Genome-Wide Significant Association Signals in *IPO11-HTR1A* Region Specific for Alcohol and Nicotine Codependence

Lingjun Zuo, Xiang-Yang Zhang, Fei Wang, Chiang-Shan R. Li, Lingeng Lu, Liefu Ye, Heping Zhang, John H. Krystal, Hong-Wen Deng, and Xingguang Luo

Background: Alcohol and nicotine codependence can be considered as a more severe subtype of alcohol dependence. A portion of its risk may be attributable to genetic factors.

Methods: We searched for significant risk genomic regions specific for this disorder using a genome-wide association study. A total of 8,847 subjects underwent gene–disease association analysis, including (i) a discovery cohort of 818 European American cases with alcohol and nicotine codependence and 1,396 European American controls, (ii) a replication cohort of 5,704 Australian family subjects with 907 affected offspring, and (iii) a replication cohort of 449 African American cases and 480 African American controls. Additionally, a total of 38,714 subjects of European or African descent in 18 independent cohorts with 10 other nonalcoholism neuropsychiatric disorders were analyzed as contrast. Furthermore, 90 unrelated HapMap CEU individuals, 93 European brain tissue samples, and 80 European peripheral blood mononuclear cell samples underwent *cis*-acting expression quantitative locus (*cis*-eQTL) analysis.

Results: We identified a significant risk region for alcohol and nicotine codependence between *IPO11* and *HTR1A* on chromosome 5q that was reported to be suggestively associated with alcohol dependence previously. In the European American discovery cohort, 381 single nucleotide polymorphisms (SNPs) in this region were nominally associated with alcohol and nicotine codependence ($p < 0.05$); 57 associations of them survived region- and cohort-wide correction ($\alpha = 3.6 \times 10^{-6}$); and the top SNP (rs7445832) was significantly associated with alcohol and nicotine codependence at the genome-wide significance level ($p = 6.2 \times 10^{-9}$). Furthermore, associations for 34 and 11 SNPs were replicated in the Australian and African American replication cohorts, respectively. Among these replicable associations, 4 reached genome-wide significance level in the meta-analysis of European Americans and European Australians: rs7445832 ($p = 9.6 \times 10^{-10}$), rs13361996 ($p = 8.2 \times 10^{-9}$), rs62380518 ($p = 2.3 \times 10^{-8}$), and rs7714850 ($p = 3.4 \times 10^{-8}$). *Cis*-eQTL analysis showed that many risk SNPs in this region had nominally significant *cis*-acting regulatory effects on *HTR1A* or *IPO11* mRNA expression. Finally, no markers were significantly associated with any other neuropsychiatric disorder examined.

Conclusions: We speculate that this *IPO11-HTR1A* region might harbor a causal variant for alcohol and nicotine codependence.

Key Words: *GWAS*, Alcohol and Nicotine Codependence, *cis*-eQTL, *IPO11*, *HTR1A*.

ALCOHOL AND NICOTINE are the most commonly misused substances in the United States. Nearly 20 million Americans are alcohol abusing or dependent, and almost 50 million Americans smoke cigarettes (Substance Abuse and Mental Health Services Administration (SAMHSA, 2010). Alcohol dependence and nicotine dependence frequently co-occur in the same individuals. Furthermore, nicotine-dependent individuals are 4 times more likely than the general population to be alcohol dependent and people who drink are 3 times more likely than the general population to smoke (Grant et al., 2004). Identical twins are twice as likely as fraternal twins to become alcohol and nicotine dependent if the other twin is dependent (Carmelli et al., 1993; Swan et al., 1997). Alcohol and nicotine may enhance motivation to use either drug by activating common brain targets that are responsible for their reinforcing effects. They

From the Department of Psychiatry (LZ, FW, C-SRL, JHK, XL), Yale University School of Medicine, New Haven, Connecticut; Menninger Department of Psychiatry and Behavioral Sciences (X-YZ), Baylor College of Medicine, Houston, Texas; Department of Epidemiology and Public Health (LL), Yale University School of Medicine, New Haven, Connecticut; Department of Urology (LY), Fujian Provincial Hospital, Fuzhou, Fujian, China; Department of Biostatistics (HZ), Yale University School of Public Health, New Haven, Connecticut; and Department of Biostatistics (H-WD), School of Public Health and Tropical Medicine, Tulane University, New Orleans, Louisiana.

Received for publication March 11, 2012; accepted August 31, 2012.

Reprint requests: Xingguang Luo and Lingjun Zuo, Yale University School of Medicine, 300 George Street, New Haven, CT 06511; Tel.: 203-932-5711 ext. 3590; Fax: 203-937-4741; E-mail: xingguang.luo@yale.edu and lingjun.zuo@yale.edu

DOI: 10.1111/acer.12032

may also exert synergistic effects on behaviors which may contribute to their concurrent use.

Alcohol and nicotine codependence may represent a more severe subtype of alcohol dependence. A large number of risk loci have been associated with both alcohol dependence and nicotine dependence by candidate gene approach, including many genes that are involved in the dopaminergic, serotoninergic, GABAergic, glutamatergic, cholinergic, opioid, and endocannabinoid systems. However, none of these genes have been confirmed by recent genome-wide association studies (GWASs) of alcohol dependence (Bierut et al., 2010; Edenberg et al., 2010; Heath et al., 2011; Johnson et al., 2011; Treutlein et al., 2009). Only 2 of them (*CHRNA6-CHRNB3* [Thorgeirsson et al., 2010] and *CHRNA5-CHRNA3-CHRNB4* [Liu et al., 2010]) were confirmed by meta-analysis GWASs of nicotine dependence. GWASs of alcohol dependence or alcohol consumption reported multiple other potential risk loci (Bierut et al., 2010; Edenberg et al., 2010; Heath et al., 2011; Schumann et al., 2011; Treutlein et al., 2009), so did most GWASs of nicotine dependence. To date, there has been only 1 GWAS (Lind et al., 2010) directly studying the phenotype of alcohol and nicotine codependence. That study identified 3 risk genes including *ARHGAP10*, *MARK1*, and *DDX6*. However, those findings have not been replicated independently yet.

