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Evidence of common and specific genetic effects: association of the muscarinic acetylcholine receptor M2 (*CHRM2*) gene with alcohol dependence and major depressive syndrome

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Several correlated phenotypes, alcohol dependence, major depressive syndrome, and an endophenotype of electrophysiological measurements, event-related oscillations (EROs), have demonstrated linkage on the long arm of chromosome 7. Recently, we reported both linkage and association between polymorphisms in the gene encoding the muscarinic acetylcholine receptor M2 (*CHRM2*) and EROs. In this study, we evaluated whether genetic variation in the *CHRM2* gene is also a risk factor for the correlated clinical characteristics of alcoholism and depression. The *CHRM2* gene contains a single coding exon and a large 5′ untranslated region encoded by multiple exons that can be alternatively spliced. Families were recruited through an alcohol dependent proband, and multiplex pedigrees were selected for genetic analyses. We examined 11 single nucleotide polymorphisms (SNPs) spanning the *CHRM2* gene in these families. Using the UNPHASED pedigree disequilibrium test (PDTPHASE), three SNPs (one in intron 4 and two in intron 5) showed highly significant association with alcoholism ($P = 0.004–0.007$). Two SNPs (both in intron 4) were significantly associated with major depressive syndrome ($P = 0.004$ and 0.017). Haplotype analyses revealed that the most common haplotype (>40% frequency), T–T–T (rs1824024–rs2061174–rs324650), was under-transmitted to affected individuals with alcohol dependence and major depressive syndrome. Different complementary haplotypes were over-transmitted in alcohol dependent and depressed individuals. These findings provide strong evidence that variants within or close to the *CHRM2* locus influence risk for two common psychiatric disorders.

INTRODUCTION

Alcohol dependence and major depressive disorder are common diseases that frequently co-occur. Multiple studies provide substantial evidence that genetic factors contribute to the development of both of these complex diseases (1–4). Family (5–7) and adoption studies (8,9) demonstrate that alcoholism and depression are jointly transmitted in families, and twin studies provide evidence that this co-transmission is in part due to common underlying genetic factors (10,11).

As part of the collaborative study on the genetics of alcoholism (COGA), families of individuals in treatment for

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alcoholism were recruited and comprehensively assessed in multiple domains (12). Genome-wide linkage analyses were conducted by COGA on a total of 2310 individuals from 262 families in which at least three first-degree members had alcoholism. These studies have provided consistent evidence for an alcoholism susceptibility locus on the long arm of chromosome 7 (13,14). More recent studies have also observed linkage with major depressive syndrome in the same region of chromosome 7 (15).

A unique feature of COGA has been the collection of electrophysiological endophenotypes, including the event-related potential (ERP) and the electroencephalogram (EEG). P300 (P3) amplitude has long been known to be reduced in both alcoholics and alcohol naive offspring of alcoholics, suggesting that this difference in P3 may underlie risk for alcoholism (16,17). More recently, it has been observed that theta and delta oscillations underlying 'Go No-Go' P3 are also reduced in alcoholics (18). Genetic analysis of electrophysiological features derived from event-related oscillations (EROs) underlying P3 (evoked theta and delta oscillations) demonstrated linkage to an overlapping region of the long arm of chromosome 7, and association with multiple single nucleotide polymorphisms (SNPs) in the gene encoding the muscarinic acetylcholine receptor subtype 2 (*CHRM2*) (19).

Muscarinic acetylcholine receptors (mAchRs) containing five subtypes (m1–m5) belong to a family of G-protein coupled receptors. They are present in neurons in the central and peripheral nervous system, cardiac and smooth muscles, and a variety of exocrine glands (20,21). Muscarinic receptors are involved in many functions in the brain, including attention, learning, memory and cognition (22). Functional studies using *CHRM2* knock-out mice have shown that *CHRM2* mediates muscarinic receptor-dependent movement, temperature control, analgesic effects, bradycardia and gallbladder contractility (23–29).

