A Genome-Wide Screen for Nicotine Dependence Susceptibility Loci

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Abstract

Genome-wide model free linkage analysis was conducted for nicotine dependence and tobacco use phenotypes in 607 members of 158 nuclear families consisting of at least two ever smokers (100 or more cigarettes smoked in lifetime). DNA from whole blood was genotyped for 739 autosomal microsatellite polymorphisms with an average inter-marker distance of 4.6 cM. A peak LOD score of 2.7 was observed on chromosome 6 for scores for the Fagerström Test for Nicotine Dependence. Exploratory analyses were conducted to determine whether sequence variation at other loci affected other measures of dependence or tobacco use. Four additional loci with LOD scores of 2.7 or more were associated with alternative measures of nicotine dependence, one with current frequency of use, and one with smoking cessation. Several of the corresponding support intervals were near putative loci reported previously (on chromosomes 6, 7, and 8) while others appear to be novel (on chromosomes 5, 16, and 19).

Keywords

nicotine; dependence; linkage; genetics; smoking

Introduction

Twin studies have identified a significant genetic component to several aspects of tobacco use initiation and persistence [Carmelli et al., 1992; Heath et al., 1995; Li et al., 2003a;

Sullivan and Kendler, 1999; Swan and Carmelli, 1997; Tyndale, 2003] and, more recently, to clinically meaningful phenotypes such as indicators of nicotine dependence (ND) [Kendler et al., 1999; Lessov et al., 2004; McGue et al., 2000; True et al., 1999], "failed cessation" [Xian et al., 2003], and nicotine withdrawal [Xian et al., 2003]. While none of the published twin studies utilized the Fagerström Test for Nicotine Dependence questionnaire (FTND) [Heatherton et al., 1991], considered by many to be the "gold-standard" for measuring ND, one study [Lessov et al., 2004] has reported high heritability (71%) for the Heaviness of Smoking Index [Heatherton et al., 1989], which is composed of two of the six FTND items. The one published non-twin study of familial aggregation of ND assessed the FTND in sibling pairs and found evidence for significant aggregation for ND with a recurrence risk of 1.7 [Niu et al., 2000].

There have been several published reports involving linkage between a variety of tobacco use phenotypes and specific genomic regions. Collectively, these investigations have relied on four population cohorts: the Collaborative Studies on the Genetics of Alcoholism cohort [Bergen et al., 1999; Bierut et al., 2004; Duggirala et al., 1999], the Framingham Heart Study cohort [Goode et al., 2003; Li et al., 2003b; Saccone et al., 2003], the Christchurch, New Zealand cohort [Straub et al., 1999; Sullivan et al., 2004], and a family study of panic disorder [Gelernter et al., 2004]. The first attempt to map susceptibility loci for ND *per se* utilized the precursor of the FTND, the Fagerström Tolerance Questionnaire [Fagerstrom, 1978] (FTQ; ND defined as a score of 7 or more) in a convenience sample of 130 families from Christchurch, New Zealand [Straub et al., 1999]. While initial results by Straub et al. showed only modest evidence for linkage with specific regions (the strongest being a sharp peak at or near D2S1326), a subsequent reanalysis of the same data using different methods detected the same peak with an estimated Z score of about 2.5 [Sullivan et al., 2004].

The present study, based on a cohort that was originally constructed to identify predictors of incident smoking behavior among adolescents, was primarily designed to map loci for ND as well as for other tobacco-related phenotypes. A LOD score of 2.7 was detected on chromosome 6 for the FTND phenotype. Because we suspect that evidence for linkage depends on the specific tobacco-related phenotype that is assessed, we explored other measures of ND and tobacco use in this analysis. The analysis of these additional phenotypes could provide insight regarding other phenotypes that also segregate at ND loci, and such knowledge could facilitate replication in the future.