In the present study, we searched for significant risk genomic regions for alcohol and nicotine codependence using a GWAS. A European American cohort was used as the discovery one, and a European Australian cohort and an African American cohort were used as the replication ones. Additionally, we used 3 independent samples of European descent to detect expression quantitative trait locus (eQTL) signals in this risk genomic region, to see whether the risk variants were functional. Finally, as contrast, we tested gene–disease associations in 18 additional independent cohorts with 10 other nonalcoholism neuropsychiatric disorders, to see whether the risk regions were specific for alcohol and nicotine codependence or not.

MATERIALS AND METHODS

Subjects

A total of 8,847 subjects underwent gene–disease association analysis, including (i) a discovery cohort of 818 European American cases with alcohol and nicotine codependence (476 males and 342 females; 38.3 ± 10.2 years) and 1,396 European American controls (422 males and 974 females; 39.4 ± 10.4 years), (ii) a replication cohort of 5,704 European Australian family subjects (1,856 families; 2,620 males and 3,084 females; 46.0 ± 10.0 years; 907 affected offspring with alcohol and nicotine codependence including 366 females), and (iii) a replication cohort of 449 African American cases (260 males and 189 females; 40.3 ± 7.8 years) with alcohol and nicotine codependence and 480 African American controls (170 males and 310 females; 39.6 ± 8.6 years). Additionally, a total of 38,714 subjects of European or African descent in 18 independent case–control or family-based cohorts with 10 other neuropsychiatric disorders were analyzed. These neuropsychiatric disorders included schizophrenia, autism, attention deficit hyperactivity disorder, major depression, bipolar disorder, Alzheimer's disease, amyotrophic lateral sclerosis, early onset stroke, ischemic stroke, and Parkinson's disease (Tables S1a and S1b).

The European American discovery cohort and the African American replication cohort came from the data set of the Study of Addiction—Genetics and Environment (SAGE) (dbGaP study accession phs000092.v1.p1) (Bierut et al., 2010), and the Australian replication cohort came from the data set of the Australian twin-family study of alcohol use disorder (OZ-ALC) (dbGaP study accession phs000181.v1.p1) (Heath et al., 2011; Lind et al., 2010). All subjects with alcohol and nicotine codependence in another data set of the Collaborative Study on the Genetics of Alcoholism (COGA) (dbGaP: phs000125.v1.p1) (Edenberg et al., 2010) have been included in this SAGE data set. These data sets were originally collected to study alcohol dependence alone. SAGE subjects were recruited from 8 different study sites in 7 states and the District of Columbia; the majority of subjects were recruited in Missouri (Bierut et al., 2010). All subjects were interviewed using the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA) (Bucholz et al., 1994). Affected subjects met lifetime Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria for both alcohol and nicotine dependence (American Psychiatric Association, 1994). Affected subjects were excluded if they had schizophrenia or other psychotic illnesses. Controls were defined as individuals who had been exposed to alcohol and nicotine (and possibly to other drugs), but had never become dependent on these substances. Additionally, controls were also screened to exclude individuals with major Axis I disorders, including schizophrenia, mood disorders, and anxiety disorders. The Australian subjects included twins and their parents, siblings, spouses, children, and other family members. The index cases reported a history of alcohol dependence and nicotine dependence (DSM-IV). More detailed demographic information is available elsewhere (Bierut et al., 2010; Edenberg et al., 2005, 2010; Heath et al., 2011). The European American discovery cohort and the African American replication cohort were genotyped on the Illumina Human 1M beadchip, and the Australian cohort was genotyped on the Illumina CNV370v1 beadchip (Illumina, Inc., San Diego, CA).

Detailed demographic information, including sample sizes, ethnicity, and diagnosis of the 18 cohorts with other neuropsychiatric disorders, is shown in Table S1 or is available in dbGaP database (http://www.ncbi.nlm.nih.gov/gap). These subjects were genotyped on different Illumina or Affymetrix (Santa Clara, CA) microarray beadchip platforms. All subjects gave written informed consent to participating in protocols approved by the relevant institutional review boards (IRBs). All subjects were deidentified in this study that was approved by Yale IRB.

Imputation

After we identified a significant risk genomic region in the European American discovery cohort, we imputed that entire region (1.5 Mb at Chr5: 61,708,573 to 63,257,546 from the transcript start site [TSS] of *IPO11* to the TSS of *HTR1A*) in all samples of 21 cohorts using the same strategy as previously (Zuo et al., in press). Rare variants with minor allele frequencies < 0.05 were excluded.

Data Analysis

Before the association analysis, we strictly cleaned the phenotype and genotype data of all data sets (see Data S1). We tested gene–disease associations in the European American discovery cohort first, to identify the significant risk genomic regions at genome-wide significance level, and then, we imputed and carefully examined this region across 21 cohorts.

