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Genomewide suggestive linkage of opioid dependence to chromosome 14q

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The genetic predisposition to addiction to opioids and other substances is transmitted as a complex genetic trait, which investigators are attempting to characterize using genetic linkage and association. We now report a high-density genome-wide linkage study of opioid dependence. We ascertained 305 DSM-IV opioid dependent affected sibling pairs from an ethnically mixed population of methadone maintained subjects and genotyped their DNA using Affymetrix 10K v2 arrays. Analysis with MERLIN identified a region on chromosome 14q with a non-parametric lod (NPL) of 3.30. Secondary analyses indicated that this locus was relatively specific to the self-identified Puerto Rican subset, as the NPL increased from 3.30 to 5.00 ($NPL_{Caucasian} = 0.05$ and $NPL_{African\ Amer.} = 0.15$). The 14q peak encompasses the *NRXN3* gene (neurexin 3), which was previously identified as a potential candidate gene for addiction. Secondary analyses also identified several regions with gender-specific NPL scores greater than 2.00. The most significant was a peak on (10q) that increased from 0.90 to 3.22 when only males were considered ($NPL_{female} = 0.05$). Our linkage data suggest specific chromosomal loci for future fine-mapping genetic analysis and support the hypothesis that ethnic and gender specific genes underlie addiction susceptibility.

INTRODUCTION

Addiction to heroin is a major public health problem both in the USA and throughout the world. Nearly four million people in the USA have tried heroin, and approximately one million are currently addicted (1). Tens of billions of dollars are spent every year on the adverse health effects and criminal consequences of heroin use. Although statistics vary in different parts of the USA, approximately one-fifth of intravenous users of heroin are infected with human immunodeficiency virus and three-quarters with hepatitis C virus (2). A substantial body of evidence from family, twin and adoption studies indicates that a genetic component underlies addiction disorders (3–11). Twin studies show a 2–4-fold difference in concordance for substance abuse disorder between monozygotic and dizygotic twins (12,13). Calculations by Pickens *et al.* (12) suggest that genetic factors contribute 31% of the vulnerability in males for drug abuse/dependence disorders. Higher values (45% and 79%) were reported by Grove *et al.* and Van den Bree *et al.*, respectively (5,13). Both drug-specific

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and common genetic factors contribute to addiction (14–16). In a study by Tsuang *et al*. (15), the 54% overall heritability for opiate dependence could be apportioned into a component accounting for unique genetic variance (38%) and another for shared liability (16%) with other substances. Kendler *et al*. (16), on the other hand, in their study on male twins, attributed all of the genetic liability for six substances, including opiates, to a common genetic factor. There is also some evidence that genetic factors may play less of a role in opiate use liability in females (12,13,17).

An underlying genetic vulnerability is consistent with current models of addiction as a brain disease, which follows three decades of neurobiological research of the dopaminergic reward circuitry (18–21). It is hypothesized that polymorphic variation in the genes involved in regulating the reward circuitry may cause interindividual differences in drug use initiation, the rewarding effects of addicting substances and the brain's adaptation to them (4,17,22,23).

An underlying genetic component has also been observed in animal models of addiction. For example, the Lewis rat strain is more vulnerable to self-administration of addicting substances, compared to the Fisher 344 and Holtzman strains (24,25). This suggests that a polymorphic behavioral trait or physiological process has emerged in the evolution of higher animals that manifests in humans (and artificially in laboratory animals) as an increased vulnerability to compulsively seek addicting substances without regard to adverse consequences, following an initial exposure to those substances.

Mendelian inheritance does not explain the pattern of drug addiction susceptibility seen in families. Rather, the inheritance is more consistent with a complex inherited trait influenced by environmental factors. Most previous studies carried out to identify genetic factors in addiction have focused on analyzing candidate genes based on knowledge of the brain dopaminergic reward pathways, and the receptor and transporter proteins targeted by addicting substances, such as *DRD2* and *OPRM1*. However, since neurotransmitter and signal transduction pathways in the brain interact with each other on many levels, and trigger complex intracellular events affecting hundreds of different proteins and genes, identifying the specific receptors and transporters targeted by addicting substances does not necessarily translate into choosing the correct candidate genes for analysis. A hypothesis-free, whole genome approach offers the potential to capture the full range of genetic possibilities in an unbiased manner.

