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Markers in the 5′-Region of *GABRG1* Associate to Alcohol Dependence and are in Linkage Disequilibrium with Markers in the Adjacent *GABRA2* Gene

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Following an initial report, there have been multiple replications of an association of alcohol dependence (AD) to markers within a haplotype block that includes the 3′-half of the gene encoding the $GABA_A$ α-2 subunit (*GABRA2*), on chromosome 4p. We examined the intergenic extent of this haplotype block and the association to AD of markers in the adjacent 5′ haplotype block in *GABRG1*, which encodes the $GABA_A$ receptor γ-1 subunit. We genotyped 15 single nucleotide polymorphisms in the *GABRG1-GABRA2* interval as well as at 34 ancestry informative markers in three samples: 435 AD and 635 screened control subjects from Connecticut and 812 participants from a multicenter AD treatment trial. We observed two large haplotype blocks in the *GABRG1-GABRA2* intergenic interval with a region of increased recombination midway between the two genes. Markers in the two haplotype blocks were in moderate linkage disequilibrium. Compared with markers in the *GABRA2* haplotype block, markers in the 5′ *GABRG1* haplotype showed greater allelic, genotypic and haplotypic association with AD in European Americans from both AD samples. Logistic regression analysis indicated that genetic elements in the *GABRG1* haplotype block likely contribute to AD risk in an additive manner, whereas those in the *GABRA2* haplotype block may act in a dominant manner in relation to risk of AD.
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INTRODUCTION

Alcohol dependence (AD (MIM 103780)) is a common psychiatric disorder, recently estimated to affect 3.8% of the adult US population during a 1-year period (Grant *et al*, 2004). A variety of adverse consequences are associated with AD, including medical, social and legal problems (Caetano and Cunradi, 2002). Based on heritability estimates ranging from 0.52 to 0.64 (Kendler, 2001), considerable efforts have been made to identify genes that increase risk for the disorder.

Genomewide linkage scans implicated a region on chromosome 4p12 that harbors a cluster of four genes encoding $GABA_A$ receptor subunits (γ-1, α-2, α-4, and β-1) (Long *et al*, 1998; Reich *et al*, 1998). As $GABA_A$ receptors have been implicated in biological processes related to the acute and chronic effects of alcohol (Koob, 2004; Krystal *et al*, 2006), the $GABA_A$ receptor genes at 4p12 are both positional and functional candidates for AD risk. Fine mapping of markers in the chromosome 4p $GABA_A$ receptor gene cluster in relation to AD involved a study of 69 single nucleotide polymorphisms (SNPs) in multiplex families from the Collaborative Study on the Genetics of Alcoholism (COGA) (Edenberg *et al*, 2004). These investigators found significant associations for multiple markers in the gene encoding the $GABA_A$ α-2 subunit (*GABRA2* (MIM 137140)) and for a single marker in the adjacent *GABRG1* (MIM 137166) gene, which encodes the $GABA_A$ γ-1 subunit. There was no evidence of association with other members of the gene cluster. This association to *GABRA2* was subsequently evaluated in three independent samples of subjects of European ancestry (Covault *et al*, 2004; Lappalainen *et al*, 2005; Fehr *et al*, 2006), and in each sample an association of AD with a haplotype block spanning the central and 3′-regions of *GABRA2* was observed.

Two other clusters of genes encoding $GABA_A$ subunits, located on chromosomes 5 and 15, have been examined for association to AD in other studies. Results for markers in the $GABA_A$ gene cluster containing genes for β-2, α-6, α-1,

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and γ-2 subunits on chromosome 5q have been mixed, with association reported in some samples (Loh *et al*, 2000; Radel *et al*, 2005), but not others (Sander *et al*, 1999; Dick *et al*, 2005). Fine mapping of a $GABA_A$ gene cluster containing genes for the α-5, β-3, and γ-3 subunits on chromosome 15q showed modest evidence of haplotypic association to AD for SNPs in *GABRG3*, encoding the γ-3 subunit (Dick *et al*, 2004).

The present study extends the findings of association of AD to the chromosome 4 $GABA_A$ gene cluster by examining the intergenic extent of the *GABRA2* 3′-region haplotype block associated with AD and by examining markers in the adjacent haplotype block in the 5′-region of *GABRG1*. We observed a stronger association of *GABRG1* 5′-upstream markers with AD in both study samples compared with markers in the *GABRA2* haplotype block.

MATERIALS AND METHODS

Subjects

Connecticut AD subjects (372 non-Hispanic Caucasians of European decent (EA) and 63 African-Americans (AA)) were recruited as part of ongoing studies of the genetics of AD or from clinical trials for the treatment of AD at the University of Connecticut Health Center (UCHC), Farmington, CT and the VA Connecticut Healthcare Center (VA-CT), West Haven, CT. Controls from CT (535 EA and 100 AA) were recruited by advertisement in the greater Hartford, CT area. Psychiatric diagnoses were made using the Structured Clinical Interview for DSM-III-R or DSM-IV (SCID) (First *et al*, 1997) or the Semi-Structured Assessment for Drug Dependence and Alcoholism (SSADDA) (Pierucci-Lagha *et al*, 2005). All controls were screened using the SCID or the SSADDA to exclude individuals with an alcohol or drug use disorder, or other major Axis I psychiatric disorder. Subjects were paid for their participation and all provided written, informed consent to participate in study protocols that were approved by the institutional review boards at UCHC, Yale University School of Medicine, and/or VA-CT. The diagnosis of AD for Project MATCH subjects (727 EA and 85 AA) was made using the Computerized Diagnostic Interview for DSM-IV (Blouin *et al*, 1988; American Psychiatric Association, 1994). For both the CT and Project MATCH samples, analysis was limited to self-identified AA and EA subjects. For analysis of AA subjects, we pooled AD subjects from the CT ($n = 63$) and Project MATCH ($n = 85$) samples.

Demographic and clinical characteristics of the participant sample are listed in Table 1. For both EA and AA samples, the control groups were significantly younger than the AD groups and included more female subjects. Similar to other samples, AD subjects had a moderate prevalence of affective/anxiety disorders, lifetime diagnosis of cocaine or opioid dependence (lifetime drug dependence diagnoses were not available for the Project MATCH sample) or antisocial personality disorder. Among the CT EA subjects, 294 controls (55%) and 264 alcoholics (71%) were examined in our prior association study of *GABRA2* SNPs A-H (Covault *et al*, 2004).

