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CHRM2 gene predisposes to alcohol dependence, drug dependence and affective disorders: results from an extended case–control structured association study

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Cholinergic muscarinic 2 receptor (CHRM2) is implicated in memory and cognition, functions impaired in many neuropsychiatric disorders. Wang *et al.* [Wang, J.C., Hinrichs, A.L., Stock, H., Budde, J., Allen, R., Bertelsen, S., Kwon, J.M., Wu, W., Dick, D.M., Rice, J. *et al.* (2004) Evidence of common and specific genetic effects: association of the muscarinic acetylcholine receptor M2 (CHRM2) gene with alcohol dependence and major depressive syndrome. *Hum. Mol. Genet.*, 13, 1903–1911] reported that variation in *CHRM2* gene predisposed to alcohol dependence (AD) and major depressive syndrome. We examined the relationships between variation in *CHRM2* and AD, drug dependence (DD) and affective disorders, using a novel extended case–control structured association (SA) method. Six markers at *CHRM2* and 38 ancestry-informative markers (AIMs) were genotyped in a sample of 871 subjects, including 333 healthy controls [287 European-Americans (EAs) and 46 African-Americans (AAs)] and 538 AD and/or DD subjects (415 with AD and 346 with DD and 382 EAs and 156 AAs). The same *CHRM2* markers were genotyped in a sample of 137 EA subjects with affective disorders. All of the six markers were in Hardy–Weinberg equilibrium in controls, but SNP3 (rs1824024) was in Hardy–Weinberg disequilibrium in the AD and DD groups. Using conventional case–control comparisons, some markers were nominally significantly or suggestively associated with phenotypes before or after controlling for population stratification and admixture effects, but these associations were not significant after multiple test correction. However, regression analysis identified specific alleles, genotypes, haplotypes and diplotypes that were significantly associated with risk for each disorder. We conclude that variation in *CHRM2* predisposes to AD, DD and affective disorders. One haplotype block within the 5′-UTR of *CHRM2* may be more important for the development of these disorders than other regions. Interaction between two specific alleles within this block and interaction between two specific diplotypes covering this block multiplicatively increased risk for AD and DD. Although interaction between these two diplotypes also increased risk for affective disorders, the magnitude of the increased risk was less than the sum of the individual risks. In addition, a specific diplotype might inversely affect risk for AD and DD and risk for affective disorders.

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INTRODUCTION

Cholinergic receptors (CHRs) are important mediators of acetylcholine response, which gives rise to a series of intracellular and intranuclear events. The CHRs include nicotinic types (CHRN) and muscarinic types (CHRM). The muscarinic receptors belong to a family of seven transmembrane domain G-protein-coupled receptors and are classified into five subtypes.

CHRM2 receptor, which is present in neurons throughout the central and peripheral nervous systems, cardiac and smooth muscles and a variety of exocrine glands, is predominantly a presynaptic autoreceptor, helping to control cAMP regulation and being responsible for acetylcholine-mediated inhibition of adenyl cyclase activity (1). CHRM2 primarily provides negative feedback on acetylcholine release from cholinergic terminals (2,3). Cholinergic pathways, in general, and CHRM2 specifically, have been widely implicated in cognition and memory (4,5). For example, in the striatum, CHRM2 has been demonstrated to play a role in long-term potentiation (LTP), which is implicated in memory and cognition (6). CHRM2 has also been reported to play a functional role in cognition via modulation of neuroelectric oscillations (i.e. the periodic electric waves in neurons measured by EEG) (7). There is other direct evidence showing that alteration of CHRM2 is involved in the development of neuropsychiatric disorders, e.g. CHRM2 density was reduced in the frontal cortex of Alzheimer's disease patients (8).

CHRM2, the gene encoding the cholinergic muscarinic 2 receptor (CHRM2), is the focus of this study. *CHRM2* is located at chromosome 7q31–35. Similar to >90% of mammalian G-protein-coupled receptor genes, *CHRM2* has an intronless open reading frame (9). The gene consists of a single coding sequence (CDS) (i.e. the coding part of exon 6), a large 5′-untranslated region (5′-UTR) including five introns, five non-coding exons and the non-coding part of exon 6 and a 3′-UTR (Fig. 1).

Within the CDS, some experimentally modeled (but not necessarily observed) functional variants can lead to amino acid substitutions, which may alter the properties of the receptor. For example, Tyr403Phe affects the ligand binding affinities of the receptor; four other amino acid substitutions at Val385, Thr386, Ile389 and Leu390 are essential for G-protein coupling specificity and G-protein activation (10,11).

The 5′-UTR of *CHRM2*, containing a large intron (i.e. intron 5, 22.6 kb) (1,3), may be alternatively spliced in some specific tissues. Within this 5′-UTR, many functional sites can influence the transcription or expression of the CHRM2 receptor (3). For example, a region in the 5′ transcription start site (TSS1) within the 5′-UTR, 146 kb upstream from the CDS, is responsible for the major transcriptional activity of *CHRM2* (1); a CA tandem repeat polymorphism in exon 1 may influence the transcription of *CHRM2* in airway smooth muscle (HASM) and in BEAS-2B cells (1).

In addition, the 3′-UTR might contain functional sites, e.g. a polymorphism (rs8191992) in that region has, in some studies, been associated with IQ and with major depression in women (12,13). This evidence suggests that variation at *CHRM2* can alter the function of the CHRM2 receptor and may thus predispose to disease, especially neuropsychiatric disorders that, at some point in their course, include impairment of cognition, such as Alzheimer's disease, major depression, alcohol dependence (AD) or drug dependence (DD) (14). The present study aimed to test the hypothesis that *CHRM2* plays a role in the susceptibility to AD, DD (including cocaine dependence and opioid dependence) and affective disorders.

AD is a disorder with a high rate of comorbidity. Comorbid illnesses may occur simultaneously or sequentially. Alcoholics are 35 and 13 times more likely than non-alcoholics to suffer from cocaine dependence and opioid dependence, respectively (15). In addition, 27.6–29.2% of alcoholics suffer from a current affective disorder (16,17). It is likely that these disorders share some susceptibility genes. For example, variation both in *OPRM1*, the gene encoding the mu-opioid receptor, and in *DRD4*, the gene encoding the dopamine-4 receptor, have been associated in some studies with susceptibility to AD and/or DD (18–24). In addition, Gorwood *et al.* (25) demonstrated that affective disorders and AD, interacting with a genetic deficiency in serotonin reuptake attributable to polymorphic variation in the serotonin transporter gene (*SLC6A4*), were associated with an increased risk for aggressive/impulsive behaviors such as suicide attempts. Nurnberger *et al.* (26) showed that a locus on chromosome 1 might predispose some individuals to alcoholism and others to depression. Huang *et al.* (27) reported that both substance use disorders and major depression were associated with the human 5-HT1B receptor gene (*HTR1B*) G861C polymorphism.

Recently, Wang *et al.* (28) reported that variants within the 5′-UTR of *CHRM2* influenced risk for both AD and major depressive syndrome. On the basis of these findings, we studied three phenotypes (i.e. AD, DD and affective disorders) in the present study to test both the general and the specific effects of *CHRM2* variation in these disorders.