1. Genome-wide association tests in the European American discovery cohort: The allele frequencies of all cleaned markers across the genome were compared between cases and controls using genome-wide logistic regression analysis implemented in the program PLINK (Purcell et al., 2007). Diagnosis served as the dependent variable, alleles served as the independent variables, and sex, age, the first 10 principal components, and the most significant marker (i.e., rs7445832) served as the covariates. The principal component scores of our samples were derived from all autosomal single nucleotide polymorphisms (SNPs) across the genome using principal component analysis implemented in the software package EIGENSTRAT (Price et al., 2006). Each individual received scores on each principal component. These principal components reflected the population structure of our samples. The first principal component (PC1) separated the self-identified European American and African American subjects very well, which was highly consistent with a previous report (Bierut et al., 2010). The second principal component (PC2) separated the self-identified Hispanic subjects from the non-Hispanic subjects. Other principal components also accounted for very small fractions of the total variance. The first 10 principal component scores accounted for >95% of variance. These PCs serving as covariates in the regression model can control for the population stratification and admixture effects on association analysis. The *p*-values derived from these association analyses are illustrated in Fig. 1. Furthermore, similar association analysis was performed on the imputed data (see below). The top-ranked ($p < 10^{-5}$) risk markers are listed in Table 1. To mitigate false-positive rates, genome-wide associations in the discovery cohort were corrected for multiple testing by Bonferroni correction ($\alpha = 5 \times 10^{-8}$).
2. Association tests for the imputed genotype data in all samples in 21 cohorts: To analyze the associations between neuropsychiatric diseases and all imputed markers in the case–control samples, we used the logistic regression analysis described above. For the family samples, we tested associations using the program FBAT (Horvath et al., 2001). Association results were corrected for multiple comparisons by the effective number of SNPs within the *IPO11-HTR1A* region and the number of cohorts examined (i.e., $n = 21$). The effective marker numbers were calculated using the program SNPSpD (Li and Ji, 2005). In the present study, the effective genetic marker number was 669 in the *IPO11-HTR1A* region; thus, the region- and cohort-wide corrected α was set at 3.6×10^{-6}. The associations that were replicable between the discovery and replication cohorts are shown in Tables 2 and 3. Meta-analysis was performed on these replicable associations, to derive the combined *p*-values using the program METAL (http://www.sph.umich.edu/csg/abecasis/metal/index.html).
3. *Cis*-acting genetic regulation of expression analysis in the lymphoblastoid cell lines: To examine relationships between all available SNPs in the *IPO11-HTR1A* region and mRNA expression levels of local genes (i.e., *HTR1A* and *IPO11*) in the lymphoblastoid cell lines, we performed *cis*-acting expression quantitative locus (*cis*-eQTL) analysis. Expression array data of 14,925 transcripts (14,072 genes) in 90 unrelated HapMap CEU individuals were assessed (Stranger et al., 2005). Differences in the distribution of mRNA expression levels between SNP genotypes were compared using a Wilcoxon-type trend test. The risk SNPs that were associated with disease in the discovery cohort and had $p < 0.05$ in this *cis*-eQTL analysis are shown in the Table S2.
4. *Cis*-eQTL analysis on all available SNPs in the *IPO11-HTR1A* region in the brain tissue samples and the peripheral blood mononuclear cell (PBMC) samples: To examine whether the SNPs in the risk region influence the local gene expression changes, we also tested the associations between the genotypes and the expression levels of exons and transcripts of local genes (i.e., *HTR1A* and *IPO11*) in 2 additional European samples (Table S2). Expression array data in 93 autopsy-collected frontal cortical brain tissue samples with no defined neuropsychiatric condition and 80 PBMC samples collected from living healthy donors obtained from a study (Heinzen et al., 2008) at Duke University were evaluated. Each of these associations was analyzed using a linear regression model by correcting for age, sex, source of tissues, and principal component scores of ancestry. The expression array data have been confirmed by quantitative real-time polymerase chain reaction previously (Heinzen et al., 2008).

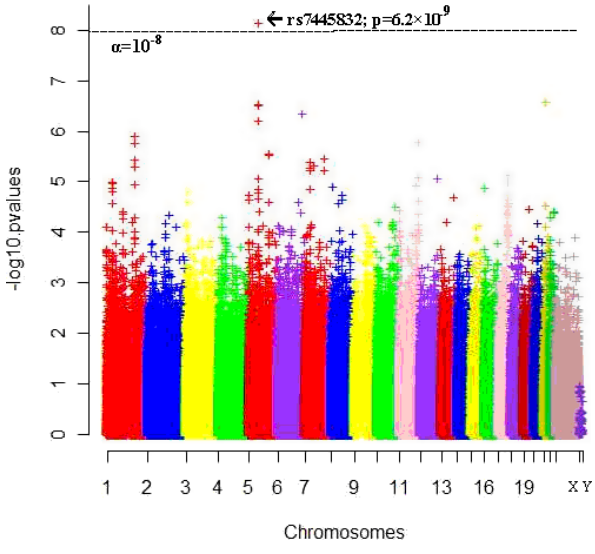

Fig. 1. Manhattan plot for the *p*-values in European American discovery cohort [*Y*-axis: $-\log 0.05 = 1.3$; $-\log 10^{-5} = 5$; $-\log (5 \times 10^{-8}) = 7.3$. *X*-axis: Chr1-22 = autosomes; X = ChrX; Y = ChrY; single nucleotide polymorphisms were ordered by physical distance within each chromosome/region].

RESULTS

We scanned the genome in the European American discovery cohort and identified a significant risk region between *HTR1A* and *IPO11* on chromosome 5q at genome-wide significance level (Figs 1–3), with the most significant SNP rs7445832 ($p = 6.2 \times 10^{-9}$). We examined the 10 Mb range surrounding this SNP, which covered the entire *IPO11-HTR1A* region (1.5 Mb), in the discovery cohort, and found a total of 13 SNPs that had association signals for alcohol and nicotine codependence with $p < 10^{-4}$ (i.e., $6.2 \times 10^{-9} \leq p \leq 9.1 \times 10^{-5}$). These SNPs were concentrated within a narrow region (0.5 Mb) surrounding the most significant SNP between *IPO11* and *HTR1A* (Fig. 2A).

We further examined the entire *IPO11-HTR1A* region (1.5 Mb) in multiple populations and detected many association and functional signals (Tables 1–3 and Table S2). In the European American discovery cohort, among 2,726 SNPs including 261 originally genotyped SNPs and 2,465 imputed SNPs, 381 SNPs were nominally associated with alcohol and nicotine codependence ($p < 0.05$) (Table 4); 57 SNPs

Table 1. *p*-Values for Top-Ranked ($p < 10^{-5}$) Risk SNPs in *IPO11-HTR1A* Region in the Discovery Cohort