An early genome-wide study relevant to addiction was carried out by Berrettini *et al*. (26), who used quantitative trait locus (QTL) mapping to identify several loci for self-administration in mice, one of which turned out to be the *Oprm1* gene. Researchers in human addiction subsequently carried out genome-wide linkage studies in alcoholism, nicotine, cocaine and opiate dependence. Positive loci on chromosomes 1, 4 and 7 were reported in the initial linkage reports in alcoholism (27–30). The chromosome 4 linkage peak at 4p13–p12 encompasses the $GABA_A$ gene cluster. Eventually, Edenberg *et al*. narrowed the search using linkage disequilibrium (LD) mapping and identified SNPs and SNP haplotypes in the *GABRA2* gene that were significantly association with alcoholism, a finding that was replicated by Covault *et al*. (31,32). Several loci for nicotine addiction have also been mapped, and eventually, *CHRN4*, which codes for the nicotinic acetylcholine receptor α4 subunit, and *GABAB2*, which codes for the type β receptor subunit 2 of the GABA receptor, were targeted as likely susceptibility genes (33,34). Recently, Gelernter *et al*. (35) published the results of a genome-wide linkage scan using a 400 marker set in 528 sibling pairs concordant for cocaine dependence. Two significant linkage peaks were identified on chromosomes 9 and 12. Glatt *et al*. (36) recently reported the first genome-wide linkage study in heroin addicts in Han Chinese families; no markers achieved genome-wide significance, although two non-parametric lod (NPL) *Z*-scores >2.0 were found (2.19 at marker D4S1644 and 2.36 at marker D17S1880).

An alternative to linkage mapping—whole genome association using LD—has also been used to map susceptibility genes for drug addiction. Using this approach, dozens of novel candidate genes have been targeted including *NRXN3* (neurexin 3) and other cell adhesion molecules involved in synaptogenesis and neuronal development (37,38).

We now report a genome-wide linkage study to map susceptibility genes in opiate dependence using concordant sibling pairs from the New York City metropolitan area. Subjects were recruited from methadone maintenance treatment programs (MMTPs), a clinical population suited for genetic studies as they are some of the most severely addicted patients in the world, exhibiting a high degree of polysubstance abuse. Cocaine use is found in 40–75% of patients, alcohol abuse in an estimated 15–30% and more than 90% smoke cigarettes (39–44). Since heritability increases sharply with a diagnosis of drug dependence, the unambiguous nature of opiate dependence in this population, combined with the high prevalence of polysubstance abuse, is likely to increase the probability of genetic loading (13).

RESULTS

A total of ∼25 000 patients from ∼60 participating MMTPs were screened. Clinical data were collected and DNA obtained from a total of 667 subjects from 296 subject-described families. Every subject met DSM-IV criteria for lifetime opioid dependence; 73% had a lifetime diagnosis of cocaine abuse/dependence. Two-thirds of the subjects in our study were of Hispanic origin (Table 1). Although Hispanics constitute a heterogeneous mix of cultures and ethnicities, our population was fairly homogenous in that the overwhelming majority were of Puerto Rican heritage, reflecting the demographics of the neighborhoods serving the clinics we used for recruitment, which were primarily in Bronx County and the lower East Side of Manhattan. Ninety-four percent of the Hispanic subjects in our study reported that all four grandparents were born in Puerto Rico; 2% had four grandparents born in Dominican Republic; 1% had four Cuban-born grandparents; and the remainder reported mixed heritage that included grandparents born in St Thomas, Venezuela, Spain, Mexico, along with Puerto Rico and Dominican Republic. The 'other' category (Table 1) consisted primarily of self-identified African Americans who reported having a White or Native American parent or grandparent.

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Table 1. Basic demographics

	Frequency (%)	Female/male	Age
Hispanic	66.46	45.8/54.2	40.6
African American (non-Hispanic)	22.62	34.9/65.1	46.5
White (non-Hispanic)	10.10	30.4/69.6	42.8
Other	0.80	50.0/50.0	39.0

Male–male, 140; female–female, 88; male–female, 77; total, 305. Full sibs, 237; half sibs, 68; total, 305.

There was a significant correlation between self-reported ethnicity and cluster analysis using STRUCTURE (Pearson Correlation Coefficient = 0.59613; $P < 0.0001$; Supplementary Material, Figure S1).