Genome-wide scan linkage studies from the Collaborative Study on the Genetics of Alcoholism (COGA) provided suggestive evidence for a risk locus for AD in a region on chromosome 7q (29,30); another COGA study based on the same data, but different diagnoses, also provided evidence for linkage with major depressive syndrome in the same region of chromosome 7 (26), as noted earlier. Motivated by the linkage studies, Wang *et al.* (28) genotyped a denser set of 31 short tandem repeat (STR) markers at this region and fine-mapped a possible risk locus for AD close to marker D7S1799 and a risk locus for major depressive syndrome between D7S1799 and D7S1817, a narrow region harboring *CHRM2.*

To identify a specific risk locus within a linked region, the association study method is used most commonly. Wang *et al.* (28) genotyped 11 single nucleotide polymorphisms (SNPs) within or close to *CHRM2*, including three markers at intron 4, three markers at intron 5, two markers at the 3′-UTR and three markers close to the 3′ end. These investigators found that the markers and their haplotypes within the 5′-UTR, but not within the 3′-UTR or further 3′, were associated with AD and major depressive syndrome. In the present study, we used an association study design to further finer-map the risk alleles for AD, DD and affective disorders by genotyping more markers within the 5′-UTR further upstream of the CDS than that examined by Wang *et al.* (28). Specifically, we included two additional markers, SNP1 and SNP2 at intron

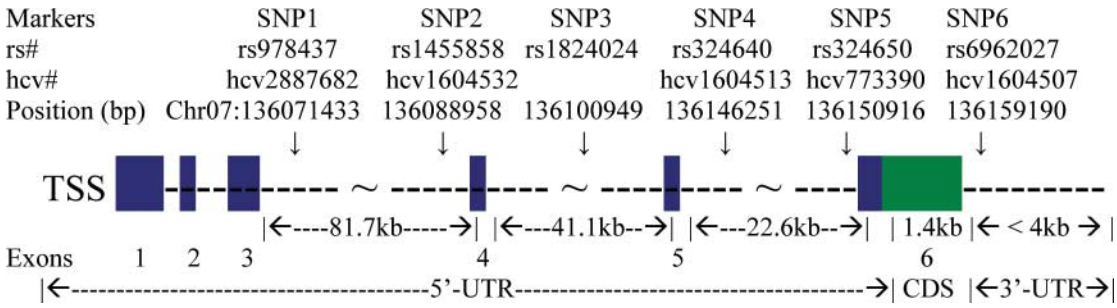

Figure 1. Markers at *CHRM2*.

3, closer to the functional 5′ TSS region described earlier (Fig. 1). We did not investigate the CDS variation in this study because this CDS is highly conserved, and no known variation in the populations included in this study (i.e. EAs and AAs) has been reported for this region of the gene.

Markers within a gene are usually in different degrees of linkage disequilibrium (LD). Different markers within the 5′-UTR of a gene might interactively affect mRNA stability or translational efficacy and contribute to the expression of differing levels of the protein. Epistasis might occur among these markers; i.e. association between one marker and phenotype might influence association between another marker and phenotype. Therefore, interactive effects on the trait from different markers should not be neglected and were, accordingly, considered in this study.

The population-based association design, especially the unmatched case–control design, is more powerful than the family-based association design for detecting gene–gene interactions, when the disease prevalence is moderate (as is the case for AD, DD and affective disorders) (31). We employed a population-based association design in this study. Our sample size in this study would, further, be sufficient for detecting gene–gene interaction as required by a population-based association design (31).

However, population-based association designs are potentially vulnerable to population stratification and admixture effects that could result in spurious findings, such as spurious associations between genotypes and phenotypes, spurious LD between unlinked markers or Hardy–Weinberg disequilibrium (HWD) at non-susceptibility markers. To exclude population stratification and admixture effects, a novel and powerful logistic regression method was employed in this study. This extended the rationale for use of the structured association (SA) method (32), which can control for the effect of population stratification on population-based data. We previously demonstrated that a set of STR markers is sufficient to the task of identifying population structure that can confound association studies and to the task of providing a measure to control for such stratification (33).

RESULTS

(1) *CHRM2 markers were located in several haplotype blocks* (Fig. 2). LD among *CHRM2* markers was stronger in EAs than in AAs (Fig. 2A and B). Pairwise LD analysis showed that, among EAs, SNPs 1, 2 and 3 belonged to one haplotype block ($D' > 0.90$), but only SNP1 and SNP2 were in a haplotype block in AAs. SNP4 and SNP5 were in one haplotype block both in EAs and in AAs. There were no significant differences in LD between cases and controls, within either EAs or AAs (data not shown).

(2) *Genotype frequency distributions of all markers were in Hardy–Weinberg Equilibrium (HWE) among both EA and AA controls (except SNP4 in EAs). However, among both EA and AA cases, some of the markers were in HWD* (Table 1). In EAs, nearly all *CHRM2* markers were in HWE in controls (except SNP4, $P = 0.019$); however, several *CHRM2* markers were in nominally significant ($P < 0.05$) HWD in cases (Table 1), including SNP1 in AD, SNP3 in DD and SNP5 in affective disorders. After correction for multiple testing using SNPSpD, where $\alpha = 0.0125$, only SNP3 remained in significant HWD in the DD group ($P = 0.010$). In AAs, all *CHRM2* markers were in HWE in controls; however, some *CHRM2* markers were in nominally significant HWD in cases ($P < 0.05$) (Table 1), including SNP3 in AD and DD and SNP6 in DD. After correction using SNPSpD, where $\alpha = 0.010$, SNP3 remained in significant HWD in the AD and DD groups ($P = 0.010$ and $P = 0.008$, respectively).

(3) *Case–control comparisons showed that the alleles and genotypes of some CHRM2 markers were nominally associated with AD and DD in AAs* (Table 2). In AAs, alleles for SNPs 2, 4 and 6 and genotypes for SNP2 were nominally significantly associated with both AD and DD ($P < 0.05$). In EAs, no alleles and genotypes were significantly associated with these disorders. After controlling for admixture effects by SA, alleles of SNPs 2, 4 and 6 were suggestively associated with AD and SNP1 genotype was suggestively associated with AD and DD. After correcting for multiple comparisons using SNPSpD, where $\alpha = 0.010$, no associations were significant. Case–control comparisons for haplotype frequency distributions executed using the program PHASE showed no significant associations between haplotypes and phenotypes (*P*-values not shown).

(4) *The risk-influencing loci for AD, DD and affective disorders were closest to either SNP2 or SNP5* (Table 3). In EAs, the highest δ values for *CHRM2* markers were

