of affected individuals) for the males and females of the replication material (Study 1) on chromosome 20 for *DSM-IV* nicotine dependence diagnosis.

permutation analyses based on 1,000 permutations; as a LOD score this high (3.3) was not reached even once (highest LOD score obtained in the simulations for females was 3.2).

Discussion

We replicated earlier findings that chromosome 20 harbors a genetic element influencing ND (Han et al., 2010). In our previous genome-wide linkage scan (Loukola et al., 2008), LOD score of 2.36 was obtained for *DSM-IV* ND at 20p13. In the current study, linkage with a LOD score of 3.8 was found with a marker residing in 20p11.21 (Study 1). Sex-specific analyses revealed that the signal was predominantly driven by females (MLOD score of 3.4), with the linkage locus shifting to 20q11. When the sample size was increased (Study 2), evidence for linkage remained invariable (MLOD score of 3.3 in 20q11.23 for females).

Multiple genes reside under the linkage peaks. Among others, chromosome 20p11.21 harbors genes coding for Type 2 cystatins (*CSTs*), extracellular secreted polypeptides that are broadly distributed and found in most body fluids (Dickinson, Thiesse, & Hicks, 2002). Interestingly, there is a resemblance between cystatins and the α subunits of nAChRs, as both contain four cysteine residues forming two disulfide bonds, which in nAChRs play a critical role in agonist binding (Steinlein, Weiland, Stoodt, & Propping, 1996). Chromosome

20p11.21 also contains *GGTLC1* (gamma-glutamyltransferase light chain), a transpeptidase crucial in the metabolism of glutathione.

The female-specific linkage locus on 20q11.23 harbors, among others, *SRC* (proto-oncogene tyrosine protein kinase *SRC*), which plays a role in the regulation of embryonic development and growth, *BLCAP* (bladder cancer-associated protein), which encodes an apoptosis stimulating tumor suppressor protein, and *NNAT* (neuronatin isoform beta), suggested to regulate ion channels during brain development and thus being one of the many forming and maintaining factors of the nervous system (Dou and Joseph 1996). There is no evidence for any of these genes mentioned to have a role in the development of ND. The genes located at the linkage peak area are presented in the Supplementary Figure 1.

A gene earlier associated with ND measures, *CHRNA4*, resides on 20q13.2–13.33, nearly 26-Mb downstream. However, as our linkage signal peak is rather wide, the multipoint 1-LOD drop region defining the 90% confidence region (Dupuis & Siegmund, 1999) covers a region of ~15 cM, and the multipoint signal peaks at 20q13.12, the possibility that the signal is caused by a genetic element around *CHRNA4*, cannot be ruled out.

The chromosome 20q signal is repetitively identified in linkage and candidate gene association studies of smoking

Figure 3. Results of single-point and multipoint linkage analyses (testing for allele sharing within pairs of affected individuals) for the combined material (Study 2) on chromosome 20 for DSM-IV nicotine dependence diagnosis.

behavior, but no single genome-wide association study so far has found an association at the region ([The Tobacco and Genetics Consortium, 2010](#)). Even the three large meta-analyses of smoking quantity, smoking persistence, and smoking initiation did not observe any association to this region ([Liu et al., 2010](#), [The Tobacco and Genetics Consortium, 2010](#), [Thorgeirsson et al., 2010](#)). Thus, the effect of the *CHRNA4* locus on ND may be heterogeneous or sex specific. Allelic heterogeneity would suggest that a locus has multiple variants affecting a phenotype, and these alleles may be rare in the population at large. Linkage analysis examining the cosegregation of marker alleles may detect such effects, but association analysis can identify only common variants influencing the phenotype.

Sex differences are apparent in the association of this chromosomal area to ND. The fact that genome-wide association meta-analyses of smoking-related traits have so far not performed sex-specific analyses may be another explanation for the lack of significant association findings for this chromosome 20 region. In a study including families of European American and African American ancestry, [Li et al. \(2005\)](#) found that the association between *CHRNA4* and ND was strongest in African American females. However, the association has also been observed in a sample of Chinese male smokers ([Feng et al., 2004](#)). Both studies used FTND ([Heatherton et al., 1999](#)) as a continuous measure of ND.

The FTND affection status measure showed no linkage with chromosome 20 region markers in our study, and only a minuscule linkage signal with FTND as continuous variable was observed.

It has been proposed that the two different measures of ND, DSM-IV ND and FTND, measure the phenomenon from partly different points of view. This is supported by the fact that in clinical trials, DSM-IV ND and FTND rarely yield consistent results ([diFranza et al., 2010](#); [Moolchan et al., 2002](#); [Piper, McCarthy, and Baker, 2006](#)). It is likely that the FTND provides a stronger measure of physical and pharmacological dependence, whereas the DSM-IV ND measures more thoroughly the behavioral and cognitive factors, for example loss of control in terms of smoking behavior, underlying ND. On the basis of these arguments, and considering that females are more prone to the pressure of social factors than males, our results are consistent with the assumption of the differences between the two ND measures.

We observed changes in LOD scores when we increased the sample size by combining two subsamples of the NAG study. However, this is not unusual. LOD scores are known to be sensitive to changes ([Hodge and Greenberg, 1992](#)). Despite the changes in LOD scores, the signal exists.

The meta-analysis of 15 linkage scans of 10,253 family members identified multiple chromosomal regions, including chromosome 20, associated (at nominal significance levels) with smoking behavior based on FTND and MaxCig24 measures ([Han et al., 2010](#)). As for genome-wide association studies, also linkage studies require large sample sizes to study complex traits. For the same reason, the information content for markers analyzed in Study 1 were slightly lower than in Study 2 (Figures 1 and 3). ND as a complex trait seems to require larger

Figure 4. Results of single-point and multipoint linkage analyses (testing for allele sharing within arbitrary groups of affected individuals) for the males and females of the combined material (Study 2) on chromosome 20 for *DSM-IV* nicotine dependence diagnosis.

samples in order to increase the information contents of the markers.

A limitation of our study is the low number of participating parents leading to incomplete family structures and decreased power in the linkage analysis. This is due to the fact that the twins were relatively old (mean age of 57 years) at the time of the data collection and thus the family members included are mostly siblings. In addition, as the three-symptom diagnostic threshold of *DSM-IV* does not provide a perfect accuracy in the diagnosis of ND, some subjects' affection status may have been misclassified. No biochemical verification of the smoking status was performed as the analysis was based on affected only; it is unlikely that any nonsmokers would have claimed to be smokers in the extensive interview. Essentially, we did not replicate our MaxCigs24 finding (Saccone et al., 2007), which suggests that the original finding was either a false positive or, among other possible explanations, the variance or patterns of transmission for MaxCigs24 was different in consequential ways once subjects were added to the linkage families. The sample may be underpowered to resolve the non-replication, and more work is needed to be done to resolve the results.

In conclusion, our results provide further evidence that chromosome 20 harbors genetic elements influencing ND. The

comparison of our results with the literature supports the hypothesis that the locus has multiple mutant alleles influencing smoking behavior.

Supplementary Material

Supplementary Figure 1 can be found online at <http://www.ntr.oxfordjournals.org>.

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Declaration of Interests

JK has served as a consultant to Pfizer in 2008 on pharmacogenetics of smoking cessation and has received a GRAND award funded by Pfizer Inc. UB has served as a consultant to Pfizer in 2008 on ND measurements.

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