After eliminating singletons whose siblings did not return for ascertainment, we genotyped 591 DNA samples for 10,204 SNPs distributed genome-wide. Nineteen samples had call rates below 94% and the assays were repeated. The overall genotype call rate after these samples had been repeated was 97.33% (~9932 genotypes/person) and data from 9769 autosomal SNPs that were both polymorphic and had call rates in the total sample of >90% were used for subsequent analysis. One hundred and sixty-nine markers were not in Hardy Weinberg equilibrium (HWE) (empirical P-value <0.001), leaving 9600 for further analysis. Of those, 1188 were found to be in LD with another marker ($r^2 > 0.4$) and were eliminated. Consequently the final set of markers for the linkage analysis contained 8412 SNPs.

After estimation of allele frequencies, the true genetic relationships between self-reported sibling pairs were examined by using RELCHECK and PREST (45,46). These analyses resulted in the deletion of 51 families in which self-reported siblings were found to be either unrelated, or parent/offspring pairs. Based on the results using RELCHECK, PREST and ALTERTEST, we also found 15 pairs of self-reported full siblings to be half-siblings, five pairs of 'siblings' to be cousins and two pairs of 'siblings' to be half sibs + cousins. The relationship in several other pairs could not be determined with certainty (more related than half-sibs, less related than full sibs) and were not included in the linkage analysis. The final sample for linkage analysis contained 490 subjects. These included 201 sibpairs, 22 sibtrios, three sibquads and two sibquints for the total of 305 affected sibling pairs. Of these, 237 were full sibs and 68 were half sibs. There were 140 male–male pairs, 88 female–female pairs and 77 male–female pairs (Table 1).

Nonparametric linkage analyses, both two-point and multipoint, were carried out on the entire data set using MERLIN (47). To estimate empirical significance levels of our data set, we used MERLIN to generate 500 replicates of the entire genome, retaining the family structure. The threshold value for observing a genome-wide type I error once per scan was a –log(P) NPL score of 3, under the hypothesis of no linkage. In our primary analysis, using all individuals with DSM-IV opiate dependence as affected, an NPL score greater than 3.00 was achieved for a wide region of markers located on chromosome 14q, suggestive of genome-wide significance (48). Two peaks ~12 cM apart were seen: 3.30 at 87.21–90.40 cM and 3.22 at 75.55–73.63 cM (Fig. 1; Supplementary Material, Figure S2 for more detailed individual chromosome data).

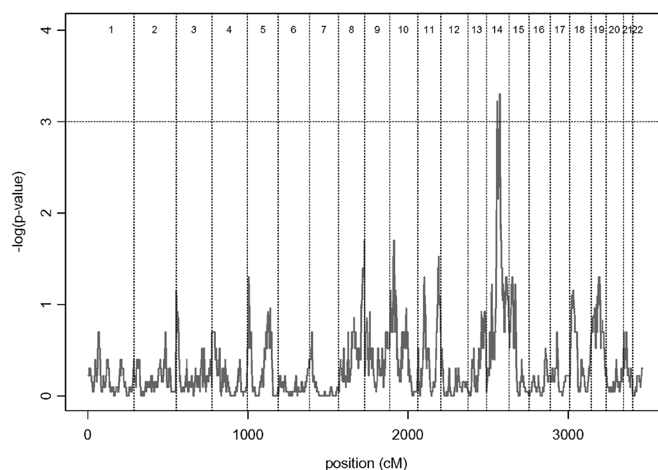

Figure 1. Linkage analysis-entire data set.

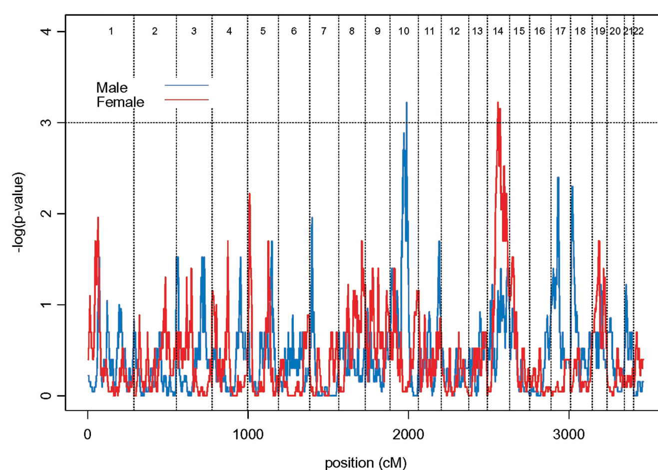

Figure 2. Linkage analysis-gender.