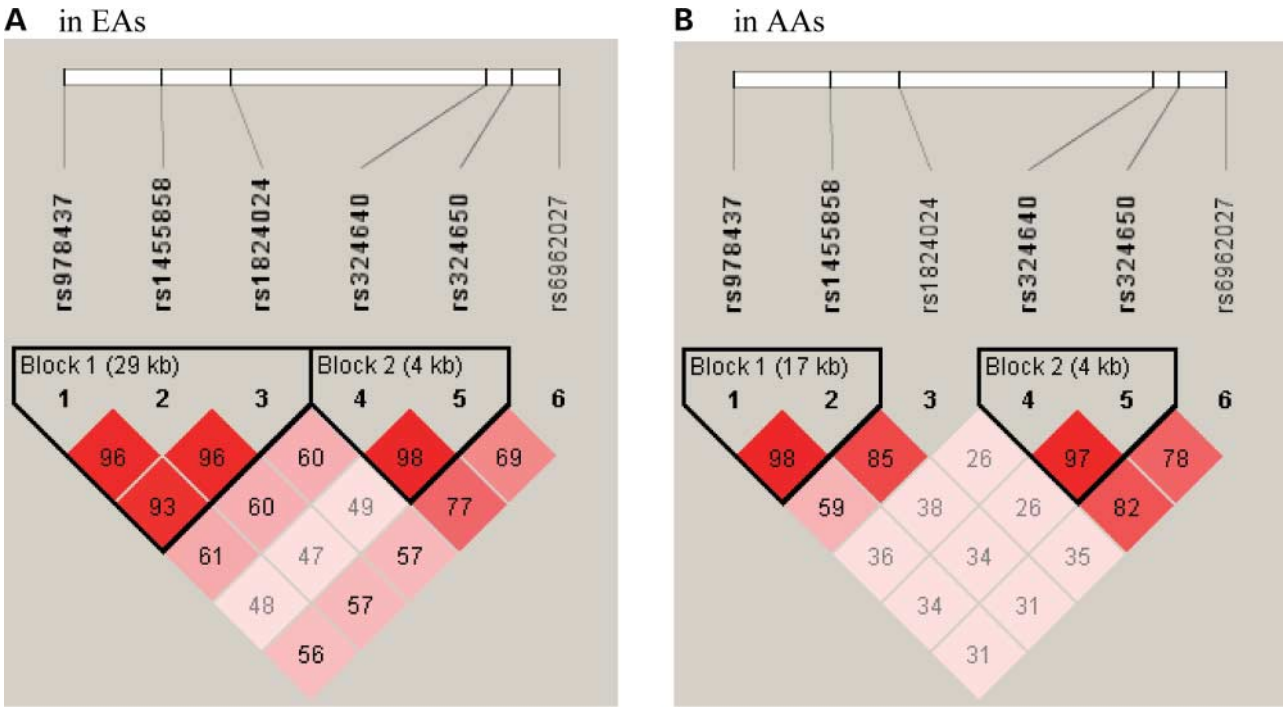

Figure 2. Pairwise LD analysis on *CHRM2* markers (**A**) in EAs and (**B**) in AAs. The numbers in the squares are D′ × 100.

Table 1. *P*-values for HWE test in different phenotype groups

Marker	EA				AA		
	AD	DD	AFD	Con	AD	DD	Con
rs978437	0.014	0.055	1.000	0.786	0.353	0.061	0.497
rs1455858	0.061	0.099	1.000	0.793	0.064	0.077	1.000
rs1824024	0.106	**0.010**	0.559	0.780	**0.010**	**0.008**	0.221
rs324640	0.439	0.667	0.203	0.019	0.798	0.703	0.771
rs324650	0.152	0.764	0.046	0.153	0.626	0.851	0.558
rs6962027	0.158	0.302	0.346	0.068	0.139	0.043	0.057

Effective SNP number in EAs is 4, $\alpha = 0.0125$ and effective SNP number in AAs is 5, $\alpha = 0.0100$. EA, European-American; AA, African-American; AD, alcohol dependence; DD, drug dependence; AFD, affective disorder; Con, Controls.

at SNP2 for AD and SNP5 for DD and affective disorders. In AAs, the highest δ value for *CHRM2* markers was observed at SNP2 for all phenotypes. Thus, the putative risk locus for AD was closest to SNP2, both in EAs and in AAs; the putative risk locus for DD was closest to SNP5 in EAs and to SNP2 in AAs. The putative risk locus for affective disorders was closest to SNP5 in EAs.

(5) *Two ancestries, i.e. European and African, were detected in our sample*. One hundred percent of 667 self-reported EAs are 'genetic' EAs (European ancestry proportion greater than 0.5). Ninety-nine percent of 204 self-reported AAs are 'genetic' AA (African ancestry proportion greater than 0.5). Within the 669 'genetic' EA subjects, the admixture degree is 1.5% (this equals the total estimated weight of African ancestry proportions divided by *N*: 10.3/669). Within the 202 'genetic' AA subjects, the admixture degree is 4.0% (this equals the total estimated weight of European ancestry proportions divided by *N*: 8.1/202). Similarly, the admixture degree is 1.5% in self-reported EA subjects and 4.6% in self-reported AA subjects. (This relatively low estimated admixture rate for AAs probably reflects the lack of a native African group in our STRUCTURE analysis for reference.)

(6) *There were correlations among different alleles, genotypes, haplotypes and diplotypes* (Fig. 3). The results from correlation analyses in different phenotype groups are similar, so only the correlations in the AD group are shown. For allele data, there were significant correlations between SNP1^T and SNP2^C and between SNP4^A and SNP5^A ($r > 0.85$, $P < 0.05$); for genotype data, there were significant correlations between SNP1^T/T and SNP3^A/A, among SNP1^C/T, SNP2^C/T and SNP3^C/A, between SNP4^A/A and SNP5^A/A and between SNP4^A/G and SNP5^A/T ($r > 0.85$, $P < 0.05$); for haplotype data, there was significant correlation between TCAAAA and CTCGTT ($r > 0.45$, $P < 0.05$); for diplotype data, there was significant correlation between TCAAAA/CTCGTT and TCAGTT/CTCAAA ($r > 0.65$, $P < 0.05$) (Fig. 3). The most common haplotypes and diplotypes are shown in Table 4.

(7) *Regression analysis demonstrated that alleles, genotypes, haplotypes and diplotypes at the CHRM2 locus affected risk for AD, DD and affective disorders* (Table 5). Regression analysis showed that allele SNP1^T protected against AD and DD ($\beta_{A1} < 0$); but alleles

Table 2. *P*-values for comparisons of allele and genotype frequency distributions between cases and controls

	Case–control comparison										SA analysis			
	EA						AA				EA + AA			
	Con versus AD		Con versus DD		Con versus AFD		Con versus AD		Con versus DD		Con versus AD		Con versus DD	
	Allele	Genotype	Allele	Genotype	Allele	Genotype	Allele	Genotype	Allele	Genotype	Allele	Genotype	Allele	Genotype
rs978437	0.610	0.147	0.842	0.290	0.809	0.914	0.073	0.062	0.084	0.051	0.148	0.068	0.225	0.095
rs1455858	0.399	0.351	0.937	0.514	0.938	1.000	0.026	0.046	0.035	0.050	0.086	0.122	0.125	0.178
rs1824024	0.570	0.287	0.540	0.097	0.549	0.715	0.099	0.285	0.195	0.403	0.210	0.313	0.296	0.193
rs324640	0.679	0.493	0.791	0.480	1.000	0.989	0.042	0.096	0.038	0.092	0.078	0.172	0.117	0.174
rs324650	0.560	0.847	0.578	0.720	0.571	0.519	0.179	0.383	0.158	0.292	0.248	0.594	0.270	0.526
rs6962027	0.305	0.661	0.586	0.870	0.927	0.983	0.037	0.169	0.048	0.158	0.067	0.280	0.145	0.404

SA, structured association analysis. Effective SNP number in AAs is 5, $\alpha = 0.0100$.