Genotyping

GABRG1 and *GABRA2* SNPs were genotyped using a closed-tube fluorescent TaqMan 5′-nuclease allelic discrimination assay using MGB-probes and primers designed using Primer Express v2.0 software (Applied Biosystems Inc. (ABI), Foster City, CA). Fluorescence plate reads and genotype calls were made using ABI 7700 and 7500 Sequence Detection Systems. Ten nanograms of genomic DNA was PCR amplified in 96-well plates using a 10 μl reaction volume for 40 cycles at 94°C for 15 s followed by 60°C for 60 s. Repeat genotyping was carried out for 16% of samples with an observed error rate of 0.5%. PCR amplifications failed or provided ambiguous genotype results from 1.5% of reactions (1.7% controls, 3.4% CT cases and 0.4% Project MATCH cases). To estimate genetic ancestry proportions for each subject, DNA samples were also genotyped using a panel of 34 short tandem repeat ancestry informative markers: CSF1PO, D2S1338, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D19S433, D21S11, FGA, TH01, TPO-9, vWA, D17S799, D1S196, D7S640, D8S1827, D7S657, D22S274, D5S407, D2S162, D10S197, D11S935, D9S175, D5S410, D7S2469, D16S3017, D10S1786, D15S1002, D6S1610, D1S2628, D12S352 as described previously (Stein *et al*, 2004; Luo *et al*, 2005; Yang *et al*, 2005).

Table 1 Demographic and Clinical Characteristics of Sample

	EA—connecticut sample			AA subjects	
	Controls ($n = 535$)	AD ($n = 372$)	EA—project match AD ($n = 727$)	Controls ($n = 100$)	AD ($n = 148$)
Age (years) (SD)	30.0 (10.6)	43.8 (9.1)***	40.6 (11.1)***	31.7 (10.4)	41.6 (8.5)***
% Male	42.8	75.3***	73.2***	34.0	66.9***
% Comorbid diagnosis (lifetime)					
Affective/anxiety disorder	0	32	25	0	22
Cocaine or opioid dependence	0	14.8	ND	0	ND
Antisocial personality disorder	0	6.5	9.5	0	7.0

ND—incomplete or no data. For the AA AD subjects in the CT sample ($n = 63$), 52% reported lifetime cocaine or opioid dependence.
***$p < 0.001$ *vs* controls (by race).

Genotype distributions were in Hardy–Weinberg equilibrium (HWE) for all 15 SNPs for each of the three EA groups (controls: mean $p=0.55$, range 0.22–1.0; CT AD subjects: mean $p=0.48$, range 0.06–1.0; Project MATCH AD subjects: mean $p=0.48$, range 0.15–1.0). For the AA groups, genotype distributions were consistent with HWE expectations for all but two markers in the control group (mean $p=0.41$, range 0.04–1.0; rs1440133 (SNP7), $p=0.04$, and rs529826 (SNP C), $p=0.04$) and two markers in the AD group (mean $p=0.63$, range 0.04–1.0; rs534459 (SNP B), $p=0.04$, and rs529826 (SNP C), $p=0.04$).

Data Analysis

Diagnostic groups were compared on age using ANOVA and on sex and allele frequencies using 2×2 contingency tables and the χ^2 test. Linkage disequilibrium (LD) plots, haplotype blocks, allele frequencies, tests of HWE, and haplotype frequencies for each population and diagnostic group were generated using the software program Haploview v3.32 (Barrett *et al*, 2005). Best-estimate haplotype pairs for each subject were generated using PHASE v2.1.1 software, which incorporates a Bayesian statistical method for reconstructing estimated haplotypes from population data (Stephens *et al*, 2001; Stephens and Donnelly, 2003). Haplotype pairs were generated separately for AA and EA populations. The software program STRUCTURE v2.1 (Pritchard *et al*, 2000; Falush *et al*, 2003) was used to generate estimates of the proportion of African *vs* European genetic ancestry for each subject based on genotype results from the 34 ancestry informative markers. Simulations used 100 000 burn-ins followed by 500 000 runs and a population parameter $K=2$. Estimated haplotype frequencies were compared for alcoholics *vs* controls and AA *vs* EA controls using a series of 2×2 contingency tables for each haplotype compared to the sum of all other haplotypes. The odds ratio (OR) is reported as a measure of effect size. Binary logistic regression analysis was used to control for age, sex, and the proportion of EA genetic ancestry in determining a corrected OR for AD as a function risk haplotype. Statistical analysis was carried out using SPSS software v14.0.

RESULTS

We genotyped 15 SNPs (average inter-marker interval of 20 530 bp, range 6598–39 849 bp) extending 287 400 bp in the *GABRG1-GABRA2* region of chromosome 4 in a sample of 1634 self-identified EA and 248 AA subjects, Table 1, comprising AD and control subjects from Connecticut (CT) and a sample of AD subjects from Project MATCH, an NIAAA-sponsored, multicenter clinical trial conducted at 10 sites throughout the US. That study sought to identify predictors of response to three psychotherapeutic treatments for alcoholism (Project MATCH Research Group, 1998). Table 2 lists the primers, MGB-probes and annealing-extension temperatures used to examine the 15 *GABRG1-GABRA2* region SNPs. The trivial names for the SNPs examined in *GABRA2* (SNPs A–H) are the same as were used previously (Covault *et al*, 2004); the additional seven SNPs in the intergenic and 5′-region of *GABRG1* are numbered 1–7.

Two main haplotype blocks were observed in EA controls (Figure 1a). One was a 94 kb region that included SNP markers 2–6 and extended from *GABRG1* intron 2–62 000 bp 5′ of the *GABRG1* transcript start site. The second haplotype block extended 137 kb and included SNP marker 7 (50 000 bp 3′ from the 3′UTR of the *GABRA2* transcript) and the seven adjacent SNP markers A–G (ending in *GABRA2* intron 3), which we had previously identified as being in high LD with one another (Covault *et al*, 2004). The LD plot and haplotype block structure for AA subjects was similar to EA subjects regarding the presence of a region of increased recombination in the 14 kb interval between SNPs 6 and 7 (Figure 1b). Among AAs, SNPs 3–6 were in high LD with one another in the *GABRG1* block and in the *GABRA2* haplotype block region SNP 7 and A–E showed high LD. The LD plot and haplotype block structure for AD subjects paralleled those for the controls of their respective racial group (data not shown).

GABRG1 allele and genotype frequencies for the three EA groups and the two AA groups are shown in Table 3. Among EAs, all SNPs in the *GABRG1* haplotype block (SNPs 2–6) showed significant association (nominal $p<0.05$) with AD for both allele and genotype frequencies in both the CT and Project MATCH samples. The strongest association ($p=0.001$) was observed for SNPs 4 and 5 in the 5′-upstream region of the gene. *GABRA2* allele and genotype frequencies (SNP7 and SNPs A–H) are shown in Table 4. For the *GABRA2* haplotype block region, the three SNPs closest to *GABRG1* (ie SNPs 7, A, and B) showed modest allelic and genotypic association with AD in the CT EA sample ($p=0.009$–0.016 for allelic association), whereas SNPs D–G showed less evidence of association ($p=0.021$–0.032). In parallel with the CT sample, MATCH EA alcoholics showed a nonsignificantly higher prevalence than controls of the minor allele for each SNP in the 3′ *GABRA2* haplotype block ($p=0.083$–0.167). None of the 15 markers showed allelic association with AD in the AA sample. Allele frequencies in the controls differed significantly by racial population for 13 of the 15 SNPs (Tables 3 and 4, right-hand column).