SNP	Position	Risk allele	OR	*p*-Values	SNP	Position	Risk allele	OR	*p*-Values
rs7445832	**62622057**	**A**	**1.53**	**6.2×10^{-9}**	rs6873148	62695568	A	1.52	1.0×10^{-6}
rs1494578	62622185	G	1.47	4.7×10^{-7}	rs6873152	62695579	A	1.52	1.0×10^{-6}
rs10471577	62623023	C	1.47	4.7×10^{-7}	rs10939966	62696069	C	1.37	6.8×10^{-6}
rs7702856	62630122	G	1.47	4.3×10^{-7}	rs10805382	62696495	C	1.38	5.1×10^{-6}
rs4700575	62631732	G	1.48	2.8×10^{-7}	rs10939967	62696691	C	1.52	1.0×10^{-6}
rs346425	62632000	T	1.36	3.2×10^{-6}	rs73119677	62698022	G	1.53	9.0×10^{-7}
rs73761305	62632087	T	1.43	4.1×10^{-6}	rs4590141	62698797	C	1.37	7.0×10^{-6}
rs1319474	62634947	G	1.46	7.4×10^{-7}	rs55701004	62699567	C	1.37	7.0×10^{-6}
rs1017776	62635216	G	1.47	6.7×10^{-7}	rs6860119	62700122	T	1.53	7.9×10^{-7}
rs1462460	62649397	T	1.53	8.3×10^{-6}	rs6860278	62700179	T	1.53	7.9×10^{-7}
rs7444332	62680629	C	1.47	5.9×10^{-7}	rs6860501	62700377	T	1.53	7.9×10^{-7}
rs4403132	62681092	T	1.39	4.8×10^{-6}	rs73119687	62701503	T	1.53	7.9×10^{-7}
rs7718679	62687582	C	1.54	6.7×10^{-7}	rs6893950	62704479	T	1.51	4.2×10^{-6}
rs73119652	62688162	T	1.54	5.1×10^{-7}	**rs62380518**	**63020823**	**A**	**1.68**	**1.5×10^{-7}**
rs57363006	62689707	G	1.50	2.8×10^{-6}	rs74829400	63027037	T	1.64	1.2×10^{-7}
rs57361220	62689740	T	1.50	2.8×10^{-6}	rs62380521	63028029	C	1.41	8.1×10^{-6}
rs59544801	62689880	T	1.52	1.1×10^{-6}	rs6887027	63046909	C	1.38	9.9×10^{-6}
rs7714594	62690438	A	1.53	6.1×10^{-7}	rs62380555	63070602	C	1.47	1.5×10^{-6}
rs7735086	62692417	A	1.52	1.0×10^{-6}	**rs7714850**	**63072093**	**C**	**1.43**	**2.7×10^{-7}**
rs7735004	62692558	G	1.38	6.2×10^{-6}	rs10042862	63072461	G	1.47	1.5×10^{-6}
rs7735451	62692702	A	1.52	1.0×10^{-6}	rs13354185	63073747	A	1.48	1.1×10^{-6}
rs4302532	62694427	C	1.55	4.4×10^{-7}	rs72766222	63074411	A	1.46	7.3×10^{-6}
rs4455508	62694538	A	1.54	5.7×10^{-7}	**rs13361996**	**63075996**	**A**	**1.65**	**6.9×10^{-8}**

All markers are in Hardy–Weinberg Equilibrium (HWE), common variants, and ordered by chromosome position; all risk alleles are minor alleles. The markers underlined are nonimputed markers. The bold are the genome-wide significant markers with $p < 5 \times 10^{-8}$ in meta-analysis (see Table 2). SNP, single nucleotide polymorphism.

were significantly associated with alcohol and nicotine codependence after region- and cohort-wide correction ($\alpha = 3.6 \times 10^{-6}$). As mentioned, 1 of the SNPs showed evidence for genome-wide significance (rs7445832; $p = 6.2 \times 10^{-9}$). All risk alleles of these markers were minor alleles ($f < 0.5$). If conditional on the most significant SNP (i.e., rs7445832), all of the associations with other SNPs became less significant (all $p > 10^{-4}$; Fig. 2C). In the Australian replication cohort, 100 SNPs were nominally associated with alcohol and nicotine codependence ($0.001 \leq p \leq 0.049$; data not shown). Thirty-four associations in the discovery cohort ($6.2 \times 10^{-9} \leq p \leq 0.049$) were replicated in the Australian replication cohort ($0.001 \leq p \leq 0.049$) (Table 2 and Fig. 2D), with the same directions of gene effects in both cohorts. Meta-analysis showed that all of these 34 replicable SNPs were associated with disease ($9.6 \times 10^{-10} \leq p \leq 0.021$; Table 2), including 4 genome-wide significant SNPs, that is, rs7445832 ($p = 9.6 \times 10^{-10}$), rs13361996 ($p = 8.2 \times 10^{-9}$), rs62380518 ($p = 2.3 \times 10^{-8}$), and rs7714850 ($p = 3.4 \times 10^{-8}$). In the African American replication cohort, 77 SNPs were nominally associated with alcohol and nicotine codependence ($0.002 \leq p \leq 0.049$; data not shown). Eleven risk SNPs in the discovery cohort ($8.1 \times 10^{-6} \leq p \leq 0.042$) were also risk SNPs in the African American replication cohort ($0.032 \leq p \leq 0.049$) (Table 3). However, all of these 11 SNPs but 1 had opposite directions of gene effects between the discovery cohort and the African American cohort. Meta-analysis showed that only this exceptional 1 SNP was associated with disease (rs10042968: OR = 1.41, $p = 8.1 \times 10^{-6}$ in European Americans; OR = 1.54, $p = 0.041$ in African Americans; OR = 1.42, $p = 8.0 \times 10^{-7}$ in meta-analysis; Table 3). Among these SNPs, rs690957 was a risk SNP across 3 cohorts ($p = 0.008$, 0.004, and 0.047 in European Americans, European Australians and African Americans, respectively). Rs690957 was also the most significant one in European Australians (Table 4). In other 18 independent cohorts, 9–261 SNPs were nominally associated with diseases, but none of them survived region- and cohort-wide correction for multiple comparisons (Table 4).

Cis-eQTL analysis showed that, among the risk SNPs for alcohol and nicotine codependence, 30 SNPs had nominal *cis*-acting regulatory effects on expression of *HTR1A* or *IPO11* mRNA in the brain, PBMC, or lymphoblastoid cell lines ($2.3 \times 10^{-13} \leq p \leq 0.05$); among all of the 65 SNPs within this region that were genotyped for eQTL analysis, 43 (66.2%) were risk markers for alcohol and nicotine codependence ($6.2 \times 10^{-9} \leq p \leq 0.048$) (Table S2). *Cis*-acting regulatory effects on *IPO11* expression were much stronger than those on *HTR1A* expression. All of the risk alleles for alcohol and nicotine codependence increased the expression of *HTR1A*. However, some of the risk alleles increased the expression of *IPO11*, but the others decreased it.