Subjects and Methods

Setting

An ongoing interdisciplinary study to identify environmental and genetic determinants of tobacco use collected data from families ascertained through index participants (probands) in the Smoking in Families Study [SMOFAM; Hyman Hops, PI, Oregon Research Institute, DA003706] [Swan et al., 2003a]. The SMOFAM study, initiated in 1981, is a comprehensive, repeated measures cohort study of environmental and psychosocial risk factors for adolescent and young adult substance use, including tobacco [Hops et al., 2000]. All methods and procedures described herein were reviewed and approved by the Institutional Review Boards of the collaborating institutions (SRI International, Oregon Research Institute, and the University of California at San Francisco) and are in accord with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and the standards of the University of California Tobacco-Related Diseases Research Program.

Subjects

The procedures and design of this study are described elsewhere [Swan et al., 2003a]. Briefly, the sample for our analysis consisted of a subset of 481 SMOFAM members who had participated in the longitudinal study. Compared to the original SMOFAM sample, the volunteers for this genetic investigation were more likely to be female, younger, and less likely to have smoked more than once. The majority (84%) of the probands identified themselves as of Caucasian origin with the remainder reporting Hispanic (2%), Native American (1%), or mixed (13%) origin.

Ascertainment

SMOFAM study participants who completed at least seven of the first 10 repeat assessments were contacted for a Family History of Tobacco Use (FHTU) interview. Four hundred and eighty one SMOFAM study participants completed the interview. The FHTU interview was used to determine pedigree structure of the nuclear family, vital status, and lifetime "ever" smoking status (i.e., 100 or more cigarettes smoked) for each first-degree relative. Using these data, families were stratified based on "density" of the ever-smoking phenotype (i.e., the number of biological members who had smoked 100 or more cigarettes in their lifetime) within the nuclear family. An essential criterion for prioritizing families was that the family be composed of at least two living, first-degree, ever-smoking members. In anticipation of the genetic analysis, eligible families had to consist of a minimum "triad" configuration, defined as two biological parents plus an ever-smoking offspring, or a biological sibling pair plus a biological parent (with one ever-smoker among them). Regardless of their individual ever-smoking status, the proband and all first-degree relatives within these high priority families were invited to complete the Smoking History Questionnaire (SHQ) and to provide a blood sample [Swan et al., 2003a]. ND and tobacco use-related phenotypes examined in the linkage analyses were derived from the SHQ.

Phenotype Assessment

A consensus has emerged in which ND is viewed as multidimensional and, therefore, should be assessed and quantified accordingly [Colby et al., 2000; Hudmon et al., 2003; Lombardo et al., 1988; Moolchan et al., 2002; Pomerleau et al., 1993]. Our primary interest was to identify loci that segregate with ND as determined by the FTND. Additional measures were also included to capture the complexity of the tobacco dependence phenotype (Table 1). These included: (a) elements from the DSM-IV dependence criteria [American Psychiatric Association 1994]; (b) smoking frequency and quantity; and, (c) quitting history. Individuals who never tried even a puff of a cigarette are excluded from all definitions. Individuals who tried smoking and those who smoked 100 or more cigarettes lifetime but were never daily smokers are included in the zero category of DSM-IV related measures. All other measures included lifetime daily smokers only. The footnote to Table 1 provides explicit definitions for the phenotypes used in this study grouped into three main categories.

Laboratory Analysis

Genotyping was done as described previously [Wilhelmsen et al., 2003]. DNA was isolated from frozen blood and DNA concentration was determined by optical density. Genotypes were determined for 739 autosomal microsatellite polymorphisms [Weber and May, 1989] (HD5, Applied Biosystems, 850 Lincoln Center Drive, Foster City, CA 94404). The average marker-to-marker distance was 4.6 cM with an average heterozygosity of greater than 77% in a Caucasian population. The sizes of marker amplimers were determined blind to pedigree structure and phenotype. Allele frequencies were estimated from the study population.