There is also evidence that variations in the *CHRM2* gene are associated with several diseases. A recent study reported that *CHRM2* receptor density is higher in the frontal and temporal cortices of Alzheimer's disease patients with psychotic symptoms, than in those without psychotic symptoms (30). A polymorphism in the 3′ untranslated region (3′-UTR) of the *CHRM2* gene has been associated with IQ and with major depression in women (31,32). Like the other muscarinic receptors, human *CHRM2* has an intron-less open reading frame and contains a large 5′-UTR region encoded by multiple exons with complex tissue-specific alternative splicing patterns (33,34). Given the prior reports of association between SNPs in the *CHRM2* gene and EROs as well as depression, the evidence of linkage in this same region for alcoholism and depression, and the strong correlation between these traits, we have undertaken a comprehensive analysis of SNPs within and flanking the *CHRM2* gene to test for association between this gene and alcoholism and major depressive syndrome.

RESULTS

Linkage analysis

Linkage analyses using a map including newly typed markers resulted in a peak LOD score of 2.9 at D7S1799 with alcohol dependence (488 sib pairs, IBD sharing = 56.5%, Fig. 1). Similar analyses with major depressive syndrome (259 sib pairs, IBD sharing = 58.1%, Fig. 1) produced an overlapping peak with a maximum LOD score of 2.3 between D7S1799 and D7S1817. The composite phenotype, alcohol dependence and major depressive syndrome (144 sib pairs, IBD sharing = 61.0%, Fig. 1) resulted in a peak of the same magnitude and at the same location as that observed with major depressive syndrome alone (LOD score of 2.3 at 135 cM, Fig. 1). The highest LOD score (3.4) was observed when the combined phenotype 'alcohol dependence or major depressive syndrome' (639 sib pairs, IBD sharing = 56.2%, Fig. 1) was used. This peak was in the same position as the alcohol dependence linkage at D7S1799. These results suggest that a gene(s) in this region of chromosome 7 influence(s) susceptibility to both disorders.

SNP analyses of *CHRM2*

Because of our prior evidence of linkage and association between SNPs in the *CHRM2* gene and theta and delta EROs, and the correlation between alcohol dependence and these EROs for the P3 (18,19), we analyzed linkage disequilibrium between SNPs in the *CHRM2* gene and alcohol dependence and major depressive syndrome. Eleven SNPs spanning a 70 kb region within and flanking the *CHRM2* gene were genotyped (Fig. 2, Table 1). All of the SNPs were in Hardy–Weinberg equilibrium in the founders. Allele frequencies and the distance between markers are shown in Table 1. The program Transmit was used to determine the pair-wise disequilibrium between the SNPs (Table 1). The SNPs from rs324640 (intron 5) to rs324656 (10 kb downstream of the 3′-UTR), which flank the single coding exon and cover 25 kb, are all in very strong LD ($D' \geq 0.75$), whereas the three SNPs in intron 4 show high levels of LD with each other but lower LD with SNPs in the 3′-UTR region (Fig. 2, Table 1).

We used a family-based program, UNPHASED pedigree equilibrium test (PDTPHASE), to examine the association between the SNPs and the phenotypes. One SNP (rs1824024) in intron 4 and two SNPs (rs324640 and rs324650) in intron 5 showed significant association ($P < 0.05$) with alcohol dependence using the SUM and AVE statistics (Table 2). Two additional SNPs (rs8191992 and rs1378650) at the 3′ end of the gene showed significant association with alcohol dependence using the AVE statistic only. Some of these SNPs also showed significant association with alcohol dependence defined by ICD-10 (35) and DSM-IV (36) criteria ($P < 0.05$).

Major depressive syndrome also demonstrated significant association with multiple SNPs in *CHRM2*. Using the SUM statistic, all SNPs in intron 4 were significantly associated with major depressive syndrome. The three SNPs in intron 5 also demonstrated significant association or a trend towards significance ($P = 0.05$–0.06) (Table 2). In addition, two SNPs in the 3′ end of the gene exhibited a trend towards association ($P = 0.06$–0.08). Results using the AVE statistic were similar, though not quite as significant. In contrast to alcohol dependence, major depressive syndrome demonstrated stronger association when the SUM statistic was used rather than AVE, suggesting that large families may contribute more strongly to the *CHRM2* association with major depressive syndrome.