Table 3. Genotype and allele frequencies in EAs and AAs

		EA										AA							
		AD ($n = 315$)			DD ($n = 202$)			AFD ($n = 137$)			Con ($n = 287$)	AD ($n = 100$)			DD ($n = 144$)			Con ($n = 46$)	
		n	*f*	δ	*n*	*f*	δ	*n*	*f*	δ	*n*	*f*	*n*	*f*	δ	*n*	*f*	δ	*n*	*f*
rs978437	T/T	141	0.491		89	0.461		54	0.429		123	0.438	61	0.656		87	0.644		21	0.457
	T/C	108	0.376	0.042	76	0.394	0.011	57	0.452	0.016	128	0.456	27	0.290	0.346	39	0.289	0.306	22	0.478
	C/C	38	0.132		28	0.145		15	0.119		30	0.107	5	0.054		9	0.067		3	0.065
	T	390	0.679		254	0.658		165	0.655		374	0.665	149	0.801		213	0.789		64	0.696
	C	184	0.321		132	0.342		87	0.345		188	0.335	37	0.199		57	0.211		28	0.304
rs1455858	C/C	153	0.502		93	0.474		60	0.451		125	0.448	70	0.722		99	0.712		24	0.522
	T/C	116	0.380	0.070	77	0.393	0.007	59	0.444	0.013	123	0.441	22	0.227	0.393	33	0.237	0.378	19	0.413
	T/T	36	0.118		26	0.133		14	0.105		31	0.111	5	0.052		7	0.050		3	0.065
	C	422	0.692		263	0.671		179	0.673		373	0.668	162	0.835		231	0.831		67	0.728
	T	188	0.308		129	0.329		87	0.327		185	0.332	32	0.165		47	0.169		25	0.272
rs1824024	A/A	153	0.503		92	0.474		60	0.438		126	0.455	51	0.560		68	0.519		19	0.413
	A/C	117	0.385	0.053	72	0.371	0.028	60	0.438	0.032	124	0.448	27	0.297	0.256	44	0.336	0.200	18	0.391
	C/C	34	0.112		30	0.155		17	0.124		27	0.097	13	0.143		19	0.145		9	0.196
	A	423	0.696		256	0.660		180	0.657		376	0.679	129	0.709		180	0.687		56	0.609
	C	185	0.304		132	0.340		94	0.343		178	0.321	53	0.291		82	0.313		36	0.391
rs324640	A/A	81	0.264		55	0.281		28	0.301		77	0.302	47	0.495		64	0.464		16	0.348
	A/G	146	0.476	0.027	95	0.485	0.015	40	0.430	0.001	109	0.427	40	0.421	0.305	62	0.449	0.265	21	0.457
	G/G	80	0.261		46	0.235		25	0.269		69	0.271	8	0.084		12	0.087		9	0.196
	A	308	0.502		205	0.523		96	0.516		263	0.516	134	0.705		190	0.688		53	0.576
	G	306	0.498		187	0.477		90	0.484		247	0.484	56	0.295		86	0.312		39	0.424
rs324650	A/A	94	0.308		64	0.332		40	0.333		84	0.327	47	0.495		65	0.481		18	0.391
	A/T	139	0.456	0.030	92	0.477	0.039	48	0.400	0.035	116	0.451	38	0.400	0.220	57	0.422	0.214	20	0.435
	T/T	72	0.236		37	0.192		32	0.267		57	0.222	10	0.105		13	0.096		8	0.174
	A	327	0.536		220	0.570		128	0.533		284	0.553	132	0.695		187	0.693		56	0.609
	T	283	0.464		166	0.430		112	0.467		230	0.447	58	0.305		83	0.307		36	0.391
rs6962027	A/A	101	0.337		61	0.323		21	0.304		82	0.311	66	0.710		95	0.688		26	0.578
	A/T	135	0.450	0.063	86	0.455	0.039	31	0.449	0.006	117	0.443	22	0.237	0.381	35	0.254	0.335	13	0.289
	T/T	64	0.213		42	0.222		17	0.246		65	0.246	5	0.054		8	0.058		6	0.133
	A	337	0.562		208	0.550		73	0.529		281	0.532	154	0.828		225	0.815		65	0.722
	T	263	0.438		170	0.450		65	0.471		247	0.468	32	0.172		51	0.185		25	0.278

n, individual number (for genotype) or chromosome number (for allele); *f*, frequency; δ, the population attributable risk.

SNP1^T × SNP2^C multiplicatively ($|\beta_{A1^*A2}| > |\beta_{A1} + \beta_{A2}|$) increased risk for ($\beta_{A1^*A2} > 0$) AD and DD. Further, genotype SNP1^C/T protected against AD and genotype SNP2^C/T protected against DD ($\beta s < 0$).

Regression analysis showed that haplotype TCAGTA, which included alleles SNP1^T × SNP2^C, increased risk for AD ($\beta > 0$); diplotype TCAGTT/CTCAAA protected against AD and DD ($\beta_{D1} < 0$), increased risk for affective disorders

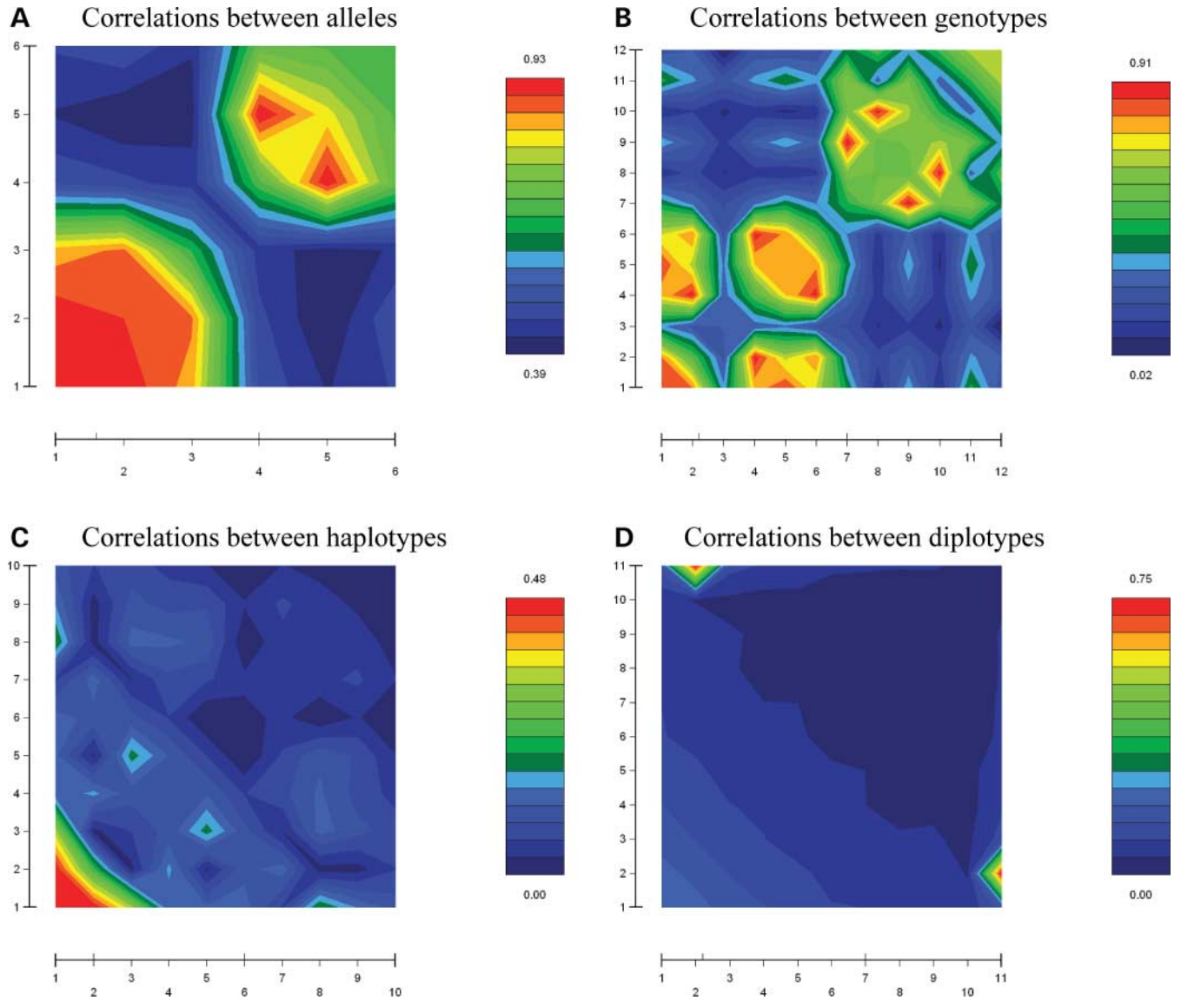

Figure 3. Pairwise correlations between any two alleles, genotypes, haplotypes or diplotypes. In Figure **B**, 1 and 2 in axes denote the two genotypes of SNP1, and 3 and 4 denote the two genotypes of SNP2; as analogy. Marker numbers correspond to the order as presented in Table 1; haplotype and diplotype numbers correspond to the order as presented in Table 4. Scale denotes correlation coefficent (r).