We also carried out secondary analyses stratified by gender and ethnicity (Figs 2 and 3 respectively; Supplementary Material, Figures S3 and S4). Even though the number of female–female pairs ($n = 88$) is less than the number of male–male pairs ($n = 140$), the majority of the contribution to the 14q peak observed in using all pairs appeared to be coming primarily from the female siblings. NPL scores of 3.22 (73.52–73.73 cM) and 3.16 (78.15–78.41 cM) were found in females, whereas the corresponding NPL scores in this region for males were 1.15 and 1.40 (Fig. 2). Likewise, a putative male-specific locus on chromosome 10q was observed with an NPL score of 3.22 (103.94 cM). A region ~18 cM away was also positive in males (2.89 at 86.02–86.93 cM). The female-specific scores in this region of chromosome 10q were essentially zero (Fig. 2).

The ethnic-stratified results also showed differential contributions to the 14q linkage peak (Fig. 3). An NPL score of 5.00 was observed for the Hispanic group at 87.22–87.46 cM on chromosome 14q, while the scores in the region were well below 1.0 for the Caucasian and African American groups.

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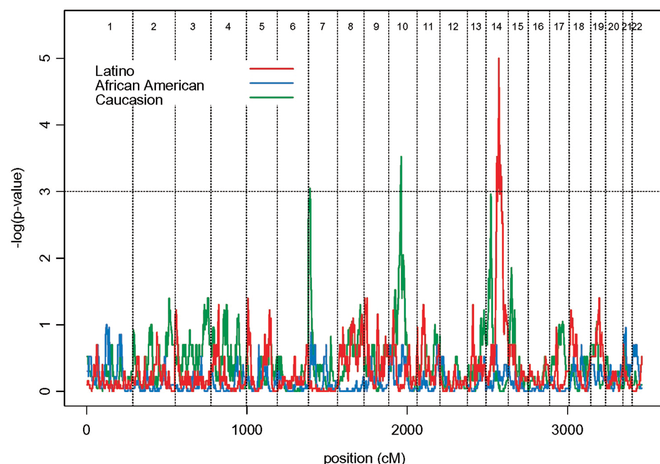

Figure 3. Linkage analysis-ethnicity.

After applying a Bonferroni correction for three genome scans, the NPL score of 5.00 is 'suggestive' of genome-wide evidence for linkage using the Lander and Kruglyak significance criteria (48).

Other NPL scores greater than ~2.0 are shown in Table 2. The most significant were found in the Caucasian subgroup. However, considering that less than 11% of the subjects in this study were Caucasian, these findings could easily be due to type I error. Indeed, the reduction in sample size necessitated by the secondary analysis of subgroups suggests that even the more robust gender and ethnic-specific findings shown in Table 2 should be viewed with caution.

DISCUSSION

In the past few years, several groups have used non-parametric linkage analysis and whole genome association to map loci in various addiction disorders and identify positional candidate genes. One of the earliest was the Collaborative Study of the Genetics of Alcoholism (COGA), which culminated in the identification of *GABRA2*, and more recently, *GABRA1*, as positional candidate genes for alcoholism, alcoholism-related behaviors, co-morbid drug use and antisocial personality (27,31,49–52). Later, other groups identified potential nicotine, cocaine and heroin addiction loci (33–36,53–55). Two positional candidate genes for nicotine addiction, which are also feasible physiological and pharmacological candidates—GABBR2 and CHRNA4—were subsequently identified.