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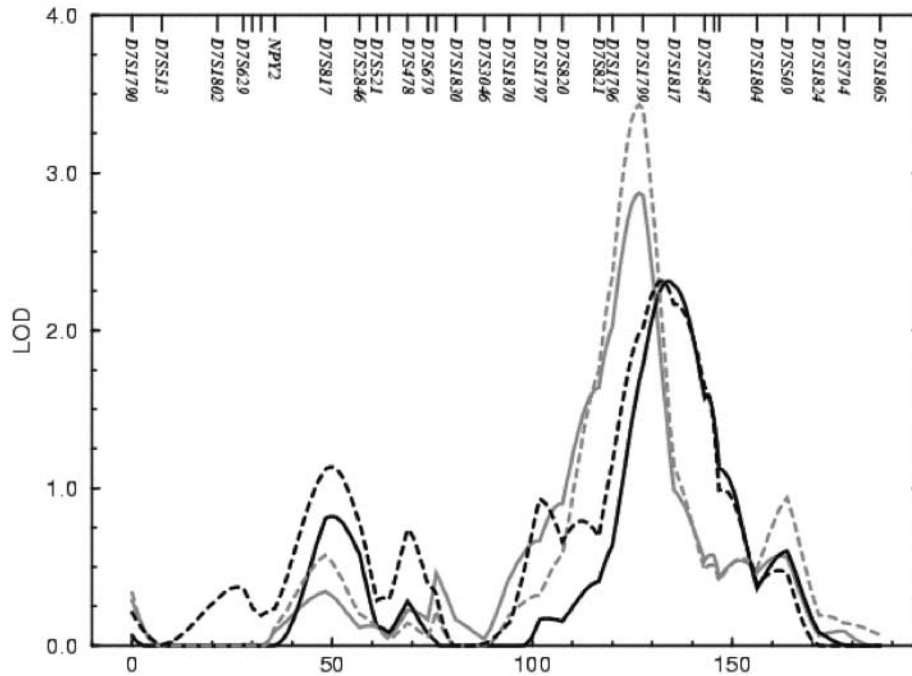

Figure 1. Multipoint linkage analyses on chromosome 7. Non-parametric multipoint linkage analysis of independent ($N-1$) affected sibling pairs was conducted using ASPEX. Solid gray line represents 'alcohol dependence,' solid black line represents 'major depressive syndrome,' dashed black line represents 'alcohol dependence and major depressive syndrome,' and dashed gray line represents 'alcohol dependence or major depressive syndrome'.

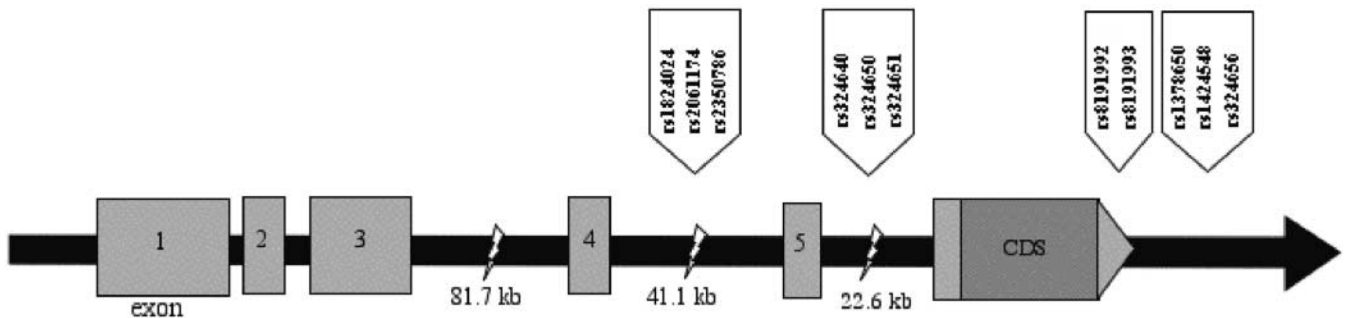

Figure 2. Location of SNPs within and flanking the *CHRM2* gene on chromosome 7. This figure is not drawn to scale. Dark gray box represents coding sequence (CDS), light gray boxes represent exons encoding untranslated sequences, and the black bars represent intronic sequences.

Significant association was also detected between SNPs in introns 4 and 5 and the composite diagnosis of 'alcohol dependence or depression' (Table 2). For the combined phenotype 'alcohol dependence and depression,' seven SNPs within the gene showed significant association even with the reduced sample defined by this narrower phenotype. This more severe phenotype exhibited the highest IBD sharing in the linkage study (61%) and the strongest evidence of association.

