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ACN9 and Alcohol Dependence: Family-Based Association Analysis in Multiplex Alcohol Dependence Families

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A previous genome-wide linkage study of alcohol dependence (AD) in the Pittsburgh-based multiplex family study found suggestive evidence for linkage on Chromosome 7q, a region in which the ACN9 gene is located. Using the same two generation Pittsburgh family data in which linkage was found, data for a third generation was added. The expanded sample included 133 pedigrees with 995 individuals. Finer mapping was undertaken using six SNPs extending from rs1917939 to rs13475 with minor allele frequency (MAF) $\geq$0.15 and pair-wise linkage disequilibrium (LD) of $r^2 < 0.8$ using the HapMap CEU population. Binary affection status, visual, and auditory P300 data were tested for family-based association. Family-based analyses found all six SNPs associated with affected status. Three SNPs are located upstream of the gene, two SNPs are within intron 1 and one is in Exon 4. FBAT *P*-values for the six SNPs ranged between 0.05 and 0.0005. Haplotype analysis revealed one four-SNP block formed by rs10499934, rs7794886, rs12056091, and rs13475 with an overall significant association at $P = 0.0008$. Analysis of visual P300 amplitude data, a known endophenotype of alcohol dependence risk, revealed a significant association for SNPs within intron 1 and exon 4 under a dominant model of transmission. Family-based association analysis shows the ACN9 gene significantly associated with alcohol dependence and P300 amplitude variation. The potential importance of the ACN9 gene for AD risk may be related to its role in gluconeogenesis which may be involved in the regulation of alcohol metabolism.

Key words: alcohol dependence; ACN9; family-based association; glucose metabolism

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INTRODUCTION

Excessive use of alcohol is the third leading cause of preventable death [Mokdad et al., 2004] in the US. The economic and social costs have been estimated to be $184 billion due to alcohol-related accidents, lost productivity, incarceration, and other alcoholrelated morbidity [Harwood, 2000]. In spite of the fact that use of alcohol is quite common, a smaller proportion of the population drink in sufficient quantity and with associated health, family, and work-related problems to be considered alcohol dependent (AD). Data from the National Comorbidity Survey, a survey of respondents ages 15–54 found 20.1% of men and 8.2% of women meeting criteria for alcohol dependence (AD) [Kessler et al., 1997]. There is now evidence that those individuals with the greatest propensity for AD may carry an increased genetic risk for developing alcohol dependence.

Although there is considerable heritability for alcohol dependence (0.49–0.64) in males [Caldwell and Gottesman, 1991; Heath et al., 1997] and females (0.56–0.59), [Kendler et al., 1992; Prescott et al., 1999], few genes have been identified that reliably confer susceptibility. However, studies employing well-designed sampling strategies that over sample families with a high density of cases have revealed important clues for gene finding. Two multiplex family studies of alcohol dependence have been developed with this strategy. The Pittsburgh-based multiplex family study and the Collaborative Study on the Genetics of Alcoholism (COGA) studies [Reich et al., 1998; Edenberg et al., 2004].

A region on 7q has shown consistent evidence for linkage in both the COGA sample [Reich et al., 1998; Foroud et al., 2000] and in the Pittsburgh-based series [Hill et al., 2004] from genome-wide scans

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of multiplex families ascertained through a pair of affected probands.

The initial sample studied by COGA consisted of 105 pedigrees and utilized DSM-IIIR and Feighner Criteria of alcohol dependence to define affected status. The initial report found a LOD score of 3.49 near D7S1793. Additional pedigrees (N = 157) were analyzed for linkage in this region with the maximal LOD score revealed at 1.3 [Foroud et al., 2000]. However, a combined sample of 262 families found a LOD score of 2.9 at D7S1799 (Wang et al., 2004). Additional genotyping of 143 densely affected pedigrees for the Genetics Workshop 14 was conducted revealing a LOD score of 4.1 [Dunn et al., 2005].

The present report is based on finer mapping of the 7q region first identified in our sample of densely affected pedigrees in which the two highest LOD scores on Chromosome 7 included one at marker D7S517 (LOD =3.54 $P = 0.002$) and another at marker D7S515 (LOD = 1.63 $P = 0.012$) [Hill et al., 2004]. These LOD scores were obtained using a binary alcohol dependence phenotype and including relevant covariates (age, gender, and P300 amplitude).