Pedigree Structure and Genotype Assessment

Genotypes were determined for 607 of the 867 participants who completed the SHQ assessment. Accuracy of pedigree structure and genotype determination was crucial for the planned genetic analyses, because it has been suggested elsewhere that a genotype error rate as small as 1% can significantly affect the results of a genomic linkage scan [Douglas et al., 2000]. A multistage data analysis approach was used to minimize errors in pedigree structure, sample identity, and genotypes. All available genotypes for all of the autosomal markers were analyzed for each family using PREST [McPeek and Sun, 2000] to detect sample and pedigree structure errors. Both phenotype and genotype data were excluded from further analysis when genotyping did not confirm the recorded pedigree structure (fewer than 1% of all participants). One hundred and fifty eight families had individuals with both phenotype and genotype information. In most families, siblings and some of their parents participated. An average of 3.8 (SD = 1.2) individuals participated per family; 269 sibling-sibling, 570 parent-child, 3 half-sibling and 112 spousal pairs with complete genotype and phenotype data were included in the analysis.

Pedcheck was used to detect non-Mendelian inheritance patterns [O'Connell and Weeks, 1998]. Relevant genotypes were reviewed blind to phenotypic status. For each Mendelian inconsistency, genotypes for the nuclear family were removed. To further reduce errors, the probability that each genotype was correct was assessed in the context of all other available genotypes using the maximum-likelihood error-checking algorithm implemented in Merlin [Abecasis et al., 2002]. Genotypes with a probability of less than 0.025 of being correct were removed from further consideration. Less than 0.5% of all genotypes were excluded.

Linkage Analysis

Multipoint LOD scores were determined using the QTL statistic implemented in Merlin (Abecasis et al. 2002). Merlin uses sparse binary trees to represent patterns of gene flow in general pedigrees to calculate exact multipoint estimates of IBD. The Merlin QTL statistic is a non-parametric statistic implemented in the general framework of Whittemore and Halpern (1994) as extended by Kong and Cox (1997). Discrete traits were coded as 2 for affected individuals and 1 for unaffected individuals but otherwise treated as continuous variables. In this approach a score, based on one of many possible functions, is computed for each family for the observed inheritance vector as well as all possible inheritance vectors. A family specific Z score is calculated based on the observed score and the mean and variance of scores for all possible inheritance vectors, and the weighted family specific Z scores are combined to produce an overall score. Merlin calculates a founder allele score for each family, which is the sum of the phenotype (less the sample mean) for each family member that carries the founder allele. The score function used by the QTL statistic is the sum of the square of the founder allele scores. As with all scores, the family scores are compared to the alternative scores for that family for all possible inheritance vectors. The contribution to the QTL statistic of individual families is weighted by family size. The QTL statistic is less likely to be affected by extreme phenotypes and the distribution of the phenotypes since the scores for each family are normalized by the mean of all possible scores for that family. In contrast, the maximum-likelihood based variance-component (Blangero et al. 2000) method is more likely to be biased by outliers. The use of the QTL statistic in this case allowed for a greater proportion of the phenotypic information than the more commonly used affected sib pair methods (e.g. NPL and ALL, Kruglyak et al. 1996) because information from both affected and unaffected individuals could be used.

The robustness of the QTL statistic to violations of multivariate normality, as implemented in Merlin (Abecasis et al. 2002) has not been reported. To assess the statistical significance of the observed QTL LOD scores, 10000 genomes of genotype data were constructed

independently of the measured phenotypes using the simulation option in Merlin. The algorithm constructed haplotypes for the founders based on the estimated allele frequencies in the original sample and then allowed them to segregate through the families. Only genotypes consistent with the measured sample were retained for analysis. The Merlin QTL option normalizes phenotypes to the population mean and constructs a score statistic, which is robust to non-normality. Genome-wide multipoint LOD scores were then calculated using the original measured phenotypes. All LOD score peaks were identified and used to estimate the number of times that a peak was observed above a specific LOD score/per simulated random genome.