Haplotype analyses

On the basis of the high level of significance observed throughout the gene, we constructed haplotypes and estimated the haplotype probabilities using PDTPHASE. Only four SNPs showed P-values <0.01 for any phenotype or PDT measure (SUM or AVE). Two of these SNPs (rs324640 and rs324650) had a correlation of 0.89 (Table 1). We therefore used three of these SNPs, rs1824024, rs2061174 and rs324650 for haplotype construction. This haplotype analysis showed significant global Chi-squared statistics using the PDT with all four phenotypes examined (Tables 3–6). The strongest evidence of association was detected with major depressive syndrome (SUM $P = 0.0001$, AVE $P = 0.0009$) (Table 4).

Three common haplotypes, (rs1824024–rs2061174–rs324650) T–T–T, G–C–A and T–T–A were observed, totaling 80% of the observed haplotypes. Individual haplotypes for major depressive syndrome and the composite phenotype 'alcohol dependence and major depressive syndrome' show similar patterns of association. The most common haplotype (>43%), T–T–T was under-transmitted to affected individuals, whereas the complementary haplotype, G–C–A was over-transmitted to affected individuals (Tables 4 and 5). In addition,

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Table 1. Pair-wise disequilibrium between SNPs in the *CHRM2* gene

SNP (allele frequencies)	rs1824024 (17 kb[a])	rs2061174 (15 kb)	rs2350786 (12 kb)	rs324640 (5 kb)	rs324650 (6 kb)	rs324651 (2 kb)	rs8191992 (0.2 kb)	rs8191993 (3 kb)	rs1378650 (5 kb)	rs1424548 (4 kb)	rs324656
rs1824024 (G:0.33/T:0.67)		0.75	0.66	0.53	0.46	0.62	0.62	0.43	0.60	0.61	0.25
rs2061174 (C:0.34/T:0.66)	0.51		0.94	0.51	0.44	0.76	0.76	0.51	0.62	0.75	0.27
rs2350786 (A:0.29/G:0.71)	0.40	0.72		0.45	0.44	0.71	0.46	0.46	0.63	0.78	0.23
rs324640 (C:0.45/T:0.55)	0.16	0.16	0.11		0.96	0.98	0.81	0.75	0.63	0.57	0.67
rs324650 (A:0.44/T:0.56)	0.13	0.13	0.10	0.89		0.97	0.77	0.67	0.58	0.64	0.63
rs324651 (G:0.88/T:0.12)	0.03	0.04	0.03	0.11	0.10		0.97	0.92	0.84	0.86	0.74
rs8191992 (A:0.41/T:0.59)	0.22	0.21	0.13	0.53	0.51	0.09		0.94	0.79	0.81	0.79
rs8191993 (C:0.70/G:0.30)	0.17	0.21	0.21	0.29	0.24	0.05	0.56		0.76	0.71	0.59
rs1378650 (C:0.58/T:0.42)	0.24	0.29	0.24	0.33	0.30	0.07	0.60	0.35		0.82	0.74
rs1424548 (A:0.38/G:0.62)	0.10	0.17	0.15	0.16	0.19	0.06	0.26	0.12	0.27		0.75
rs324656 (C:0.35/T:0.65)	0.05	0.07	0.04	0.30	0.28	0.04	0.51	0.27	0.43	0.18	

[a]Distance between two SNPs; D' values are shown in upper triangle; Δ^2 values are shown in lower triangle.

a relatively rare haplotype, G−C−T was over-transmitted to affected individuals for major depressive syndrome and another rare haplotype T−C−A was under-transmitted (Table 4).

Likewise, the T−T−T haplotype was under-transmitted to alcohol dependent individuals (Table 3). A rare haplotype, G−T−A, was over-transmitted to alcohol dependent individuals. For individuals affected with ‘alcohol dependence or major depressive syndrome,’ the T−T−T haplotype showed only weak evidence of under-transmission. Two rare haplotypes G−T−A and T−C−A were over-transmitted and under-transmitted to the affected individuals, respectively (Table 6).

Under-transmission of the haplotype is primarily driven by the T allele of SNP rs1824024, and over-transmission is primarily driven by the G allele in the same SNP. Although the transmission of the protective haplotype is similar for all of the phenotypes we examined, the component alleles of the risk haplotype for alcohol dependence appear to be different from the alleles for major depressive syndrome.

Sequencing of *CHRM2*

We sequenced the coding region of the *CHRM2* gene to determine whether there were coding polymorphisms that may provide a stronger indication for association. no coding SNP was observed; however, a novel SNP located within the 3′-UTR of the *CHRM2* gene was detected and added to our association studies (rs8191993).