P300 amplitude is thought to index susceptibility to alcohol dependence [Hill et al., 1990; Porjesz et al., 2005; Hill et al., 2009]. Interestingly, this 7q region has now shown evidence of linkage to visual P300 amplitude in an Australian sample (Wright et al., 2008). The present report targeted SNPs within the ACN9 gene based on its proximity to D7S515 and a previous report using COGA data in which ACN9 was significantly related to alcohol dependence [Dick et al., 2008]. Because P300 amplitude appears to be a robust endophenotype for alcohol dependence, analyses of auditory and visual P300 across our three generation sample was conducted using family-based association tests for SNPs located either near or within the ACN9 gene.

MATERIALS AND METHODS

Study Sample

All members of the multiplex families who participated in the study gave their written consent to do so after the nature and purpose of the study was fully explained to them. (Consent forms were approved by the University of Pittsburgh Institutional Review Board.)

Multiplex Families

Multiplex families were selected on the basis of the presence of a pair of alcohol dependent brothers or sisters. The probands were selected from among individuals in treatment for alcohol dependence in the Pittsburgh area. Probands were eligible if they met DSM-III criteria for AD and had a same sex sibling who similarly met criteria for AD. All proband pairs and their cooperative relatives (siblings and parents) were personally interviewed using a structured psychiatric interview (Diagnostic Interview Schedule [DIS]). The DIS provides good reliability and validity [Helzer et al., 1985] for alcohol dependence and alcohol abuse by DSM-III and IIIR criteria [American Psychiatric Association, 1980; American Psychiatric Association, 1987] and alcoholism by Feighner Criteria [Feighner et al., 1972], an early diagnostic set of criteria used in the Collaborative Studies on the Genetics of Alcoholism (COGA) family [Reich et al., 1998].

Families were excluded if the probands or any first-degree relative were considered to be primary for drug dependence (preceded alcohol dependence onset by at least 1 year), or the proband or first-degree relative met criteria for schizophrenia, or a recurrent major depressive disorder. Probands and relatives with mental retardation or physical illness precluding participation were excluded. Complete details regarding participant selection may be seen in Hill et al. (2004). The majority of probands (80%) had three or more siblings who contributed DNA, consented to a clinical interview, and provided family history. These large sibships resulted in a total of 418 sib pairs of all types (201 Affected-Affected, 172 Unaffected-Affected, and 45 Unaffected-Unaffected within Generation II). One or both parents of Generation II have been genotyped in 86% of the families representing an average of 5.1 individuals per family who were genotyped. This data set was utilized in a previous genome-wide linkage analysis [Hill et al., 2004].

A follow up of the third generation offspring of Generation II individuals was initiated in 1990 at a time when offspring were at an average age of 11 years. Currently, there are 248 individuals with phenotypic data that resulted from both childhood and young-adult follow-up that included structured psychiatric interviews at approximately annual follow-up in childhood and every other year in young adulthood. Third generation offspring were coded as affected if they met criteria for alcohol dependence based on either DSM-IV or Feighner criteria. Unlike Generations I and II that were specifically selected to have minimal drug dependence, diagnoses for Generation III was free to vary based as it was on long term follow up of children from the multiplex families. Due to the high prevalence of substance use disorder in Generation III, secondary analyses were also performed using any alcohol or drug abuse or dependence as an indicator of affected status. Genotyping has been performed for 247 of these individuals.

Child/Adolescent Assessment for DSM-III Diagnoses

Children between the ages of 8–19 years were adminstered an age-appropriate diagnostic instrument. Each child/adolescent and his/her parent were separately administered the Schedule for Affective Disorders and Schizophrenia (K-SADS) [Chambers et al., 1985] by trained, Masters level, clinical interviewers, and an advanced resident in child psychiatry at each annual evaluation. Using DSM-III criteria that have been used throughout the follow up, K-SADS interviewers and the resident independently provided scores for each diagnosis. A best estimate diagnosis based on these four blinded interviews was completed in the presence of a third clinician who facilitated discussion to resolve diagnostic disagreements if needed.

Young Adult Assessment for DSM-IV Diagnoses

Offspring who had reached their 19th birthday were evaluated by Masters level clinical interviewers using the Composite International Diagnostic Interview (CIDI; [Janca et al., 1992]) to determine the presence or absence of a DSM-IV Axis I diagnosis. The CIDI-SAM

(Substance Abuse Module; [Cottler et al., 1989]) was also administered in order to determine quantity, frequency, and pattern of drug and alcohol use. Interrater reliability for interviewers on the diagnostic instruments used in this study exceeded 90%.