Haplotype frequencies for the two major haplotype blocks in the *GABRG1-GABRA2* interval were estimated using markers defining the slightly shorter core of each block based on the haplotype structure for AAs (Figure 1). Four markers (SNPs 3–6) were used to define the *GABRG1* haplotype block and six markers (SNPs 7 and A–E) for the *GABRA2* haplotype block. Estimated haplotype frequencies were compared with the sum of all other haplotypes for alcoholics *vs* controls and for AA *vs* EA controls using a series of 2×2 contingency tables (Tables 5 and 6). Among EAs, two haplotypes comprised $>90\%$ of chromosomes for the *GABRG1* haplotype block and $>95\%$ of chromosomes for *GABRA2*. AA subjects had a third common haplotype for both haplotype blocks. Haplotype frequencies differed significantly between EA and AA controls. For *GABRG1*, there was a significantly greater frequency of the ATCC haplotype in AD than controls in both EA groups ($\Delta=0.078$ in CT EA cases, $p<0.001$; 0.064 in MATCH EA cases, $p=0.002$), but this did not reach significance in AA cases ($\Delta=0.059$). For the 6-SNP *GABRA2* 3′-region haplotype, there was a nonsignificantly greater frequency of the AGACTC minor haplotype in EA alcoholics than controls

Table 2 *GABRA2* SNPs Examined in Samples of Alcohol-Dependent and Control Subjects

SNP trivial name (this paper)	NCBI SNP (dbSNP build 126)	Genome assembly position[a]	Location relative to *GABRA2*	Location relative to *GABRG1*	PCR amplicon primers	Taqman MGB probes (SNP position in bold)
1	rs2350439	45779174		Intron 3	TTCTCTACTCCCATACTCTTATCTCATCTTC GGGAGTGAGTGGGAATGTAGATTTAA	Vic-CCATGTTTCTCAAAAT**A**GTAGTT 6FAM-CATGTTTCTCAAAAT**G**GTAGTT
2	rs1497577	45788470		Intron 2	TCCACTTGCGGGTATGTTTGA AATAAAGAGCAGATTGAAACACAAATAGG	Vic-TGCTATGTC**T**TTACATCTC[b] 6FAM- TGCTATGTC**A**TTACATCTC[b]
3	rs1391166	45800624		Intron 1	GTTTTGCTATCCCTATTAGTGCTCATTTA CTTGAATTTGGCTCCTGATTGC	Vic- TGGTTAGAATGA**A**ACAAAAA 6FAM- TGGTTAGAATGA**T**ACAAAAA
4	rs7654165	45821824	125 kb 3′	1 kb 5′	GCTTTCTCTGCCTAGCCATCTTC GGTGGATTAACAGCTAAAGACTCCAA	Vic-TCTTAAAACGCC**C**TCCC 6FAM- TCTTAAAACGCC**T**TCCC
5	rs10033451	45847089	99 kb 3′	26 kb 5′	CACTGACACATTACCCCGAAATT AAGAAAAGGGAAATCAGCGTACAAT	Vic- CTTCTTAAACTTG**T**AATCAATT 6FAM- CTTAAACTTG**C**AATCAAT
6	rs4280776	45883288	63 kb 3′	62 kb 5′	CTTTAAATTTTCATCTCTCTTACCATTCAG GAAAAATCACCTATAATCCCTCCTCTCTA	Vic- AGTTTTGTTTCC**C**AATCCA 6FAM- AGTTTTGTTTCC**T**AATCCA
7	rs1440133	45896677	50 kb 3′	76 kb 5′	CACAATGTGCTTTCTCATGAATTCA TCCTAATAGTCCAGGGAACTGTAATGTT	Vic- CTACACAACCTC**T**TTTTC[b] 6FAM- CTACACAACCTC**C**TTTTC[b]
A	rs567926	45936526	10 kb 3′	116 kb 5′	TTTTGTCCATGCTCCTGTCATTT TTGAACTGTGTTGCCTGTTCTATAGAA	Vic- TCTGTCTGAC**G**TTACATTT 6FAM- ATTTCTGTCTGAC**A**TTACATTT
B	rs534459	45951562	Intron 9		CCGGAATTCTTAACCACTAAATTTCA GCACTTCTGGACATTTTTTTGCTT	Vic- TCTACCTAC**A**CATCCC 6FAM- TCTACCTAC**G**CATCCC
C	rs529826	45966409	Intron 8		ACACCCAGCTGGATTTATATATCAATAT ACCACATTCTTCCCCAAAATGA	Vic- CAGAAAAGAAACA**C**CTACAGTTA 6FAM- CAGAAAAGAAACA**T**CTACAGTTA
D	rs279869	46002752	Intron 6		TGAAAGGTGAAGAGCAAATTTCTTG TGGCCTAAAGGAGATGTTATAATCATCT	Vic- AGAGTCTGTTA**G**AATCACT 6FAM- AGAGTCTGTTA**T**AATCACTG
E	rs279858	46009350	Exon 5 (K132K)		GAAGCAACTTATTTGGCATTGTCA TCTGGACTCCAGATACCTTTTTTCA	Vic-TGAGCTACTGATTT**C**TTCCCAT 6FAM-TGAGCTACTGATTT**T**TTCCCAT
F	rs279844	46024412	Intron 4		GAAGCTACTGGGATATTAATTAGTTCAGTAGTTA CAAATGGCAACTTCTGATAAAGGA	Vic- AGTTGTGAG**T**TTTAATATCT[b] 6FAM- AGTTGTGAG**A**TTTAATATCT[b]
G	rs279837	46034080	Intron 3		CAATGGTCCTGAGCATCCTCTAAT GTGGGACTTGACTTTTGCACAA	Vic- CAAAGAGCA**A**AGTAAATAT 6FAM- CAAAGAGCA**G**AGTAAATAT
H	rs9291283	46066590	Intron 3		ABI SNP assay 8262290	TTAGAGTTATATTAAATATAGTAAC[**A**/**G**] GGAGATTTGTTCCAAAGGAAATGTG

[a]Nucleotide position using the March 2006 version of the human genome chromosome 4 annotation (www.genome.ucsc.edu).
[b]Probe sequence from chromosome 4 negative strand.