Adding to this growing list of addiction susceptibility genes and loci, we now report the results of a genome-wide linkage study in heroin addiction. In our primary analysis, two peaks on 14q with NPL scores greater than 3.0 were identified, although it is not clear at this time whether these represent two distinct regions of interest on the chromosome. In addition, our gender-specific analysis identified several other regions of potential interest. None of these loci map in the vicinity of genes previously considered as potential candidates for opioid dependence susceptibility, including *OPRM1, DRD2, OPRD1, OPRK1, PENK* and *PDYN* (56). In addition, with one possible exception, there was no significant overlap in our findings with regions mapped in the two other genome-wide linkage studies carried out in drug-dependent populations that most closely resemble ours (35,36). This could be due to the presence of drug, ethnic or gender-specific genetic factors. For example, these other linkage studies were carried out in European and African Americans (35) and in a Han Chinese cohort (36), whereas our study population was largely of Puerto Rican ancestry. In addition, the linkage study reported by Gelernter *et al*. (35) was primarily carried out in cocaine-dependent sibling pairs. Although some subjects were also opiate abusers, the number with this diagnosis, their cluster 4, was relatively small ($n = 151$) and, therefore, may have been underpowered to find significant linkage signals at the loci we identified (35). Alternatively, unique behavioral features characteristic of the patient populations used in each study could account for the heterogeneity in the linkage findings. Patients enrolled in MMTPs, the primary recruitment source for opioid-dependent subjects in this study, are characterized by polysubstance abuse and a high prevalence of personality disorders, notably antisocial personality, social dysfunction and axis 1 co-morbidity, factors that all influence drug use initiation (57). Thus, the apparent genetic heterogeneity across studies could be due to the contribution of the different genetic factors influencing underlying co-morbid behavioral traits in the various cohorts, as well as ethnicity. Replication in sufficiently powerful data sets and a closer examination of co-morbid traits using a case control approach will be needed to resolve these alternative hypotheses.

The exception to the heterogeneity in the linkage findings may occur on chromosome 17. An NPL score of 2.40 was found in our male cohort at 51.41–56.78 cM, which, as seen in Table 3, overlaps with a region mapped by Glatt *et al*. their Han Chinese opioid-dependent cohort (36). It is interesting to note that 73% of the subjects in the Glatt *et al*. study were male.

While there are more differences than similarities when the different linkage studies are compared, potential regions of overlap were found between our study and the whole genome association studies reported by Liu *et al*. (37,38). In their initial report involving an analysis of 11 482 SNPs, associations were found between 38 markers and heavy drug use in European American and African American subjects. Two of these, in the *NRXN3* and *SYT16* genes, fall within the chromosome 14 linkage signals reported here (Table 4). In fact, the highest NPL score in our study was found with markers that map only 50 kb upstream of the *NRNX3* gene. Neurexins are presynaptic transmembrane proteins that bind to postsynaptic neuroligans, an interaction that aligns synaptic termini and dendrites, bringing them into close juxtaposition (58). *SYT16* is a member of the synaptotagmin family, which are transmembrane proteins intimately associated with the vesicle fusion machinery, an interaction that culminates in exocytosis.

Liu *et al*. (38) expanded their whole genome association study by using 639 401 SNPs and identified an additional two-dozen genes encoding cell adhesion proteins associated with high drug use. Adhesion proteins involved in regulating synapse formation and memory—cadherins and NrCAM—have previously been implicated in the pathogenesis of drug addiction and the effects of prenatal cocaine (59,60). Interest-

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Table 2. Summary of linkage findings

Chr	−log(*P*)	Genetic map (cM)	SNP interval	Physical map (Mb)	Sample
14	3.30	87.21–90.40	rs1861891-rs961097	77.58–80.99	ALL
14	3.22	73.55–73.63	rs2877455-rs2180770	67.83–67.84	ALL
10	3.22	103.94	rs951203	84.04	MALE
14	3.22	73.52–73.73	rs1751382-rs2180770	67.76–67.84	FEM
14	3.15	78.15–78.41	rs1859551-rs728497	71.23–71.50	FEM
14	3.15	87.18–87.38	rs724263-rs1950265	77.39–77.66	FEM
10	2.89	86.02–86.93	rs2394335-rs1900018	68.55–69.72	MALE
17	2.40	51.41–56.78	rs1551359-rs722374	26.89–30.77	MALE
18	2.30	9.92–13.29	rs556831-rs727615	4.38–5.22	MALE
5	2.22	14.92–15.67	rs400328-rs1845852	6.85–7.01	FEM
1	1.96	64.45–65.13	rs883805-rs514262	36.65–37.06	FEM
7	1.96	17.87–18.22	rs1074416-rs2189344	10.96–11.31	MALE
14	5.00	87.22–87.46	rs1861891-rs1950265	77.39–77.66	HISP
14	3.52	74.21	rs52701	68.28	HISP
10	3.52	77.38–77.64	rs4267019-rs1459990	60.56–60.69	CAUC
7	3.05	12.65–13.35	rs929542-rs2190772	7.33–7.46	CAUC
14	2.96	36.77–37.38	rs18897496-rs3905371	32.93–33.20	CAUC