P300 ASSESSMENT

Event-Related Potential Assessments

Each subject performed an auditory (Choice Reaction Time) and a visual event-related potential (ERP) oddball task with electrodes placed at frontal, vertex, parietal, and occipital locations (Fz, Cz, Pz, Oz, P3, P4). Each subject performed two blocks of 80 trials of a Choice Reaction Time task. Auditory ERPs were elicited with "high" (1500 Hz) and "low" pitched (800 Hz) tones, presented every 3 sec (70 dbA intensity; 40 msec duration with an abrupt (2 μs rise time) in a modified oddball paradigm in which subjects are asked to press a button (right or left) corresponding to the presence of a high or low tone as previously described [Hill et al., 1990; Hill et a., 1995; Steinhauer and Hill, 1993]. The visual task consisted of presentation of a brief (.33 msec) target (stick-figure "head" with a nose and only one ear) or nontarget (blank head - no nose or ears screen) stimulus. The subject responded to the position of the ear with a button press.

Electrophysiological data were amplified by 20 k using a Grass Model 12 Neurodata system set to a bandpass of .01–30 Hz. Each trial was sampled for 1200 ms at 8 ms intervals beginning with a 200 ms prestimulus baseline. Ag/AgCl electrodes were placed at frontal, vertex, parietal, and occipital locations (Fz, Cz, Pz, Oz, P3, P4). An additional electrode was placed under the left eye and referred to linked ears for recording eye movement and blink artifacts. All active electrodes were referred to linked ears with a forehead ground. Eye blinks were tracked online using an oscilloscope. Any trial affected by eye artifact (blinks or eye movements greater than 50 μV) were excluded online. Average ERPs were computed from artifact-free trials.

A computer algorithm was used to search for the maximum peak amplitude for each component within predefined latency ranges (80–136 ms at Cz for N100, 136–240 ms at Cz for P200, 200–320 ms at Cz for N250, and 264–424 ms at Pz for P300). Peaks were verified offline by two trained raters blind to diagnosis of the subject. Through this interactive algorithm, P300 and other ERP components were checked. The peak amplitude was computed as the deviation from the median voltage during the 200 ms prestimulus baseline.

SNP Selection

Our previous genome-wide linkage analysis found potentially important linkage results for multiple regions including Chromosome 7 [Hill et al., 2004]. The present study focused on genotyping in a region on Chromosome 7q that centered on the microsatellite marker D7S515.

DNA Isolation and Genotyping

Genomic DNA was extracted from whole blood with a second aliquot prepared for EBV transformation and cryopreservation. PCR conditions were as described in [Hill et al., 2004]. Genotyping was completed on a Biotage PSQ 96MA Pyrosequencer (Biotage AB, Uppsala, Sweden). Each polymorphism was analyzed by PCR amplification incorporating a biotinylated primer. Thermal cycling included 45 cycles at an annealing temperature of 60°C. The Biotage workstation was used to isolate the biotinylated single strand from the double strand PCR products. The isolated product was then sequenced using the complementary sequencing primer.

Quality Control

SNP genotyping quality control involved ongoing monitoring of SNP signals provided by Qiagen software. Output is provided using three categories for each SNP: pass, fail, and check. Data analysis was performed for only those signals meeting the "pass" criterion. Signals that failed or were returned as needing further checking were rerun. If after three attempts the SNP did not meet the "pass" criterion, it was eliminated from the analysis and another SNP chosen as a replacement.

STATISTICAL METHODS

Sample Characteristics

For the present study, the original sample consisting of 380 first and second generation individuals from 65 two generation pedigrees were expanded to include a third generation of offspring who have been followed since 1990. Because most are now young adults, diagnostic status could be characterized as either affected or unaffected using DSM-IV or Feighner criteria for alcohol dependence. Accordingly, the three-generation sample consisted of 133 pedigrees with 995 individuals (48.1% male and 51.8% female). Among the 995 individuals 544 were affected, 440 were unaffected and 11 were of unknown status (Table I).