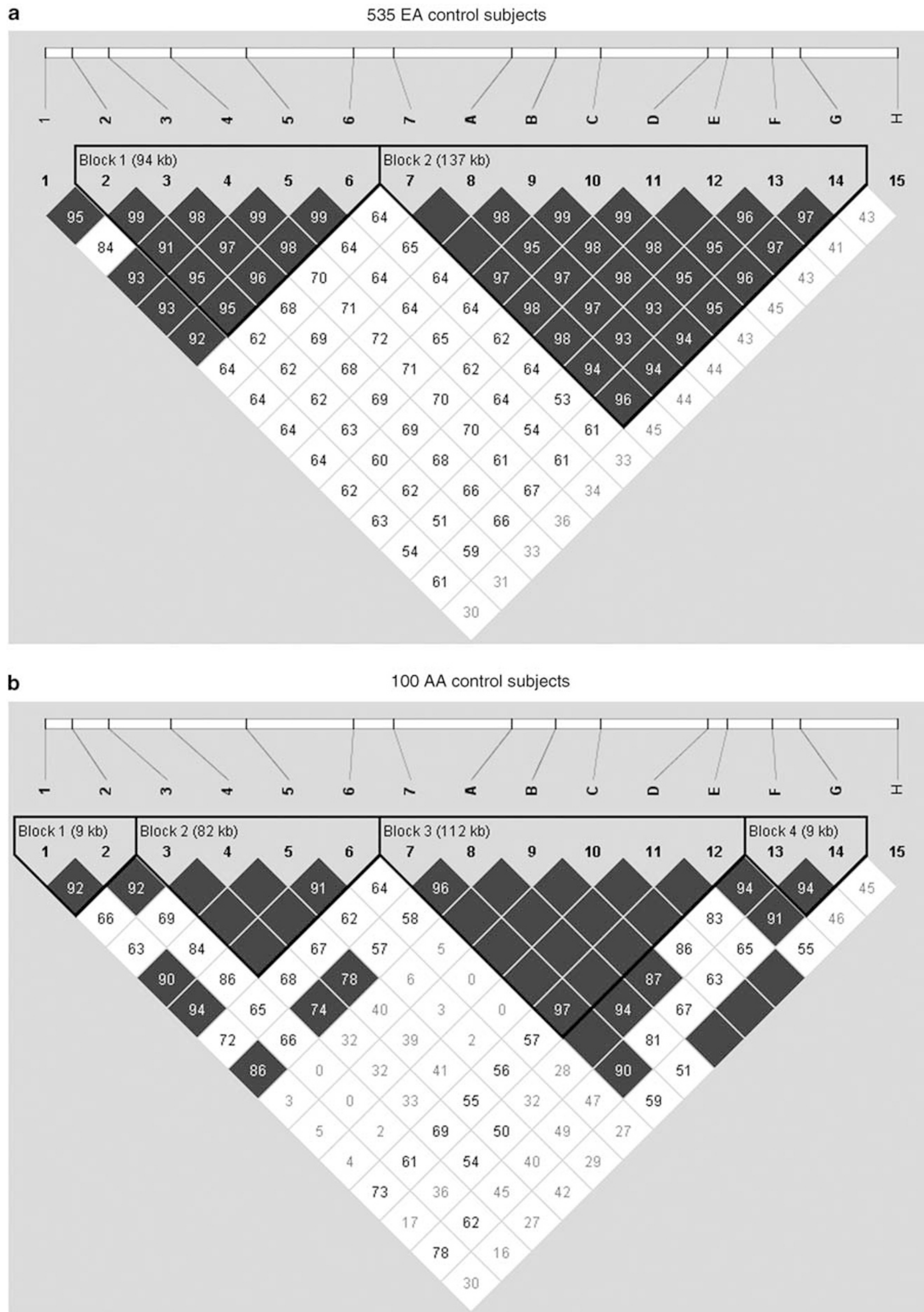

Figure 1 LD plot from Haploview 3.32 for EA control subjects (a) compared with that for AA control subjects (b). Pairwise SNP |D′| values (× 100) of linkage are shown together with haplotype blocks identified using the four-gamete rule. Marker pairs in complete LD are indicated by an empty box. Darkened blocks indicate SNP pairs without evidence of extensive recombination (ie 4-gamete rule for haplotype block characterization with one (or two) 2-SNP haplotypes having a frequency <0.02).

Table 3 *GABRG1* SNP Allele and Genotype Frequencies for Self-Identified EA and AA Subjects

SNP rs# location	Allele/ genotype[a]	EA—connecticut sample Controls ($n=535$)	AD ($n=372$)	χ^2; p-value[b]	EA—PROJECT MATCH AD ($n=727$)	χ^2; p-value[c]	AA subjects Controls ($n=100$)	AD ($n=148$)	χ^2; p-value[d]	EA c/w AA controls χ^2; p-value[e]
rs2350439-1	G	0.508	0.583	9.72	0.555	3.37	0.535	0.555	0.19	0.50
GABRG1-	A	0.492	0.417	**0.002**	0.455	0.066	0.465	0.445	0.665	0.479
intron 3	G/G	0.261	0.373		0.297		0.290	0.308		
	G/A	0.494	0.421	12.54	0.496	3.40	0.490	0.493	0.20	
	A/A	0.245	0.206	**0.002**	0.207	0.183	0.220	0.200	0.906	
Rs1497577-2	T	0.544	0.473	8.46	0.493	6.28	0.636	0.583	1.41	5.78
GABRG1-	A	0.456	0.527	**0.004**	0.507	**0.012**	0.364	0.417	0.234	**0.016**
intron 2	T/T	0.305	0.258		0.243		0.424	0.345		
	T/A	0.477	0.430	10.03	0.499	6.62	0.424	0.476	1.56	
	A/A	0.218	0.312	**0.007**	0.257	**0.037**	0.152	0.179	0.45	
Rs1391166-3	A	0.505	0.563	5.40	0.564	8.41	0.696	0.728	0.52	21.3
GABRG1-	T	0.495	0.437	**0.020**	0.436	**0.004**	0.304	0.272	0.470	**<0.001**
intron 1	A/A	0.254	0.351		0.312		0.512	0.537		
	A/T	0.502	0.425	9.24	0.505	8.71	0.369	0.381	0.87	
	T/T	0.244	0.225	**0.010**	0.184	**0.013**	0.119	0.119	0.650	
Rs7654165-4	C	0.534	0.456	10.56	0.467	11.09	0.321	0.270	1.50	29.9
GABRG1-	T	0.466	0.544	**0.001**	0.533	**0.001**	0.679	0.730	0.221	**<0.001**
1 kb 5′	C/C	0.289	0.233		0.209		0.112	0.095		
	C/T	0.490	0.446	11.76	0.515	11.97	0.418	0.351	1.69	
	T/T	0.221	0.321	**0.003**	0.276	**0.003**	0.469	0.554	0.43	
Rs10033451-5	T	0.559	0.479	10.89	0.496	9.69	0.703	0.639	2.17	13.9
GABRG1-	C	0.441	0.521	**0.001**	0.504	**0.002**	0.297	0.361	0.140	**<0.001**
26 kb 5′	T/T	0.323	0.251		0.244		0.521	0.405		
	T/C	0.472	0.457	10.68	0.503	10.28	0.365	0.466	3.22	
	C/C	0.205	0.292	**0.005**	0.253	**0.006**	0.115	0.128	0.200	
Rs4280776-6	T	0.554	0.490	6.86	0.495	8.39	0.717	0.652	2.22	17.0
GABRG1-	C	0.446	0.510	**0.009**	0.505	**0.004**	0.283	0.348	0.136	**<0.001**
62 kb 5′	T/T	0.317	0.257		0.252		0.522	0.414		
GABRA2-	T/C	0.475	0.464	6.71	0.506	9.08	0.391	0.476	2.65	
63 kb 3′	C/C	0.208	0.278	**0.035**	0.242	**0.011**	0.087	0.110	0.266	

$p<0.05$ bolded text.
[a]Allele nucleotide designation refers to the chromosome plus strand sequence.
[b]EA Controls compared with EA Connecticut Alcoholics.
[c]EA Controls compared with EA MATCH Alcoholics.
[d]AA Controls compared with AA Alcoholics (Connecticut and MATCH samples combined).
[e]EA Controls compared with AA Controls.