DISCUSSION

Genetic linkage studies in the COGA dataset have previously reported evidence of linkage with alcohol dependence on the long arm of chromosome 7 (13,14). Fine mapping reported here has increased the LOD score and narrowed the region of linkage for alcohol dependence. We also show that major depressive syndrome is linked to an overlapping region of chromosome 7 (Fig. 1). Although the peak LOD score was increased, by broadening the phenotype to include ‘alcoholism or major depressive syndrome,’ the IBD sharing was highest in individuals with the narrowest phenotype definition, ‘alcoholism and major depressive syndrome’. This suggests that genes underlying both phenotypes are likely to be located in this region and raises the possibility of pleiotropy.

It should be noted that the *CHRM2* gene is located directly under the linkage peak for theta ERO (19), but is at some distance from the linkage peak for alcoholism. Furthermore, the families that contribute to the theta ERO linkage peak are different from the families that contribute to the alcoholism linkage signal at D7S1799. These observations, together with our unpublished analyses of polymorphisms in other candidate genes in the same region of chromosome 7 suggest that multiple genes contribute to our linkage signals.

Interest in the *CHRM2* gene as a candidate gene resulted from the strong linkage findings in the same dataset with a novel ERO phenotype, frontal theta oscillations underlying P3 (19), and evidence that muscarinic receptors influence P3 generation and the underlying oscillatory processes (37). Moreover, cholinergic muscarinic genes play a major role in memory and cognition (38). In the present study, we analyzed two SNPs in the 3′-UTR of the *CHRM2* gene along with nine other SNPs flanking the single coding exon to test for genetic association with alcohol dependence and major depressive syndrome. The results from PDTPHASE demonstrate that SNPs located upstream of the coding sequence of the gene are strongly

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Table 2. Association of 11 SNPs within and flanking the *CHRM2* gene with alcoholism, major depressive syndrome, 'alcoholism or depression' and 'alcoholism and depression'

		Alcohol dependence ($N = 1034$)		Major depressive syndrome ($N = 773$)		Alcohol dependence or major depressive syndrome ($N = 1346$)		Alcohol dependence and major depressive syndrome ($N = 461$)	
		SUM	AVE	SUM	AVE	SUM	AVE	SUM	AVE
Intron 4	rs1824024	**0.018**	**0.007**	**0.003**	**0.004**	**0.033**	**0.009**	**0.001**	**0.005**
	rs2061174	0.225	0.187	**0.002**	**0.017**	0.104	0.184	**0.011**	**0.019**
	rs2350786	0.758	0.826	**0.034**	0.103	0.642	0.985	**0.037**	0.069
Intron 5	rs324640	**0.010**	**0.007**	0.064	0.182	**0.029**	0.062	**0.012**	**0.011**
	rs324650	**0.016**	**0.004**	**0.047**	0.116	**0.027**	**0.029**	**0.012**	**0.009**
	rs324651	0.119	0.195	0.065	0.084	**0.032**	0.102	0.108	0.159
3′-UTR	rs8191992	0.103	**0.036**	0.078	0.108	0.131	0.168	**0.049**	0.064
	rs8191993	0.598	0.876	0.056	0.173	0.596	0.855	**0.018**	**0.053**
Downstream of 3′-UTR	rs1378650	0.100	**0.026**	0.122	0.206	0.191	0.178	0.087	0.193
	rs324548	**0.046**	0.062	0.711	0.823	0.337	0.381	0.590	0.541
	rs324656	0.472	0.503	0.486	0.468	0.509	0.908	0.564	0.642

SUM and AVE P-values are shown. Bold numbers indicate P-value < 0.05.

associated with alcohol dependence and major depressive syndrome in the COGA families, whereas SNPs that are located immediately downstream of the gene show moderate association with alcohol dependence only (Table 2). In contrast to previous reports (31) we did not observe association between SNP rs8191992 in the 3′-UTR and depression. Furthermore, the SNPs (e.g. rs1824024) that are significantly associated with major depressive syndrome did not show gender-specific differences in the association. The strong association observed with SNPs upstream of the *CHRM2* coding sequence in this study is similar to that observed in the same dataset with theta and delta frequency band EROs (19).