Mendelian Inconsistency

The PedCheck program [O'Connell and Weeks, 1998] was used to evaluate individual SNPs for Mendelian inconsistencies based on the pedigree structures. As a result of the evaluation, 14 marker genotypes from among 4,554 were coded as missing to resolve the reported inconsistencies.

Hardy-Weinberg Equilibrium (HWE)

Estimates of population allele frequencies were calculated using MENDEL version 11 [Lange et al., 2001]. Files required by the MENDEL program were generated via the program Mega2 [Mukhopadhyay et al., 2005]. Marker allele frequencies were tested for departures from Hardy-Weinberg equilibrium using the allele frequency option in MENDEL. None of the six SNPs analyzed were found to have *P*-values below the Bonferroni adjusted threshold (<0.008) that would indicate significant HWE departures.

Genetic Maps

The Genetic Map Interpolator (GMI) software [Mukhopadhyay et al., 2010] was used to retrieve current physical map positions from Ensembl (Ensembl 73); these physical positions were then

TABLE I. Phenotypic Characteristics by Generation Including the Number Genotyped

	Mean age (s.d.)	Unaffected[a] Count with Genotype[b]			Affected[c] Count with Genotype[d]			Phenotypic status unknown	Total with phenotpic data	Total genotyped
		Male	Female	Total	Male	Female	Total			
Generation I	59.29 ± 9.27	42[a], 23[b]	87, 58	129, 81	79[c], 25[d]	39, 14	118, 39	1, 1	248	121
Generation II	36.54 ± 7.86	69, 41	96, 81	165, 122	178, 126	156, 140	334, 266	0, 0	449	388
Generation III	23.88 ± 5.71	59, 59	87, 87	146, 146	47, 46	45, 45	92, 91	10	248	247
		170	270	440	304	240	544	11	995	756

Total Genotyped reflects only those genotypes that passed PedCheck analysis.

used to linearly interpolate genetic map positions based on the Rutgers Combined Linkage-Physical Map [Kong et al., 2004; Matise et al., 2007].

Family Based Association Test (FBAT)

Transmission rates of marker alleles were examined using the family-based association test program, FBAT [Laird et al., 2000; Rabinowitz and Laird, 2000], assuming an additive genetic model with robust variance estimation (-e option) to account for the relatedness. This family-based method is a generalization of the Transmission Disequilibrium Test (TDT) (Spielman et al., 1993) which provides a valid test of association even if admixture is present. FBAT converts each pedigree into nuclear families which are then treated as independent families for the test statistic calculation. Informative families consisting of parent-child trios are utilized in the FBAT analysis. To maximize power, the offset option of FBAT was used to include unaffected offspring in the analysis. Affected status was analyzed using presence or absence of alcohol dependence for Generations I, II, and III. A second analysis was performed in which presence of SUD in Generation III was coded as affected.

Analysis of P300 data, a quantitative trait, was performed using FBAT. P300 characteristics by generation and gender may be seen in Table II. Because FBAT does not support inclusion of covariates directly, residuals were calculated using age, gender, and familial relatedness, with these residuals then entered as a quantitative trait.

Gamete-Competition (GC)

We also considered the gamete-competition model (Sinsheimer et al., 2000), a generalization of the transmission disequilibrium test (TDT), to investigate association of marker alleles with affected status. The gamete-competition model can be used to test for differences in transmission of marker alleles to affected individuals.

Genotypic Transmission Disequilibrium Test (gTDT)

The genotypic transmission disequilibrium test [Schaid, 1996] was used to estimate genotypic risk for alcohol dependence from the case-parent trios using the R/Bioconductor package trio, version 3.2.1 [Schwender et al., 2011]. Each pedigree was converted into case-parent trios and treated as independent families for the calculations. We assumed an additive model with the major allele as the risk allele with affected status defined as the presence or absence of alcohol dependence for Generations I, II, and III.

Haplotype Analysis

Linkage disequilibrium (LD) analysis was performed using the HAPLOVIEW program version 4.2 [Barrett et al., 2005]. The LD block structure was defined by calculating D' values pairwise between SNPs. SNP haplotype blocks were created using the HAPLOVIEW four gamete rule [Wang et al., 2002]. A within-family association analysis between alcohol dependence and the revealed haplotypes was performed using haploytype FBAT (Horvath et al., 2004) assuming an additive genetic model and using a robust estimate of variance (e-option).