($\Delta=0.046$ in CT cases, $p=0.052$ and 0.028 in MATCH cases, $p=0.20$). The frequency of the *GABRA2* AGACTC minor haplotype did not differ by diagnosis among AAs.

There was a moderate degree of LD between markers in the two haplotype blocks ($r=0.51$–0.59 for EA subjects and $r=0.00$–0.53 for AA subjects). To examine whether variation in one or the other of the two adjacent genes accounted for the association to AD, we examined the phase and pattern of linkage of the adjacent *GABRG1* and *GABRA2* haplotype blocks by estimating the frequency of extended haplotypes defined by the 10 SNPs from these two blocks. Table 7 lists the frequency of the most common 10-SNP haplotypes covering the larger 208 000 bp interval. The most common extended haplotype among EAs (TCTT-GAGTGT) was under represented in both of the EA alcoholic samples ($\Delta=0.098$ in CT cases and $\Delta=0.059$ in MATCH cases; $p<0.001$ and 0.002, respectively). Chromosomes with the *GABRG1* risk haplotype (ATCC) were overrepresented in EA alcoholics, irrespective of the *GABRA2* haplotype in both EA samples. The distortion was greatest when the less common *GABRG1* risk haplotype was paired with the major *GABRA2* haplotype (GAGTGT) ($\Delta=0.042$ in CT cases, and $\Delta=0.044$ in MATCH cases; $p=0.04$ and $p=0.005$, respectively). In contrast, the *GABRA2* haplotype defined by the minor allele at each marker (AGACTC), which we previously found to be

Table 4 GABRA2 SNPs Allele and Genotype Frequencies for Self-Identified EA and AA Subjects

		EA—connecticut sample			EA—project match		AA subjects			
SNP rs# location	**Allele/ genotype**[a]	**Controls ($n=535$)**	**AD ($n=372$)**	**χ^2; *p*-value**[b]	**AD ($n=727$)**	**χ^2; *p*-value**[c]	**Controls ($n=100$)**	**AD ($n=148$)**	**χ^2; *p*-value**[d]	**EA *vs* AA controls χ^2; *p*-value**[e]
rs1440133-7	G	0.607	0.549	5.81	0.580	1.91	0.773	0.755	0.21	19.4
GABRA2-	A	0.393	0.451	**0.016**	0.420	0.167	0.227	0.245	0.648	< **0.001**
50 kb 3′	G/G	0.380	0.291		0.324		0.639	0.559		
	G/A	0.453	0.515	7.30	0.512	4.97	0.268	0.393	5.03	
	A/A	0.166	0.194	**0.026**	0.165	0.083	0.093	0.048	0.08	
rs567926-A	A	0.609	0.546	6.56	0.575	2.94	0.755	0.741	0.12	14.4
GABRA2-	G	0.391	0.454	**0.010**	0.425	0.087	0.245	0.259	0.727	< **0.001**
9 kb 3′	A/A	0.384	0.302		0.320		0.587	0.532		
	A/G	0.451	0.488	6.55	0.510	5.88	0.337	0.417	1.83	
	G/G	0.165	0.210	**0.038**	0.170	0.053	0.075	0.050	0.40	
rs534459-B	G	0.597	0.534	6.81	0.570	1.93	0.281	0.303	0.27	66.9
GABRA2-	A	0.403	0.466	**0.009**	0.430	0.165	0.719	0.697	0.601	< **0.001**
intron 9	G/G	0.365	0.283		0.311		0.112	0.051		
	G/A	0.466	0.503	7.03	0.517	4.18	0.337	0.504	7.76	
	A/A	0.170	0.214	**0.030**	0.172	0.123	0.551	0.445	0.02	
rs529826-C	T	0.600	0.557	3.02	0.576	1.39	0.279	0.298	0.18	61.4
GABRA2-	C	0.400	0.443	0.82	0.424	0.238	0.721	0.702	0.669	< **0.001**
intron 8	T/T	0.365	0.306		0.319		0.128	0.050		
	T/C	0.496	0.502	3.24	0.514	3.09	0.302	0.496	10.33	
	C/C	0.166	0.192	0.197	0.166	0.213	0.570	0.454	0.006	
rs279869-D	G	0.590	0.536	4.91	0.568	1.21	0.281	0.292	0.08	63.8
GABRA2-	T	0.410	0.464	**0.027**	0.432	0.272	0.719	0.708	0.782	< **0.001**
intron 6	G/G	0.357	0.285		0.310		0.112	0.049		
	G/T	0.467	0.503	5.23	0.517	3.57	0.337	0.486	6.96	
	T/T	0.176	0.212	0.073	0.174	0.168	0.557	0.465	0.031	
rs279858-E	T	0.598	0.542	5.29	0.570	2.00	0.760	0.762	0.00	18.6
GABRA2-	C	0.402	0.458	**0.021**	0.430	0.157	0.240	0.236	0.956	< **0.001**
exon 5	T/T	0.365	0.297		0.312		0.612	0.574		
	T/C	0.465	0.490	5.21	0.515	4.16	0.296	0.376	2.76	
	C/C	0.169	0.213	0.074	0.173	0.125	0.092	0.050	0.252	
rs279844-F	A	0.579	0.525	4.85	0.546	2.67	0.478	0.525	0.97	6.50
GABRA2-	T	0.421	0.475	**0.028**	0.454	0.103	0.522	0.475	0.324	**0.011**
intron 4	A/A	0.341	0.288		0.284		0.280	0.281		
	A/T	0.476	0.472	4.87	0.524	4.71	0.398	0.489	2.81	
	T/T	0.183	0.239	0.088	0.192	0.095	0.323	0.230	0.246	
rs279837-G	A	0.600	0.548	4.60	0.568	2.67	0.732	0.736	0.01	12.4
GABRA2-	G	0.400	0.452	**0.032**	0.432	0.102	0.268	0.264	0.930	< **0.001**
intron 3	A/A	0.367	0.301		0.312		0.566	0.535		
	A/G	0.467	0.494	4.62	0.512	4.19	0.333	0.401	1.87	
	G/G	0.166	0.205	0.099	0.177	0.123	0.101	0.063	0.393	
Rs9291283-H	G	0.744	0.718	1.36	0.732	0.45	0.693	0.691	0.00	2.21
GABRA2-	A	0.256	0.282	0.244	0.268	0.501	0.307	0.309	0.977	0.138
intron 3	G/G	0.558	0.518		0.533		0.479	0.482		
	A/G	0.372	0.399	1.35	0.398	0.91	0.427	0.418	0.03	
	A/A	0.070	0.083	0.510	0.069	0.636	0.094	0.099	0.985	

$p < 0.05$ bolded text.
[a]Allele nucleotide designation refers to the chromosome plus strand sequence.
[b]EA Controls compared with EA Connecticut Alcoholics.
[c]EA Controls compared with EA MATCH Alcoholics.
[d]AA Controls compared with AA Alcoholics (Connecticut and MATCH combined).
[e]EA Controls compared with AA Control.