($\beta_{D1} > 0$); TCAAAA/CTCGTT protected against AD, DD and affective disorders (all $\beta_{D2} < 0$); the contribution of diplotype TCAAAA/CTCGTT to the risk for these disorders was much less than that of diplotype TCAGTT/CTCAAA ($|\beta_{D2}| \ll |\beta_{D1}|$); interaction of these two diplotypes (i.e. TCAGTT/CTCAAA × TCAAAA/CTCGTT) multiplicatively ($|\beta_{D1*D2}| > |\beta_{D1} + \beta_{D2}|$) increased risk for ($\beta_{D1*D2} > 0$) AD and DD, but antagonistically ($|\beta_{D1*D2}| < |\beta_{D1} + \beta_{D2}|$) increased risk for ($\beta_{D1*D2} > 0$) affective disorders; diplotypes TCAGTT/CTCGTT and TCAAAA/TCAAAA modestly ($P = 0.046$) or suggestively ($P = 0.079$) protected against DD ($\beta_{D3} < 0$), but their contributions to this protective effect was much less than that of the diplotype TCAGTT/CTCAAA ($|\beta_{D3}| \ll |\beta_{D1}|$). These diplotypes were constructed by a combination of TCA or CTC from first three SNPs and GTT or AAA from last three SNPs.

Most of the associations between phenotype and alleles, genotypes, haplotypes or diplotypes were most significant for AD (minimum $P = 0.003$), less significant for DD (minimum $P = 0.011$) and only suggestively or modestly significant for affective disorders ($0.011 < P < 0.085$). Decomposing these associations by population, i.e. separating the combined sample (EAs + AAs) into EAs and AAs and using the same regression methods, we found that all these associations remained significant in both populations. However, these associations were highly significant in AAs, but only modestly significant in EAs (data not shown).

DISCUSSION

In this study, we found that polymorphic variation at *CHRM2* predisposed to AD, DD and affective disorders. The 5′-UTR of

Table 4. Frequencies of common haplotypes and diplotypes in the combined sample

Haplotype	Frequency	Diplotype	Frequency
TCAAAA	0.400	TCAAAA/TCAAAA	0.188
CTCGTT	0.184	TCAAAA/CTCGTT	0.127
TCAGTT	0.134	TCAAAA/TCAGTT	0.097
TCAGTA	0.062	TCAGTT/CTCGTT	0.071
CTCAAA	0.045	TCAAAA/TCAGTA	0.060
TCCAAA	0.023	CTCGTT/CTCGTT	0.042
TCAAAT	0.021	TCAAAA/CTCAAA	0.036
CTCGTA	0.020	TCAAAA/TCAAAT	0.024
CTCAAT	0.016	TCAGTT/TCAGTT	0.022
CTCGAT	0.014	CTCAAA/CTCGTT	0.013
		TCAGTT/CTCAAA	0.012

Only the haplotypes and diplotypes with frequencies >0.010 are listed.

CHRM2, which contains one haplotype block harboring SNPs 1, 2 and 3, was more important for susceptibility to these disorders than other regions. Interaction between two specific alleles within this haplotype block and interaction between two specific diplotypes covering this haplotype block multiplicatively increased risk AD and DD, but the latter antagonistically increased risk affective disorders; a specific diplotype might inversely affect the risk for AD and DD and the risk for affective disorders.

In our sample, the genotype frequency distributions of some markers deviated from HWE in some phenotype groups, even after correction for multiple testing (34). For example, SNP3 was in HWD among AAs with AD, and in HWD among both EAs and AAs with DD, which might suggest that this SNP is associated with AD and DD (35–42). This finding also affected the subsequent analytic strategy; HWD violates the assumptions of the EM algorithm that conventional haplotype-construction programs are based upon, so a Bayesian approach and the partition ligation algorithm were used in this study to construct haplotypes and diplotypes and estimate their probabilities. HWD might also imply that genotypewise and diplotypewise analyses would be more powerful than allelewise and haplotypewise analyses, as demonstrated previously (41–43) and also in the present study.

Case–control comparisons on allele and genotype frequency distributions showed some nominally significant ($P \leq 0.05$) associations between SNP2, SNP4 or SNP6 and AD and DD, mainly in AAs. After controlling for admixture effects via the SA analysis, most of these associations remained, although their level of significance declined (Table 2). After correction for multiple tests, no associations remained significant, which suggests that this case–control comparison lacked adequate statistical power.

Fine-mapping using the δ value, an LD measure based on the case–control data, showed that SNP2 was closest to the putative risk locus for AD both in EAs and in AAs and for DD in AAs and that SNP5 was closest to the putative risk locus both for DD and for affective disorders in EAs.

Case–control comparisons, including the SA method, are limited by their failure to take into account marker–marker interactions. This limitation is important in the present study, where such interactions are strong. Analysis showed strong correlations among the alleles or genotypes of SNPs 1, 2 and 3 and among the alleles or genotypes of SNP4 and SNP5 ($r > 0.85$), which was completely consistent with the results from pairwise LD analysis ($D' > 0.9$). The haplotypes and diplotypes incorporate the LD information from different markers, so that correlations among haplotypes or among diplotypes are attenuated (maximum $r = 0.48$ and $r = 0.75$, respectively) (Fig. 3). But given their strength, the interactions among them should still be taken into account. For example, the highest correlation between diplotypes TCAAAA/CTCGTT and TCAGTT/CTCAAA ($r = 0.75$) is nearly four times that of the second highest correlation between other diplotypes ($r = 0.2$). These correlations reflect the finding that the correlated haplotypes are more likely to occur in subjects (individually) with a certain given trait (diagnosis), than would be expected at random. This suggests that positively correlated haplotypes or diplotypes may affect risk for that trait similarly.

We used a backward stepwise logistic regression analysis to improve the power by using allele, genotype, haplotype and diplotype probabilities instead of categorical values. This approach also allows uncertainty for haplotype inference, obviates the HWE assumption, increases sample size by combining different populations and phenotypes in a single model, controls for population stratification and admixture effects and the potential confounding by sex, takes marker–marker interactions into account and avoids multiple testing that would accrue to the inclusion of multiple populations and markers. This analytic approach showed that allele T and genotype C/T of SNP1 and/or genotype C/T of SNP2 protected against AD and/or DD; but alleles SNP1^T × SNP2^C multiplicatively increased risk for these disorders. These two SNPs were located at intron 3, close to a functional region at the 5′ transcriptional start site (TSS1) (discussed earlier). The findings from regression analysis were much stronger than those yielded by the HWD test, case–control comparison or SA, which supports the idea that the regression method is more powerful than the other methods. These findings partially overlap with those from the HWD test, case–control comparison, SA and fine-mapping; all provide evidence that SNPs 1, 2 and/or 3 might be in LD with the putative disease-influencing loci for AD and/or DD. These three SNPs were in a single haplotype block, so that the findings obtained using different analysis methods that appear to vary in statistical power and that differ in their handling of the interactions could reasonably be expected to vary among these three SNPs (while maintaining consistency in the context of the haplotype view), especially when locus main effects are weak but the interaction effects are strong. (We acknowledge that our analysis on the interaction effects with regression methods might be somewhat speculative.)