RESULTS

Family-Based Association Results

Analysis of six SNPs covering a 93.9 Kb region on chromosome 7 extending from rs1917939 to rs13475 within the three generation

TABLE II. Auditory and Visual P300 Amplitude (μV) Mean ± Standard Deviation by Generation and Sex

	Auditory					Visual				
		Male		Female			Male		Female	
Generation	Age	N	μV	N	μV	Age	N	μV	N	μV
I	60.5 ± 7.1	38	8.0 ± 5.7	53	13.2 ± 6.9	60.7 ± 8.0	19	3.1 ± 6.5	23	8.2 ± 7.4
II	35.1 ± 7.6	147	14.2 ± 7.0	140	15.7 ± 7.2	35.0 ± 7.5	73	11.7 ± 7.2	136	12.8 ± 7.6
III	23.9 ± 5.6	113	16.9 ± 7.2	133	19.0 ± 8.4	23.9 ± 5.7	113	16.9 ± 9.1	132	19.5 ± 9.4

sample revealed that all six SNPs with *P*-values between 0.05 and 0.0005. Two SNPs rs7794886 and rs12056091 located within intron 1 of the gene showed an association with affected status (FBAT $P = 0.0030$; and $P = 0.0026$, respectively). Three additional SNPs upstream of the ACN9 gene were significant with *P*-values ranging from 0.0005 to 0.0014 with a fourth located in Exon 4 significant at $P = 0.0153$. A separate analysis was performed using the presence or absence of SUD in Generation III revealing *P*-values between 0.045 and 0.007. This analysis revealed somewhat better statistical significance with five of the six SNPs with *P*-values <0.004 as may be seen in Table III. Correction for the number of SNPs tested was performed using the method of Moskvina and Schmidt (2008). Taking into account the linkage disequilibrium between the six ACN9 SNPs, the effective number of independent SNPs (N_{eff}) was determined to be 2.97 with an adjusted *P*-value of 0.017 ($0.05 \times 2.97 = 0.017$). Using this effective *P*-value, we conclude that all six SNPs were statistically significant.

Results of the Gamete Competition analyses are also summarized for comparison in Table III. LocusZoom [Pruim et al., 2010] was used to generate a plot of the association test results (Fig. 1). Pairwise linkage disequilibrium between SNPs and the LD block structure are shown in Figure 2.

Estimates of the risk for AD from the gTDT are shown in Table III. The highest AD risk for heterozygotes relative to homozygous minor allele carriers was seen in rs1917939 with OR = 2.58, 95% CI = (1.52, 4.38).

Haplotype Results

Pair-wise linkage disequilibrium between SNPs and the LD block structure are shown in Figure 2. This analysis revealed onefour-SNP block consisting of rs10499934, rs7794886, rs12056091, and rs13475 that was significantly associated with alcohol dependence. This four-SNP block showed an overall significant association at $P = 0.0008$ (Table IV). Haplotype specific transmission revealed GCAG nucleotides to be significantly associated with alcohol dependence with $P = 0.001$ and the ATGA combination at $P = 0.013$, indicating over-transmission of the major alleles to affected offspring and under transmission of the minor alleles to affected offspring.

The analysis was then repeated using third generation offspring who were coded as affected if they carried any substance use disorder (alcohol or drug abuse or alcohol or drug dependence). This analysis also showed a significant association of this haplotype with risk for alcohol dependence in the first and second generations and the broader phenotype that included drug abuse and dependence in the third generation offspring. The overall significance was $P = 0.0045$ with haplotype specific *P*-value for GCAG of 0.013 and $P = 0.040$ for ATGA.

P300 and ACN9 Variation

An FBAT analysis was performed using P300 visual and auditory amplitude as potential modifiers of the relationship between ACN9 variation and alcohol dependence within families. Under a dominant model, significant effects were seen for visual P300 for 3 of the 4 SNPs identified in block 1 of the ACN9 gene that showed a significant association with alcohol dependence (Table V).