The frequency peaks above specific thresholds per simulated random genome were calculated. It was observed that with this set of families and markers, independent of phenotype, the number of times a peak above a threshold was observed per random genome was equivalent across phenotypes. This was true for both continuous variables as well as for dichotomous traits. LOD scores of 2.7 and 3.4 occurred an average of 1.0 and 0.1 times per random simulated genome, respectively. The uniformity of the number of peaks above specific thresholds for all phenotypes suggested that the QTL statistic is robust to deviations from multivariate normality in a moderately large set of nuclear families with dense genotyping data.

Results

Description of Members of the Proband and Parental Generations

Participating SMOFAM members of the proband generation were 28.2 years of age (SD = 4.4), while members of the parental generation averaged 54.3 years of age (SD = 4.9). Approximately 51% of the proband generation was female. The prevalence of education beyond high school was 63.3% among the proband generation and 76.9% among parents. Twenty percent of the proband generation and 41% of parents earned $50,000 or more annually. Consistent with the initial construction of the SMOFAM cohort and subsequent recruitment of families, there was an overall high prevalence of current smoking among both generations with a higher prevalence among probands and siblings (39.1%) than among their parents (21.5%). The study sample exhibited a higher prevalence of lifetime DSM-IV-like ND, 74.0% and 70.3% for members of the parental and proband generations, respectively, than does the general U.S. population (24.1%); [Kandel et al., 1997; Kandel and Chen, 2000].

Table 1 provides descriptive statistics for the phenotypes used in this analysis for both parental and proband generations as well as for the probands themselves. The minimum and maximum sample size available for characterization of each generational grouping is given at the top of each column. The sample sizes vary due to the inclusion of data from lifetime smokers only for some of the phenotypes. The table reveals higher FTND scores for parents (M = 4.7) than for members of the proband generation (M = 3.4).

Primary Linkage Analysis

For the FTND summary score a maximum LOD score of 2.7 was seen at 178 cM on chromosome 6 (Figure 1 and Table 2). The marker closest to the peak was D6S446. The support interval (defined as the region in which LOD scores are greater than the maximum LOD score – 1) included 156 to 191 cM (D6S1581-D6S281). The next largest LOD score peak had a maximum of 2.4 at 88 cM on chromosome 20 (data not shown).

Secondary Linkage Analysis

In subsequent analyses we examined additional tobacco use phenotypes for evidence of linkage. To minimize the reporting of results that may be due to chance, we report individual LOD scores of 2.7 or greater (see Table 2).

Other measures of ND—The support interval for Withdrawal Severity overlapped the FTND support interval on chromosome 6 (Figure 1) with a peak LOD score of 2.7. Also shown in Figure 1 is a quitting history phenotype, Short-Term Quit that had a peak LOD score of 1.9 in the same region.

The largest LOD score for any ND phenotype (LOD score = 3.0) was observed for DSM-IV-like ND Severity (see footnotes [4 and 5], Table 1 for a list of the criteria) near D7S636 (164 cM; support interval 159-167 cM; Table 2). For the dichotomous DSM-IV-like ND measure, a maximum LOD score of 2.7 was observed on chromosome 8 at 31 and 35 cM (near marker D8S258).

Tobacco use—Cigarette Quantity produced a peak LOD score of 2.9 on chromosome 5 near marker D5S1969. A peak LOD score of 2.9 was observed on chromosome 19 (at 90 cM near D19S572) for Current Smoking Frequency (Table 2).

Quitting history—The largest linkage peak (LOD score = 4.0) in the entire study was observed for Short-Term Quit at D16S415 (Table 2).