Upper panel: summary of entire linkage data; middle panel: gender breakdown; lower panel: ethnic breakdown. –Log P is maximum non-parametric lod score in region. Abbreviations: FEM, female; HISP, Hispanic; CAUC, Caucasian; Mb, megabases; cM, centimorgan.

Table 3. Summary of other addiction linkage studies

Population	chr	cM	Lod scores/candidate genes
Cocaine (35)			
EA	3	42.1	2.45 (CD)
EA	3	71.4	2.59 (CD)
AA	9	117.4	3.65 (CIP)
AA	10	2.1	2.53 (CIP)
EA	12	104.7	4.66 (cluster 1)
EA,AA	6	53.8	2.79 (cluster 1 and cluster 3)
AA	2	221.1	2.43 (cluster 3)
AA	18	52.9	3.35 (cluster 4)
EA,AA	10	166.2	2.70 (CD, NPL, multipoint)
Heroin (36)			
CH	4	143.3	2.19 (NPL)
CH	17	53.4	2.36 (NPL)
Alcohol (27,31)			
EA, AA	1	168.9	2.93
	7	93.9	3.49
	4	46Mb	*GABRA2*
Nicotine (33,34,53,55)			
EA, AA	9q22	~98 Mb	*GABBR2*
EA, AA, CH	20q13	~61.4 Mb	*CHRNA4*
EA, mixed	8	164 cM	
	7	164 cM	

CD, cocaine dependence; CID, cocaine induced paranoia; EA, European Americans; AA, African Americans; CH, Han Chinese; cluster 1, cocaine predominant; cluster 3, cocaine heavy use, late onset; cluster 4, moderate cocaine and opioid use. NPL, non-parametric lod score.

ingly, in our secondary analysis to identify gender-specific loci, we detected several putative linkage peaks that map closely to three adhesion genes associated with high drug use by Lui *et al*. (38) in their expanded association study; *CTNNA3*, *CTNND2* and *CSMD2* (Table 4). These observations are consistent with novel models of addiction focusing on alterations in neuronal plasticity and synaptic architecture, and in the process, new candidate genes in addiction vulnerability have been targeted, beyond the level of dopamine and opioid receptor/transporter pathways that have received most of the attention over the past two decades (37,38,61–65).

Table 4. Regions of overlap with whole genome association studies

Chr	~pos (Mb)	−log(*P*)	Population	Overlapping genes
14	77.39–77.66	5.00/3.30	Hisp/All	*NRXN3* (77.71–79.40 Mb)
14	67.83–68.28	3.52/3.22	Hisp/All	*SYT16* (61.5–61.6 Mb)
10	68.55–69.72	2.89	Male	*CTNNA3* (67.35–69.13)
5	6.85–7.01	2.22	Female	*CTNND2* (11.02–11.96)
1	36.65–37.06	1.96	Female	*CSMD2* (33.75–34.40)

The chromosome position, −log(*P*) values, and population refer to this study; overlapping genes refers to potential candidate genes identified in Liu *et al*. (37,38).

Overall, the findings reported here are consistent with a heterogeneous genetic model of addiction generated by ethnic, gender and phenotypic differences, similar to the genetic heterogeneity found in the numerous linkage studies carried out in schizophrenia, major depression and bipolar disorder.

MATERIALS AND METHODS

Subjects

The primary recruitment was carried out in MMTPs located in the New York City Metropolitan area, primarily in Bronx County, Kings County and lower Manhattan. Potential probands were approached in their home clinics by Masters level research assistants and answered a simple screening questionnaire to determine eligibility. The primary criterion was having one or more siblings (or half-siblings) currently enrolled in a MMTP in the New York City metropolitan region. We also included siblings who were previously enrolled in a MMTP, if they were able to provide proof of prior enrollment, such as an old picture ID. In order to

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maintain patient confidentiality, probands were asked to have their siblings contact either a study coordinator or their clinic counselor to assess eligibility. Siblings were ascertained either in their home clinic or in the proband's home clinic. All subjects who were not interviewed in their home clinic were told to bring their MMTP identification cards. Many were also asked to bring a birth certificate to reduce the risk of deception for the financial incentive being offered (especially half siblings with different surnames, and opposite sex sibling pairs). If eligible for ascertainment, subjects signed informed consents that had been approved by the parent academic institution or local institutional review boards.