Because the HWD test, case–control comparison and SA did not take into account the marker–marker interactions, the conclusions drawn from regression analysis should be more valid. Furthermore, the only positive finding for a haplotype by regression analysis is that the haplotype TCAGTA (which harbored alleles SNP1^T × SNP2^C) modestly increased risk for AD. Haplotype analysis was much less powerful than diplotype analysis for this locus, consistent

Table 5. Results of backward stepwise regression analysis on allele, genotype, haplotype and diplotype probabilities in three disorders

	EA + AA						EA		
	AD			DD			Affective disorder		
	Covariates	*P*-value	β	Covariates	*P*-value	β	Covariates	*P*-value	β
Model 1									
Ancestry				European ancestry	1.2E-08	−1.285			
Sex	Male	2.9E-20	1.658	Male	4.8E-11	1.216			
allele	SNP1^T	0.026	−2.096	SNP1^T	0.071	−1.778			
	SNP1^T × SNP2^C	0.006	2.145	SNP1^T × SNP2^C	0.031	1.753			
Model 2									
Ancestry	European ancestry	0.045	−0.474	European ancestry	9.2E-09	−1.290			
Sex	Male	5.0E-20	1.650	Male	4.2E-11	1.220			
Genotype	SNP1^C/T	0.003	−0.548	SNP2^C/T	0.020	−0.446			
Model 3									
Ancestry	European ancestry	6.1E-04	−0.755	European ancestry	2.2E-14	−1.616			
Sex	Male	1.3E-21	1.554	Male	1.9E-11	1.136	Male	0.054	0.402
Haplotypes	TCAGTA	0.029	1.170						
Model 4									
Ancestry	European ancestry	8.3E-04	−0.771	European ancestry	2.8E-12	−1.566			
Sex	Male	9.3E-21	1.525	Male	6.0E-11	1.122	Male	0.085	0.366
Diplotypes	TCAGTT/CTCAAA	0.007	−104.610	TCAGTT/CTCAAA	0.011	−99.965	TCAGTT/CTCAAA	0.059	161.260
	TCAAAA/CTCGTT	0.093	−2.532	TCAAAA/CTCGTT	0.081	−2.574	TCAAAA/CTCGTT	0.011	-18.475
	(TCAAAA/CTCGTT × TCAGTT/CTCAAA)	0.007	148.496	(TCAAAA/CTCGTT × TCAGTT/CTCAAA)	0.014	140.126	(TCAAAA/CTCGTT × TCAGTT/CTCAAA)	0.032	58.665
				TCAGTT/CTCGTT	0.046	−0.767			
				TCAAAA/TCAAAA	0.079	−0.399			

Ancestry, European ancestry proportion; *P*, *P*-value; β, regression coefficient; 'E', scientific format of number; regression model 1, 2, 3 and 4 involve in alleles, genotypes, haplotypes and diplotypes, respectively.

with our findings elsewhere and theoretical expectations that under HWD, diplotypewise analysis is the more powerful approach (42,43). This reflects sensitivity of diplotype methods to HWD, which itself reflects recessively acting risk loci. The findings from haplotypewise and diplotypewise analyses are consistent with those from allelewise and genotypewise analyses. The susceptibility diplotypes were constructed by a combination of TCA, which harbored SNP1^T × SNP2^C, or their counterparts (i.e. CTC) from the first three SNPs and GTT or their counterparts (i.e. AAA) from the last three SNPs. Nearly, all of these diplotypes protected against AD, DD and affective disorders, except that TCAGTT/CTCAAA increased risk for affective disorders; among these diplotypes, TCAGTT/CTCAAA contributed to the protection against or risk for these disorders much more than any of the other diplotypes. Diplotypes TCAGTT/CTCAAA × TCAAAA/CTCGTT multiplicatively increased risk for AD and DD, but antagonistically increased risk for affective disorders. Associations were most significant for AD, less significant for DD and only suggestively or modestly significant for affective disorders. Decomposing these associations by population, we found that these associations were highly significant in AAs, but only modestly significant in EAs—a trend consistent with that by case–control comparisons. Diplotype trend regression (DTR) analysis is a powerful regression method that uses diplotype probability as the predictor variable (42,44). In the case of affective disorders, association could not be detected by any other association methods (probably due to smaller sample size for this phenotype).

Interestingly, our findings are consistent with those of Wang *et al.* (28). First, both studies found that SNP3 (rs1824024) at intron 4 upstream of the CDS was one of the most important susceptibility SNPs for AD and affective disorders. Secondly, both studies found that the large 5′-UTR upstream of the CDS was more important than the 3′-UTR downstream of the CDS, for susceptibility to the disorders studied. The haplotypes or diplotypes covering the SNPs at the 5′-UTR were risk or protective factors for AD and affective disorders. The SNPs in the 3′-UTR, i.e. SNP6 (rs6962027) in our study and rs8191992 in Wang *et al.* (28), which are 627 bp apart, were not associated with any of the phenotypes examined. Thirdly, some variants and the haplotypes or diplotypes that include these variants exerted inverse effects on AD and affective disorders.

Regression analyses that examined AD and DD were conditional on ancestry proportions for each subject, in order to control for population stratification and admixture effects. The ancestry proportions for individuals in the sample of EAs with affective disorders were not examined, so that the regression analysis for affective disorders did not control for admixture effects. However, our study of the first part of the sample, including the controls and the subjects with AD and DD, showed that 100% of self-reported EAs were 'genetic' EAs and their admixture degree was very low (only 1.5%). Together, these findings indicate that the self-reported EA population can provide an excellent proxy for a 'genetic' EA population and its admixture effects were so weak that they can reasonably be ignored. Thus, the findings in EAs with affective disorders should be reliable, despite the fact that admixture effects were not considered. Together with

findings reported by Wang *et al.* (28), this study provides evidence of a role for variation in *CHRM2* in the risk for AD, DD and affective disorders.

MATERIALS AND METHODS

Subjects

Two sets of samples were included in the present study. The first set consists of 871 subjects which included 333 healthy controls [287 European-Americans (EAs) and 46 African-Americans (AAs)] and 538 affected subjects [382 EAs and 156 AAs; 415 with AD; 346 with DD, including dependence on cocaine ($n = 306$) or opioids ($n = 148$)]. Five hundred twenty-six subjects were males and 345 were females. Males constituted 72.9% of the cases and 40.2% of the controls. Age data were available for 97.6% of controls and 98.1% of cases; of those for whom such data were available, the average ages were 28.1 ± 9.1 years for controls and 39.4 ± 9.2 years for cases. The cases met lifetime DSM-III-R criteria (45) for AD, DD (cocaine or opioid) or a combination of these disorders. Diagnoses were made using the Structured Clinical Interview for DSM-III-R (SCID) (46), the computerized Diagnostic Interview Schedule for DSM-III-R (C-DIS-R) (47) or a checklist composed of DSM-III-R symptoms. The control subjects were screened using the SCID, the C-DIS-R or the Schedule for Affective Disorders and Schizophrenia (48), to exclude major Axis I mental disorders, including alcohol or drug abuse or dependence, psychotic disorders (including schizophrenia or schizophrenia-like disorders), affective disorders and major anxiety disorders. The populations and the population groups (races) for individual subjects were classified by ancestry proportions rather than self-report (see Materials and Methods).