Additionally, a total of 2,058 SNPs in *ARHGAP10*, *MARK1*, *DDX6*, *KIAA1409*, *CTBP2*, *GRM3*, *TBC1D2B*, *BACH2*, and *CNTNA* that were significant risk genes for alcohol dependence, alcohol and nicotine codependence, or nicotine dependence identified by Lind and colleagues (2010) were also tested in our samples. We listed all *p*-values <0.01 in the Table S3. We found that none of these markers were

Table 2. *p*-Values for Replicable Risk SNPs Between European American Discovery and Australian Replication Cohorts

			European Americans		Australians		Meta-analysis	
SNP	Position	Risk Allele	OR	*p*	OR	*p*	z	*p*
rs6861297	62212972	T	1.62	0.024	2.67	0.008	2.57	0.010
rs6884324	62213017	C	1.60	0.026	2.67	0.008	2.54	0.011
rs350306	62469018	T	1.38	0.012	1.56	0.005	3.18	0.001
rs1494622	62491395	T	1.27	0.021	1.17	0.009	2.92	0.004
rs1494623	62493473	C	1.20	0.037	1.18	0.004	2.82	0.005
rs691234	62496771	C	1.37	0.003	1.23	0.013	3.48	0.001
rs690957	62502634	C	1.38	0.008	1.67	0.001	3.20	0.001
rs681342	62507733	T	1.28	0.012	1.18	0.015	3.04	0.002
rs181156	62509099	A	1.39	0.002	1.23	0.007	3.66	2.6×10^{-4}
rs350309	62509354	T	1.38	0.002	1.23	0.007	3.66	2.6×10^{-4}
rs350311	62510417	C	1.39	0.003	1.17	0.019	3.43	0.001
rs350312	62511201	A	1.37	0.004	1.17	0.019	3.34	0.001
rs690816	62514088	G	1.38	0.003	1.17	0.020	3.43	0.001
rs114705639	62536627	A	1.35	0.022	1.46	0.019	2.71	0.007
rs17481124	62574713	G	1.23	0.048	1.21	0.048	2.30	0.021
rs72758793	62588647	A	1.37	0.018	1.80	0.046	2.62	0.009
rs55860379	62593868	A	1.36	0.010	1.78	0.034	2.85	0.004
rs72760718	62615268	G	1.35	0.016	1.80	0.027	2.67	0.008
rs7445832	**62622057**	**A**	**1.53**	**6.2×10^{-9}**	**1.15**	**0.049**	**6.12**	**9.6×10^{-10}**
rs9291778	62622713	A	1.51	4.0×10^{-5}	2.08	0.012	4.50	6.7×10^{-6}
rs56051136	62630434	A	1.41	0.003	3.33	0.001	3.51	4.6×10^{-4}
rs60685959	62630435	C	1.41	0.003	3.33	0.001	3.51	4.6×10^{-4}
rs73761304	62632086	T	1.37	4.7×10^{-4}	2.37	0.001	4.14	3.4×10^{-5}
rs6882265	62702209	T	1.28	0.001	1.58	0.026	3.82	1.3×10^{-4}
rs62380518	**63020823**	**A**	**1.68**	**1.5×10^{-7}**	**1.33**	**0.028**	**5.59**	**2.3×10^{-8}**
rs7714850	**63072093**	**C**	**1.43**	**2.7×10^{-7}**	**1.27**	**0.042**	**5.52**	**3.4×10^{-8}**
rs10042862	63072461	G	1.47	1.5×10^{-6}	1.24	0.039	5.18	2.2×10^{-7}
rs13354185	63073747	A	1.48	1.1×10^{-6}	1.33	0.034	5.21	1.9×10^{-7}
rs13361996	**63075996**	**A**	**1.65**	**6.9×10^{-8}**	**1.78**	**0.002**	**5.76**	**8.2×10^{-9}**
rs17180095	63104352	C	1.52	0.001	1.64	0.033	3.69	2.2×10^{-4}
rs10061598	63113748	T	1.14	0.048	1.59	0.033	2.51	0.012
rs989049	63114801	C	1.19	0.012	1.73	0.039	2.94	0.003
rs2202266	63117281	G	1.14	0.049	1.79	0.039	2.44	0.015
rs1478493	63119942	G	1.26	0.008	1.53	0.011	3.60	3.2×10^{-4}

The bold are the genome-wide significant markers with $p < 5 \times 10^{-8}$ in meta-analysis. SNP, single nucleotide polymorphism.

Table 3. *p*-Values for Replicable Risk SNPs Between European American Discovery and African American Replication Cohorts

			European American		African American		Meta-analysis	
SNP	Position	Risk Allele	OR	*p*	OR	*p*	OR	*p*
rs10514949	62068672	C	1.15	0.042	0.78	0.049	1.05	0.376
rs58617906	62332704	A	1.24	0.020	0.61	0.032	1.12	0.185
rs690957	62502634	C	1.38	0.008	0.64	0.047	1.16	0.177
rs10042968	63069474	G	1.41	8.1×10^{-6}	1.54	0.041	1.42	8.0×10^{-7}
rs7700448	63124338	A	1.17	0.025	0.81	0.048	1.05	0.407
rs17795292	63125355	G	1.17	0.018	0.81	0.036	1.04	0.424
rs35393059	63125728	C	1.17	0.020	0.81	0.044	1.05	0.369
rs13159097	63125873	A	1.17	0.022	0.81	0.044	1.05	0.381
rs6876878	63127666	A	1.17	0.016	0.81	0.045	1.06	0.333
rs2365875	63128094	G	1.17	0.019	0.80	0.040	1.05	0.363
rs10939982	63128760	G	1.17	0.018	0.82	0.048	1.05	0.378

SNP, single nucleotide polymorphism.

significantly associated with alcohol and nicotine codependence in our samples after Bonferroni correction.

DISCUSSION

In the European American population, we identified a genome-wide significant risk marker at the *IPO11-HTR1A* region specific for alcohol and nicotine codependence. The region surrounding this marker was enriched with many association signals and functional signals. We speculated that this region might harbor a causal variant for alcohol and nicotine codependence.

Several pieces of evidence supported our conclusion. First, within 10 Mb range surrounding this genome-wide