Table 5 *GABRG1* 4-SNP Haplotype (SNPs 3–6; rs1391166, 7654165, 10033451, 4280776) Frequencies for Self-Identified EA and AA Subjects

	EA—connecticut sample			EA—project match		AA subjects			
Haplotype[a]	**Controls ($n=535$)**	**AD ($n=372$)**	**χ^2; p-value**[b]	**AD ($n=727$)**	**χ^2; p-value**[c]	**Controls ($n=100$)**	**AD ($n=148$)**	**χ^2; p-value**[d]	**EA c/w AA controls χ^2; p-value**[e]
TCTT	0.490	0.394	**15.74; <0.001**	0.426	**10.43; 0.001**	0.291	0.261	0.57; 0.45	**26.13; <0.001**
ATCC	0.432	0.510	**11.47; <0.001**	0.496	**9.77; 0.002**	0.261	0.320	1.93; 0.16	**19.23; <0.001**
ATTT	0.026	0.024	0.07; 0.79	0.025	0.01; 0.92	0.359	0.359	0.00; 0.96	**255.4; <0.001**

$p<0.05$ bolded text.
[a]Only those haplotypes with frequency >0.05 in at least one subgroup are shown.
[b]2×2 χ^2 table (haplotype (y) *vs* all others) EA Controls compared with EA Connecticut Alcoholics.
[c]EA Controls compared with EA MATCH Alcoholics.
[d]AA Controls compared with AA Alcoholics (Connecticut and MATCH combined).
[e]EA Controls compared with AA Controls.

Table 6 *GABRA2* 6-SNP Haplotype (snps 7, A-E; rs1440133, 567926, 534459, 529826, 279869, 279858) Frequencies for Self-Identified EA and AA Subjects

	EA—connecticut sample			EA—project match		AA subjects			
Haplotype[a]	**Controls ($n=535$)**	**AD ($n=372$)**	**χ^2; p-value**[b]	**AD ($n=727$)**	**χ^2; p-value**[c]	**Controls ($n=100$)**	**AD ($n=148$)**	**χ^2; p-value**[d]	**EA c/w AA controls χ^2; p-value**[e]
GAGTGT	0.585	0.524	**6.18; 0.013**	0.561	1.94; 0.16	0.281	0.278	0.00; 0.95	**60.50; <0.001**
AGACTC	0.386	0.432	3.78; 0.052	0.414	1.63; 0.20	0.229	0.228	0.00; 0.98	**17.13; <0.001**
GAACTT	0.005	0.001	N/A	0.001	N/A	0.453	0.440	0.16; 0.69	**469.8**; **<0.001**

$p<0.05$ bolded text.
N/A—χ^2 table cell expected counts <5.
[a]Only those haplotypes with frequency >0.05 in at least one subgroup are shown.
[b]2×2 χ^2 table (haplotype (y) *vs* all others) EA Controls compared with EA Connecticut Alcoholics.
[c]EA Controls compared with EA MATCH Alcoholics.
[d]AA Controls compared with AA Alcoholics (Connecticut and MATCH combined).
[e]EA Controls compared with AA Controls.

associated with AD (Covault *et al*, 2004), when paired with the *GABRG1* non-risk haplotype (TCTT), did not differ in frequency between EA alcoholics and controls in the present analysis. A more formal restatement of this analysis is to examine the ratio in cases *vs* controls of the two putative risk haplotypes with each conditional on the presence of the other. Both haplotype ratios, f(AB)/f(Ab) and f(aB)/f(ab), are expected not to differ in cases and controls if the 'A *vs* a' component of the haplotype identifies a disease-associated marker and the 'B *vs* b' site represents a neutral marker, irrespective of genetic mode of inheritance (Valdes and Thomson, 1997). The χ^2 test of the null hypothesis that these ratios are equivalent is rejected ($p<0.01$) in EA AD samples, if the *GABRA2* AD-associated haplotype is considered the risk marker and the *GABRG1* haplotype is considered the neutral marker. In this context, markers in the *GABRA2* haplotype block do not capture all of the disease risk associated with this chromosomal region. In contrast, the null hypothesis that the ratios are equivalent is sustained ($p>0.2$) when the *GABRG1* AD-associated haplotype block is treated as the conditional risk marker. Equivalent results were obtained examining 2-SNP haplotypes using a single representative SNP for the *GABRG1* and *GABRA2* blocks (eg SNPs 4 and A). These observations suggest that the allelic and haplotypic association of *GABRA2* SNPs with AD in our CT sample in both this and our prior study (Covault *et al*, 2004) may in part be secondary to LD of these markers with risk-related variants in the adjacent *GABRG1* 5′-region. In AAs, although not statistically significant, the extended 10-SNP haplotype pattern was qualitatively similar to the pattern in EAs, in that there was a greater frequency of chromosomes with an extended haplotype containing the *GABRG1* ATCC motif.

Significant racial population differences were observed in the allele frequencies for 13 of the 15 SNPs, as well as for the most common haplotypes for the *GABRG1* and *GABRA2* blocks (Tables 3–6). To evaluate population genetic stratification, we used the program STRUCTURE v2.1 (Pritchard *et al*, 2000; Falush *et al*, 2003) to generate estimates of the proportion of African *vs* European genetic ancestry using genotype results from a panel of 34 ancestry informative markers (Stein *et al*, 2004; Luo *et al*, 2005; Yang *et al*, 2005). We found no differences in the degree of EA genetic ancestry in the three EA samples (controls $=0.980\pm0.043$, CT alcoholics $=0.980\pm0.043$, and MATCH alcoholics $=0.983\pm0.029$; $F(2,1631)=1.56$, $p=0.21$).