The second set of subjects included 137 EA patients with affective disorders, of whom 68 were males and 69 were females. The diagnosis in this group was made using the Structured Clinical Interview for DSM-III-R (SCID) (46) or the SCID version 2.0 (49). The specific affective disorders included bipolar affective disorder (34.3%), major depression (56.9%) and seasonal affective disorder (8.8%).

We used the SA method for the controls and substance-dependent subjects in this study. That is, we genotyped ancestry-informative markers (AIMs) and applied the SA approach (described subsequently). Because the ancestry proportions were not available for the subjects with affective disorders, their population group (race) was identified by self-report.

The subjects were recruited at the University of Connecticut Health Center, the VA Connecticut Healthcare System-West Haven Campus or the Connecticut Mental Health Center. All subjects gave informed consent before participating in the study, which was approved by the Institutional Review Board at the respective institutions.

Marker inclusion

Six markers flanking the *CHRM2* coding sequence were selected, including two markers (SNP1: rs978437 and SNP2: rs1455858) at intron 3, one marker (SNP3: rs1824024) at intron 4, two markers (SNP4: rs324640 and SNP5: rs324650) at intron 5 and one marker (SNP6: rs6962027) at the 3′-UTR. The allele frequencies and polymerase chain reaction (PCR) conditions for five of these markers (which have an hcv no.) have been validated by Applied Biosystem, Inc. (ABI, Foster City, CA, USA). SNP3, SNP4 and SNP5 were also studied by Wang *et al.* (28). These six markers span a total of 87 757 bp, with an average intermarker distance of 15 kb (Fig. 1).

Thirty-eight AIMs unlinked to *CHRM2*, including 37 STRs and one FY SNP marker, were genotyped to examine the population structure of our first set of sample. These markers were employed by several studies (42,43,50,51), and their characteristics are described in the study by Yang *et al.* (33).

Genotyping

By TaqMan technique. All *CHMR2* SNPs were genotyped with the TaqMan technique, i.e. a fluorogenic 5′ nuclease assay method (52), using the ABI PRISM 7900 Sequence Detection System (ABI). Five markers (with hcv no.) were genotyped using 'assays-on-demand' and one marker (without hcv no.) was genotyped using 'assay-by-design'. Detailed PCR conditions are reported elsewhere (41). All genotyping was performed in duplicate and compared to ensure validity of the data. Mismatched genotypes, which constituted $<0.5\%$ of the total number of duplicate genotypes performed, were discarded.

By PCR–restriction fragment length polymorphism (RFLP) technique. The Duffy antigen gene (FY) marker (rs2814778), being highly informative of the ethnic ancestry of the subject, was genotyped by the PCR–RFLP technique as described previously (53).

By fluorescence capillary electrophoresis (FCE) technique. The 37 STR markers were genotyped by a fluorescence capillary electrophoresis (FCE) technique using the ABI PRISM 3100 semiautomated capillary fluorescence sequencer as described in detail elsewhere (33).

Statistical analysis

LD analysis. Pairwise LD between any two *CHRM2* markers was analyzed separately by population, i.e. EAs and AAs. The value of the standardized disequilibrium coefficient, D', for each LD pair was calculated and is shown in Figure 2 [using the program Haploview (54)]. The haplotype block was defined by Gabriel *et al.* (55).

HWE test. HWE of the genotype frequency distribution for each marker was tested within different populations and separately in cases and controls. The *P*-values for HWE tests, which were calculated using the program PowerMarker (56), are shown in Table 1. The HWD in cases, i.e. the deviation from HWE expectations, sometimes indicates a valid disease–gene association or the possible existence of population stratification, selection or insufficient power. This test

is the prerequisite for determining valid choice of haplotype-reconstruction programs (some of which produce valid results only in the context of HWE).

Case–control comparisons for allele and genotype frequency distributions. The allele and genotype frequencies of the *CHRM2* markers in different phenotype groups are shown in Table 3. Associations among the alleles, genotypes and the phenotypes were analyzed by comparing the allele and genotype frequency distributions between cases and controls (within EAs and AAs, respectively) with exact tests implemented in the program PowerMarker and the *P*-values are listed in Table 2.

Fine-mapping the risk locus. Assuming that there is a risk locus within *CHRM2*, LD between this putative risk locus (possibly not observed) and the marker loci (observed) can be calculated. Under ideal scenarios, evolutionary forces acting on the markers and disease-influencing variant can be ignored. If the time from the initial generation, in which the disease effect was first introduced (i.e. when the disease-influencing mutation occurred), to the current generation is not very long, then the strength of LD between disease locus and marker locus will directly reflect the genetic distance between them. If multiple markers are tested, the distribution of values of LD between disease locus and marker locus will exhibit a single peak that occurs at the disease locus; i.e. the closer the marker is to the disease locus, the stronger the LD between them. However, the peak of the LD distribution might not distinguish reliably among the markers around the putative disease locus when using different LD measures such as the correlation coefficient Δ, Lewontin's D', the robust formulation of the population attributable risk δ, Yule's Q and Kaplan and Weir's proportional difference d (57) (based on a case–control sample) or the values of F, F', J and J' (35,37) (based on a case-only sample), because these measures fluctuate with the allele frequencies of marker locus and disease locus. Allowing for this, the δ value, which controls for the influence of allele frequency, is the best measure for fine-mapping the risk locus. $\delta = (ad - bc)/[(a + c)d]$, where a, b, c and d are the numbers of the risk and the protective alleles in cases and controls, respectively (58). δ is equivalent to J (37).

SA analysis. Usually, to reduce population stratification effects, statistical analyses are preformed separately for different populations. However, this may be not sufficient when the populations are themselves admixed. The admixture within a population can lead to spurious findings, especially when the disease prevalence varies between the ancestral populations and the ancestry proportions vary between individuals. In the USA, many populations are admixed. A prototypical example is the AA population, which has been shown in many studies to be admixed primarily between native African and EA ancestry (59,60). Recently, admixture within EAs has also been confirmed by some studies (61,62), although the extent of that admixture is much less than that in AAs. Thus, the admixture effects for these two populations should be controlled.

In general, to detect admixture and to measure the extent of admixture in a population, the ancestry information content from AIMs can be extracted through a Bayesian approach (63). For example, allele 196 of marker D11S935 has a frequency of 0.432 in the ancestral European population and 0.161 in the ancestral African population (as estimated by STRUCTURE, on the basis of observed AA and EA allele frequencies) (43). If an individual carries one copy of allele 196, the probability is 72.8% that the individual is of European ancestry and 27.2% that the individual is of African ancestry (calculated through the Bayesian approach, assuming otherwise equal prior probability of being European or African). Through the use of additional AIMs, one can assign ancestry with high confidence. For this purpose, the 38 AIMs permit the assignment of all individuals into different genetic populations (33) and further the classification of each according to ancestry proportions. This is accomplished using the program STRUCTURE, which is based on the Bayesian approach (32,64). We have successfully applied this approach in several studies (33,42,43,50,51).

The ancestry proportions were entered into the program STRAT (65) to perform the SA analysis among 44 markers (including 38 AIMs and six *CHRM2* markers) and phenotypes (AD or DD) conditional on the ancestry proportions to exclude admixture effects. In addition, the ancestry proportions were entered into the regression model described subsequently for an extended analysis.