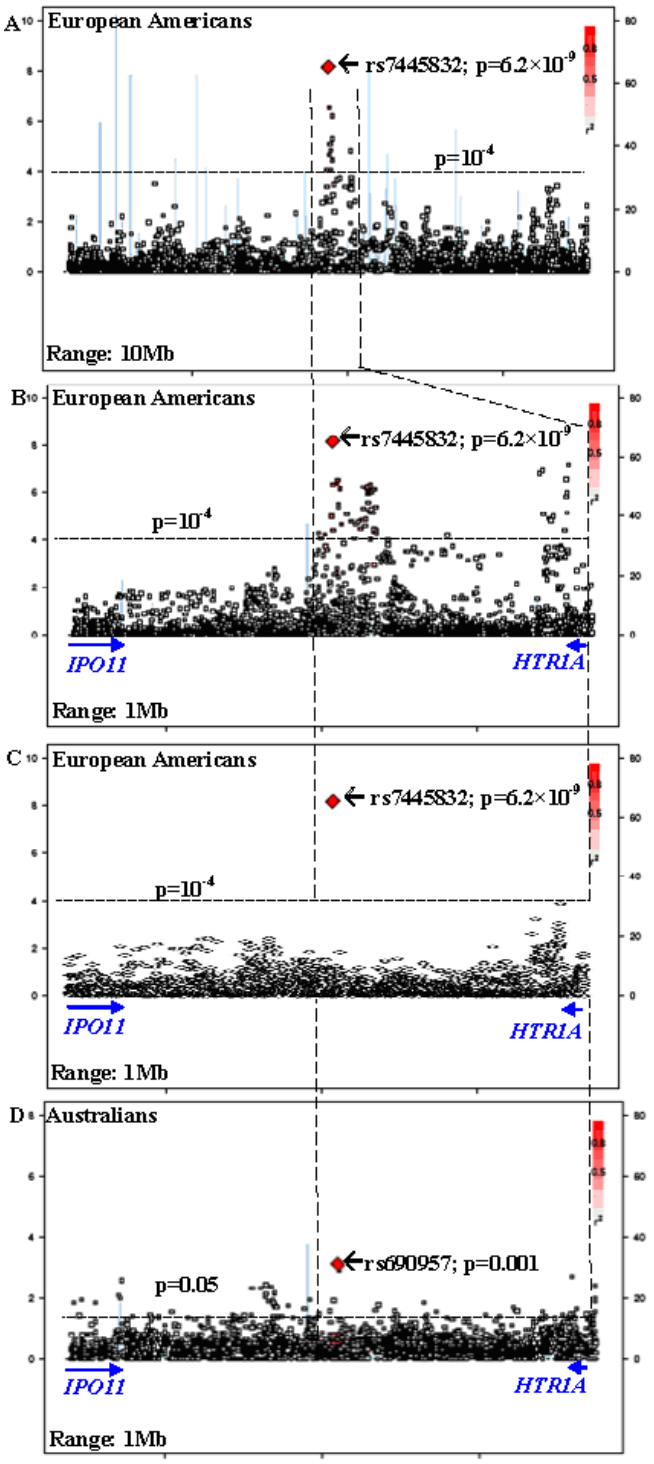

Fig. 2. Regional association plots [left *Y*-axis corresponds to –log (*p*) value; right *Y*-axis corresponds to recombination rates; quantitative color gradient corresponds to r^2; squares represent peak single nucleotide polymorphisms (SNPs). (**A**) Regional association plot in European American discovery cohort for a 10 Mb region around the peak association SNP (rs7445832); (**B**) regional association plot in European American discovery cohort for a 1 Mb region around the peak association SNP (rs7445832) [without conditioning on rs7445832]; (**C**) regional association plot in European American discovery cohort for a 1 Mb region around the peak association SNP (rs7445832) [conditional on rs7445832]; (**D**) regional association plot in Australian replication cohort for a 1 Mb region around the peak association SNP (rs7445832)].

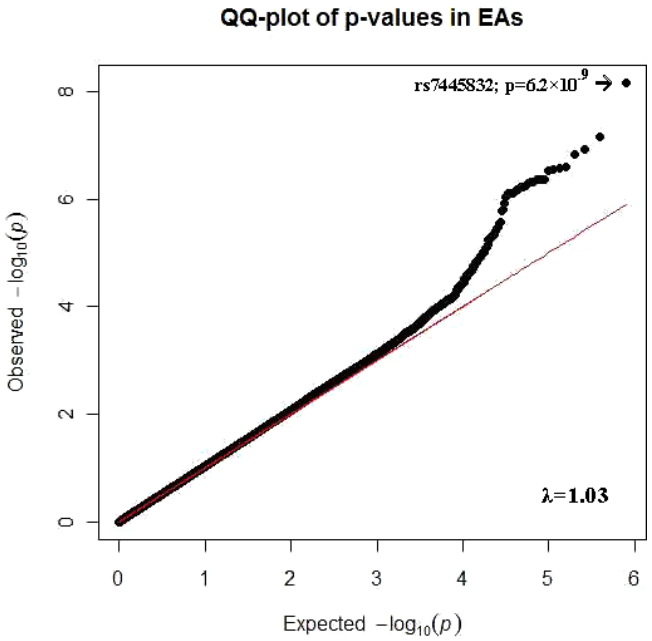

Fig. 3. QQ-plot for the *p*-values in European American discovery cohort [*X*-axis: expected –log (*p*) values; *Y*-axis: observed –log (*p*) values; *p*-values correspond to associations between single nucleotide polymorphisms and alcohol and nicotine codependence; $\lambda = 1.03$].

significant risk SNP, all association signals for alcohol and nicotine codependence with $p < 10^{-4}$ were concentrated within a narrow region surrounding this SNP. This region was completely located between *HTR1A* and *IPO11*. It is, thus, highly likely that the putative causal variant for alcohol and nicotine codependence was located within this region. Second, many risk SNPs in this region had significant *cis*-acting regulatory effects on mRNA expression both in the PBMC and in the brain, increasing the possibility that the *IPO11-HTR1A* region plays a direct functional role in the disorder. Third, many associations discovered in European Americans were replicated in European Australians, and meta-analysis showed that 4 SNPs reached the genome-wide significance level. Some associations in European Americans were also replicated in African Americans. Finally, this region was specific for alcohol and nicotine codependence, not for any other nonalcoholism neuropsychiatric disorder examined. This region has been suggestively associated with alcohol dependence (75.6% nicotine dependence) in the same data set before ($p = 2.3 \times 10^{-6}$ by Bierut et al., 2010; $p = 2.8 \times 10^{-7}$ by Zuo et al., 2011), but not genome-wide significant ($\alpha = 5 \times 10^{-8}$). The association was genome-wide significant only in the subgroup with alcohol and nicotine codependence ($p = 6.2 \times 10^{-9}$), which might suggest that this region is associated with a more severe subtype of alcohol dependence.