The results for major depressive syndrome and the composite phenotypes 'alcohol dependence and depression' and 'alcohol dependence or depression' were more significant using the PDT SUM statistics, whereas the results for alcoholism were more significant using the PDT AVE statistic. Significant genetic heterogeneity is expected for psychiatric disorders, resulting in different genes acting in different families. The distribution of family size in the informative subset of families for each phenotype may thus influence the relative significance of the PDT SUM versus the PDT AVE statistic. In the presence of genetic heterogeneity, it is therefore not unexpected that these statistics vary depending on the informative subset of families for the particular phenotype/gene under study. Overall, we have added confidence in the results with the consistency of the findings across diagnoses and statistics.

Haplotype analyses showed that the most common haplotype (rs1824024–rs2061174–rs324650 = T–T–T) protects against risk for alcohol dependence, major depressive syndrome and the composite phenotypes, whereas the vulnerability haplotypes varied for these disorders. This leads us to speculate that protective factors provide a general protection against the development of multiple disorders. We note that different variants of the *CHRM2* haplotype contribute to the risk of alcoholism or depression and so vulnerability factors appear to be more specific. Interestingly, there is strong association with the composite phenotype 'alcoholism and depression,' despite the smaller sample size. This may represent a unique syndrome and not the simple co-occurrence of two illnesses. Our linkage results support this hypothesis, as the IBD sharing for 'alcohol dependence and major depressive syndrome' is higher than for each of the single diagnoses

Table 3. Haplotype analysis of three associated SNPs in the *CHRM2* gene with alcohol dependence

rs1824024–rs2061174–rs324650	Haplotype frequencies	SUM PDT		AVE PDT	
		Z	P-value	Z	P-value
G–C–A	0.195	1.525	0.127	1.762	0.078
G–C–T	0.069	−0.283	0.777	−0.342	0.733
G–T–A	0.024	1.654	0.098	2.448	**0.014**
G–T–T	0.027	0.616	0.538	0.421	0.674
T–C–A	0.037	−1.362	0.173	−1.939	0.053
T–C–T	0.038	0.908	0.364	0.822	0.411
T–T–A	0.180	0.992	0.321	1.250	0.211
T–T–T	0.431	−2.601	**0.009**	−2.504	**0.012**

Global test: $X^2 = 13.95$ SUM $P = 0.05$. Global test: $X^2 = 18.95$ AVE $P = 0.008$. Bold numbers indicate P-value < 0.05.

Table 4. Haplotype analysis of three associated SNPs in the *CHRM2* gene with major depressive syndrome

rs1824024–rs2061174–rs324650	Haplotype frequencies	SUM PDT		AVE PDT	
		Z	P-value	Z	P-value
G–C–A	0.195	2.538	**0.011**	2.038	**0.042**
G–C–T	0.069	2.958	**0.003**	2.808	**0.005**
G–T–A	0.024	1.298	0.194	1.523	0.128
G–T–T	0.027	−1.931	0.054	−1.715	0.086
T–C–A	0.037	−2.163	**0.031**	−2.142	**0.032**
T–C–T	0.038	0.690	0.490	0.696	0.486
T–T–A	0.180	0.859	0.390	0.412	0.680
T–T–T	0.431	−2.727	**0.006**	−2.342	**0.019**

Global test: $X^2 = 29.69$ SUM $P = 0.0001$. Global test: $X^2 = 24.52$ AVE $P = 0.0009$. Bold numbers indicate P-value < 0.05.

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Table 5. Haplotype analysis of three associated SNPs in the *CHRM2* gene with the composite phenotype 'alcohol dependence and major depressive syndrome'

rs1824024–rs2061174–rs324650	Haplotype frequencies	SUM PDT		AVE PDT	
		Z	P-value	Z	P-value
G–C–A	0.195	2.459	**0.014**	2.437	**0.015**
G–C–T	0.069	1.763	0.078	1.818	0.069
G–T–A	0.024	1.031	0.303	0.917	0.359
G–T–T	0.027	−1.327	0.184	−1.618	0.106
T–C–A	0.037	−1.062	0.288	−0.831	0.406
T–C–T	0.038	−0.484	0.628	−1.010	0.313
T–T–A	0.180	1.176	0.240	0.983	0.326
T–T–T	0.431	−3.055	**0.002**	−2.677	**0.007**

Global test: $X^2 = 21.05$ SUM $P = 0.004$. Global test: $X^2 = 19.73$ AVE $P = 0.006$. Bold numbers indicate P-value < 0.05.