The primary and secondary linkage analyses were re-run after limiting the sample to only those individuals of Caucasian or Hispanic origin (a total of 86% of the entire sample). The primary LOD score for the FTND on chromosome 6 remained unchanged (2.7). The linkage peaks observed on chromosomes 5 (Cigarette Quantity) and 16 (Short-Term Quit) were somewhat reduced but remained above 2.7 and were 2.8 and 3.1, respectively. In the restricted sample, the remaining four secondary linkage peaks fell below the 2.7 cutoff (Withdrawal Severity, DSM-ND Severity, dichotomous DSM-ND, and Current Smoking Frequency).

Discussion

A search for autosomal loci affecting ND and a variety of tobacco-related phenotypes was conducted in nuclear families with a high prevalence of smoking. In discussing these results, we indicate, where possible, their proximity to other reported linkage peaks or candidate genes. Potential candidate genes were selected on the basis of previously reported significant association with tobacco use phenotypes [Tyndale, 2003] or their location within relevant receptor, metabolic, or signal transduction pathways [Sullivan et al., 2004]. Linkage peaks that may not have been reported previously are noted as novel.

A maximum LOD peak on chromosome 6 was detected for the FTND summary score, and this peak was supported by a nearby peak for Withdrawal Severity. Previous work has identified linkage peaks at or near the support interval reported here for FTND [Bergen et al., 1999; Sullivan et al., 2004]. Moreover, the support interval is very close to the *OPRM1* gene (mu 1 opioid receptor) and contains *MAP3K4* (mitogen-activated protein kinase 4) and *LPAAT-delta* (lysophosphatidic acid acyltransferase, delta), both candidate genes for ND [Sullivan et al., 2004]. We therefore report this finding with increased confidence.

We are encouraged that several loci reported here have been detected in other linkage studies as well. The support interval on chromosome 7 observed here for DSM-IV-like ND Severity is near the linkage peak, D7S1804, reported previously for the FTQ [Sullivan et al.,

2004], and is near the candidate gene *HTR5A* (5-hydroxytryptamine [serotonin] receptor 5A). The support interval seen in the present study on chromosome 8 for DSM-IV-like ND is near previously reported linkage peaks for the ever smoking phenotype [Bergen et al., 1999] and is close to the candidate genes *CHRNA2* (alpha 2 nicotinic acetylcholine receptor) and *ADRA1A* (alpha 1A adrenergic receptor).

Heterogeneity inherent in ND is apparent when viewing these results. DSM-IV and FTND definitions of ND are poorly correlated with a kappa value of agreement of 0.2 [Moolchan et al., 2002]. The development of the DSM classification of drug dependence was largely based on evidence from the alcohol field and may not be specific to ND. The parent instrument of the FTND, the FTQ, on the other hand, was specifically developed to measure ND. In our study, the FTND was assessed for the heaviest period of smoking in the respondent's life [Hudmon et al., 2005] and, therefore, can be thought of as a lifetime assessment of ND severity that may have a physiological basis. Whether the heterogeneity across chromosomes for indices of ND derives from genetic or measurement sources cannot be determined from the present study and will need to be addressed in future research.

The few previous biometric or measured genetic studies that were designed to evaluate the role of genetic influences on the ability to stop smoking and/or to maintain abstinence support the notion of a genetic substrate. Carmelli et al. [Carmelli et al., 1992] found epidemiological evidence suggestive of genetic involvement in relapse following cessation. Significant heritability estimates have been reported for "failed cessation" (54%) [Xian et al., 2003] and for difficulty quitting (54-68%) [Lessov et al., 2004]. Previous work has determined that variation in dopaminergic (*DRD2*), opioidergic (*OPRM1*), and cytochrome P450 metabolic (*CYP2B6*) genes is associated with the ability to stop smoking following pharmacological treatment [Lerman et al., 2003; Lerman et al., 2004; Miksys et al., 2003; Swan et al., 2005]. The support interval reported on chromosome 16 for Short-Term Quit may be novel and is near several candidate genes proposed recently [Sullivan et al., 2004] including *PRKCB1* (Protein kinase C, beta 1), *CBFB* (core binding factor beta subunit), and *ATP6V0D1* (vacuolar H$^+$-ATPase, V0 subunit d isoform 1).