It is worth noting that the "causal" variants may not be identical to the "risk" markers, which is actually a common limitation of most association studies. There were other reasons for this inconsistency between the "causal" variants

Table 4. Associations Between *IPO-11-HTR1A* Gene Region and Different Neuropsychiatric Disorders

Human diseases	Data set no.	SNP no. (total)	SNP no. ($p < 0.05$)	Minimal *p*-value	Most sig. SNP	Gene	Minor allele frequency	
							Affected	Unaffected
AD + ND	1	2,726	381	6.2×10^{-9}	rs7445832	*Sig. region*	0.289	0.211
AD + ND	2	2,605	100	0.001	rs690957	*Intergenic*	0.084	0.076
AD + ND	3	2,901	77	0.002	rs1353270	*Intergenic*	0.184	0.126
ADHD	4	2,716	67	2.8×10^{-4}	rs1478498	*Intergenic*	0.229	0.236
Schizophrenia	5	2,087	216	0.001	rs923963	*Intergenic*	0.372	0.326
Schizophrenia	6	1,997	62	0.001	rs9283703	*Intergenic*	0.392	0.493
Schizophrenia	7	2,013	126	0.001	rs7707596	*Sig. region*	0.059	0.094
Autism	8	2,587	86	0.003	rs1319474	*Sig. region*	0.128	0.134
Major depression	9	2,743	143	0.005	rs16892399	*Intergenic*	0.255	0.226
Bipolar disorder	10	2,015	9	0.018	rs35509126	*Intergenic*	0.101	0.068
Bipolar disorder	11	2,015	164	0.001	rs347670	*Sig. region*	0.093	0.060
Bipolar disorder	12	1,948	95	0.002	rs260991	*Intergenic*	0.052	0.117
Alzheimer's disease	13	2,678	170	3.0×10^{-4}	rs4449492	*Intergenic*	0.473	0.457
Alzheimer's disease	14	1,570	175	0.001	rs1160346	*Intergenic*	0.216	0.119
ALS	15	2,492	143	0.002	rs1422301	*Sig. region*	0.197	0.281
Early onset stroke	16	2,559	79	0.002	rs16891019	*Intergenic*	0.196	0.140
Early onset stroke	17	2,817	144	0.001	rs56280615	*Sig. region*	0.054	0.118
Ischemic stroke	18	2,435	261	1.2×10^{-4}	rs13186191	*Intergenic*	0.319	0.184
Parkinson's disease	19	2,614	95	0.001	rs1851333	*Intergenic*	0.109	0.135
Parkinson's disease	20	2572	149	3.1×10^{-4}	rs34606485	*Sig. region*	0.070	0.132
Parkinson's disease	21	2,683	95	0.004	rs6888308	*Intergenic*	0.324	0.278

AD + ND, alcohol and nicotine codependence; ADHD, attention deficit hyperactivity disorder; ALS, amyotrophic lateral sclerosis; SNP, single nucleotide polymorphism.

Only the most significant risk markers are listed. Data set no. refers to Table S1a. "Sig. Region," a 0.5 Mb significant risk region for alcohol and nicotine codependence (see Fig. 2A).

and the "risk" markers implicated in the current study. First, none of the risk SNPs presented here were nonsynonymous. Rather, they appeared to have implications for risk and function by virtue of their being in linkage disequilibrium (LD) with a putative causal variant and/or due to their location in the regulatory region that may in turn regulated transcription of the causal variant. Second, the SNPs employed by GWAS are common, but not rare, variants. Numerous studies have shown that many gene–disease associations are not due to a single common variant, but rather due to a constellation of more rare, regionally concentrated, disease-causing variants. Thus, the signals of association credited to our common SNPs might be synthetic associations resulting from the contributions of multiple rare SNPs in the *IPO11-HTR1A* region, which needs to be identified by sequencing. Third, the associations in the European American discovery cohort, the associations in the replication cohorts, and the functional signals in the eQTL analysis did not perfectly match, which was probably because these risk markers were not the causal variants per se, but rather in LD with a common putative causal variant. Fourth, current evidence, including the effect sizes and the significance strength of associations, was not sufficient to fine-map the putative causal variant to any 1 of the 4 genome-wide significant risk markers, although the most significant one (i.e., rs7445832) was most likely. Sequencing is warranted to detect the actual causal variant. Finally, after conditioning on rs7445832, all association signals for other markers were significantly reduced, which might suggest that there exists only 1 putative causal locus in this region.

Our study is the first to detect the association between *HTR1A* and alcohol and nicotine codependence at a genome-wide significance level. *HTR1A* is located in 5q11.2-q13. It encodes the 5-HT1A receptor that binds the endogenous neurotransmitter serotonin (5-hydroxytryptamine, 5-HT). This receptor is a G-protein-coupled receptor that is coupled to Gi/Go and mediates inhibitory neurotransmission. In the central nervous system, 5-HT1A receptors exist in the cerebral cortex, hippocampus, septum, amygdala, and raphe nucleus in high densities. The activation of 5-HT1A receptor has been shown to increase dopamine release in the medial prefrontal cortex, striatum, and hippocampus, to impair cognition, learning, and memory by inhibiting the release of glutamate and acetylcholine in various areas of the brain, or to increase impulsivity and inhibition of addictive behaviors. This activation is therefore likely to be related to the development of alcohol dependence or nicotine dependence. This is consistent with our findings that the risk alleles of the variants in the *IPO11-HTR1A* region for alcohol and nicotine codependence increased the expression of *HTR1A*. Additionally, a well-known and functional promoter SNP of *HTR1A*, C-1016G (rs6295), displays differential binding to repressors and affects transcription (Lemonde et al., 2003; Strobel et al., 2003). Its minor allele G has been reported to increase risk for alcohol dependence (Lee et al., 2009) or increase the relapse rate of alcohol dependence (Wojnar et al., 2006), which is consistent with our conclusion that minor alleles in this region are risk alleles.

IPO11 is a flanking gene of *HTR1A*. It encodes the importin 11 that is a member of the karyopherin/importin-beta

family of transport receptors. This receptor mediates nucleocytoplasmic transport of protein and RNA cargoes (Plafker and Macara, 2000). It has been reported that, in mice, *IPO11* expression was significantly regulated by ethanol in the prefrontal cortex (Kerns et al., 2005) and in the whole embryos (Zhou et al., 2011). In the present study, we found many alcohol and nicotine codependence-associated markers had significant *cis*-acting regulatory effects on *IPO11* mRNA expression both in the brain and in the PBMC. Thus, *IPO11* might play important roles in alcohol and nicotine codependence too.