Subjects were then interviewed by the research assistants with an instrument to collect basic demographic information and were administered the Structured Clinical Interview for DSM-IV (SCID) to confirm the diagnosis of opioid dependence, and establish comorbid drug or alcohol dependencies (66). The SCID is a widely used structured diagnostic interview designed to assess the presence of current and lifetime DSM-IV diagnosis, and has been demonstrated to have acceptable levels of internal consistency and interater reliability (67). Masters level research assistants were trained in the administration of the SCID during a 3-month training period using practice subjects recruited from MMTPs in the Beth Israel Medical Center system. Phlebotomy was carried out and blood samples were sent to the Rutgers University Cell and DNA Repository for processing (DNA extraction and EB-virus transformation).

Genotyping

DNA samples were genotyped for 10 204 SNPs distributed genome-wide using GeneChip® mapping 10K 2.0 arrays (Affymetrix, Inc.). DNA samples were processed for hybridization to the arrays in 96-well plates, according to the manufacturer's protocol (https://www.affymetrix.com/support/downloads/manuals/10k2_manual.pdf, registration required), with one exception. We found that increasing the amount of purified PCR product from 20 to 23 μg into the fragmentation step of the protocol resulted in a more robust reaction, thereby increasing call rate (>98%). Arrays were hybridized in a GeneChip® Hybridization Oven, washed on a GeneChip® Fluidics Station 400 and scanned with a GeneChip® Scanner 3000, according to the manufacturer's protocol (Affymetrix, Inc.). SNP genotype calls were made using GeneChip® DNA Analysis Software (GDAS) (Affymetrix, Inc.), then stored and output in file formats suitable for analysis using Progeny Lab 6 (Progeny Software, LLC).

Genotyping data can be accessed at the NIDA web site through the following link: http://www.Nidagenetics.org/Results/data2.html.

Statistical analysis

Due to the lack of parental genotypic information, allele frequencies were estimated by randomly choosing one affected member from each family. The allele frequencies used in the linkage analysis were the average from 20 such repeated estimations. A goodness of fit test to determine Hardy–Weinberg equilibrium (HWE) was carried out by using the SAS/Genetics 9.1.3 'PROC ALLELE' module. The critical *P*-value used to determine departure from HWE was set at 0.001, the default setting on Haploview, which takes into consideration an adjustment for multiple testing. Relationships between self-reported sibpairs were examined by using RELCHECK and PREST and the results were very highly correlated (45,46). ALTERTEST was used to assign true familial relationships when self-reported siblings were neither full nor half-sibs (46). LD between markers was determined using SNPLINK (68), as LD between markers can produce type I error in multipoint linkage studies when dense sets of markers are used and no parental information is available (69). The between marker LD cutoff was $r^2 > 0.4$, which is the default value of SNPLINK (68). Non-parametric linkage analyses were performed using MERLIN (47). Both two-point and multipoint analyses were carried out. The $-\log(P)$ from the NPL score that Merlin calculates was extracted to generate genome-wide scan graphical plots where the x-axis corresponds to the Marshfield genetic map. The primary analysis considered all individuals with DSM-IV opiate dependence as affected. We also performed secondary analyses stratified by sex, and then by self-reported ethnicity. Self-reported ethnicity was highly correlated with cluster, which was determined by structure analysis computed using an admixture model with $K = 3$, a Markov Chain Monte Carlo burning period of 5000, and 5000 iterations (STRUCTURE; Pearson Correlation Coefficient = 0.59613; $P < 0.0001$) (70,71) (see Supplementary Material, Figure S1).

To estimate empirical significance levels of our data set, we used MERLIN to generate 500 replicates of the entire genome, retaining the family structures. The threshold value for observing a genome-wide type I error once per scan corresponds to an NPL score of 3 under the hypothesis of no linkage.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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