DISCUSSION

The current study of the ACN9 gene was undertaken to follow up on a region of chromosome 7q first identified in our genome-wide linkage analysis of our two-generation data set [Hill et al., 2004]. Two previous linkage analyses in this region of 7q have also given support for the possibility that this region may harbor genes involved in alcohol dependence susceptibility [Reich et al., 1998; Wang et al., 2004]. Maturation of a third generation of offspring (currently at an average age of 23.8 years) from our multiplex families provided the opportunity to expand the data set and to pursue variation within the ACN9 gene that might be related to alcohol dependence. Family based association analyses (FBAT and GC) were performed for six SNPs in the ACN9 region of Chromosome 7 using three-generation multiplex families. FBAT analysis

TABLE III. SNP Position and Location with Significance Levels. Within-Family Association Analyses were Completed Using FBAT, GC, and R/trio, for Three-generation Pedigrees with 995 Alcohol Dependence Phenotypes

Marker	SNP location[a]	Alleles[b]	Position[c]	MAF	Number of Informative Families[d]	FBAT *P*-value[e]	GC *P*-value[e]	FBAT *P*-value Gen III SUD[f]	R/trio GRR (95% CI)[g]
rs1917939	Upstream	**G**/A	96,716,864	A = 0.270	45(46)	0.0005	0.0041	0.0075	2.58(1.52,4.38)
rs10246622	Upstream	**A**/G	96,734,057	G = 0.445	49(54)	0.0069	0.0044	0.0218	1.93(1.24,3.00)
rs10499934	Upstream	**A**/G	96,737,576	G = 0.208	39(42)	0.0014	0.0051	0.0150	2.32(1.35,3.97)
rs7794886	Intron 1	**T**/C	96,749,012	C = 0.381	49(55)	0.0030	0.0069	0.0109	1.96(1.25,3.10)
rs12056091	Intron 1	**G**/A	96,758,671	A = 0.376	49(55)	0.0026	0.0018	0.0080	1.96(1.25,3.10)
rs13475	Exon 4	**A**/G	96,810,729	G = 0.458	49(55)	0.0153	0.0265	0.0454	1.83(1.16,2.87)

[a]SNP locations are based on NCBI build 104 and Ensembl Release 74 (GRCH 37. p13).
[b]Risk allele in bold.
[c]Positions are based on NCBI build 104 and Ensembl Release 74 (GRCH 37. p13).
[d]Number of informative families with AD Phenotype. SUD # in parentheses.
[e]Generation I and II.
[f]Generation I, II, and III.
[g]Genotype relative risk for AD – major allele additive genotypic model.

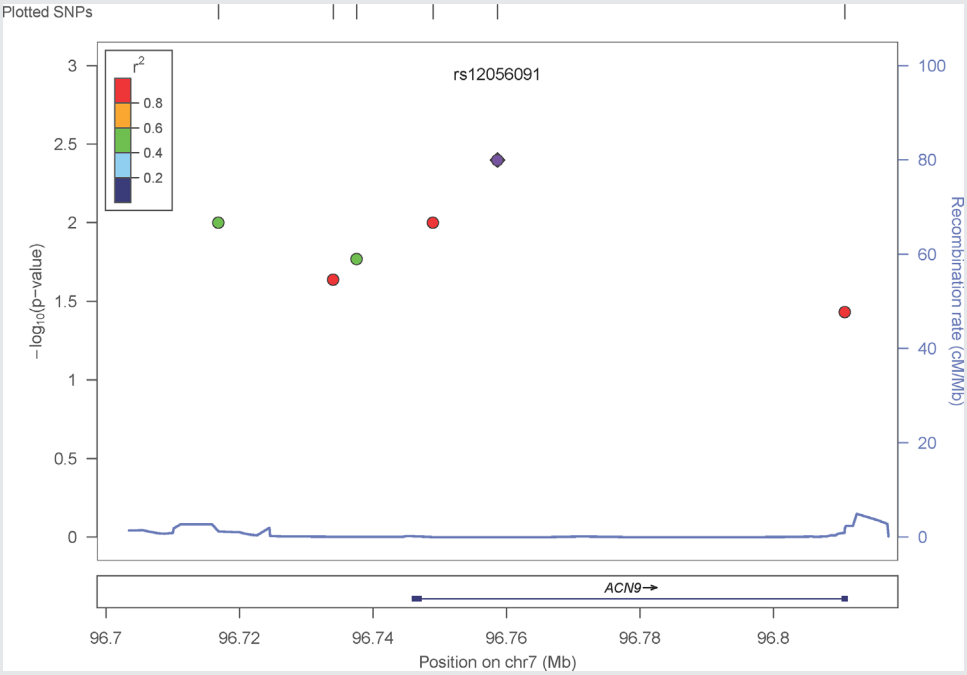

FIG. 1. The figure depicts the region of Chromosome 7q21.3 that includes the human ACN9 gene and single nucleotide polymorphism (SNP) sites included in the present analyses. Distances are based on physical maps from Ensembl 73.