Among *CHRNA6-CHRNB3* and *CHRNA5-CHRNA3-CHRNB4* regions that have been widely associated with both alcohol and nicotine dependence before (Bierut et al., 2007; Edenberg et al., 2004; Liu et al., 2010; Ray et al., 2009; Saccone et al., 2007; Thorgeirsson et al., 2010), we only found that *CHRNA6*^rs6474421 was modestly associated with alcohol and nicotine codependence in the European American discovery cohort ($p = 0.005$). Furthermore, this modest association was not replicated in the Australian and African American replication cohorts, nor did the marker make the top-ranked gene list in the present study, consistent with previous results using the same SAGE data set (Bierut et al., 2010; Wang et al., 2011). Additionally, the risk genes identified by Lind and colleagues (2010) were not significantly associated with alcohol and nicotine codependence in our samples after Bonferroni correction. Critical difference between the study of Lind and colleagues (2010) and ours might result from the sample heterogeneity. Finally, in the present study, only the region between the TSS of *IPO11* and the TSS of *HTR1A* was studied. The 5′ regulatory regions, which boundaries are hard to defined, of both genes were excluded. Some information in these 5′ regulatory regions might be lost.

ACKNOWLEDGMENTS

We thank Dr. Weissbecker for helpful comments. This work was supported in part by National Institute on Drug Abuse (NIDA) grants K01 DA029643 and R01DA016750, National Institute on Alcohol Abuse and Alcoholism (NIAAA) grants R01 AA016015 and R21 AA020319, and the National Alliance for Research on Schizophrenia and Depression (NARSAD) Award 17616 (LZ). We thank NIH GWAS Data Repository, the Contributing Investigator(s) who contributed the phenotype and genotype data from his/her original study (e.g., Drs. Bierut, Edenberg, Heath, Singleton, Hardy, Foroud, Myers, Gejman, Faraone, Sonuga-Barke, Sullivan, Nurnberger, Devlin, Monaco, etc.), and the primary funding organization that supported the contributing study. Funding and other supports for phenotype and genotype data were provided through the National Institutes of Health (NIH) Genes, Environment and Health Initiative (GEI) (U01HG004422, U01HG004436, and U01HG004438); the GENEVA Coordinating Center (U01HG004446); the NIAAA (U10AA008401, R01AA013320, P60AA011998); the NIDA (R01DA013423); the National Cancer Institute (P01 CA089392); the Division of Neuroscience, the NIA National Institute of Neurological Disorders and Stroke (NINDS); the NINDS Human Genetics Resource Center DNA and Cell Line Repository; the NIH contract "High throughput genotyping for studying the genetic contributions to human disease" (HHSN268200782096C); the Center for Inherited Disease Research (CIDR); a Cooperative Agreement with the Division of Adult and Community Health, Centers for Disease Control and Prevention; the NIH Office of Research on Women's Health (ORWH) (R01NS45012); the Department of Veterans Affairs; the University of Maryland General Clinical Research Center (M01RR165001), the National Center for Research Resources, NIH; the National Institute of Mental Health (R01MH059160, R01MH59565, R01MH59566, R01MH59571, R01MH59586, R01MH59587, R01MH59588, R01MH60870, R01MH60879, R01MH61675, R01MH62873, R01MH081803, R01MH67257, R01MH81800, U01MH46276, U01MH46282, U01MH46289, U01MH46318, U01MH79469, U01MH79470, and R01MH67257); the NIMH Genetics Initiative for Bipolar Disorder; the Genetic Association Information Network (GAIN); the Genetic Consortium for Late Onset Alzheimer's Disease; the Autism Genome Project, the MARC: Risk Mechanisms in Alcoholism and Comorbidity; the Molecular Genetics of Schizophrenia Collaboration; the Medical Research Council (G0601030) and the Wellcome Trust (075491/Z/04), University of Oxford; the Netherlands Scientific Organization (904-61-090, 904-61-193, 480-04-004, 400-05-717, NWO Genomics, SPI 56-464-1419), the Centre for Neurogenomics and Cognitive Research (CNCR-VU); Netherlands Study of Depression and Anxiety (NESDA) and the Netherlands Twin Register (NTR); and the European Union (EU/WLRT-2001-01254), ZonMW (geestkracht program, 10-000-1002). Assistance with phenotype harmonization and genotype cleaning, as well as with general study coordination, was provided by the Genetic Consortium for Late Onset Alzheimer's Disease, the GENEVA Coordinating Center (U01 HG004446), and the National Center for Biotechnology Information. Genotyping was performed at the Johns Hopkins University Center for Inherited Disease Research, and GlaxoSmithKline, R&D Limited. The data sets used for the analyses described in this manuscript were obtained from dbGaP at http://www.ncbi.nlm.nih.gov/sites/entrez?Db=gap through dbGaP accession numbers listed in Table S1. XL had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

CONFLICT OF INTEREST

XL has read the journal's policy and the following authors have conflicts of interest. Dr. Krystal has been a paid consultant for Aisling Capital, LLC, AstraZeneca Pharmaceuticals, Brintnall & Nicolini, Inc., Easton Associates, Gilead Sciences, Inc., GlaxoSmithKline, Janssen Pharmaceuticals,

Lundbeck Research USA, Medivation, Inc., Merz Pharmaceuticals, MK Medical Communications, F. Hoffmann-La Roche Ltd, SK Holdings Co., Ltd, Sunovion Pharmaceuticals, Inc., Takeda Industries, and Teva Pharmaceutical Industries, Ltd. He serves as a member of Scientific Advisory Boards for Abbott Laboratories, Bristol-Myers Squibb, Eisai, Inc., Eli Lilly and Co., Forest Laboratories, Inc., Lohocla Research Corporation, Mnemosyne Pharmaceuticals, Inc., Naurex, Inc., Pfizer Pharmaceuticals, and Shire Pharmaceuticals. He is the Editor of *Biological Psychiatry*, a member of Board of Directors of Coalition for Translational Research in Alcohol and Substance Use Disorders, and the President Elect for American College of Neuropsychopharmacology. He also receives support from Tetragenex Pharmaceuticals.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Demographic Data of All Cohorts

Table S2. *p*-Values for *cis*-eQTLs in the *IPO11-HTR1A* Region

Table S3. *p*-Values for Associations Between Alcohol and Nicotine Co-Dependence and Risk Genes Previously Identified by the Study of Lind and colleagues (2010)

Table S4. Data Cleaning for Subjects and Genetic Markers in the Discovery Sample

Data S1. Supplemental materials and methods.