Table 7 *GABRG1-A2* 10-SNP Extended Haplotype (SNPs 3–7 & A–E; rs1391166, 7654165, 10033451, 4280776, 1440133, 567926, 534459, 529826, 279869, 279858) Frequencies for Self-Identified EA and AA Subjects

	EA—connecticut sample			EA—project match		AA subjects			
Haplotype[a] g1/a2 risk	**Controls ($n=535$)**	**AD ($n=372$)**	**χ^2; p-value[b]**	**AD ($n=727$)**	**χ^2; p-value[c]**	**Controls ($n=100$)**	**AD ($n=148$)**	**χ^2; p-value[d]**	**EA c/w AA controls χ^2; p-value[e]**
−/− TCTT-GAGTGT	0.425	0.327	**14.46; <0.001**	0.366	**9.29; 0.002**	0.148	0.146	0.07; 0.79	**48.52; <0.001**
+/+ ATCC-AGACTC	0.309	0.346	3.26; 0.07	0.334	1.69; 0.19	0.135	0.165	0.41; 0.52	**17.86; <0.001**
+/− ATCC-GAGTGT	0.109	0.141	**4.06; 0.044**	0.153	**8.02; 0.005**	0.081	0.095	0.41; 0.52	1.56; 0.21
−/− ATTT-GAACTT	0.003	0.000	N/A	0.001	N/A	0.294	0.280	0.09; 0.76	**315.5; <0.001**
−/− TCTT-GAACTT	0.003	0.003	N/A	0.001	N/A	0.114	0.073	1.43; 0.23	**103.8; <0.001**
−/+ TCTT-AGACTC	0.055	0.052	0.21; 0.64	0.047	0.91; 0.34	0.026	0.024	0.08; 0.78	2.66; 0.10

$p<0.05$ bolded text.
N/A—χ^2 table cell expected counts <5.
[a]Only those extended haplotypes with frequency >0.05 in at least one subgroup are shown. Risk haplotype blocks overrepresented in alcoholics are indicated by + (ATCC for *GABRG1* and AGACTC for *GABRA2*), all other haplotype blocks are noted with a−sign.
[b]2×2 χ^2 table (haplotype (y) *vs* all others) EA Controls compared with EA Connecticut Alcoholics.
[c]EA Controls compared with EA MATCH Alcoholics.
[d]AA Controls compared with AA Alcoholics (Connecticut and MATCH combined).
[e]EA Controls compared with AA Controls.

Consequently, population stratification is unlikely to be an explanation for the observed allele frequency differences between EA alcoholics and controls. Similarly, for AAs, there was no significant difference in estimated European genetic admixture for alcoholics and controls (0.084 ± 0.149 and 0.054 ± 0.143, respectively; $F(1,246)=1.61$, $p=0.11$).

Quantitative estimates of genetic ancestry proportion from STRUCTURE were also used as a covariate in binary logistic regression analysis (together with age and sex, which differed by diagnosis). This yielded corrected ORs for AD as a function of *GABRG1* or *GABRA2* AD-associated haplotype copy number as determined using PHASE. Dominant, recessive, and additive models were compared using dummy coding: 0, 1, 1; 0, 0, 1; and 0, 0.5, 1, respectively, for 0, 1 or 2 copies of the *GABRG1* or *GABRA2* AD-associated haplotype (ie ATCC or AGACTC, respectively). For EAs (combined MATCH and CT alcoholics), an additive genetic model provided the best fit for the *GABRG1* ATCC haplotype (Table 8), with an OR = 1.26 (95% CI = 1.06–1.50) for one copy (ie ATCC/x) and OR = 1.60 (95% CI = 1.13–2.25) for two copies of the AD-associated haplotype (ie ATCC/ATCC). Qualitatively similar results were seen when age, sex, and genetic ancestry were omitted from the model. For the *GABRA2* AGACTC haplotype, a dominant genetic model provided the best fit and an OR = 1.39 (95% CI = 1.08–1.80) for carriers. These models did not show interactive effects of haplotype with either gender or genetic ancestry proportion. Owing to the collinearity of the two risk haplotypes, we were unable simultaneously to examine the independent effects on AD risk of the *GABRG1* and *GABRA2* haplotype blocks. There was no evidence that the association of AD with *GABRG1* or *GABRA2* markers in the CT sample was due to comorbid drug dependence (relevant data were not available for Project MATCH). Considering the impact of drug dependence, ORs for the AD risk haplotypes were marginally greater (*GABRG1*) or unchanged (*GABRA2*) for subjects in the CT EA sample with no comorbid lifetime diagnosis of cocaine, opioid, or cannabis dependence compared with all CT EA alcoholics (*GABRG1* additive model OR = 1.35 (95% CI = 1.05–1.74) *vs* 1.25 (95% CI = 0.99–1.58) for one copy and OR = 1.82 (95% CI = 1.09–3.04) *vs* 1.56 (95% CI = 0.98–2.48) for two copies of the *GABRG1* risk haplotype; *GABRA2* dominant model OR = 1.45 (95% CI = 0.99–2.14) *vs* 1.40 (95% CI = 0.98–1.99) for carriers).

Table 8 Binary Logistic Regression Analysis of *GABRG1* and *GABRA2* AD-Risk Haplotypes as a Function of the Genetic Model (Including Age, Sex, and % EA Genetic Heritage as Covariates)

Risk haplotype	Genetic model	B	SE	Wald	p-value
GABRG1	Additive	0.47	0.18	7.1	0.008
ATCC haplotype	Dominant	0.27	0.14	4.0	0.45
	Recessive	0.37	0.15	5.7	0.016
GABRA2	Additive	0.32	0.18	3.1	0.079
AGACTC haplotype	Dominant	0.33	0.13	6.3	0.012
	Recessive	0.00	0.17	0.0	0.99

Finally, based on the apparent difference in genetic mode of action of risk elements potentially represented in the two haplotype blocks, we used binary logistic regression analysis to derive corrected measures for association of individual SNPs with AD in the combined EA sample using variable coding for both additive and dominant genetic models. Figure 2 illustrates the $\log_{10}$ (*p*-value) from binary logistic regression analyses in which age, sex, and genetic ancestry were used as covariates. The strongest association with AD was seen for SNPs 4 and 5 (rs7654165 and rs10033451) in the 5′-upstream region of *GABRG1* assuming an additive genetic model followed by SNPs 7, A, and B (rs1440133, rs567926, and rs534459) in the 3′region of *GABRA2* assuming a dominant genetic model. This difference in the best-fit genetic model for markers in the two haplotype block regions suggests that there may be