Table 6. Haplotype analysis of three associated SNPs in the *CHRM2* gene with the composite phenotype 'alcohol dependence or major depressive syndrome'

rs1824024–rs2061174–rs324650	Haplotype frequencies	SUM PDT		AVE PDT	
		Z	P-value	Z	P-value
G–C–A	0.195	1.693	0.091	1.650	0.099
G–C–T	0.069	1.769	0.077	1.514	0.130
G–T–A	0.024	2.317	**0.021**	2.536	**0.011**
G–T–T	0.027	−1.922	0.055	−1.443	0.149
T–C–A	0.037	−2.294	**0.022**	−2.279	**0.023**
T–C–T	0.038	0.212	0.832	0.255	0.799
T–T–A	0.180	0.994	0.320	0.470	0.638
T–T–T	0.431	−1.799	0.072	−1.711	0.087

Global test: $X^2 = 21.52$ SUM $P = 0.003$. Global test: $X^2 = 19.20$ AVE $P = 0.007$. Bold numbers indicate P-value < 0.05.

(61% versus 56% for alcohol dependence or 58% for major depressive syndrome).

Mutagenesis studies have shown that the amino acid substitution Tyr403Phe in the *CHRM2* gene affects the ligand binding affinities of the receptor and that four other amino acid substitutions at Val386, Thr386, Ile389 and Leu390 are essential for G-protein coupling specificity and G-protein activation (39,40). However, sequencing of the entire coding region of the gene on 180 chromosomes failed to identify any coding variants in our study subjects. Thus, the coding region for the *CHRM2* gene is highly conserved and even the silent coding SNPs found in asthmatics are uncommon in our population (41,42). The significant association of this gene with alcohol dependence and major depressive syndrome was primarily observed with SNPs in introns of the 5′-UTR. The recent report demonstrating that the 5′-UTR of *CHRM2* is encoded by five exons that exhibit tissue-specific alternative splicing (34) suggests that thorough sequence analysis of the 5′ end of the gene is needed to fully assess its impact on the phenotypes studied in the COGA dataset.

In summary, we have observed strong association between SNPs at the 5′ end of the *CHRM2* gene and both alcohol dependence and major depressive disorder, consistent with the observation of common genetic factors in the development of alcoholism and depression in twin studies (10,11). Given our previous report of association between SNPs in the *CHRM2* gene and theta and delta EROs (19), we hypothesize that the underlying neural processes that alter theta and delta EROs may also result in the differences in susceptibility to depression and alcoholism.

MATERIALS AND METHODS

Study subjects

Probands were systematically recruited from inpatient and outpatient alcohol treatment units and were required to meet DSM-IIIR criteria for alcohol dependence (43) and Feighner criteria for definite alcoholism (44). The combined endorsement of a lifetime history of DSM-IIIR alcohol dependence and Feighner definite criteria has been termed COGA alcohol dependence (referred to as alcohol dependence) and was the phenotype used in our initial linkage studies (13). All first-degree relatives of probands were invited to participate, and all subjects were assessed by direct interview using the semi-structured assessment for the genetics of alcoholism (45). Informed consent was obtained from all subjects. Families with at least three alcoholic first-degree relatives underwent further assessment with neurophysiological tests, and blood samples were obtained for genetic samples. A total of 2310 individuals in 262 families was selected for genetic analyses (14). Approximately 82% of the sample is Caucasian, 15% is African-American and 3% consists of other ethnicities. Owing to the relatively small sample size of non-Caucasians, we did not perform any analyses stratified by ethnicity.

Major depressive syndrome was defined by a lifetime history having five or more symptoms of depression for 2 weeks or more (criterion A for a major depressive episode, DSM-IIIR). As it is often clinically difficult to determine if depressive symptoms precede or result from alcohol problems, all episodes of depression were included regardless of the attribution to alcohol, bereavement or other medical disorders. Subjects who reported a manic episode in addition to depression, consistent with the diagnosis of bipolar affective disorder, were excluded from the depression analyses. To examine the relationship between alcohol dependence and major depressive syndrome, two composite phenotypes were also constructed: 'alcohol dependence or depression' in which affection status was defined as having either disorder; and 'alcohol dependence and depression' in which affection was defined by requiring both disorders. Results were then compared with analyses of individuals who had only one disorder or the other which removes any confounding issue due to comorbidity. In our dataset, 573 individuals were alcohol dependent only, 312 individuals had major depressive syndrome only and 461 individuals had both disorders.