Correction for multiple tests. When the same data set is tested multiple times from different independent perspectives, the threshold for statistical significance (α) should be adjusted to reduce type I error. In the present study, the HWE tests, the case–control comparisons for allele and genotype frequencies and the SA, all were performed six times marker-by-marker. Although the Bonferroni correction is most commonly used to adjust α, because it treats every test as an independent test, it is overly conservative for genotype data in which markers are in LD, thereby neglecting the LD information that reflects correlation among markers. One approach used to avoid multiple comparisons is a simulation method, which tests all of the markers in one omnibus test to derive an empiric global *P*-value (40,41). However, this simulation method is computationally intensive and time-consuming. To overcome the disadvantages of both methods, Nyholt (34) recently developed a novel web-based program SNPSpD (http://genepi.qimr.edu.au/general/daleN/SNPSpD), which provides an adjusted Bonferroni correction method. The results generated by SNPSpD agree well with those provided by simulation methods, especially when the number of markers does not exceed seven. SNPSpD calculates the effective independent marker number from the non-independent markers, incorporating the LD information from them. For example, six *CHRM2* markers that are in LD provide information equivalent to four and five independent markers in EA cases and in AA cases, respectively. On the basis of the information, the α level is set at 0.0125 and 0.010 for EAs and AAs, respectively.

Haplotype reconstruction, case–control comparisons for haplotype frequency distributions and individual haplotype

and diplotype probability estimation. The program PHASE was used to reconstruct haplotypes in this study. This program was developed by Stephens *et al.* (66,67), on the basis of a Bayesian approach and the partition ligation algorithm. These algorithms have been claimed to be more accurate in reconstructing haplotypes than the expectation-maximum (EM) algorithm, especially when the HWE does not hold among some markers, as is the case for our data (Table 1) (66–68). Haplotype frequencies were estimated and their distributions were compared between cases and controls directly by PHASE.

PHASE was also used to estimate the probabilities of all likely pairs of haplotypes (i.e. diplotypes) for every individual. The individual-phased diplotypes that could be unambiguously inferred by PHASE have a probability of 1.0; the unphased diplotypes that were ambiguously inferred by PHASE have probabilities between 0.0 and 1.0. These haplotype and diplotype probabilities were entered into the regression model discussed subsequently for analysis. Uncertain haplotype data cannot be accurately analyzed by the SA method, but can be analyzed by the regression method described subsequently.

To reconstruct the haplotypes more accurately, our sample (except those with affective disorders) was separated into two subgroups, that is, the genetically inferred EAs (European ancestry proportion greater than 0.5) and the genetically inferred AAs (African ancestry proportion greater than 0.5) haplotypes were reconstructed within the 'genetic' EAs and AAs, respectively, rather than within the self-reported EAs and AAs. Haplotypes in the patients with affective disorders were reconstructed within the self-reported EAs, because their ancestry proportions were not available. The haplotype frequency distributions were compared between cases and controls separately within populations; but different populations were combined as one admixed population in the aforementioned SA analysis and in the after mentioned correlation analysis and regression model.

Marker–marker interaction effect analysis. Different markers within *CHRM2* were in LD to varying degrees (Fig. 2). Markers in the same haplotype block could exert interaction effects on trait. Identification of such an interaction could increase our understanding of the mechanisms through which the gene acts to modify risk for expression of the trait; ignoring an existing interaction might make the main effects of the markers appear non-significant or lead to incorrect conclusions with respect to determination of the mode of inheritance and erroneous estimation of the magnitude of the effects of the markers (69–71). One commonly used method for evaluation of marker–marker interaction effects is stratification analysis (72–74), e.g. the sample can be stratified into three groups according to the three genotypes of the first SNP, and then, within each genotype group, the relationship between the genotypes of the second SNP and the phenotype can be evaluated. However, when many markers (i.e. subgroups) are involved, stratification analysis can reduce the sample size in each subset unacceptably. Furthermore, the stratification analysis uses the unphased genotype data directly, but not the phased diplotype data, thereby ignoring the LD among markers and the diplotype frequency distribution in the whole population, and thus inflates the type I error rate (because of maximal subdivision of the sample). Another method commonly used for analyzing marker–marker interaction effects is regression analysis, which directly models all of the variables in a single analysis using the entire data set, thereby optimizing the statistical power (75–78).

The interaction effect depends on the correlation between markers and is related to the traits. The correlation *per se* between markers (such as LD) depends on the physical distance between markers, the allele frequencies of markers, population history and the nature of traits (including the definition of phenotypes, the sample size and the ethnicity). Before considering the interaction effects in the regression model, a correlation matrix of the markers was created. Only when the marker–marker correlations were strong, the interaction effects were considered in the regression model, because the strong marker–marker correlations suggested likely marker–marker interaction effects on trait. The marker–marker correlation analysis includes allele–allele correlation and genotype–genotype correlation (between different markers), which is equivalent to pairwise LD analysis between single markers. Incorporating information on the LD among markers into haplotypes, the haplotype–haplotype correlation and the diplotype–diplotype correlation might more likely suggest an interaction effect on trait than marker–marker correlation, because haplotypes or diplotypes are mutually exclusive in genetic structure (i.e. a single chromosome expresses exactly one haplotype). Furthermore, haplotypes and diplotypes might be more informative than single markers in the analysis of interaction effects, because haplotypes and diplotypes reflect information from more closely mapping unknown markers on the same haplotype background that, although not detected, might be involved in interaction effects. Thus, in this study, we analyzed haplotype–haplotype correlation and diplotype–diplotype correlation and considered their interaction effects in the regression model.

Haplotypes or diplotypes themselves reflect LD information among markers; the correlations among haplotypes or diplotypes should be attenuated, because their correlations on genetic structure have been excluded. Thus, only the strongest haplotype–haplotype and diplotype–diplotype correlations (which were nevertheless not as strong as the marker–marker correlations) were considered in the regression model (see red color in Fig. 3). The correlation coefficients (r) were visualized through application of the program GOLD (79) (Fig. 3).

Regression analysis. A general backward stepwise logistic multivariate regression analysis was used to test associations between gene and diseases. We modeled the analysis with the following equation:

$$ln[P/(1-P)] = \beta_0 + \beta_a X_a + \beta_s X_s + \Sigma\beta_i X_i + \Sigma\beta_j X_i X_j + \Sigma\beta_k X_i X_j X_k,$$

where P is the probability of disease; β_0 corresponds to the intercept; β_a, β_s, β_i, β_j and β_k are regression coefficients, among which β_a, β_s and β_i correspond to the main effects and β_j and β_k correspond to the two-way and three-way

interaction effects, respectively; X_a denotes the European ancestry proportions predicted by the program STRUCTURE (omitted for the second set of samples); X_s denotes the sex of individuals and X_i, X_j and X_k denote the ith, jth and kth ($i < j < k$) allele probabilities (model 1), genotype probabilities (model 2), haplotype probabilities (model 3) or diplotype probabilities (model 4). In the matrix X_i, for allele data, only one common allele from each SNP was entered into the regression model with probability 0, 0.5 or 1; for genotype data, only two genotypes among the three from each SNP were entered into the model with probability 0 or 1 and for haplotype or diplotype data, only the haplotypes and diplotypes with population frequencies greater than 0.01 were entered into the model. The backward stepwise regression method could reduce the variable number to a minimum in the final step of the equation (Table 5).

Ancestry proportions were included in the model for the first set of samples to exclude both population stratification and admixture effects. Sex was included in the model to exclude its potential confounding on associations, because it was highly asymmetrically distributed between cases and controls, and a sex-specific role of *CHRM2* in depression has been reported (12). The probabilities, instead of the categories, of alleles, genotypes, haplotypes and diplotypes were included, because the probabilities preserve more information than does the direct use of categorical variables.

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