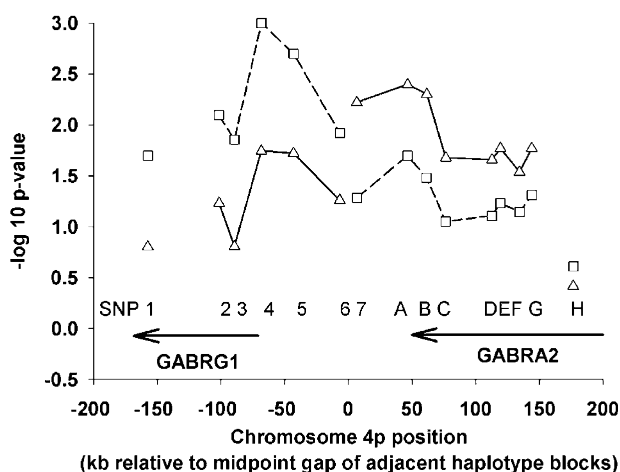

Figure 2 Negative $\log_{10}$ of binary logistic regression significance (*p*-value) for allelic association with AD after correction for age, sex, and proportion of EA genetic ancestry among all EA subjects (1099 AD and 535 controls) as a function of *GABRG1* and *GABRA2* SNP location assuming additive (– –□– –) or dominant (—△—) genetic models. SNPs within each of the two haplotype blocks identified in Figure 1a for EA subjects are joined by a line. Marker positions are shown relative to the mid-point between these two haplotype blocks. Consistent with results from haplotype analysis regarding potential mode of risk transmission, individual markers in the *GABRG1* haplotype block show a larger effect under an additive model, whereas those in the *GABRA2* block are more consistent with a dominant genetic effect model.

separate contributions to risk for AD by *GABRG1* and *GABRA2*.

DISCUSSION

The findings reported here suggest that the association of *GABRA2* with AD, as reported in four independent studies (Covault *et al*, 2004; Edenberg *et al*, 2004; Lappalainen *et al*, 2005; Fehr *et al*, 2006), may be complicated by a moderate degree of LD between markers in the 3′-region of *GABRA2* with those in the 5′-region of *GABRG1*. Although prior studies showed consistent evidence for association of AD to markers and haplotypes in the middle and 3′-region of *GABRA2*, the extent of the AD-associated *GABRA2* haplotype block and the potential association of AD to markers in the adjacent haplotype block were not well described. Edenberg *et al* (2004) examined six SNPs in the adjacent *GABRG1* gene (0.4 kb upstream to IVS8) and found nominal evidence for association ($p = 0.05$) to AD for one of the markers within the 5′-region haplotype block of *GABRG1*, which was examined in this report. Seeking to define the extent of the *GABRA2* 3′-region haplotype block, we genotyped markers extending into the 5′-region of *GABRG1*, where we found evidence among EAs of association to AD that exceeded the evidence for allelic and haplotypic association for *GABRA2*. Indeed, in the Project MATCH EA sample, the distortion of allele and haplotype frequencies related to AD for *GABRA2* markers was not statistically significant, whereas allele and haplotype frequencies in the haplotype block in the *GABRG1* 5′-region showed significant evidence of association in both EA samples. These findings were unaffected when corrections for age, sex, and ancestry proportion were included in the analysis. These results suggest that our prior reported findings of association of AD with the *GABRA2* gene (Covault *et al*, 2004) were partly due to LD of *GABRA2* markers with functional genetic variation in the adjacent *GABRG1* gene.

It is of interest to note that in the three published case–control studies (Covault *et al*, 2004; Lappalainen *et al*, 2005; Fehr *et al*, 2006), AD was associated with the minor allele and haplotype for markers in the 3′-region of *GABRA2*, consistent with the findings reported here for the EA alcoholic sample from CT. In contrast, in the COGA multiplex family sample, the more common haplotype was overrepresented among alcoholics (Edenberg *et al*, 2004). Re-analysis of the COGA data set revealed that the association of the major allele at markers in the 3′-region of *GABRA2* with AD was observed only among subjects dependent on both alcohol and drugs (Agrawal *et al*, 2006).

We observed large differences in allele frequencies for AA *vs* EA subjects at 13 of the 15 SNPs examined. Population differences in marker frequencies have been observed in other candidate genes related to substance use disorders (Gelernter *et al*, 1997, 1999; Covault *et al*, 2001; Luo *et al*, 2003; Herman *et al*, 2006). Such population differences highlight the need to consider population genetic stratification as a potential source of artifact in genetic association studies. Further, results from a recent gene expression microarray study noted that differences in frequency of *cis*-acting SNPs among ethnic groups may also be associated with significant differences in RNA expression levels of the associated genes (Spielman *et al*, 2007).

Limitations of our study include the lack of a sample of controls collected at each of the Project MATCH treatment sites, which required comparison of allele and haplotype frequencies for both samples of AD subjects with a common control sample recruited exclusively from Connecticut. However, we found no evidence of differences in genetic admixture between the CT and MATCH alcoholic samples. Although we observed a similar magnitude of increase in frequency of the *GABRG1* risk haplotype ATCC in AA and EA subjects, our small sample of AA subjects yielded limited statistical power. The sample of female alcoholics also limited our capacity to examine with high statistical power interactive effects of sex × genotype. Finally, the lack of data on lifetime drug dependence diagnoses in the Project MATCH sample limited our ability to examine whether in the CT AD sample, the association of AD to *GABRG1* markers was independent of comorbid drug dependence.

The findings from this study provide important new information regarding the likely physical location of functional genetic variants responsible for the reported association of AD to the $GABA_A$ receptor subunit gene cluster on chromosome 4p. The region of strongest association to AD that we observed includes potential regulatory regions immediately upstream of the *GABRG1* gene. The markers with the strongest association, rs7654165 and rs10033451, are located 1 and 26 kb upstream of the 5′end of the *GABRG1* transcript, respectively, suggesting that functional variants related to AD in this interval might alter the patterns of regional, cellular, or temporal expression of *GABRG1*. The γ-1 subunit is notable, in that unlike most $GABA_A$ subunits, its expression is limited to very few brain areas, including the pallidum, septum, bed nucleus of the

stria terminalis and the central and medial amygdaloid nuclei (Ymer *et al*, 1990; Araki *et al*, 1992; Persohn *et al*, 1992; Wisden *et al*, 1992; Pirker *et al*, 2000). Other brain regions that show selective expression of γ-1 (eg hypothalamic medial preoptic area) *vs* γ-2 subunits (eg ventromedial nucleus of the hypothalamus) (Herbison and Fenelon, 1995; Nett *et al*, 1999) differ in $GABA_A$ pharmacologic properties and show opposite effects with respect to receptor modulation by anabolic steroids (Jorge-Rivera *et al*, 2000). Additional differences in the pharmacology of γ-1-containing receptors include an insensitivity to the benzodiazepine antagonist flumazenil (Ymer *et al*, 1990; Khom *et al*, 2006) and an increased sensitivity of γ-1-containing receptors to the neuroactive steroid allopregnanolone in transfected human embryonic kidney (HEK293) cells compared with γ-2-containing receptors (Puia *et al*, 1993). Pharmacologic agents that are selective for γ-1-containing $GABA_A$ receptors may be of clinical interest given the limited CNS distribution of this receptor subunit.

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CONFLICTS OF INTEREST STATEMENT

None of the authors have conflicts of interest related to this work and have not had any conflict related to the work within the past 3 years.

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