The support interval on chromosome 19 for Current Smoking Frequency also appears to be new and is not near any previously reported peaks. Several candidate genes map near the interval including *CYP2A6* (cytochrome P450 2A6), *CYP2B6* (cytochrome P450 2B6), and *PTPRH* (protein tyrosine phosphatase, receptor type, H).

Heritability estimates for most or all of these phenotypes have been reported previously, including: the number of cigarettes smoked per day (range of estimates, 49-72%); [Carmelli et al., 1990; Hettema et al., 1999; Kaprio et al., 1984; Lessov et al., 2004; Swan et al., 1990], FTND (71% for a composite of two items from the scale) [Lessov et al., 2004], DSM-IV ND (present/absent; range of estimates, 56-60%) [Lessov et al., 2004; True et al., 1999], and current smoking (range of estimates 27-53%) [Heath and Martin, 1993; Madden et al., 1999, 2004].

The LOD scores reported here are among the highest reported for ND and related phenotypes. The higher LOD scores observed were in part due to the sample size and ascertainment strategy used. In addition, the linkage analysis was conducted with the QTL score statistic to use all of the available information for each trait. Simulation analysis indicated that the QTL score statistic is robust to significant deviations from normality even to the extent that it can be used for dichotomous traits. Unlike other commonly used methods, the QTL score statistic allows information to be used from both affected and unaffected individuals. Implicit in our use of the QTL score statistic is the assumption that affected and unaffected individuals are equally informative. We think it is likely that non-

genetic factors have as big an impact on *not* smoking as they do on smoking [Heath and Martin, 1993; Madden et al., 1999; Swan et al., 2003a; Swan et al., 2003b; True et al., 1999].

In summary, the present results are encouraging and provide support for the importance of taking into account the phenotypic complexity of ND in genetic investigations. Rather than one global measure of ND as used in previous studies, the use of multiple measures more accurately reflects the current state-of-the-art of the measurement of ND. The genetic contributions to different components of ND appear to be due to different loci. This conclusion is supported by previously published studies. That different loci are associated with different components of ND may be important to the eventual understanding of the underlying genetic architecture of ND.

Acknowledgments

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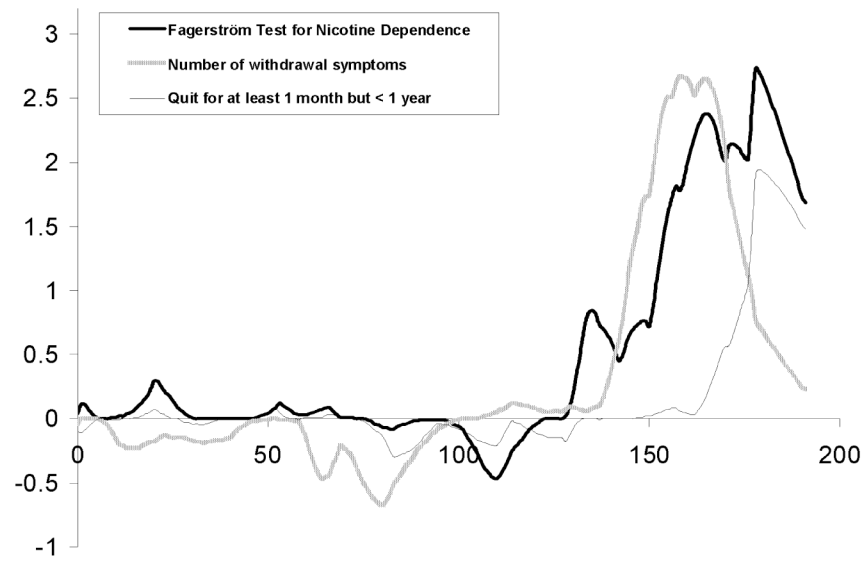
Fig. 1.
Multipoint LOD scores are shown for chromosome 6 FTND (thick solid black line), Withdrawal Severity (thick gray line), and Short-Term Quit (gray thin line) phenotypes.