Statistical methods

Linkage analyses. Three additional microsatellite markers were genotyped in the region of chromosome 7 in which linkage was previously reported with alcohol dependence (13,14).

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Non-parametric multipoint linkage analysis of independent ($N - 1$) affected sibling pairs was conducted using ASPEX (46), which allows large sibships to be included in analyses. Linkage analyses were performed using the SIBPHASE option that infers allele sharing when there is ambiguity between identity by state and identity by descent by using marker frequencies in the sample. As estimating marker frequency from the data set can lead to biases because of ethnic stratification, we re-ran analyses using families whose genotypic data was available from both parents in order to minimize false positive results because of biased allele frequency estimations. Although this type of analysis results in greater accuracy in the estimates of marker allele sharing, this occurs at the expense of a reduction in the sample size.

Association analyses. Linkage disequilibrium (LD) between markers was analyzed using the program Transmit (47). To examine association between the SNPs and the phenotypes of alcohol dependence and major depressive syndrome, we used the program PDTPHASE within the UNPHASED (http://www.hgmp.mrc.ac.uk/~fdudbrid/software/unphased/) suite of programs. The PDT is a family-based association test and thus avoids problems of false positives arising from population stratification, which can occur in population-based association approaches (48,49). Two statistics are reported with the PDT, SUM and AVE. The SUM statistic weighs all affected individuals equally, so that large families with multiple affected individuals contribute more to the statistic than do smaller families with fewer affected subjects. In contrast, the AVE statistic gives equal weight to all families, so that large families do not contribute disproportionately to the statistic.

As we were focusing on two correlated characteristics, alcohol dependence and depression, we were conservative with the definition of unaffected. Unaffected individuals in the alcoholism analyses were drinkers who did not endorse any symptoms for alcohol dependence. This 'pure' unaffected individual offers the greatest contrast with affected alcohol dependent individuals. For the depression assessment, a threshold of 2 weeks of low mood was required for individuals to be fully assessed for depression. Given this threshold, the decision was made to focus only on affected individuals. Unaffected individuals contribute to the assignment of genotypes but not to the association test statistic for depression. Finally, we used PDTPHASE to carry out haplotype analysis with significantly associated SNPs.

SNP assays

Publicly available databases, dbSNP (http://www.ncbi.nlm.nih.gov/SNP) and LocusLink (http://www.ncbi.nim.nih.giv/LocusLink/refseq.html) were used to identify SNPs within and flanking the *CHRM2* gene. SNP rs8191993 was identified by our sequencing analysis; rs8191992 was reported by Fenech *et al.* (41) and Comings *et al.* (31). For SNP genotyping, PCR primers were selected using the MacVector 6.5.3 program (Oxford Molecular Group, Inc.) to give 200–500 bp genomic fragments containing the SNP. Ten of the 11 SNPs were genotyped using a Pyrosequencing method (Biotage AB) with sequencing primers designed using the Pyrosequencing Primer Design program (http://www.pyrosequencing.com). To screen putative SNPs without available frequency data, we attached a universal primer to one of the PCR primers and included a third biotinylated universal primer in the PCR. For SNPs with validated frequency data, we labeled one of the PCR primers with biotin. Standard PCR procedures were followed to generate PCR products. We used an restriction fragment length polymorphism (RFLP) assay for one SNP, rs1424548 that did not optimize for Pyrosequencing. Genotypes were tested for Mendelian inheritance. Missing genotypes and genotypes inconsistent with Mendelian inheritance were retyped. Any further inconsistency errors were discarded. On an average 2297/2310 (99.4%) individuals had a genotype for each marker.

Sequence analysis

To examine whether there were coding or splice-site polymorphisms within the *CHRM2* gene, the entire coding region was sequenced in both directions in DNA from 90 individuals, including carriers of the major haplotypes. Publicly available sequence databases were used to select PCR primers to amplify the coding exon plus at least 60 bp of flanking intronic sequence. Each fragment was amplified separately from genomic DNA using standard PCR procedures. PCR products were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) to remove excess primers. Purified PCR products were sequenced using the BigDye Terminator Cycle Sequencing method and then electrophoresed on an ABI3100 automated DNA sequencer (ABI, Foster City, CA, USA). Electropherograms were analyzed using ABI DNA sequencing analysis software (Navigator and Factura), version 3.4.

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