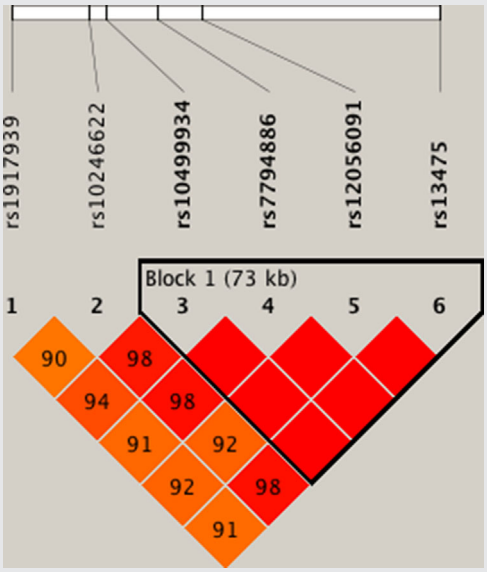

FIG. 2. Linkage disequilibrium analysis was performed using HAPLOVIEW (version 4.2). The block structure was defined by calculating D' values pairwise between SNPs. Using the four gamete rule, one four-SNP block consisting of rs10499934. rs7794886, rs12056091, and rs13475 was significantly associated with alcohol dependence. This four-SNP block showed an overall significant association, $P = 0.0008$.

requires heterozygosity in parents for families to be informative and included in the analysis. Accordingly, the FBAT analysis utilized only a subset of families potentially reducing the power to detect within-family variation. Nevertheless, significant results were found in the three-generation families that was also seen when the analysis included any SUD in Generation III.

These results would appear to have potential importance. Using the same alcohol dependence criterion used in the present study (DSM-IIIR and Feighner Criteria), Reich et al. (1998) reported a LOD score of 3.49 near marker D7S1793 in 105 pedigrees. Subsequently, the full COGA data set of 262 families was utilized and a LOD score of 2.9 revealed at marker D7S1799. Using 143 COGA families that were more densely affected with alcohol dependence, and similar in density to the present sample, a LOD score of 4.1 was seen with microsatellite D7S1799 [Dunn et al., 2005]. Finer mapping of Chromosome 7 in 262 COGA families revealed eight SNPs in the vicinity of D7S1799 with *P*-values <0.01 [Dick et al., 2008]. Therefore, the present results that are based on a multiplex sample of families ascertained in Pittsburgh replicate the results found for multiplex families selected through the COGA initiative. Estimates of relative risk within our multiplex families show an elevation in risk ranging from 1.8 to 2.6 times higher for family members who carry a particular risk allele.

The ACN9 gene was first identified as a novel protein involved in gluconeogenesis in yeast [Dennis and McCammon 1999]. The yeast homologue to the human ACN9 gene appears to be involved in utilization of acetate. Yeast mutants defective in the ACN9 gene were shown to display phenotypes identical to those found in yeast that were defective in metabolic enzymes required to utilize acetate

TABLE IV. Haplotype Analysis of the Four SNPs within Block 1

			Block		Overall	
Markers	**Haplotype**	**Freq.**	***P*-value[a]**	***P*-value[b]**	***P*-value[a]**	***P*-value[b]**
rs10499934–rs7794886–rs12056091–rs13475	A-T-G-A	0.653	0.013	0.040	0.0008	0.0045
	G-C-A-G	0.174	0.001	0.013		
	A-C-A-G	0.138	0.439	0.439		

[a]Affected status for Generation III is alcohol dependence only.
[b]Affected status for Generation III includes any SUD.

as an energy source along with a deficiency in the de novo synthesis of glucose from ethanol. This inability to assimilate acetate or ethanol into carbohydrate seen in yeast may have implications for alcohol metabolism in humans though currently this gene has not been widely studied in man.