Table I

Phenotype Means (SD) for Continuous Measures and Prevalence Estimates (%) for Categorical Measures in the Parental and Proband Generations and Among Probands Only.

Phenotype (range)	Parental generation	Proband generation[1]	Proband
N (range)[2]	178-275	213-336	105-160
Dependence			
FTND (0-10)[3]	4.7 (2.8)	3.4 (2.4)	3.2 (2.4)
DSM-IV-Like Severity: sum of 7 ND criteria (lifetime) (0-7)[4]	3.6 (2.2)	3.3 (2.1)	3.4 (1.9)
DSM-IV-Like Dependence: 3 or more of 7 ND criteria (lifetime) (% yes)[5]	74.0	70.3	73.9
Withdrawal Severity: sum of 9 withdrawal symptom scores (0-27)[6]	10.4 (7.2)	8.7 (6.5)	8.5 (6.4)
Tobacco use			
Quantity: number cigarettes smoked per day (past 3 months) (0-80)*	4.7 (11.0)	6.0 (9.0)	6.5 (9.0)
Current Frequency: smoking one or more cigarettes on most days (% yes)*	21.5	39.1	43.7
Quitting history			
Quit smoking cigarettes completely (% yes)*	65.3	33.8	32.8
Short-Term Quit: ever quit smoking for > 1 month but < 1 year (% yes)*	54.1	56	49.5

[1] The sample size for the proband generation includes the probands themselves.

[2] The minimum and maximum sample size available for characterization of each generational grouping is given at the top of each column. The sample sizes vary due to the inclusion of data from lifetime smokers only for some of the phenotypes.

[3] Fagerström Test for Nicotine Dependence summary score assessed during the heaviest period of smoking lifetime.

[4,5] DSM-IV criteria were constructed using items from the DSM-based Nicotine Dependence Scale [Snedecor et al., 2004] in combination with other questionnaire items. DSM-IV ND was defined as a continuous severity summary score across seven ND criteria[4] (tolerance, withdrawal, ever smoked more than meant to, difficulty quitting, great deal of time spent obtaining cigarettes, give up important social or occupational activities in order to smoke, continue smoking despite health problems caused or exacerbated by smoking), as well as a categorical measure to indicate the reporting of 3 or more of 7 ND criteria lifetime[5].

[6] Summation of nine withdrawal symptom scores [Hughes and Hatsukami, 1986; Jarvis et al., 1982]; depressed mood, trouble falling asleep, irritability/frustration/anger, anxiety, difficulty concentrating, restlessness, increased appetite/weight gain, cravings to smoke, decreased heart rate) rated on a 4-point scale, 0 = not at all, 3 = severe.

* Definitions of these four phenotypes are self-explanatory.

Table II

Multipoint Linkage Results for Nicotine Dependence Phenotypes.

Chr	Phenotype (Category)	Peak cM (Support Interval)	Max LOD	Closest marker to peak
5	Quantity: number cigarettes smoked per day (past 3 months)	62 (57-70)	2.9	D5S1969
6	FTND	178 (156-190)	2.7	D6S446
	Withdrawal Severity: sum of 9 withdrawal symptom scores	159 (148-173)	2.7	
7	DSM-IV-Like Severity: sum of 7 ND criteria (lifetime)	164 (159-167)	3.0	D7S636
8	DSM-IV-Like Dependence: 3 or more of 7 ND criteria (lifetime)	31 (23-39)	2.7	D8S258
16	Short-Term Quit: ever quit smoking for > 1 month but < 1 year	70 (52-80)	4.0	D16S415
19	Current Frequency: smoking one or more cigarettes on most days	90 (83-97)	2.9	D19S572