Several human studies have reported a preference for highly sweet tastes and alcohol use disorders [Kampov-Polevoy et al., 1997] and an association between alcohol and sweet preference in rats [Sinclair et al., 1992] and in mice [Belknap et al., 1993]. One predominant interpretation of these findings has been that they represent aspects of a reward deficiency syndrome in which it is thought that tonically low levels of reward in preferring strains of animals or individuals with a predisposition to alcohol dependence demands greater stimulation to elicit a normal level of reward or reinforcement [Blum et al., 1995], However, another explanation of the overlap may be rooted in the effects of alcohol on gluconeogenesis. Gluconeogenesis is the process that produces needed glucose from glycogen in the liver or muscle and when depleted utilizes non-carbohydrate sources such as protein. However, alcohol inhibits gluconeogenesis so that blood levels of glucose may fall in response. Early work using positron emission tomgraphy showed that alcohol inhibits cortical and cerebellar metabolism of glucose with this inhibition being more pronounced in alcoholics [Volkow et al., 1990]. A hypoglycemic state may induce craving for sugar but may also stimulate craving for more alcohol. Interestingly, a maxim offered by AA for recovering alcoholics is to eat regularly and avoid becoming too hungry as this thought to promote going off the wagon. A recent study, Volkow et al. (2013) has confirmed the relationship between alcohol use and hypoglycemia by showing that alcohol intoxication decreases glucose metabolism but increases acetate uptake in the human brain.

Some limitations of this work should be mentioned. First, the third generation subjects have not reached middle age so that some individuals considered to be unaffected in the current analysis that was based on the last follow-up interview may convert to affected status at a later time. Also, because secular trends in the use of drugs other than alcohol has increased in the third generation in comparison to the first and second generation, it was necessary to also consider the possibility that those without alcohol abuse or dependence by DSM-IV criteria should be considered affected if they met criteria for drug abuse or dependence. Accordingly, analyses were performed twice, once with alcohol dependence across all three generations, and then using affected status to include drug abuse or dependence in the third generation.

Although genome wide association studies (GWAS) have not reported significant results for the ACN9 gene, this may not necessarily be a limitation. GWAS studies even with thousands of cases and controls have limited power to detect alleles with modest effect sizes (e.g., odds ratios of 1.2) and accordingly loci reaching genome-wide significance often have weak additive predictive power. Severely affected families (multiplex) enriched for cases are often able to reveal genes having a major effect with a greater proportion of explained variance [McClellan and King, 2010; Galvan et al., 2009].

TABLE V. Results of FBAT Analyses of Visual and Auditory P300. Each Subject's P300 Value was Adjusted for Age and Sex to Obtain the Appropriate Residual Value before Entering into the FBAT Analyses

Marker	**SNP location[a]**	**Alleles[b]**	**Number of Informative Families with Visual P300 Data[c]**	**Dominant Model Visual P300 FBAT *P*-value**	**Dominant Model Auditory P300 FBAT *P*-value**
rs1917939	Upstream	**G**/A	49(50)	0.096	NS
rs10246622	Upstream	**A**/G	54(54)	0.049	NS
rs10499934	Upstream	**A**/G	46(48)	NS	NS
rs7794886	Intron 1	**T**/C	54(54)	0.011	NS
rs12056091	Intron 1	**G**/A	53(53)	0.011	NS
rs13475	Exon 4	**A**/G	53(53)	0.018	NS

[a]SNP locations are based on NCBI build 104 and Ensembl Release 74 (GRCH 37. p13).
[b]Risk allele in bold.
[c]Number in parentheses is the number of informative families available for the auditory P300 analysis.

The P300 results showed a relationship between visual P300 at the parietal (Pz) electrode that was not seen with auditory P300 at Pz. Due to the inconsistency of results across modalities it is uncertain how robust these findings may be. There is some evidence that visual P300 may be more likely to associate with familial risk for alcohol dependence than auditory P300 at least in childhood [Polich et al., 1994]. Additionally, a 7q linkage for visual P300 has been reported at the central (vertex) electrode with a maximal LOD score at marker D7S2204 (LOD = 3.88 $P = 0.00024$) [Wright et al., 2008]. However, it should be noted that our previous work showed auditory P300 to be a discriminating covariate in our linkage analysis. We are uncertain why the current analysis finds visual but not auditory P300 at the parietal electrode significantly related to ACN9 variation. Accordingly, the results for P300 may need to be viewed with some caution.

In spite of our reservation regarding the P300 results, we view the results for the binary alcohol dependence phenotype to hold promise for medication development in view of the agreement across studies for the importance of the ACN9 variation especially in multiplex families including our own and those derived from the COGA study. Whether these results will generalize to families that are less densely affected is unknown.

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